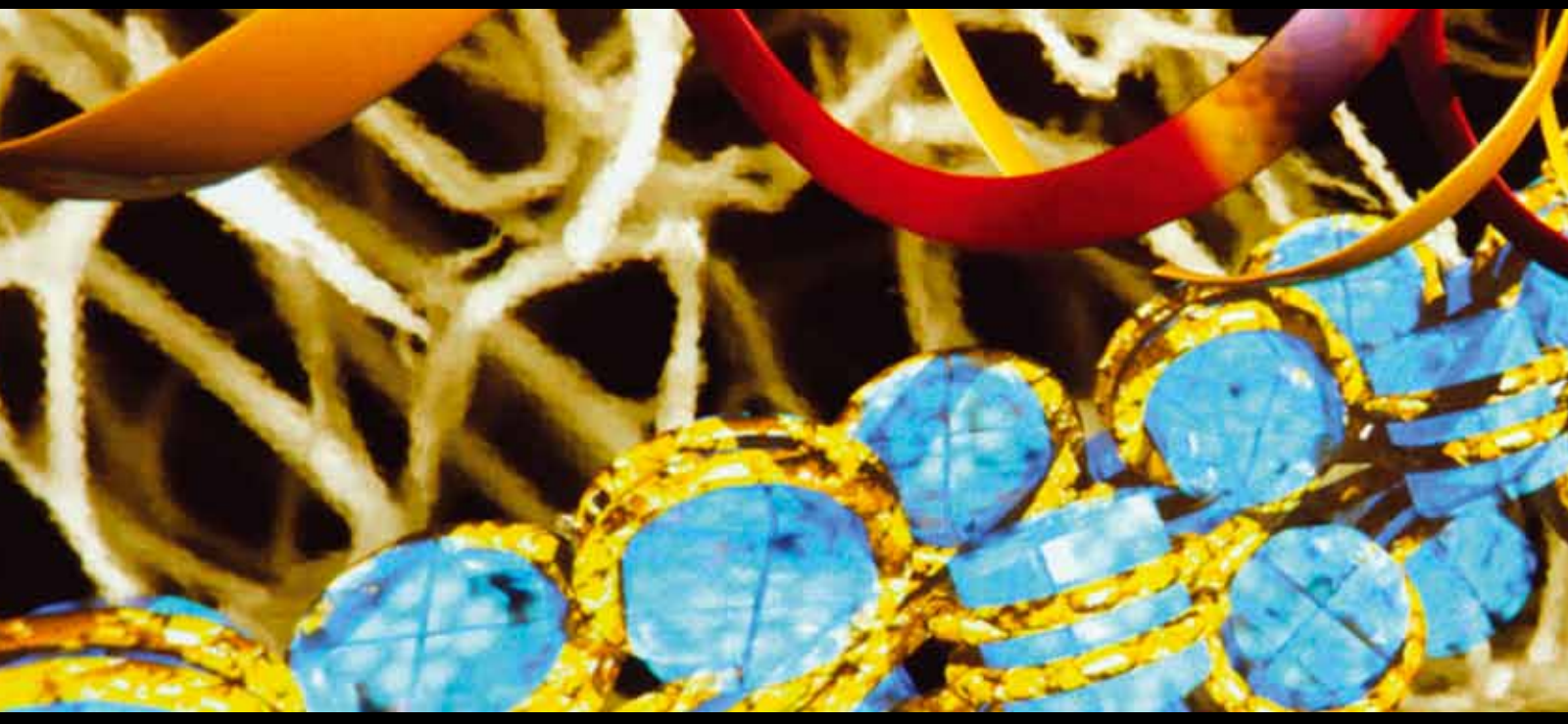
Molecular Biology International

Host-Pathogen Interactions of Retroviruses

Guest Editors: Abdul A. Waheed, Abraham L. Brass, Suryaram Gummuluru, and Gilda Tachedjian



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Editorial Board

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Editorial

Host-Pathogen Interactions of Retroviruses

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Retroviruses, such as HIV-1, are enveloped RNA viruses that use the enzyme reverse transcriptase (RT) to make a DNA copy of their RNA genome during replication in the host cell. The retrovirus life cycle is generally divided into two distinct phases: the early and late phase. The early phase encompasses virion entry into the host cell, reverse transcription of the viral RNA, nuclear import of the pre-integration complex (PIC), and integration of viral DNA into the host chromosome. The late phase involves transcription of viral DNA to multiple copies of viral RNA, translation of viral proteins, trafficking of viral proteins and genome to assembly sites, budding of viral particles, and, finally, maturation. A number of host factors have been implicated in specific steps of virus replication, and identification of such factors is a rapidly growing field. Recently, many host proteins were identified in genome-wide siRNA screens as being required for HIV-1 replication [1–3].

Over 25 antiretroviral drugs are currently in clinical use for treating HIV-1, and except for the fusion inhibitor that targets the viral envelope glycoprotein gp41 or the coreceptor CCR5, these drugs target the activity of the viral enzymes RT, integrase (IN), and protease (PR) [4–8]. The advent of highly active antiretroviral therapy (HAART) has made a significant impact on the natural history of HIV/AIDS by dramatically prolonging the life of HIV-infected individuals [9]. However, besides long-term drug toxicity and drug-drug interactions leading to treatment failures, significant limitations of antiviral therapy include the emergence of drug-resistant viral variants [10]. Further, the success of topical and oral preexposure prophylaxis (PrEP) in preventing the sexual transmission of HIV in a clinical trial setting presents potential concern because antiretrovirals or drugs with similar resistance profiles are used both for therapy and prevention [11]. This, in a PrEP setting, could either result in the transmission of drug-resistant viral strains or the generation of such viral strains in individuals taking PrEP unaware of their HIV infection status, thereby limiting future therapeutic options. Such concerns warrant efforts to identify novel inhibitors of HIV. Understanding the role of host proteins in viral replication could potentially lead to the development of new therapeutic strategies to combat this deadly pathogen.

This special issue brings together 17 reviews by experts on various aspects of the HIV-1 life cycle, highlighting the significant roles played by host factors in virus replication, and the antiviral agents that act on the viral and cellular targets. These reviews do not necessarily represent an exhaustive inventory of the current state of research or opinion in the field. Instead, the reviews cover the widely studied host-factors in each step of the HIV-1 replication cycle and antiviral therapy targeting viable cellular and viral targets. We, the guest editors, would like to sincerely thank all the authors for their contribution to this special issue and the reviewers for their time and expertise.

In his review "*TRIM5 and the regulation of HIV-1 infectivity*," Jeremy Luban offers an in-depth analysis of how TRIM5 impedes retroviral infection, including the recent exciting data concerning TRIM5's innate immune signaling capacity that permits the host factor to recognize HIV-1's capsid (CA) lattice and subsequently signal to

downstream antiviral effectors. This review also presents a comprehensive picture of a major challenge facing the field today—understanding the structural basis of TRIM5's recognition of HIV-1 CA.

Esposito and colleagues review the structure and function of the HIV-1 RT and the mode of action of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs). The authors discuss novel RT inhibitors that are currently in development, including NRTIs that act as chain terminators and those that act by blocking RT translocation or delaying DNA chain termination. New NNRTIs designed to inhibit HIV-1 mutants resistant to first-generation NNRTIs such as nevirapine and efavirenz, and those that block RT by competing with nucleotide substrate, a mechanism distinct from classical NNRTIs, are also covered in this review. Further, the authors highlight RNaseH inhibitors and pyrophosphate analogues and molecules that disrupt the essential RT subunit interaction.

Sheehy and Erthal in their exceptionally well-written review "*APOBEC3 versus retroviruses, immunity versus invasion: Clash of the Titans*" deftly touch on the major advances in understanding the role of this fascinating antiretroviral protein, and highlight some compelling future topics for research. The authors also cover the latest *in vivo* observations on APOBEC3 functions in HIV-infected patients.

Macrophages are a key source of HIV persistence *in vivo*. The review by Gavegnano and colleagues describes how nucleotide pools differ in macrophages compared to actively dividing T lymphocytes. Specifically, dNTP levels are limited relative to high levels of rNTPs and this disparity, shaped by the myeloid-cell-specific restriction factor SAMHD1, leads to preferential incorporation of rNTPs compared to dNTPs during reverse transcription. The authors discuss how the incorporation of rNTPs in the nascent viral DNA strand, which dispels the dogma that RT can only incorporate dNTPs, can be exploited in the design ribonucleoside chain terminator inhibitors that block HIV replication specifically in macrophages.

Felipe Diaz-Griffero shifts our attention to a host dependency factor that is required for HIV-1 infection by expertly discussing recent advances in elucidating the role of the karyopherin, TNPO3, in lentiviral replication. Specifically, this timely review covers recent genetic and biochemical data showing that the HIV-1's CA protein is the viral determinant for the requirement of TNPO3 during infection. Although the precise role of TNPO3 in lentiviral infection is a hotly debated topic in the field, this review succinctly frames the current state of this discussion, thereby providing a much-needed overview of this fast-moving topic.

The Debyser group has presented a comprehensive overview on HIV-1 dependency factors (HDFs) involved in viral integration and nuclear import. The work primarily discusses LEDGF, an HDF which is critical for mediating lentiviral integration; we are taken from the early days when biochemical approaches implicated LEDGF via its physical association with HIV-1, into the current era, where high-throughput small-molecule screens have identified novel inhibitors of this now-well-established host-viral interaction. The growing field of HIV-1 nuclear import is also covered, demonstrating the exciting work done in this area after a transformative whole genome siRNA screen first catalyzed interest in this fascinating topic. A recapping of the subsequent contributions by many laboratories to determine the mechanism of numerous HDFs in the nuclear import of the virus is also provided.

Schiralli Lester and Henderson in their review masterfully integrate the vast amount of data investigating HIV-1 proviral transcription. With deft skill, the pair interprets the role played by viral transcription in the rapidly expanding field of HIV-1 latency wherein viral reservoirs resist eradication after long-term antiviral therapy. Important topics in proviral transcriptional regulation covered in this selection include host transcriptions factors, chromatin, transcriptional interference, and elongation, the latter with special emphasis on the actions of the viral accessory protein, Tat. Tat is defined as a critical regulator and therapeutic target for the alleviation of latency and a potential cure. Multiple groups in both academia and industry are now reporting their fascinating investigations of HIV-1 latency, thus providing a dynamic stage for this prescient effort.

HIV-1 RNA interacts with numerous proteins including the viral nucleocapsid (NC) protein, and the structure of the RNA genome is linked to HIV-1 replication. However, the higher-order structure of the viral RNA is poorly understood. Sztuba-Solinska and Le Grice have come up with an excellent review on the utilities of the selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) technology, which can resolve the structure and quantify the flexibility of RNA at single-nucleotide resolution. The authors provide an overview of the SHAPE methodology- and also discuss the benefits and limitations of this technology in studying the structure of short RNAs. Shape technology has enabled the resolution of the structure of the entire HIV-1 genome (~9750 nucleotides) at the single-nucleotide level, and such detailed structural understanding could elevate the viral RNA as a viable target for small-molecule therapeutic intervention.

Expression of the HIV-1 Gag precursor protein, $Pr55^{Gag}$, is sufficient to produce virus-like particles. Nascent Gag traffics to the assembly sites—predominantly the plasma membrane, where Gag multimerization promotes virus budding, and finally the host-mediated scission leads to release of immature particles. Ono and colleagues present a comprehensive review on the dynamic association of Gag with membrane microdomains- and survey the role of lipid rafts and tetraspanin-enriched membrane microdomains in HIV-1 assembly. Besides discussing the more recent understanding of Gag multimer-driven reorganization of membrane microdomains, the authors also highlight the role of plasma membrane microdomains in cell-cell spread through virological synapses in T cells.

Hattleman et al. in their review on retroviral restriction factors, "*TRIM22: a diverse and dynamic antiviral protein,*" investigate another fascinating TRIM family member, TRIM22. The authors first relate TRIM22's evolutionary history including gene expansion/loss and the evidence revealing that the gene has experienced strong positive selection.

Interestingly, the authors describe the growing list of viruses restricted by TRIM22, including encephalomyocarditis virus, hepatitis B virus, and HIV-1. Lastly, the authors focus on the latest developments in the cell biology of TRIM22, including its role in cell proliferation and differentiation, and in cancer and autoimmune disease.

HIV-1 Gag, via the C-terminal PTAP motif known as the "late domain" hijacks the cellular protein Tsg101, a component of endosomal sorting complexes required for transport (ESCRT-1) complex during virus budding. Erlich and Carter review the role of ESCRT and non-ESCRT proteins in virus budding and release. The authors describe the role of PI(4,5)P2 in Gag targeting to the plasma membrane and the late domain-mediated recruitment of ESCRT machinery in HIV-1 budding. Recently, the Carter Group reported the activation of the inositol 1,4,5-triphosphate receptor (IP3R), which gates intracellular calcium ion stores, as a determinant in Gag trafficking and virus release.

Hammonds, Wang and Spearman provide an excellent state-of-the-art overview of the rapidly advancing field of tetherin biology, with a focus on recent advances in the understanding of the structure and function of this transmembrane protein. The authors begin by describing the historical details of the relationship between tetherin and the HIV-1 accessory factor, Vpu, and then discuss the relevance of tetherin in the replication and spread of other retroviruses. Further, the authors present a balanced synopsis of evidence for and against the model that proposes tetherin localization to membrane microdomains as a critical determinant of its antiretroviral activity.

The Env glycoprotein associates with Gag during virus assembly to form infectious virus particles. Murakami in his review describes the biosyntheseis, trafficking, and incorporation of Env glycoproteins into virus particles. In this review, he surveys various proposed models for Env incorporation into virus particles. The Env incorporation can be passive or via direct or indirect Gag-Env interaction, which reportedly occurs at specific membrane microdomains and is mediated by specific host factors. Murakami's review covers in detail the host cellular factors implicated in Gag-Env interactions and their specific role in virion incorporation.

The HIV-1 PR activity converts immature particles to infectious mature particles. In her review, Adamson details the sequential cascade of events that accompany the PR-mediated cleavage of the Gag polyprotein. Inhibiting PR activity by protease inhibitors (PIs) results in the production of noninfectious virus particles, and nine PIs are currently approved for clinical use. In contrast, maturation inhibitors bind to Gag and specifically block the individual cleavage events or alter the order of cleavage events, thereby resulting in the production of aberrant particles. In this review, Adamson provides an overview of the mechanism of action of PIs and maturation inhibitors- and highlights the problems associated with drug-resistant mutants.

In their contribution, Hartman and Buckheit review the HIV inhibitors currently in clinical use, novel HIV RT inhibitors in the pipeline, and drugs that target additional viral proteins including the gp41 involved in viral fusion, the zinc fingers of NC required for viral genome encapsidation and reverse transcription, the IN inhibitors that block insertion of the viral cDNA into the host cell chromosome, and the PIs that target viral maturation. The authors also review molecules that target the HIV-1 regulatory and accessory proteins Tat, Rev, Vpu, Vpr, and Vif. The review also examines strategies for targeting host cells proteins (Tsg101 and LEDGF/p75) that are hijacked by HIV for replication, and ways to exploit intracellular host cell restriction factors (i.e., APOBEC3 and tetherin) that block HIV replication. Immunotherapy, gene therapy, and strategies to eliminate the latent reservoirs of HIV are also described.

Microbicides are chemical entities formulated in a gel, cream, ring, film, or tablet that can prevent or reduce transmission of sexually transmitted infections including HIV infection, when applied to the vagina or rectum. In their review, Buckheit and Buckheit provide a comprehensive assessment of the HIV microbicide field and the preclinical tests that are required for progression of a candidate microbicide through the development pathway. The authors also highlight gaps that exist in product development that relate to product dosing, formulation and delivery, and pharmacokinetics and pharmacodynamics, which all must be addressed to improve prioritization of candidate microbicides for clinical testing. Besides vaginal microbicides, the development and formulation of dual compartment use microbicides for both vaginal and rectal use are discussed. The emerging area of multipurpose prevention technologies with the premise to prevent unplanned pregnancies, HIV, and other sexually transmitted infections that can increase HIV acquisition are also described.

A consequence of suboptimal antiretroviral therapy is the emergence of drug-resistant strains of HIV-1, which can lead to therapy failure. Much of our knowledge regarding the type of mutations that emerge during therapy and their role in decreasing drug susceptibility is derived from studies with HIV-1 subtype B. However, 90% of HIV-infected individuals worldwide harbour nonsubtype B variants that contain distinct polymorphisms. Wainberg and Brenner review the ability of such polymorphisms in nonsubtype B HIV to affect the level of resistance mediated by major drug-resistance mutations, and to modulate the evolution of certain drug resistance mutations in the presence of drug. The authors also propose studies that would increase our understanding of the role of polymorphisms in drug resistance and, thereby, promote more informed use of first, second and third-line antiretroviral drugs in different geographical settings.

There are few research areas that are not covered explicitly in this special issue, such as retrovirus entry, and the role of receptors and coreceptors in virus entry. However, this issue offers a comprehensive view of our understanding of the HIV-1 life cycle, host factors involved in virus replication, and viral and cellular antiviral drug targets.

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Review Article

Cellular Cofactors of Lentiviral Integrase: From Target Validation to Drug Discovery

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To accomplish their life cycle, lentiviruses make use of host proteins, the so-called cellular cofactors. Interactions between host cell and viral proteins during early stages of lentiviral infection provide attractive new antiviral targets. The insertion of lentiviral cDNA in a host cell chromosome is a step of no return in the replication cycle, after which the host cell becomes a permanent carrier of the viral genome and a producer of lentiviral progeny. Integration is carried out by integrase (IN), an enzyme playing also an important role during nuclear import. Plenty of cellular cofactors of HIV-1 IN have been proposed. To date, the lens epithelium-derived growth factor (LEDGF/p75) is the best studied cofactor of HIV-1 IN. Moreover, small molecules that block the LEDGF/p75-IN interaction have recently been developed for the treatment of HIV infection. The nuclear import factor transportin-SR2 (TRN-SR2) has been proposed as another interactor of HIV IN-mediating nuclear import of the virus. Using both proteins as examples, we will describe approaches to be taken to identify and validate novel cofactors as new antiviral targets. Finally, we will highlight recent advances in the design and the development of small-molecule inhibitors binding to the LEDGF/p75-binding pocket in IN (LEDGINs).

1. Introduction: Cofactors of Integration as Potential Antiviral Targets

Infection with the human immunodeficiency virus type 1 (HIV-1) remains a substantial public health as well as a socioeconomic problem worldwide [1]. Although highly active antiretroviral therapy (HAART) effectively halts HIV replication and profoundly increases survival of patients, it has not been possible yet to achieve a cure. Interruption of HAART typically results in a rebound of virus replication. This is primarily due to the fact that HIV has evolved mechanisms to escape from the continuous immune surveillance in a small pool of latently infected cells that are not susceptible to drug therapy. These latently infected cells reside in reservoirs where the distribution of antiretroviral (ARV) drugs is extremely variable and often lower than the expected maximal inhibitory concentration (for recent reviews see [2–4]). Moreover, the rapid replication rate and the generation of an extensive genetic diversity fuel the emergence of drug-resistant viral strains resulting in treatment failure [5, 6]. Therefore, there is a continuous demand to search for novel and better ARVs for a better control of the HIV pandemic with the hope to eventually induce permanent remission of the disease.

HIV relies on the host cellular machinery to complete its replication cycle. HIV hijacks several biological processes and protein complexes of the host cell through distinct virus-host protein-protein interactions (PPIs) [7, 8]. Since these host-pathogen interactions directly mediate viral replication and disease progression, their specific disruption can provide alternative targets for therapeutic intervention. PPIs represent an attractive group of biologically relevant targets for the development of small-molecule protein-protein interaction inhibitors (SMIPPIs) [9–11]. Since protein-protein interfaces are often based on extended, flat, barely defined, and large hydrophobic surfaces, overcoming binding energy with small

molecules is hard to achieve. Therefore, obtaining validated starting points for chemical optimization of SMIPPIs has been difficult [11]. Moreover, the applicability of PPIs as therapeutic targets is not only defined by their physicochemical properties but also by the biological properties of the protein-protein interaction and requires meticulous target validation prior to drug discovery.

In recent years, our understanding of the HIV-host interaction has dramatically increased, opening the possibility for the discovery of novel classes of therapeutics [8, 12–14]. Not surprisingly, there are numerous interactions between HIV and cellular proteins involved in all stages of virus replication [8]. In principle, any distinct interaction between virus-encoded proteins and host cofactors has the potential to be a target for drug design. The CCR5 antagonist, maraviroc, was approved as the first ARV targeting a host factor [15]. Maraviroc binds to the CCR5 coreceptor on the surface of cells and prevents interaction with the gp120 envelope protein of the virus [16]. Successful targeting of host-virus PPIs demonstrates that HIV-1 therapeutic drug targets are not limited to virus-encoded enzymes and that understanding of the virus-host interactome can be the basis for future HIV therapeutics [17–20]. In theory, this antiviral strategy is expected to make it more difficult for the virus to develop resistance. Since the host factor is genetically conserved in a biologically relevant host-virus interaction, resistance is less likely to occur increasing the clinical potential of these drugs. Alternatively, drug-induced mutations at a conserved interface may reduce viral fitness [21].

In recent years, HIV-1 integrase (IN) joined the selection of important therapeutic targets to treat HIV infection (for a review see [22]). The enzyme orchestrates the insertion of the viral DNA into the host chromatin [23, 24]. HIV IN is a 32-kDa protein containing 3 canonical structural domains connected by flexible linkers: the N-terminal (NTD, residues 1–50), the catalytic core (CCD, residues 51–212), and the C-terminal domain (CTD, 213–270) (Figure 1(a)). All 3 domains are required for 3′ processing and DNA strand transfer. The solution structure of the N-terminal HHCC domain revealed a three-helix bundle stabilized by zinc [25]. The central catalytic core domain contains the DD(35)E motif conserved among retroviruses and retrotransposons. D64, D116, E152 residues coordinate 2 Mg^{2+} ions necessary for catalysis [26]. The C-terminal domain has a SH3-like fold [27]. Full-length HIV-1 IN is a multimeric enzyme and forms stable tetramers in solution [28].

Despite the recent release of the crystal structure of full-length IN of the prototype foamy virus (PFV) [29], we still lack a crystal structure of full-length HIV-1 IN. The main obstacle for structural studies of HIV IN is its propensity to aggregate. The published two-domain crystal structures of HIV-1 IN (comprising the N-terminal and the catalytic core or the catalytic core and the C-terminal domain) [30, 31] as well as the crystal and NMR structures of individual domains (for review see [32]) represent valuable, but incomplete information on the functional structure of the HIV intasome. HIV integrase was the last HIV enzyme to be effectively targeted with small molecules. Reasons were

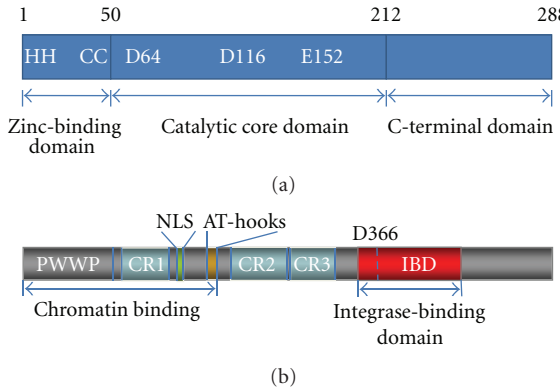

Figure 1: Domain organization of HIV-1 IN and LEDGF/p75. (a) HIV-1 IN is composed of an N-terminal domain (NTD), a catalytic core domain (CCD), and a C-terminal domain (CTD). The CCD contains the catalytically essential DD(35)E motif and the hot spots for interaction with the IBD in LEDGF/p75. The Asp and Glu residues of the CCD coordinate one or two Mg^{2+} ions and are involved in 3′ processing and DNA strand-transfer activities. (b) LEDGF/p75 has several structural motifs involved in chromatin tethering and protein-protein interactions. The PWWP domain, the charged regions (CRs), and AT-hooks are involved in chromatin binding. The C-terminus contains the well-characterized IN binding domain (IBD) and acts as a protein interaction playground. Asp residue 366 critical for HIV-1 IN binding is indicated.

the lack of homologous disease targets, as opposed to well-studied DNA polymerases and aspartyl proteases and the absence of a crystal structure. Indeed, nowadays structural information is playing a central role in successful drug development. HIV protease (PR) was already recognized as a target in the early nineties [33], and soon after the first crystal structure of HIV-1 PR was published [34]. Publication of the structure of HIV-1 PR complexed with the inhibitor MVT-101 preceded only by six years the approval of the first PR inhibitor as an anti-HIV drug [35, 36].

After completion of reverse transcription, the so-called preintegration complex (PIC) is formed. Along with viral cDNA and IN, the PIC contains viral reverse transcriptase (RT), nucleocapsid (NC), matrix (MA), and Vpr. RT and NC are involved in the synthesis of viral cDNA, while MA and Vpr may affect nuclear import of the PIC. The PIC also contains host cell proteins, and nuclear import is mediated by the interaction with transport factors and nucleoporins. In the nucleus, HIV IN catalyzes the stable insertion of the viral cDNA into a host chromosome.

The recent success in the application of structure-based rational drug design in the discovery and development of allosteric HIV-1 integrase (IN) inhibitors, the LEDGINs [37], was possible due to 7 years of intensive basic research on the cofactor lens epithelium-derived growth factor/p75 (LEDGF/p75). LEDGINs inhibit the interaction between LEDGF/p75 and HIV-1 IN and will be used as an example to discuss approaches, challenges, and future perspectives of SMIPPIIs.

2. Identification and Validation of Cofactors as Novel Antiviral Targets

Purified proteins from diverse sources could rescue the intermolecular integration activity of retroviral PICs isolated from infected cells and salt-stripped of associated host factors. This observation opened a new field in retrovirology focused on the so-called cellular cofactors of retroviral integration (for review see [38]). Farnet and Bushman noticed that a factor important for integration activity *in vitro* was removed upon gel filtration of HIV-1 PICs in the presence of high salt [39]. The activity could be restored by addition of protein extracts from uninfected human SupT1 cells. The factor was identified as the high-mobility-group chromosomal protein A1 (HMGA1, HMG I(Y) protein) [39]. HMGA1 is a nonhistone DNA-binding protein involved in the regulation of inducible gene transcription and microRNA expression [40] in both benign and malignant neoplasias [41]. The same method led to discovery of another cellular cofactor of HIV, barrier-to-autointegration factor (BAF) [42]. By combining antibodies against known viral and cellular PIC components (MA, Vpr, Ku-80) with anti-BAF antibodies, Lin and Engelman proved that human BAF is a component of PIC [43]. Their functional coimmunoprecipitation strategy was based on examining different fractions obtained from HIV-1-infected C8166 T-cells for the presence of integration activity, viral IN and endogenous BAF [43]. Although BAF was suggested to protect retroviral DNA from autointegration and also to promote the association of PICs with target DNA [44], knockdown of BAF by siRNA in HeLaP4 cells did not affect HIV-1 replication [45]. Validation of the role of cellular cofactors in lentiviral infection, thus, requires multiple independent experimental approaches.

The initial discoveries of HMGA1 and BAF were not the result of a systematic search for cellular cofactors of lentiviral integration. The increasing interest in the interactomics of HIV integration and replication has resulted in algorithms for the identification and proper validation of cofactors (Figure 2). Discovery of novel HIV-1 cofactors as potential antiviral targets can be accomplished by different techniques and is often based on the search for specific and direct protein interaction partners by yeast two-hybrid (Y2H) screen or high-throughput coimmunoprecipitation (co-IP) followed by mass spectroscopy. Alternatively, full-genome RNA interference (RNAi) screens can be used to identify genes/proteins involved in HIV integration/replication.

Physical protein-protein interactions between viral protein and cofactor (Y2H and co-IP) need validation in a phenotypic assay. After specific RNAi-mediated depletion of the specific host factor, the impact on HIV replication is determined. If depletion of the candidate cofactor, verified by western blotting and RT qPCR, has no deleterious effect on HIV replication, the cofactor can be dismissed as an important cofactor of HIV replication. If depletion results in a stimulation of HIV replication, the binding partner may represent a restriction factor. In parallel, colocalization of viral protein (IN) and host protein in the cell can be verified by microscopy. Phenotypic assays measure single and multiple rounds of infection in both laboratory immortalized cell lines (e.g., HeLaP4) and primary CD4+ T cells and macrophages. In our expertise, multiple round replication represents the best assay system to validate cofactors. Use of multiple siRNAs targeting the same cofactor and back-complementation with siRNA-resistant cofactor encoding plasmids should avoid offtarget effects. Growth curves of cells depleted of cofactor should reveal major toxicity effects. An alternative method which can be also conveniently combined with RNAi to validate a cellular cofactor as a target for antiviral drug development is the use of dominant negative mutants, originally successfully exploited for interference with functions of viral proteins [46–48]. Overexpression of the integrase-binding domain (IBD) of LEDGF/p75, for example, blocks HIV-1 replication which was instrumental in studying the role of LEDGF/p75 in the HIV-1 life cycle [49, 50].

In case of discovery through a siRNA screen, co-IP or pull down experiments should be carried out to investigate the direct physical interaction between cofactor and viral protein. Quantitative PCR (qPCR) analysis of the different HIV DNA species (reverse transcripts, 2-LTR circles, integrants) in cells depleted for the cofactor may reveal the replication block hinting to the potential interacting viral protein (RT, IN,...). However, the expertise with siRNA screens so far has taught us that cellular pathways rather than specific PPIs are highlighted by this approach [51]. The recent efforts to use high-throughput co-IP and MS to unravel the HIV interactome should reveal more specific HIV cofactors than the siRNA screens [8].

For HIV, efficient strategies for large comprehensive Y2H screens of different cDNA libraries have been developed [52]. In the primary screen, HIV-1 IN fragments serve as baits. By combination of random and oligo-dT cDNA priming techniques, Rain et al. significantly increased the confidence of the hits by requiring identification of the same positive clone from the two independent cDNA libraries. Confirmation of the specificity of the interactions with HIV IN is done in rebound screens, where hits from the primary screen (potential cellular cofactors) are used as baits against a library of random HIV-1 protein prey fragments. This also allows mapping of respective IN binding domains [52]. By Y2H, IN interactor 1 (INI1)/hSNF5 and transportin-SR2 (TRN-SR2) were identified as IN cofactors [53, 54].

Three RNAi-based whole-genome screens for HIV infection in mammalian cells were reported in 2008 [13, 55, 56], and a meta-analysis of these studies was published in 2009 [57]. Drawbacks of these screens are the use of HeLa or HEK293T cells that are not natural host cells of HIV-1 infection. Later Thys et al. [58] demonstrated that VSV-G pseudotyping of HIV may confound interactions with natural host factors during early steps of the replication. Use of mutated or cell-line adapted viruses in the screens can be another source for false negatives and positives. The necessity of proper validation of potential cofactors derived from siRNA screens is underlined by comparison of the results of 2 large siRNA screens performed for HIV. Brass et al. [55] identified 284 genes, whereas Zhou et al. [56] picked

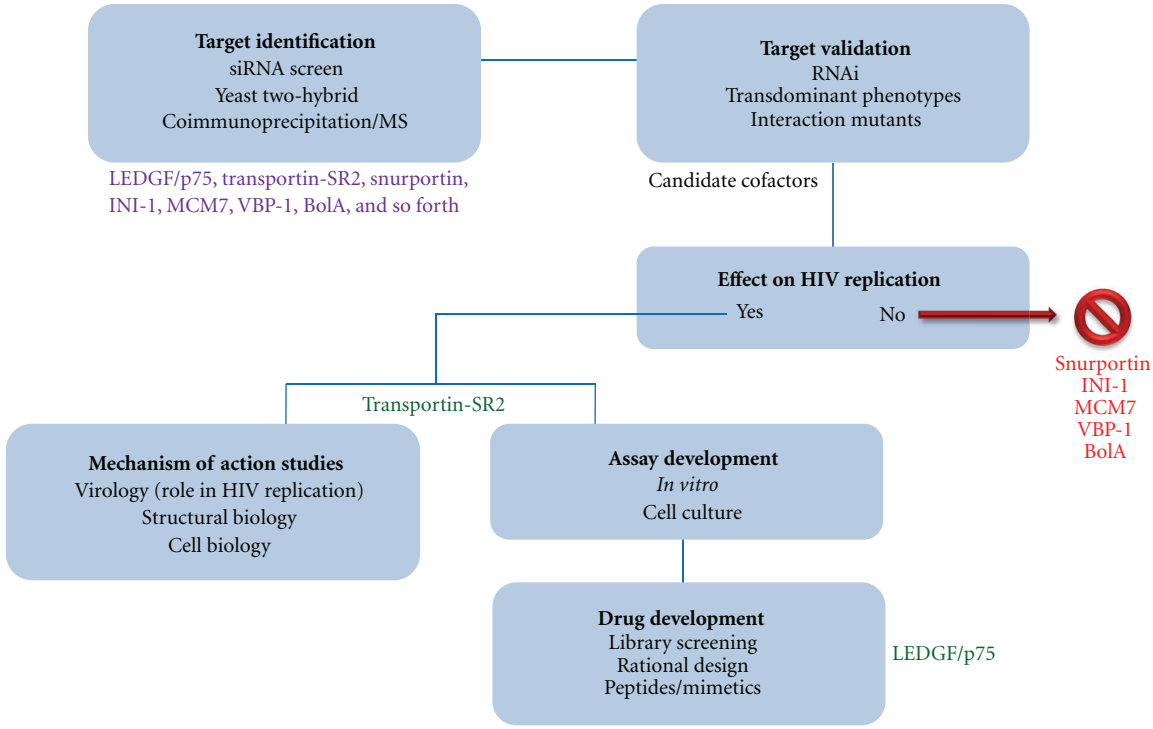

Figure 2: Algorithm to identify and validate novel cofactors as antiviral targets with examples of candidate and validated HIV-1 IN cellular cofactors at particular stages of validation. The algorithm was used in the validation of LEDGF/p75 and TRN-SR2 as cellular cofactors of HIV-1 IN and in validating LEDGF/p75 as an antiviral target. In case of some candidate cofactors, the experimental intervention verifying affect on HIV replication was accompanied by toxicity. These candidates were excluded from follow-up steps of drug target validation. These proteins can still be involved in the HIV life cycle but were not considered priority targets.

up 232 genes. Only 15 genes overlapped between both studies [56]. LEDGF/p75 was not identified in either of them.

Nuclear import is an important step in lentiviral infection. The classical technique to study nuclear import of cellular proteins with recombinant import factors is based on digitonin-permeabilized cells [59]. The method was also adapted to study nuclear import of snRNA [60] and DNA [61]. This technique is of limited use for the study of lentiviral nuclear import since NLSs of individual viral proteins can be masked within the PIC, and the data obtained for isolated proteins do not need to fit the real situation during viral infection. There are now better approaches available for studies of lentiviral nuclear import (and early postentry steps in general) based on advances in fluorescence microscopy: real-time *in vivo* tracking [62–64] and the so-called PIC import assay [54, 65]. The PIC import assay is based on fluorescently labeled viral particles containing IN fused to eGFP (HIV-IN-eGFP) *trans*-incorporated in the particle through a fusion with HIV-1 Vpr [66].

After validation of the interaction between host factor and viral protein, drug discovery can be initiated, facilitated by high throughput screening (HTS) and high-content screening (HCS) technologies developed since the 1990s, as for example, amplified luminescent proximity homogeneous assay (AlphaScreen) technology, high-throughput FLIM for protein-protein interaction screening, enhanced chemiluminescence, fluorometric microvolume assay technology (FMAT), LeadSeeker, scintillation proximity assays (SPA), and so forth. These screening technologies allow screens to be performed efficiently, cost-effectively, and with low amounts of material. Nowadays there is a trend to move from labeled reporter assays towards label-free assays [67–69]. If structural biology approaches (crystallography, NMR, SAXS, etc.) can reveal the interface of the PPI aided by site-directed mutagenesis to corroborate the hot spots of the interaction, structure-based drug design can be embarked upon. For the discovery of LEDGINs, AlphaScreen technology and structure-based drug design were used.

3. The Interaction between LEDGF/p75 and HIV-1 IN Is a Novel Anti-HIV Target

Today, LEDGF/p75 represents the classical example of a viral cofactor validated as druggable target for antiviral therapy. Basic academic research on the role of LEDGF/p75 in HIV infection ultimately led to development of LEDGINs, first-in-class allosteric HIV-1 integrase inhibitors [37].

LEDGF/p75 was originally identified in Leuven in 2002 by coimmunoprecipitation as a binding partner of HIV-1 IN [28]. LEDGF belongs to the hepatoma-derived growth factor (HDGF) family. Together with HDGF-related proteins (HRPs), this family is composed of chromatin-associated proteins. The N-terminal part of these proteins is highly

conserved and contains a characteristic PWWP (Pro-Trp-Trp-Pro) domain [70, 71] (Figure 1(b)). HDGF and its homologues display between 54% and 78% sequence identity among the 91 N-terminal amino acids. Because of this similarity the amino-terminal region has been termed *Homologue to Amino Terminus of HDGF* (HATH region) [70, 71]. LEDGF/p75 is implicated in the regulation of stress response proteins. There are two splice variants of LEDGF/p75 expressed from the PSIP1 (PC4- and SFRS-interacting protein 1) gene: LEDGF/p75 and p52. They share the same N-terminal 325 amino acid residues, but have different C-termini; 205 amino acid residues in the case of p75 and 8 in the case of p52. LEDGF/p75 (530 amino acid residues) was identified as a binding partner of HIV-1 IN by immunoprecipitation of IN tetramer complexes from nuclear extracts of 293T cells expressing IN from a synthetic gene [28]. Colocalization studies with constructs of IN and LEDGF/p75 fused to GFP or HcRed1 revealed that the N-terminal and the central core domains of HIV IN are involved in the interaction with LEDGF/p75 [72]. The IN-binding domain of LEDGF/p75 was mapped to the C-terminal part of the protein and is absent from LEDGF/p52 [72]. RNAi-mediated knockdown of endogenous LEDGF/p75 expression abolished nuclear/chromosomal localization of IN [72]. This observation led to the hypothesis that LEDGF/p75 is the main chromatin-tethering factor for IN that hence determines integration site selection of *Lentivirinae* [73–75]. Through the interaction with LEDGF/p75, integration of HIV into the host cell chromatin is preferentially targeted to the body of active genes [74]. A dynamic scan-and-lock mechanism for the chromatin tethering mediated by the LEDGF/p75 PWWP domain was evidenced by a later study of Hendrix et al. [76].

Soon an evolutionary highly conserved protein-binding domain spanning amino acids 347–429 was identified by means of limited proteolysis and deletion mutagenesis [77]. This domain was coined integrase binding domain or IBD (Figure 1(b)). In the HRP family, the IBD is only present in the hepatoma-derived growth factor-related protein 2 (HRP2). In spite of the identification of the interaction between HIV-1 IN and LEDGF/p75, definition of the IBD in LEDGF/p75, a clear phenotype of IN relocalization after LEDGF/p75 knockdown, and the role of LEDGF/p75 in HIV infection remained disputed for some years, especially after one publication dismissing such role [78]. Multiple lines of increasingly solid evidence were reported in subsequent years 2005–2012 (for more extensive reviews see [7, 79, 80]). A role of LEDGF/p75 in integration and replication of HIV-1 was first suggested by the study of mutants of IN identified by Y2H screening [81]. A single mutation in IN, Q168A, disrupted the interaction with LEDGF/p75 without major effect on the catalytic activity *in vitro*. Viruses containing IN-Q168A were defective for replication and the replication block was mapped to the integration step using qPCR. Simultaneously, it was proven that LEDGF/p75 is not required for active nuclear import of the HIV PIC [81]. Using transient and stable knockdown of LEDGF/p75, Vandekerckhove et al. were first to demonstrate a close correlation between LEDGF/p75 levels and extent of HIV integration and replication [82]. Back-complementation of LEDGF/p75 restored viral replication to nearly wild-type levels [82].

In 2005, the solution structure of the IBD of LEDGF/p75 was published [83] and amino acid residues essential for the interaction with HIV-1 IN were identified: Ile365, Asp366, Phe406, and Val408. The IBD is a compact right-handed bundle composed of five α-helices. Residues essential for the interaction with IN are localized in the interhelical loop regions of the structure. The crystal structure of the IBD in complex with a dimeric CCD of IN was a major advance in defining the structural properties of the IBD-CCD interface [84]. The LEDGF/p75 binding pocket in IN is formed at the dimeric interface of the CCD of IN. The structure was confirmed by mutagenesis studies of Busschots et al. [85]. Two regions of the IN CCD dimer were identified to be involved in the interaction with LEDGF/p75: one centers around residues Trp131 and Trp132 while the second extends from Ile161 up to Glu170 [85].

In 2006, it was demonstrated that stable overexpression of the IBD reduces HIV replication 100-fold [49]. By competing with endogenous LEDGF/p75 for IN binding, IBD fused to eGFP was able to block HIV-1 replication at the integration step [49]. This result provided proof of concept that the HIV-1 IN/LEDGF/p75 interaction constitutes a novel target for antiviral therapy. Serial passaging of the virus in IBD overexpressing cells yielded a resistant virus with IN mutations at positions 128 and 170, located at both sides of the LEDGF/p75 binding pocket [21]. Al-Mawsawi et al. subsequently showed that a LEDGF/p75-derived oligopeptide containing the IN interacting residues Ile355 and Asp366 blocked interaction between LEDGF/p75 and IN [86]. Even though peptides and natural products have been shown to modulate PPIs in several therapeutic areas, their physicochemical properties make them less amenable for drug development [9]. Therefore, small molecule inhibitors that bind to the LEDGF/p75 binding pocket in HIV-1 IN were proposed as novel therapeutic strategy [17]. Du et al. [87] reported that a benzoic acid derivative, D77, allegedly disrupted the LEDGF/p75-IN interaction and inhibited HIV replication, albeit with cellular toxicity. Subsequently, structure-based rational drug design resulted in the identification of small molecules (CHIBA-3002 and its analogs) that reduce LEDGF/p75-IN interaction [88]. However, the first potent and selective inhibitors of HIV replication that act by disrupting LEDGF/p75-IN interaction were reported in 2010. We coined the class of small molecule inhibitors that bind to the LEDGF/p75 binding pocket in HIV-1 IN as LEDGINs. The first molecules of this class, quinolinylacetic acid derivatives, were discovered by rational drug design [37]. The reported LEDGINs have potent antiviral activity and are now in advanced preclinical development.

From the drug discovery point of view, the interactions of LEDGF/p75 with other cellular proteins are of importance. Perturbation of these interactions while targeting LEDGF/p75-IN interaction could potentially deregulate the normal cellular role of LEDGF/p75 and lead to cellular toxicity. By Y2H screens with the C-terminal domain (aa 341–507) of LEDGF/p75 as the bait, JPO2 and pogZ were

identified as LEDGF/p75 binding partners and their interactions were extensively characterized [89, 90]. Maertens et al. demonstrated that interaction of JPO2 with LEDGF/p75 is mediated by LEDGF/p75 IBD, and recombinant IN competes with JPO2 for binding to LEDGF/p75 *in vitro* [91]. A positively charged patch on the surface of the IBD structure is involved in an interaction with another LEDGF/p75 binding partner, Cdc7-activator of S-phase kinase (Cdc7-ASK) [92]. LEDGF/p75 is also a crucial cofactor required for both the oncogenic and tumor suppressor functions of mixed lineage leukaemia protein (MLL)/menin complexes. MLL chimeric oncoproteins in complex with menin are dependent on the association with LEDGF/p75 [93]. Recently, the crystal structure of the ternary complex of menin-N-terminal fragment of MLL1-LEDGFIBD has been published [94].

4. Rational Design of LEDGF/p75-IN Interaction Inhibitors

Different approaches have been employed to design and identify small-molecule inhibitors of the LEDGF/p75-IN interaction. These include large-scale screening of chemical libraries [87, 95], computational three-dimensional (3D) database screening of chemical libraries and structure-based *de novo* design [37, 88]. High-throughput screening of large libraries of chemicals against a biological target is the prevailing method for the identification of new hit compounds in modern drug discovery. Alternatively, virtual screening is based on a computer-aided survey of large libraries of chemicals that complement targets of known structure and on experimentally testing of a limited set of compounds predicted to bind well. In order to obtain *bona fide* LEDGF/p75-IN interaction inhibitors, we embarked in 2007 upon structure-based drug design [37]. Drug design is based on a virtual screen of large libraries of small molecules to fit a consensus pharmacophore docked into the region of interest. The consensus pharmacophore consists of chemical groups critical for interaction with amino acid residues or peptide backbones in the proposed drug-binding pocket. In our case the pharmacophore was designed to bind to the LEDGF/p75 binding pocket located at the interface of a dimer of the CCD of HIV-1 IN. In principle, any drug discovery project requires design, prioritization, analysis, and interpretation of results of consecutive experiments to ultimately facilitate the development of new therapeutic compounds. The rational drug design work flow used during the discovery and hit-to-lead optimization process of LEDGINs was a combination of methods. The *in silico* screen for LEDGINs integrates a multidisciplinary approach where existing structural bioinformatics and chemoinformatics were employed in combination with a validated target-based PPI assay [37]. Different crystal structures of the HIV-1 CCD [96] and cocrystal structures with the IBD of LEDGF/p75 [84] or ligand [97] bound to the CCD were superpositioned to refine and construct more precisely a consensus pharmacophore model. Most important features in the final predictive pharmacophore model constructed for virtual screening were a "hydrophobic/aromatic" moiety overlapping with Ile365 of the IBD, a "hydrophobic/aromatic" feature overlapping with Leu368 of the IBD, "acceptor" features mimicking the acid functionality of Asp366, and a "hydrophobic/aromatic" feature overlapping with the Lys364 side chain of LEDGF/p75. 200,000 commercially available and structurally diverse compounds were filtered using the established 3D-pharmacophore query. After stringent sequential scoring and filtering of the initial libraries, 25 promising molecules with the best scoring were ordered for biological evaluation in a bead-based *in vitro* LEDGF/p75-HIV-1 IN protein-protein interaction assay in the AlphaScreen format. AlphaScreen is a bead-based medium throughput assay optimized to measure the interaction between LEDGF/p75 and HIV-1 IN [37, 89, 95]. Hits emerging from the screening were optimized by reiterative chemical refining and biological profiling in AlphaScreen and in a cell-based antiviral assay, MTT/MT4. Of the 25 molecules retained from the initial screening, four hit molecules inhibited the LEDGF/p75-HIV-1 IN interaction. One of the hit molecules, LEDGIN **1**, inhibited the PPI by 36% at 100 μM and served as a starting point for structure-activity relationship (SAR) investigations aimed at the identification of more potent LEDGINs (Figure 3) [37]. Deduced SARs were used to guide synthesis of analogues with enhanced activity. The resulting early lead compounds were then further optimized in an integrated lead optimization strategy while the molecular mechanism of action was investigated in cell culture. Medicinal chemistry optimization, aided by structural information provided by high-resolution cocrystals of LEDGIN **3** soaked into the HIV-1 CCD (Figure 4), generated congeners of LEDGIN **3** (including LEDGIN **6** and **7**) with improved biological activity (Figure 3).

Furthermore, LEDGINs did not interfere with the interaction between LEDGF/p75 and its cellular binding partners JPO2 or pogZ, conforming their specificity. Of note, Hou et al. [95] identified several compounds inhibiting the LEDGF/p75-IN interaction through high-throughput screening of a compound library of more than 700,000 small molecules with AlphaScreen. However, the quinolinylacetic acid derivatives are the first examples of potent and specific inhibitors of HIV-1 replication which have been extensively evaluated for their therapeutic potential and mechanism of action in cell-based antiviral assays (including in primary cells) [37].

5. LEDGINs as Therapeutics

A critical evaluation of the mechanism of action and therapeutic potential of LEDGINs requires investigation of different drug characteristics: (a) a high binding affinity and specificity to HIV-1 IN, (b) potent and broad spectrum anti-HIV activities in cell-based antiviral assays, (c) lack of toxicity, and (d) a optimal pharmacokinetic (PK) and pharmacodynamic (PD) profile allowing a once a day administration in patients. We could demonstrate that inhibition of the LEDGF/p75-HIV-1 IN interaction by LEDGINs blocks HIV

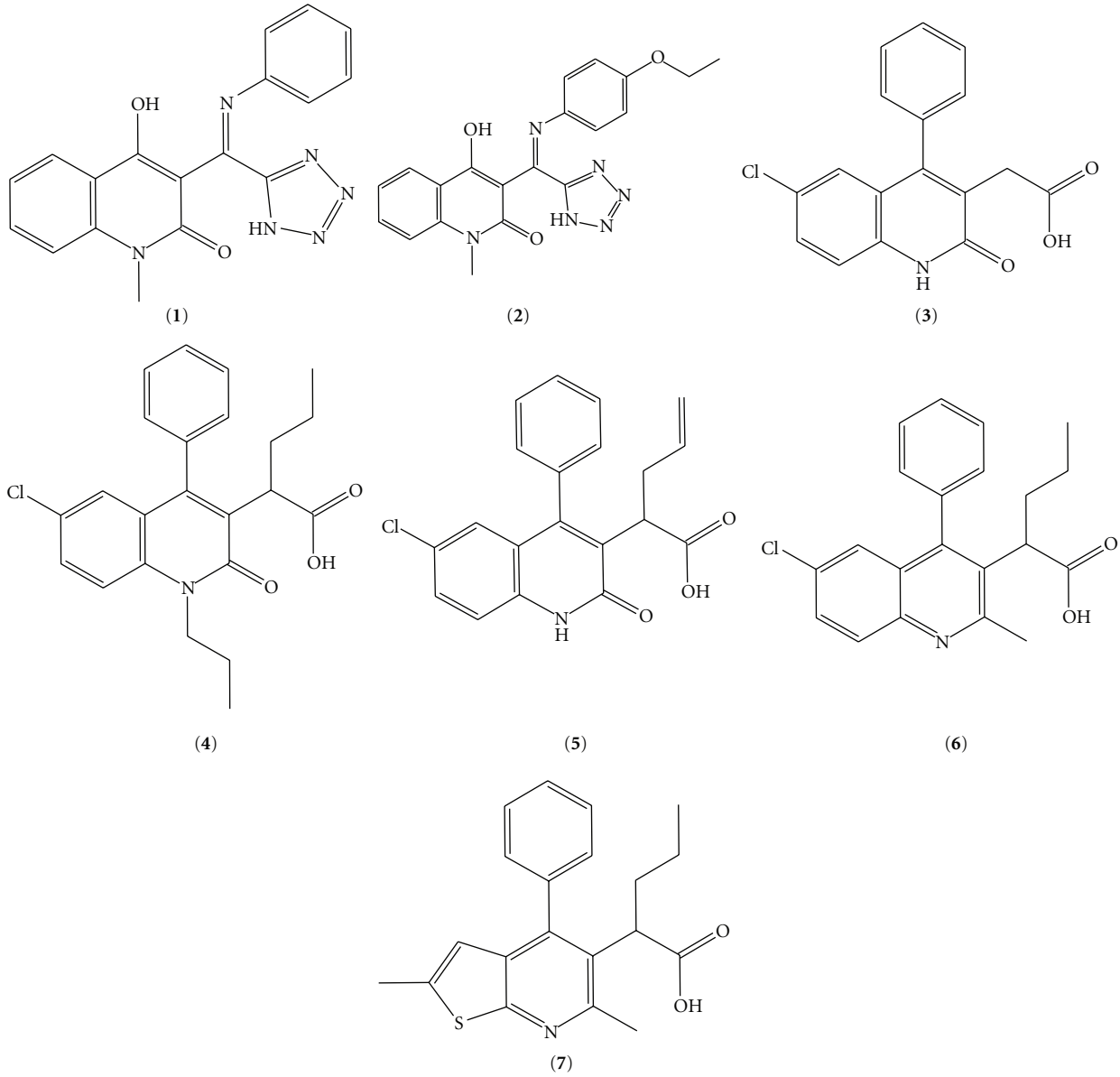

Figure 3: Chemical structures of the LEDGINs. Of the 25 molecules tested in AlphaScreen, compound **1** was identified as the initial hit with *in vitro* activity. Compounds **2** and **3** are commercial congeners of **1**. Compounds **4**–**7** are newly synthesized compounds with improved *in vitro* and *in vivo* activities. After serial rounds of optimization by medicinal chemistry, the early lead compounds **6** and **7** were identified with potent and selective anti-HIV activity. Compound **7** has submicromolar antiviral activity [37].

integration [37]. Integration inhibitors are characterized by a typical pattern of viral DNA species as measured by qPCR. 2-LTR circles are the dead-end byproduct of nonintegrated viral DNA; their number is increased upon integration block if upstream steps are not hampered [98]. We showed that both the classical integrase strand transfer inhibitor (INSTI) raltegravir and LEDGINs reduce the number of integrated proviral DNA and increase the number of 2-LTR circles without effect on reverse transcription. Resistance selection in cell culture against a new class of antiviral agents ultimately corroborates the antiviral target. By serial passaging of HIV-1 in increasing concentrations of LEDGIN **6**, we selected a resistant strain with the A128T substitution in IN. The A128 residue is a hot spot of the IN-LEDGF/p75 interface and was included in the predictive pharmacophore model for the virtual screen. The resistance mutation, thus, corroborates the specificity of LEDGINs. The A128T mutation in integrase is not associated with resistance to INSTIs and LEDGINs lack cross-resistance with other ARV classes corroborating their novel mode of action. Of note, it was recently shown that LEDGINs can also block the interaction between HRP-2 and HIV IN in the absence of LEDGF/p75 [99].

In conclusion, there are obvious advantages of drugs targeting LEDGF/p75-IN interaction. LEDGINs show a pathway of resistance development that is different from that of the INSTIs and lacks cross-resistance with ARV in the

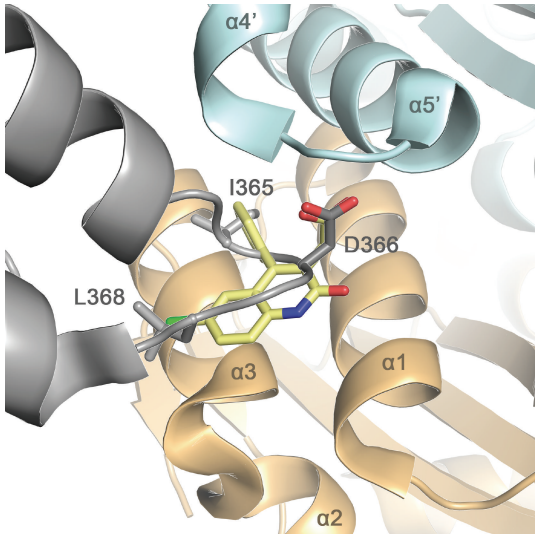

Figure 4: Cartoon representation of the LEDGIN **3** (yellow) superimposed with the LEDGF/p75 IBD (gray) in the pocket at the interface of the IN CCD dimer (light blue and orange). LEDGINs bind to the LEDGF/p75 binding pocket in HIV-1 IN and thereby block the interaction of the IBD of LEDGF/p75 with the dimer of the CCD, thereby interfering with tethering of the HIV-1 PIC to the host cell chromatin.

clinic [100]. Discovery of LEDGINs is a good example of structure-based rational drug design targeting a well-defined and biologically relevant PPI.

6. HIV Integrase Cofactors and Nuclear Import

To accomplish their life cycle, retroviruses need to integrate their genetic material into the host DNA in the nucleus. For this purpose, retroviruses developed distinct strategies to overcome the nuclear membrane barrier. Gammaretroviruses such as murine leukemia virus (MLV), for example, cannot pass nuclear pore complexes and only integrate during mitosis after breakdown of the nuclear membrane [101]. Lentiviruses such as HIV in contrast are able to infect both dividing and nondividing cells [102, 103]. Many factors, both from viral and host cell origin, have been suggested to take part in the nuclear import of the lentiviral preintegration complex (PIC) (for reviews see [104, 105]). Nuclear import is a bottleneck in lentiviral infection, and cellular cofactors of this process are attractive targets for anti-HIV therapy. Although recent studies shed light on lentiviral PIC transport to the nucleus, general consensus on the importance of particular viral and cellular players still has to be established. From the viral proteins present in the PIC IN, matrix (MA) and viral protein R (Vpr) were suggested to affect nuclear import, and several nuclear localization signals (NLSs) were identified in these proteins (for review see [105]). A *cis*-acting central polypurine tract (cPPT), a sequence present almost exclusively in the lentivirus genus and used for initiation of plus-strand synthesis, may as well affect the efficiency of nuclear import [106–108]. However, HIV with mutations in each of the NLSs still remained infectious in nondividing cells [109]. Yamashita and Emerman, using HIV chimeric viruses in which the entire IN sequence was replaced by that of MLV, and all the other NLSs in MA, Vpr, and cPPT were eliminated, demonstrated that neither of these NLSs is essential for the ability of HIV to infect nondividing cells [110]. Despite the fact that none of the above-mentioned viral elements appears absolutely required for nuclear import, a major effect of the cPPT on the kinetics of viral DNA entry into the nucleus was demonstrated [108, 111, 112]. After excluding a role for the previously reported viral NLSs in lentiviral nuclear import, two major explanations for the cell cycle independence of lentiviral nuclear entry prevail. Limited uncoating of the gammaretroviral capsid may interfere with importin-mediated transport through the nucleopore, whereas timely disassembly of the lentiviral capsid may allow interaction with importin(s). Alternatively, interaction with components of the nuclear import machinery may be restricted to proteins present in the lentiviral PIC. For a discussion on the impact of the lentiviral capsid on nuclear import, we refer to [113–115].

Several nuclear import factors and nucleoporins (Nups) have been implicated in HIV nuclear import: importin α1* [116–119], importin α3* [120, 121], importin 7* [122, 123], Nup153* [13, 55, 124–127], Nup62* [128], Nup54 [129], Nup85 [55, 125], Nup98 [13, 130–132], Nup107 [55, 125], Nup133 [55, 125], Nup155 [130], Nup160 [55, 125], Nup210 [130], Nup214 [13], and Nup358/RanBP2 [13, 55, 115, 125, 133, 134] (proteins with * were shown to interact with IN).

Importin α1/Rch1 was the first karyopherin shown to interact with HIV-1 IN [117]. The study was initiated by the observation that the growth defect of a HIV-1 MA/Vpr double deletion mutant in terminally differentiated macrophages was masked at high MOI. These data pointed to an activity that can substitute for MA and Vpr in the nuclear import of the HIV-1 PIC. Authors showed that HIV-1 IN is a karyophilic protein, detected IN-Impα1 interaction, and defined two NLSs (one around positions 186–189 (KRK^{188}) and one encompassing residues 211–219 ($KELQKQITK^{219}$)) in the C-terminal region of HIV-1 IN as responsible for the interaction [117]. The IN-Impα1 interaction was initially confirmed by *in vitro* binding studies [119, 135], but questioned later by work of Ao et al. [123]. The Impα family contains 6 isoforms grouped into 3 subfamilies with a primary sequence identity between 50 and 85% [136]. *In vitro* studies suggest that various isoforms can recognize the same NLS-containing proteins, although with different binding efficiency [120]. Therefore, Ao et al. [120] investigated the contributions of the different Impα isoforms to HIV-1 replication. Via shRNA, mediated knockdown Impα3 was shown to be required for efficient HIV infection of HeLaP4 cells, T cells, and primary macrophages. qPCR analysis revealed that Impα3-knockdown resulted in a significantly reduced level of 2-LTR circles, suggesting a role in HIV nuclear import. By immunoprecipitation, the HIV-1 IN-Impα3 interaction was attributed to the C-terminal domain (CTD aa 250–270) of IN. Impα1 and Impα5 also affected HIV infection [120]. The importance of importin α isoforms for HIV nuclear import was questioned by

work of Depienne et al. [137] who studied nuclear import in digitonin-permeabilized HeLa cells. According to these authors, nuclear accumulation of IN (as a protein) does not involve karyopherins α, β1, and β2-mediated pathways and is also independent of GTP hydrolysis and Ran [137]. Here, we raise again the question whether nuclear import of IN is relevant for the nuclear entry of the HIV PIC. Importin 7 (Imp7) has also been implicated in HIV-1 nuclear import. Originally, it was proposed as a HIV-1 nuclear import factor by Fassati et al. based on nuclear import of purified HIV-1 reverse transcription complexes in digitonin-permeabilized HeLa cells and primary human macrophages [122]. However, when Zielske and Stevenson depleted Imp7 by 80–95% in primary macrophages and HeLa cells using RNAi, neither the rate nor the extent of HIV-1 or SIV cDNA synthesis or nuclear translocation was affected [138]. In a direct comparison using coimmunoprecipitation, HIV-1 IN was found to interact with Imp7, but not with Impα1/Rch1 [123]. Finally, the Fassati group admitted that Imp7 is not essential for HIV-1 infection but maximizes nuclear import [139].

In a full-genome siRNA screen, Nup153, Nup214 and Nup358 were found to play a role in the nuclear import and Nup98 in the integration of HIV [13], although detailed validation still had to be performed. Nup153 and Nup358/RanBP2 are the most extensively studied Nups in the context of HIV infection. Nup153 has been shown to interact with HIV-1 IN, and the interaction is mediated by its C-terminal domain rich in FxFG repeats [124]. When added in excess to the semipermeabilized import assay, the C-terminal domain of Nup153 inhibited the nuclear import of HIV-1 IN [124]. Interestingly, codepletion of Nup153 and transportin-SR2 (TRN-SR2) yielded synergistic effects, that outweighed those calculated based on individual knockdowns, indicating potential interdependent roles for these factors during HIV-1 infection [127]. Nups requirement for HIV-1 infection was further studied by Lee et al. [125]. HIV-1 infection was impaired by Nup358/RanBP2, Nup153, or Nup160 knockdown. In contrast, infection by the HIV-1 CA N74D mutant (see below) was less dependent on Nup358/RanBP2 and Nup153, suggesting that these proteins interact, directly or indirectly, with CA during infection [125].

Nup62 has been shown to act at several steps during HIV-1 replication. Monette et al. first showed that HIV-1 replication markedly alters the localization of Nup62 and that its expression is linked to the nuclear export of the unspliced viral genomic RNA [140]. Later proteomics and immunogold electron microscopy studies showed that HIV-1 infection induces extensive changes in the composition of the nuclear envelope and its associated proteins and identified Nup62 as a component of purified virus [141]. Former observation is particularly important for consideration of the involvement of individual Nups and Nups-interacting partners (like importins) in HIV infection. HIV-1 can via remodeling of the nuclear pore complexes (NPCs) make accessible Nups which facilitate nuclear import and/or integration, and the process of remodeling can have impact not only on late stages of infection (production of the progeny), but also on the mentioned early steps. Encapsidated Nup62 may be required for efficient nuclear import of the PIC in newly infected cells [141].

Nup62 has recently been proposed as a binding partner of HIV-1 IN [128]. GST-tagged IN was able to pull down Nup62. The specificity of the interaction was further proven by co-IPs. Nup62 knockdown in CD4+ T cells and macrophages significantly inhibited HIV-1 infection and by qPCR analysis, the block of the infection was pinpointed to viral integration and in a much lesser extent to the nuclear import step. Subcellular protein fractionation showed that Nup62 binds to chromatin, interacts with HIV-1 IN both in the nuclear and chromatin bound extracts, and knockdown of Nup62 significantly reduced the association of the IN with chromatin causing impaired HIV-1 integration observed also by qPCR. Finally, expression of the C-terminal domain of Nup62 in CD4+ T cells reduced the association of IN with chromatin and did inhibit HIV-1 infection [128].

HIV integration is favored in chromosomal regions rich in active transcription units and associated features such as CpG islands, DNAaseI hypersensitive sites, and high G/C content [142]. Integration site sequencing offers a new view on how HIV-1 uses the host nuclear import machinery to reach its integration sites [115, 134]. Wild-type HIV-1 in the presence of cyclosporine (Cs), HIV-1 CA mutants deficient for CypA interaction (CA G89V or P90A), and chimeric HIV-1 containing SIVmac CA, all integrate in genomic areas of high gene density/activity. On the contrary, HIV-1 capsid mutants that are less sensitive to TRN-SR2, Nup358 or Nup153 depletion by RNAi (CA N74D or N57A) integrate in genomic areas of low gene density/activity. Both groups of CA mutants were impaired in replication in HeLa cells and human macrophages. In accord with the observed differences in integration pattern, a block of engagement of CypA/Nup358 by mutating the virus CA or by inhibiting cellular CypA with cyclosporine force HIV-1 to use for nuclear import and integration a Nup358/Nup153-independent pathway [115].

In 2008, transportin-SR2 (TRN-SR2, TNPO3) was independently identified as a cellular cofactor of HIV-1 replication in two siRNA screens [13, 55] and as a HIV-1 IN binding partner by Y2H screening [54]. Although its exact role in HIV-1 infection has not been fully clarified, several independent studies confirmed TRN-SR2 as a genuine cellular cofactor to the extent that it is now being used as a positive control in HIV-1 interaction studies [143].

7. Transportin-SR2 as a Cofactor of HIV Nuclear Import.

TRN-SR2 belongs to the importin-β superfamily of karyopherins [144]. The protein has 975 amino acid residues and is composed of α-helical HEAT repeats. TRN-SR2 is known to import essential splicing factors, the serine/arginine-rich proteins (SR proteins), to the nucleus and is, therefore, involved in the regulation of both constitutive and regulated precursor mRNA splicing. The recognition of the SR-proteins by TRN-SR2 relies on the conserved RS-domain

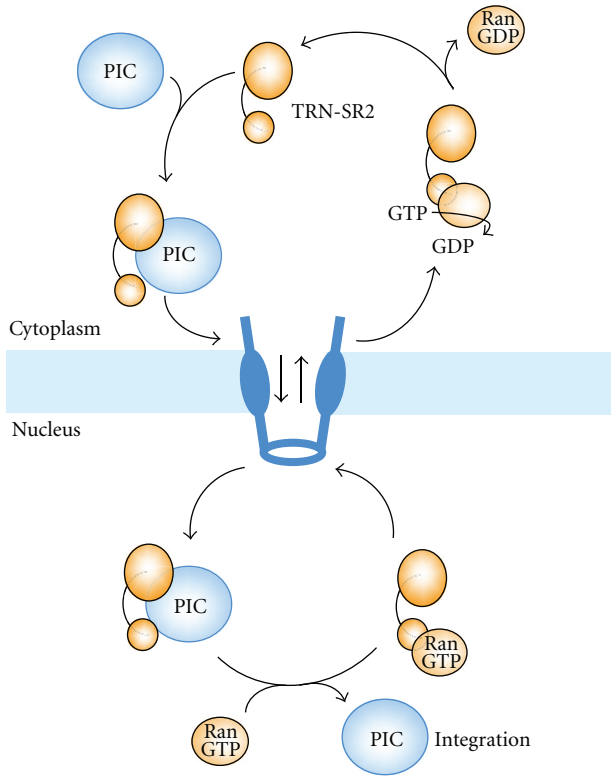

Figure 5: Scheme of nuclear import of the PIC and TRN-SR2 recycling.

and requires phosphorylation [144–146] although TRN-SR2 is known to import as well proteins not belonging to the SR protein family [147, 148]. The RS domain of SR proteins serves both as an NLS and a subnuclear localization signal [149, 150]. A TRN-SR2 mutant deficient in Ran binding colocalized with SR proteins in nuclear speckles [146]. TRN-SR2 binds its cargo in the cytoplasm and via its interaction with the nuclear pore proteins translocates with cargo to the nucleus (Figure 5). The import is linked to the RanGTP/RanGDP cycle. The small protein Ran GTPase is a member of the Ras protein superfamily and the motor of nuclear protein import. Interaction between Ran and karyopherins is modulated by the state of the bound nucleotide (GTP or GDP). In the nucleus, RanGTP binds to TRN-SR2, displaces the cargo, and then shuttles together with TRN-SR2 to the cytoplasm, where GTP is hydrolyzed to GDP. In the GDP-bound state, Ran dissociates from TRN-SR2 enabling a new round of nuclear import [151]. TRN-SR2 has been shown to interact with Nup62 or its associated complex [146]. Of note, Nup62 is translocated to the cytoplasm and encapsidated into HIV-1 virions during HIV-1 infection [140, 141].

ASF/SF2 has been proven to affect the splicing pattern of HIV RNA transcripts [152, 153]. The nuclear import of this splicing factor is mediated by TRN-SR2 and this was the first indication of a possible involvement of TRN-SR2, in HIV replication. TRN-SR2 was identified as a cellular cofactor of HIV-1 in the RNAi genome-wide screens [13, 55], but not in the Zhou screen [56]. TRN-SR2 knockdown had little or no effect on murine leukemia virus (MLV) transduction [54, 55]. Interaction of TRN-SR2 with HIV-1 IN was originally detected in a Y2H screen of a random primed CEMC7 cDNA library with HIV-YU2 IN as bait [54]. Exclusivity of the interaction with viral IN was verified in a reverse screen against a library of HIV genome DNA fragments. The specificity of the interaction of HIV-1 IN with TRN-SR2 was confirmed in pulldown assays [54]. SiRNA-mediated knockdown of TRN-SR2 resulted in a 6-fold inhibition of HIV replication in HeLaP4 cells [54]. TRN-SR2 specific shRNA reduced infectivity of both HIV-1 (~8- to 10-fold) and SIVmac (~20-fold) [54, 115]. Interestingly, IN inhibitor-resistant viruses are still susceptible to TRN-SR2 knockdown [54]. By real-time qPCR, the block in HIV replication was mapped to a moment after reverse transcription and prior to integration, which coincides with nuclear import [54]. The import assay with IN-eGFP labeled virus [65] was used to corroborate the role of TRN-SR2 in HIV nuclear import [54]. After depletion of TRN-SR2 using red fluorescent siRNA, the treated cells were infected by HIV-IN-eGFP. In cells positive for the red fluorescent label, the numbers of PICs present in the nucleus versus the cytoplasm were counted. The nuclear/cytoplasmic ratio of PICs dropped 5-fold in the TRN-SR2 depleted cells [54].

A possible role of lentiviral capsid in TRN-SR2-mediated nuclear import was suggested by the finding that both a chimeric HIV virus, carrying MLV capsid (CA), MA and p12 proteins, and a HIV-1 strain, carrying the CA N74D mutant, apparently were insensitive to TRN-SR2 knockdown [58, 125, 154, 155]. Authors concluded that the viral capsid and not IN determines TRN-SR2 dependency of HIV infection. One should be careful with interpretation of some data. Some studies [154, 156] were done with pseudotyped HIV virus carrying the vesicular stomatitis virus G envelope (VSV-G), known to induce receptor-mediated endocytosis instead of membrane fusion as a way to enter the cell. Moreover, VSV-G pseudotyped HIV does not engage chemokine coreceptors (CCR5, CXCR4) known to induce signal transduction cascades in the cell [157]. When TRN-SR2 knockdown cells were infected with viruses carrying the wild type HIV-1 envelope, the HIV-1 N74D CA mutant regained sensitivity to TRN-SR2 knockdown [58].

TRN-SR2 is not used to the same extent as a nuclear import factor by all lentiviruses [58, 154, 156] but a direct correlation between the phenotype in cell culture and the *in vitro* PPI has not yet been documented. Logue et al. showed that the *Drosophila* TRN-SR2 can substitute for its human counterpart and defined the cargo-binding domain of TRN-SR2 as required for lentivirus infection [156]. From the IN part of the interaction, IN mutations previously characterized to impair LEDGF/p75 binding (W131A, Q168L) were insufficient to affect nuclear import [158]. Zhou et al. recently proposed a model in which CA along with tRNAs is export cargoes for TRN-SR2 in a RanGTP-dependent way [159]. According to this hypothesis, TRN-SR2 modulates nuclear uncoating of imported PICs by removing any remaining CA proteins and tRNAs blocking the integration step and promotes nuclear export of these

viral components. The model suggests that efficient HIV-1 integration depends on this TRN-SR2 activity [159]. Another study hinted at a role for TRN-SR2 prior to integration. HIV integration site selection was modified by depletion of TRN-SR2 and Nup358/RanBP2 [134]. However, this observation can alternatively be explained by the fact that correct trafficking through the NPC may facilitate the subsequent integration step. Although a clear understanding of HIV nuclear import and on the role of TRN-SR2 requires more experimentation, all data are consistent with a close link between HIV uncoating in the cytoplasm and nuclear import on the one hand, and nuclear import and integration on the other hand.

8. Conclusions

This paper highlights the importance of research on cellular cofactors of HIV replication as potential targets for anti-HIV drugs. The interaction between LEDGF/p75 and IN is crucial for HIV replication, and the rational design of LEDGINs as novel antivirals represents an important achievement in translational research. Efficient targeting of host-virus PPIs expands the possible arsenal of targets beyond HIV-encoded enzymes. This novel paradigm can be extended to other viral diseases. Increased understanding of the virus-host interactome can be the basis for plenty of future antivirals. Since PPIs have pivotal roles in virtually all physiological and disease-related intracellular macromolecular complexes, development of SMIPPIs can benefit many therapeutic areas. While the example described here is particularly relevant to the field of virology, applications of SMIPPI technology to other fields will increase as our knowledge on the role of PPIs in human diseases expands.

Since the nuclear import of PICs still represents a black box in our knowledge of HIV infection and since IN plays an active role at this stage, study of the IN interactome may also shed light on this process. The discovery that the importin TRN-SR2 is a binding partner of IN can provide the lever to open this box. Research on HIV nuclear import not only provides us with insights in basic virology, but also has great potential for drug discovery especially since nuclear import is a bottleneck in HIV replication. There is increasing evidence that lentiviral chromosomal target site selection for integration is linked to nuclear import of PICs. Moreover, proper illumination of the lentiviral route to the nucleus and of the impact on integration site selection will aid the design of safer gene therapy approaches.

Conflict of Interests

The authors disclose any other conflict of interests.

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Review Article

Protease-Mediated Maturation of HIV: Inhibitors of Protease and the Maturation Process

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Protease-mediated maturation of HIV-1 virus particles is essential for virus infectivity. Maturation occurs concomitant with immature virus particle release and is mediated by the viral protease (PR), which sequentially cleaves the Gag and Gag-Pol polyproteins into mature protein domains. Maturation triggers a second assembly event that generates a condensed conical capsid core. The capsid core organizes the viral RNA genome and viral proteins to facilitate viral replication in the next round of infection. The fundamental role of proteolytic maturation in the generation of mature infectious particles has made it an attractive target for therapeutic intervention. Development of small molecules that target the PR active site has been highly successful and nine protease inhibitors (PIs) have been approved for clinical use. This paper provides an overview of their development and clinical use together with a discussion of problems associated with drug resistance. The second-half of the paper discusses a novel class of antiretroviral drug termed maturation inhibitors, which target cleavage sites in Gag not PR itself. The paper focuses on bevirimat (BVM) the first-in-class maturation inhibitor: its mechanism of action and the implications of naturally occurring polymorphisms that confer reduced susceptibility to BVM in phase II clinical trials.

1. Introduction

Human Immunodeficiency Virus Type 1 (HIV-1) is the causative agent of the worldwide Acquired Immunodeficiency Syndrome (AIDS) epidemic. Approximately 34 million people were estimated to be living with HIV at the end of 2010. The number of people infected is a consequence of continued large numbers of new HIV-1 infections together with a reduction in AIDS-related deaths due to a significant expansion in access to antiretroviral drug therapy [1]. In the absence of an effective vaccine or cure, antiviral drugs are currently the only treatment option available to HIV-infected patients. Therapeutic regimes commonly termed HAART (highly active antiretroviral therapy) suppress viral replication but do not eradicate the virus; therefore, treatment must be administered on a lifelong basis [2, 3]. HAART consists of the simultaneous use of a combination of three or four different antiretroviral drugs. This combinational approach is required due to the ease with which HIV-1 can acquire drug resistance to a single drug administered as monotherapy [3, 4]. Drug resistance arises due to the high degree of HIV-1 genetic diversity within the virus population (quasi-species) infecting an individual patient. This genetic diversity is created as a consequence of a rapid rate of viral replication combined with the error prone nature of the viral reverse transcriptase (RT), which copies the viral RNA genome into a double-stranded DNA copy and the frequent recombination events that occur during genome replication [3, 5, 6]. HAART is possible due to the successful development and clinical use of more than 20 antiretroviral drugs, which belong to six different mechanistic classes. These drugs primarily target the viral enzymes: RT inhibitors (which fall into two classes based on their mode of action: the nucleoside-analog RT inhibitors (NRTIs) and nonnucleoside-analog RT inhibitors (NNRTIs)), protease (PR) inhibitors (PIs), and

an integrase (IN) inhibitor [7–10]. Most clinical treatment regimens use a combination of either a PI or NNRTI with two NRTIs, though since its approval for clinical use in 2007 the first IN inhibitor (insentress) has increasingly been used in therapy regimens. The remaining two mechanistic drug classes each contain one approved drug and target the viral entry process by either blocking viral fusion by targeting the viral gp41 envelope protein or acting as an antagonist against the host cell coreceptor CCR5 [11]. The viral entry inhibitors are in general reserved for salvage therapy. Salvage therapy is required upon treatment failure primarily due to the emergence of drug resistance and to be effective should ideally include at least one new drug targeting a novel site of action. Until a cure for HIV infection is achieved, the continued threat of drug resistance makes the identification and development of a continuous pipeline of new drugs with a novel mechanism of action an ongoing requirement [12]. In this paper we discuss protease-mediated maturation of HIV-1 particles and the strategies to target this step in HIV-1 replication for therapeutic intervention.

2. Proteolytic Maturation and Its Role in HIV-1 Replication

Proteolytic maturation is essential for the production of infectious HIV-1 virus particles and has been extensively reviewed [16–18]. Particle assembly is driven by the Gag ($Pr55^{Gag}$) polyprotein, which is transported to the cellular plasma membrane where it undergoes higher-order Gag-Gag multimerization. A second polyprotein Gag-Pol ($Pr160^{Gag-Pol}$) is also incorporated into the assembling particle through Gag-Gag interactions. Gag-Pol is expressed via a -1 ribosomal frameshift during approximately 5–10% of Gag translation events. The Pol domain encodes the viral PR, RT, and IN proteins. Gag-Gag multimerization forces membrane curvature and assembly is completed upon budding of the particle from the plasma membrane. Initially, the newly formed particles have a noninfectious immature morphology. However, concomitant with virus budding, PR is activated to facilitate particle maturation. The exact mechanism of PR activation is not clearly understood, but it is known to require Gag-Pol dimerization. Once PR is liberated from the polyprotein through autocatalysis, it cleaves Gag and Gag-Pol into their respective proteins. Cleavage of the Pol domain results in the enzymatic proteins PR, RT, and IN. Cleavage of Gag results in four protein domains: matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), p6, and two spacer peptides SP1 (p2) and SP2 (p1) (Figure 1(a)). Gag cleavage follows a sequential cascade that is kinetically controlled by the differential rate of processing at each of the five cleavage sites in Gag. The first cleavage creates an N-terminal fragment that contains the MA-CA-SP1 domains and a C-terminal fragment that contains the NC-SP2-p6 domains. Subsequent cleavage events occur at the MA-CA and SP1-p6 sites and finally the CA-SP1 and NC-SP2 sites are cleaved.

The physical consequence of Gag cleavage is a morphological rearrangement of the non-infectious immature particle to a mature infectious particle containing a conical core, which is generated by a second assembly event upon release of the CA domain (Figure 1(b)). The conical CA core contains the RT and IN enzymes along with the dimeric viral RNA genome in complex with NC and is essential for virus replication upon infection of a new cell. Therefore, correct core formation is essential for the production of infectious particles and this has been shown to be dependent on accurate proteolytic processing of Gag as mutations that disrupt the cleavage of individual sites or alter the order in which sites are cleaved result in aberrant particles that have significantly reduced infectivity. The fundamental role of proteolytic maturation in the generation of infectious particles makes inhibiting this process an attractive target for therapeutic intervention. In this paper we discuss how this has been approached by (i) the successful development and clinical use of PIs which target the PR enzyme itself and (ii) research to develop a novel class of antiretroviral drug termed maturation inhibitors which target the Gag cleavage sites that act as the substrate for PR.

3. Protease Inhibitors

3.1. Introduction. Protease inhibitors (PIs) target and inhibit the enzymatic activity of the HIV-1 PR. PIs inhibit PR activity to the extent that is sufficient to prevent cleavage events in Gag and Gag-Pol that result in the production of non-infectious virus particles. The development of PIs in the mid 1990s was a critical step forward in the successful treatment of HIV-1 patients. This is because their development provided a second mechanistic class of antiretroviral drug, which made HAART combination therapy possible. PIs have remained a key component of HIV-1 patient treatment regimens right up to the current day. To date, nine PIs have been approved for clinical use, they are saquinavir, ritonavir, indinavir, nelfinavir, fosamprenavir, lopinavir, atazanavir, tipranavir, and darunavir [8] (Table 1).

3.2. Protease Inhibitor Design. Design of PIs has been primarily driven by structural knowledge of PR (Figure 2), its substrate, and the chemical reaction of peptide bond cleavage [16]. Like other retroviruses, HIV-1 PR, is related to the cellular aspartyl PR family, which include pepsin and renin. This family of proteases are typified by an active site that uses two apposed catalytic aspartic acid (Asp) residues, each within a conserved Asp-Thr-Gly motif. To function, the cellular PRs form a pseudodimer utilizing two Asp residues from within the same molecule to create an active site. In contrast, retroviral PRs only contain one Asp-Thr-Gly motif and must therefore form a true dimer. Indeed X-ray crystallography has shown that the HIV-1 PR exists as a dimer consisting of two identical monomers [19–21]. The crystal structure of the dimer reveals that four-stranded β sheets derived from both ends of each monomer hold the dimer together. A long substrate-binding cleft is created between the monomers and the active site is situated near its centre with the two Asp residues located at its base. Two β-hairpin flaps originating from each monomer cover the

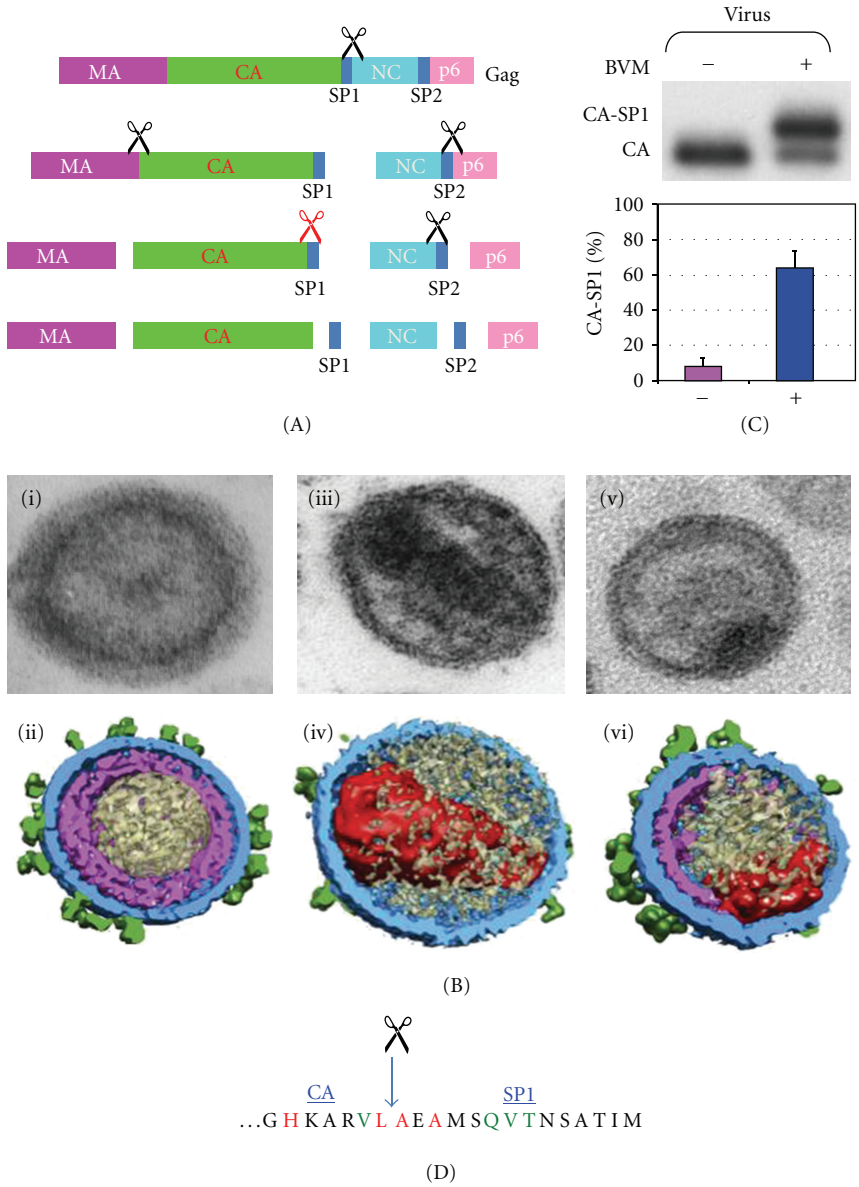

Figure 1: Proteolytic maturation of HIV-1 and its inhibition by bevirimat (BVM). (A) Gag processing cascade, illustrating the order in which the Gag precursor is cleaved by the viral protease. Each cleavage site is indicated by a scissor symbol, the red scissor symbol depicts the cleavage event blocked by BVM. (B) Virion morphology visualized by transmission electron microscopy (i, iii, v) and cryoelectron tomography models generated by segmented surface rendering. The glycoprotein spikes are coloured green, the membrane and MA layer in blue, Gag related shells in magenta, core structures in red, and other internal density in beige (ii, iv, vi). Immature particles (i and ii), mature (iii and iv), and BVM-treated (v and vi). (C) Biochemical data demonstrating accumulation of the uncleaved CA-SP1 precursor in virus particles in the presence of 1 μg/mL BVM. (D) Amino acid sequence at the CA-SP1 junction region; amino acids highlighted in green indicate the highly polymorphic residues to which reduced susceptibility to BVM in clinical trials has been mapped and amino acids highlighted in red indicate those that at which BVM resistance arises *in vitro*. Adapted with permission from Elsevier and the American Society for Microbiology [12, 13].

active site and are thought to function by stabilizing the substrate within the binding cleft.

The substrate-binding cleft interacts with multiple different substrate cleavage site sequences in Gag and Gag-Pol. The sequence of these sites are at least seven amino acids long and termed P4-P3′, with P1 and P1′ directly flanking the cleavage site [16]. There is no clear consensus amino acid recognition sequence; however, general patterns have been recognised and most substrate sites have a branched amino acid at the P2 site, a hydrophobic residue at P1, and an aromatic or proline at P1′. Instead of amino acid sequence, the topology of the cleavage site is primarily important for their recognition and interaction with PR [22]. Each of the substrate recognition sites has a super-imposable structure, known as the substrate

Table 1: FDA approved protease inhibitors. Key protease resistance mutations sourced from the 2011 data review of HIV drug resistance by the international AIDS society USA [15].

Protease inhibitor	Year of FDA approval	Key resistance mutations
Saquinavir	1995	G48V, L90M
Ritonavir	1996	Used for boosting
Indinavir	1996	M46I/L, V82A/F/T, I84V
Nelfinavir	1997	D30N, L90M
Fosamprenavir	1999	I50V, I84V
Lopinavir	2000	V32I, I47V/A, L76V, V82A/F/T/S
Atazanavir	2003	I50L, I84V, N88S
Tipranavir	2005	I47V, Q58E, T74P, V82L/T, N83, I84V
Darunavir	2006	I47V, I50V, I54M/L, V76V, I84V

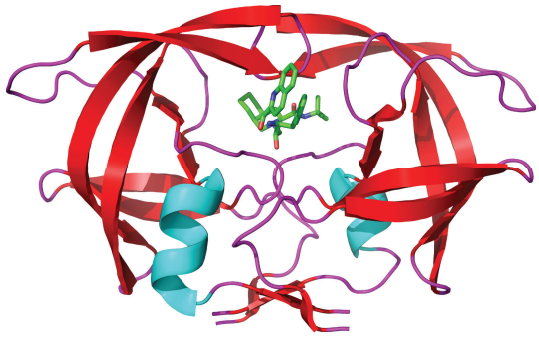

Figure 2: Three-dimensional structure of the HIV protease dimer in complex with the protease inhibitor saquinavir bound at the active site. Adapted by Jerry Alexandrators with permission from Annual Reviews [14].

envelope, which fits within the PR substrate-binding cleft. The divergent amino acid sequences of substrate recognition sites do however result in subtle structural differences, which are caused by different side chain protrusions from the substrate envelope. These side chains extend into pockets or subsites in the substrate-binding cleft. Each subsite is named for the corresponding substrate side chain, for example, the S1 subsite corresponds to the P1 side chain. These differences are thought to alter the rate at which cleavage occurs at individual sites in Gag facilitating the regulated proteolytic processing cascade of Gag that is essential for correct particle formation.

The catalytic mechanism of substrate cleavage requires the Asp residues to coordinate a water molecule that is used to hydrolyze the target peptide (scissile) bond [23]. During the reaction, a transition state intermediate is formed which has been mimicked in the design of most PIs, which are peptidomimetic transition-state analogues. The principle of this design strategy is that the normal peptide linkage [–NH–CO–] is replaced by a hydroxyethylene group [–CH_2–CH(OH)–], which cannot be cleaved by simple hydrolysis. Saquinavir was the first PI to be approved for clinical use and its design is based on this principle. The following PIs ritonavir, indinavir, nelfinavir, amprenavir, lopinaivr, atazanavir, fosamprenavir, and darunavir also all contain a central core motif of a hydroxyethylene scaffold. The exception is tipranavir, which has a coumarin scaffold and is therefore the only clinically approved PI, which is not a peptidomimetic [8]. Knowledge of the catalytic mechanism and a strategy to generate a transition state analogue was coupled with the ability to cocrystallize candidate inhibitors in complex with PR. This facilitated structure-based drug design that enabled consecutive rounds of lead optimization to develop inhibitors, which competitively bind the active site with affinities to purified PR in the low nanomolar to low picomolar range. The rational design strategy was also used to develop inhibitors that aim to combat problems encountered in the clinic, including poor bioavailability, aberrant side effects, and drug resistance.

3.3. Clinical Application and Resistance. Clinical use of PIs began in 1995 with the FDA approval of saquinavir [8]. Saquinavir's approval was closely followed by ritonavir and indinavir in 1996 and nelfinavir in 1997 [8]. *In vitro* studies demonstrated that all of these "first generation" PIs inhibit HIV-1 replication in the nanomolar range in a selection of cell types relevant to HIV-1 infection [24–27]. Initial clinical trials with these drugs were conducted as monotherapy and encouragingly demonstrated declines in HIV-1 RNA levels although the antiviral effect was not sustained for long periods of time due to the rapid acquisition of drug resistance [28–33]. Improved and more sustained reductions in viral RNA levels along with increased CD4 cell counts were obtained when a PI was included in triple therapy combinations with two NRTIs [34–40]. Importantly, these triple-drug regimens (HAART) significantly reduced disease progression and mortality in HIV/AIDS patients [8, 38]. Therefore, the development of PIs facilitated a pivotal step forward in the clinical management of HIV/AIDS and dramatically improved the clinical outcome of the disease.

Despite the successes, antiviral suppression was not always durable and these early clinical trials highlighted a number of key problems associated with PIs. As indicated above, drug resistance was problematic from the outset and the complex mechanisms of resistance will be discussed in more detail below. Acquisition of drug resistance was compounded by problems with adverse side effects (abnormal lipid and glucose metabolism) and low bioavailability (typical of peptide-like molecules), which led to suboptimal

drug concentrations, high pill burdens, and difficulties with patient adherence to treatment regimens [8]. A notable observation to help overcome the pharmacological problems was that ritonavir acts as a potent inhibitor of the cytochrome P450 3A4 metabolic pathway [41]. As a consequence it has been demonstrated that coadministering a low non-therapeutic dose of ritonavir with other PIs leads to dramatically improved bioavailability, half-life, and potency of these PIs [41]. Ritonavir boosting has become a standard procedure when using most PIs in the clinic.

The next generation of PIs aimed to improve upon the problems highlighted above. The first was amprenavir, which was approved for clinical use in 1999, next came lopinavir which was approved in 2000, followed by atazanavir in 2003, tipranavir in 2005, and lastly darunavir in 2006 [8]. In 2003 amprenavir was subsequently reformulated as the prodrug fosamprenavir, which improved drug plasma concentrations and afforded a lower pill burden [42]. Reduction in pill burden was also achieved by the coformulation of lopinavir with a low dose of ritonavir and further progress in the simplification of drug regimens came with atazanavir, which was the first PI with a once daily dosing regimen. Drug potency has also been improved, in vitro studies have shown atazanavir and darunavir to be particularly potent with IC50 values of between 1 and 5 nM however, tipranavir is the least potent because of its novel nonpeptidomimetic chemical structure [43–47]. Clinical trials demonstrated that the next generation PIs performed well with superior virological efficacy when tested against a placebo or another comparator PI in a background of two NRTIs [8, 48–52]. Finally many of these PIs acquire different drug resistance mutation profiles from the earlier PIs and/or have a higher genetic barrier to resistance.

Resistance has been encountered for all nine PIs and has been extensively reviewed [8, 53–55]. A current summary of the key mutations acquired by each of them is provided in a data review of HIV-1 drug resistance by the International AIDS Society-USA [15]. The genetic barrier for acquisition of PI resistance is relatively high, that is, it requires two or more amino acid changes to confer significant resistance. This is because PI drug resistance is a stepwise pathway that results in complex interdependent combinations of multiple mutations. All of these interdependent changes are required to act in synergy to confer drug resistance whilst simultaneously maintaining the fitness of the virus.

The mutations that arise first are referred to as primary or major mutations and they are usually located in the PR substrate-binding cleft or its immediate vicinity. Examples of primary mutations include D30N, G48V, I50L/V, V82A/F/L/S/T, I84V, and L90M [15]. These primary mutations are principally responsible for acquisition of drug resistance by causing conformational changes in and around the active site that prevent inhibitor binding [53]. More specifically, PIs bound to the substrate-binding cleft occupy a similar space as the substrate envelope, but atoms of the PI protrude from this space and interact with residues in PR. Therefore, it has been proposed that drug resistance mutations arise at PR residues involved in these points of contact to inhibit PI binding [56]. In addition to the direct mechanism of resistance described above resistance-conferring mutations may also result in conformational changes to PR beyond the active site and nonactive site mutations can also contribute to drug resistance [53]. Recently rare amino acid insertions, particularly between residues 32 and 42 have been observed to occur more frequently and in correlation with the introduction of atazanavir, lopinavir, amprenavir, and tipranavir into the clinic. The insertions have been proposed to be associated with PI resistance by imposing minor structural changes to the PR flap and substrate-binding cleft, although they always appear in combination with other well-described PI resistance mutations [57].

Primary mutations are accompanied by secondary or minor mutations, which can be preexisting polymorphisms or acquired after primary mutations. The function of many of these secondary mutations is often not to confer drug resistance *per se* but instead to compensate for the effect of primary mutations, which reduce protease catalytic efficiency and virus replication capacity or fitness [58–62]. Despite their function being less drug specific in action, they are however critical for development of high-level resistance. The secondary mutations are generally located at residues distal from the active site and occur at more than 20 residues of PR [15]. Unlike the primary mutations, which generally occur at highly conserved residues, the secondary mutations, are often polymorphic in PI treatment-naïve patient isolates, a well-documented example is the L63P substitution [58], and thus favour the selection of primary mutations in the presence of drug. Despite the presence of multiple secondary mutations almost all clinical strains of HIV-1 with high-level PI drug resistance display some degree of fitness loss [58, 61, 63, 64].

Resistance to PIs is a compromise between resistance and PR enzyme function. The mutations in PR described above primarily have an impact on inhibitor binding while still allowing the enzyme to recognise and cleave its Gag and Gag-Pol substrates to some degree. In addition to the changes in PR itself, amino acid changes in the Gag substrate have also been described [54]. These mutations are primarily located at or near to Gag cleavage sites and more specifically the sites in the NC-SP2-p6 region of Gag. Key mutations observed at the NC-SP2 cleavage site are A431V and I437V, which are commonly found in association with the PR primary mutation V82A and key mutations observed at the SP2-p6 cleavage site are L449F and P453L, which are commonly found in association with the PR primary mutations I50V and I84V [65–72]. *In vitro* selection experiments have also shown that mutations at the NC-SP2 cleavage site (A431V, K436E, and/or I437V/T) can also be selected in the presence of PIs without any accompanying resistance mutations in PR [73]. Mutations in Gag located at positions distal to cleavage sites have also been documented [74–76]. The impact of mutations in Gag has been attributed to (i) acting as compensatory mutations that improve fitness defects imposed by PI resistance-conferring mutations in PR and (ii) directly contributing to PI resistance [65–67, 73, 77]. The mechanism by which Gag cleavage-site mutations compensate for a loss in viral fitness is by improving the

interaction between the substrate and the mutant enzyme and hence increasing the ability of the mutant PR to cleave [78]. Noncleavage site mutations are thought to improve fitness by causing more broad conformational changes in Gag making cleavage sites more accessible to PR [74–76]. The mechanism by which Gag cleavage-site mutations directly contribute to PI resistance is however not clearly understood [54].

Despite the complex interdependent combinations of multiple mutations in both PR and its Gag substrate that are required to attain high-level PI drug resistance, many of the PIs have a distinctive primary mutation that can be considered a signatory of drug resistance to that particular PI. For example the D30N mutation is a signatory of nelfinavir resistance, I50L is a signatory of atazanavir resistance, the I50V mutation is a signatory of amprenavir and darunavir resistance, and the G48V mutation is a signatory of saquinavir resistance [15]. Unfortunately, however, many mutations confer drug resistance to multiple PIs leading to broad cross-resistance amongst most PIs [15]. For example, the I84V mutation is the most important as it affects all eight PIs in clinical use and acts as a key mutation for five of them (atazanavir, darunavir, fosamprenavir, indinavir, and tipranavir). Mutations at residue 82 affect all of the PIs except darunavir. The I54V substitution acts as a key mutation for darunavir but it also affects all the other PIs with the exception of nelfinavir and the L90M mutation affects PIs with the exception of darunavir and tipranavir. Cross-resistance is likely due to the fact that although chemically different, most of the PIs were designed using the same basic principle and have similar structures and interactions with the PR substrate-binding cleft. Extensive cross-resistance has serious clinical consequences that threatens the usefulness of PIs and drives an ongoing need for new PIs with improved resistance profiles.

3.4. Conclusion. The introduction of PIs into the clinic more than 15 years ago heralded the era of HAART and resulted in a significant reduction in morbidity and mortality among HIV-infected patients. Due to their clinical potency, PIs are still commonly used in treatment regimens, although only three (lopinavir, atazanavir and darunavir) of the nine approved PIs are in widespread use. Despite the clinical benefits, the usefulness of first generation PIs was particularly hampered by toxic side effects and low bioavailability, which resulted in high pill burdens and low patient adherence. A significant advance in resolving these issues was the introduction of low-dose ritonavir boosting, which increases plasma PI levels by inhibiting the cytochrome P450 metabolic pathway. Ritonavir-boosting is itself; however, associated with toxicity; therefore, alternative boosting compounds with improved properties are being developed.

PI drug resistance is a major cause of therapy failure despite the relatively high genetic barrier to resistance. Unfortunately, PR has proven to be a highly flexible and adaptable drug target due to diverse mutational profiles and the complex interplay between PR and its Gag substrate. Extensive cross-resistance to PIs has also been a key problem that has limited the overall usefulness of the drug class despite the development of new inhibitors such as darunavir with favourable resistance profiles. Therefore, there is a need to develop further novel inhibitors with improved resistance profiles to address these ongoing issues [79]. One strategy to develop such new PIs is to build on the design of existing inhibitors that target the PR active site by introducing novel modifications to established PI chemical entities. One such example is the novel inhibitor GS-8374, which is a modification of a darunavir-like analogue [80]. GS-8374 has been shown to be highly potent with a resistance profile superior to all clinically approved PIs including the parent molecule darunavir [80]. A second strategy is to identify molecules with novel chemical scaffolds, for example PPL-100 is a nonpeptidomimetic inhibitor that incorporates a new lysine-based scaffold and binds the flap region of PR via a novel mechanism [81]. PPL-100 has been shown to have a favourable resistance profile against known PI resistant HIV-1 isolates and its *in vitro* selection pattern results in two previously undocumented mutations T80I and P81S together with two previously reported compensatory mutations K45R and M46I [81, 82]. Allosteric inhibitors that bind a site other than the PR active site via a noncompetitive mechanism of action have also been identified and shown to be effective against both wildtype and PI resistant purified PR [83]. A further novel strategy, discussed below, is to design inhibitors that prevent proteolytic maturation by targeting the Gag substrate rather than the PR enzyme itself.

4. Maturation Inhibitors

4.1. Introduction. PIs directly target the PR enzyme; however, an alternative approach to inhibiting HIV-1 proteolytic maturation is to identify small molecules that bind its Gag substrate and specifically block individual cleavage events. Such a strategy would be successful because accurate proteolytic processing of Gag is essential for the production of infectious particles as mutations that disrupt the cleavage of individual sites or alter the order in which sites are cleaved result in aberrant particles that have significantly reduced infectivity. Molecules with this mechanism of action have been termed maturation inhibitors and the first-in-class is 3-O-(3′,3′-dimethylsuccinyl)betulinic acid (DSB), also known as PA-457, MPC-4326, or bevirimat (BVM).

4.2. Mechanism of Action. BVM specifically inhibits CA-SP1 cleavage, which occurs late in the Gag proteolytic cleavage cascade [84, 85]. This has been demonstrated by a number of key observations: (i) biochemical studies have demonstrated an accumulation of the uncleaved CA-SP1 intermediate in both cell and virus-associated protein fractions from HIV-1 expressing cells treated with BVM [84–86] (Figure 1(c)); (ii) viruses such as HIV-2 and SIV which have a divergent sequence at the CA-SP1 junction are not sensitive to BVM [87]; (iii) the majority of BVM drug-resistance conferring mutations map to the CA-SP1 junction or within SP1 [84–86, 88–95]. A second molecule PF-46396

has been identified that also inhibits CA-SP1 cleavage [96]. Interestingly, although PF-46396 has a similar mechanism of action as BVM, it belongs to a distinct chemical class as it is a pyridone-based compound not a betulinic acid derivative like BVM [96].

The consequence of BVM blocking SP1 cleavage from the C-terminus of CA is the formation of noninfectious particles with an aberrant morphology [84] (Figure 1(b)). Three-dimensional (3D) imaging of BVM-treated particles by cryoelectron tomography showed that they contain an incomplete protein shell, which has a hexagonal honeycomb lattice in the CA layer that is similar in structure to the Gag lattice of immature virus particles [13]. This partial shell is consistent with the aberrant electron dense crescent inside the viral membrane observed in BVM-treated particles by conventional thin-sectioning electron microscopy [84]. Both imaging techniques also showed most BVM-treated particles to contain an acentric mass, which represents an abnormal core-like structure [13, 84]. The general morphological features of BVM-treated particles are shared by particles generated by the CA5 mutant, which has two amino acid substitutions that completely block CA-SP1 cleavage [84, 97]. However, these particles have a thinner CA layer with no visible evidence of honeycomb lattice organization [13]. The presence of structural organization in the BVM-treated but not the CA5 CA layer suggests that BVM binding stabilizes the immature lattice as well as blocking CA-SP1 cleavage and that both modes of action may potentially contribute to the generation of non-infectious particles [13].

The assembly state of Gag is a determinant of BVMs activity. BVM does not inhibit CA-SP1 processing in the context of monomeric Gag in solution [84], but instead requires Gag assembly for its activity [84, 98, 99]. Therefore, it can be hypothesized that BVM binds to a pocket formed during Gag-Gag multimerization. Conversely, Gag processing disrupts the putative binding site because BVM has been shown to bind immature but not mature HIV-1 particles [99]. The BVM binding site has been mapped to the CA-SP1 junction within immature virus particles using photoaffinity BVM analogues and mass spectroscopy [100]. This provides the first direct evidence that the BVM binding site spans the CA-SP1 junction and is consistent with previous biochemical and genetic data that have implicated this region of Gag in BVM binding. Indeed, BVM binding is disrupted in a selection of BVM-drug resistant mutations with amino acid substitutions that map to the CA-SP1 junction [100, 101]. Positioning of BVM across the CA-SP1 junction supports a mechanism of action whereby binding blocks access of the viral PR to the CA-SP1 cleavage site. A second related hypothesis is that BVM binding alters the conformation, exposure, or flexibility of this region such that PR cleaves it less efficiently. The binding study [100] also identified a second BVM binding site in the major homology region (MHR) of CA, a region of Gag known to function in virus assembly [17]. The significance of a potential second BVM binding site has yet to be established but may provide an explanation for the observation that at high concentrations BVM inhibits virus particle assembly [102].

The structure of the BVM binding site remains unknown because this region of Gag has been disordered in X-ray crystallographic studies [103, 104]. The disorder has been attributed to a structural flexibility, which permits higher-order Gag-Gag multimerization during virus particle assembly [105–110]. It is, however, generally accepted that the CA-SP1 region of Gag adopts a α-helical conformation. The evidence for a helical structure is based on (i) secondary structure computer modelling predictions [111], (ii) genetic data demonstrating that mutation of key residues predicted to be helix breakers results in a disruption of virus particle assembly [106, 111] and (iii) biophysical and NMR techniques that have shown the CA-SP1 region to have a propensity to adopt a helical conformation under certain environmental conditions [110, 112, 113]. Although the interactions formed by the proposed CA-SP1 junction helices in the Gag lattice are not known, a cryoelectron tomography study of immature particles led to the hypothesis that the CA-SP1 region exists as a six-helix bundle that lies directly below the hexagonal honeycomb CA lattice [114]. Because BVM activity is known to require higher-order Gag-Gag multimerization, it has been suggested that the BVM binding pocket might involve more than one helix and hence bound BVM may occupy a cleft formed between helices [100]. The considerable technical challenges of obtaining high-resolution structural information of the CA-SP1 junction in the context of higher-order multimerized Gag make the prospect of rational drug design using inhibitor cocomplexes not currently possible. However, further understanding of the interactions involved is important for the development of second-generation maturation inhibitors. Such new molecules are now required as clinical development of BVM was suspended in 2010 due to problems with intrinsic BVM drug resistance in HIV-1 infected patients during phase II clinical trials.

4.3. Clinical Development and Resistance. BVM was considered an attractive candidate for clinical development because of its potent in vitro activity with a mean IC50 value of 10 nM and its novel mechanism of action, which makes it equally effective against viruses that have acquired resistance to key antiretroviral drugs in clinical use [84]. Additional attributes including promising pharmacological and safety studies in animal models and phase I clinical trials [115] led to the testing of BVM in HIV-1 infected patients. Initial success in these phase II clinical trials demonstrated significant BVM dose-dependent viral load reductions [115]. However, further studies quickly showed that approximately 50% of BVM-treated patients did not effectively respond to the drug and exhibited viral load reductions of less than 0.5 log [93]. Failure to respond was not due to suboptimal BVM plasma concentrations but has been attributed to virological parameters instead.

Examination of patient-derived virus revealed amino acid assignment at SP1 residues 6, 7, and 8 (Gag positions 369, 370, and 371) is associated with response to BVM [92, 93, 95] (Figure 1(d)). This trio of residues map to the C-terminal half of SP1, which is relatively nonconserved

but commonly encodes a QVT (glutamine-valine-threonine) motif in clade B HIV-1 isolates [95]. Patients most likely to respond to BVM are infected with virus encoding the QVT motif, while patients infected with virus encoding polymorphisms at SP1 residues 6–8 are less likely to respond [93]. Studies to investigate the contribution of individual substitutions at SP1 residues 6–8 have shown that mutations at SP1 residue 7 and 8 (e.g., SP1-V7A, -V7M, -T8Δ, -T8N) all confer varying degrees of reduced susceptibility to BVM [88, 92, 94, 95]. Most notably, a critical role for BVM resistance has been attributed to the SP1-V7A polymorphism as it confers full resistance to BVM [88, 92, 94, 95]. BVM susceptibility was not however reduced by mutations at SP1 residue 6 (e.g., SP1-Q6H, Q6A) or the SP1-T8A polymorphism [88, 92, 94, 95]. Therefore, any contribution of these substitutions to reduced BVM susceptibility maybe dependent on the synergistic effects of a combinations of different polymorphisms, i.e. the context of the wider Gag background. Indeed, one study identified five patient-derived virus samples with significantly reduced BVM susceptibility *in vitro* but still encoded the QVT motif [92]. In two of these isolates, BVM resistance has been demonstrated to be conferred by a polymorphism in CA (CA-V230I) situated at the P2 position of the CA-SP1 cleavage site [92] (Figure 1(d)). In the other three isolates, the determinants of reduced BVM susceptibility were not resolved [92], indicating that in some instances the factors conferring BVM susceptibility are likely to be more complex than the parameters that have been established to date.

The CA-V230I and SP1-V7A substitutions have also been acquired in in vitro BVM drug-resistance selection experiments [88, 90, 91]. In vitro studies have also identified a panel of other BVM-resistance mutations (CA-H226Y, CA-L231M, CA-L231F, SP1-A1V, SP1-A3V, and SP1-A3T) [84–86, 90] (Figure 1(d)). Unlike, the clinically important innate polymorphisms discussed above, these *in vitro* selected BVM-resistance mutations map to residues in the vicinity of the CA-SP1 cleavage site that are highly conserved throughout HIV-1 isolates [86]. As a likely consequence, these mutations have not been observed in most patient-derived virus samples either with [93, 95] or without BVM treatment [92, 95]. However, it should be noted that the most frequently acquired mutation SP1-A1V has been shown not to impose a significant defect on virus replication *in vitro* [86, 89, 90] and replicates efficiently in SCID-hu Thy/Liv mice [116]. Therefore, it remains a hypothetical possibility that the SP1-A1V mutation could be acquired over time in patients that initially respond well to BVM treatment.

Initial failure to select the key BVM-resistance conferring polymorphisms *in vitro* has been attributed to the experimental conditions utilized [91]; however, later experiments did result in selection of some of the key polymorphic mutations albeit at low frequency [88, 90]. Nevertheless, a recent study used a more sophisticated *in vitro* method of serial passage of quasi-species containing recombinant HIV-1 and deep sequencing that more accurately mimicked *in vitro* the selection of BVM-resistance observed *in vivo* [91]. In hindsight use of this *in vitro* selection method or more extensive testing of the spectrum of activity across a diverse panel of clinical isolates may have more accurately predicted the clinical response to BVM and either led to discontinuation of BVM development at an earlier stage thereby avoiding costly clinical studies or alternatively steered BVM's clinical development to include a genotyping test to screen for preexisting key polymorphisms to enable prior identification of patients most likely to effectively respond to BVM treatment [91].

The clinically important polymorphisms preexist in the HIV-1 population without prior BVM treatment. This intrinsic resistance has caused problems for BVM's clinical development, which was consequently discontinued in 2010. Genotypic analysis has demonstrated a high prevalence of polymorphisms at the QVT motif and their frequency is dependent on the genetic clade of HIV-1 [94, 95, 117]. In clade B viruses, which are predominant in the US and Europe, polymorphism frequency at the QVT motif has been reported to occur at a rate of ~30–60% [91, 95, 117]. This genotypic analysis matches BVM susceptibility rates in the *in vitro* phenotypic and clinical trial studies discussed above [92, 93, 95]. In nonclade B viruses, QVT polymorphism rates are much higher with rates of >90% [95, 117]. Typically polymorphisms occur most frequently at SP1 residue 7, followed by residue 8, and then residue 6 [91, 94, 95]. The critical SP1-V7A polymorphism has been shown to be largely predominant and occurs at a frequency of ~16% in clade B viruses and ~65–70% in clade C viruses, which are mostly found in Southern Africa [94, 95]. The high frequency of the SP1-V7A polymorphism combined with its known capacity to confer full resistance to BVM therefore poses the biggest threat to the potential effectiveness and clinical development of BVM.

The prevalence of the key polymorphisms in relation to HAART and the presence of PI resistance mutations has been investigated due to the complex interplay between PR and its Gag substrate. Being a new class of antiretroviral drug BVM was most likely in the first instance to be used as salvage therapy for patients harbouring multidrug resistant HIV-1 isolates. Studies have shown no association between the prevalence of key QVT polymorphisms and HAART treatment experience but in the absence of BVM [91, 92, 117]. One study also reported no association between prevalence of QVT polymorphisms and PI resistance-conferring mutations [92]; however, two other studies with bigger sample sizes demonstrated a higher frequency of BVM resistance mutations in PI resistant patient isolates [117, 118]. The effect of PI resistance on acquisition of BVM resistance *in vitro* has also been investigated [89, 90]. These two studies made different conclusions about the impact of the PI mutations on the temporal acquisition of BVM-resistance conferring mutations, with one study reporting a delay in the emergence of BVM-resistance [89]. The reported differences may be dependent on the type of PI mutations or the study systems used. Interestingly, the other study [90] demonstrated that the PR background influenced the type and diversity of BVM resistance conferring mutations. Viruses with a wildtype PR predominantly acquired the SP1-A1V mutation, whereas viruses with a PI resistance PR acquired a significantly higher prevalence of mutations

at the QVT motif (SP1-V7A, V7N, and SP1-T8N), at the polymorphic CA-230 residue (CA-V230I) and also a previously unreported mutation SP1-S5N. The PR genetic background was also found to effect BVM susceptibility and virus replication capacity [90]. While these studies have not fully resolved the complex interplay between PR, the Gag substrate and susceptibility to BVM they clearly demonstrate that this parameter should be considered in future development of maturation inhibitors.

4.4. Conclusion. Maturation inhibitors are a novel mechanistic class of antiretroviral drug that target PR cleavage sites in Gag. BVM is the first-in-class maturation inhibitor, which specifically inhibits cleavage of SP1 from the C-terminus of CA. A number of other small molecules that target Gag have also been identified. PF-46396 is a second maturation inhibitor, which also inhibits CA-SP1 cleavage but is chemically distinct from BVM. There are also a small number of molecules that target CA and inhibit assembly of the immature particle and/or the CA core [12]. BVM is however the only molecule that targets Gag, which has been tested in clinical trials. BVM was considered a good candidate for clinical development because of its *in vitro* potency, novel mechanism of action, and good safety profile in animal models and phase I clinical trials. Although initial results of BVM efficacy in HIV-1 infected patients were encouraging, it was quickly established that approximately 50% of patients do not effectively respond to the drug. Failure to respond is due to virological parameters, more specifically, intrinsic polymorphisms primarily located at SP1 residues 6, 7, and 8. These polymorphisms have a high prevalence, particularly in non-clade B HIV-1 isolates. The existence of BVM-resistance conferring polymorphisms in BVM-treatment naïve patients severely limits the clinical usefulness of BVM and consequently clinical development of BVM was suspended in 2010.

Halted clinical development of BVM necessitates the need for a second-generation maturation inhibitor to overcome the problem of intrinsic drug resistance encountered by BVM. BVM targets an as yet undefined drug-binding pocket, which is hypothesized to be created upon higher-order multimerization of Gag during virus particle assembly. The significant technical challenge of obtaining high-resolution structural information of this hypothetical drug target makes rational structure-based drug design unfeasible at the current time. However, the need to develop improved maturation inhibitors has highlighted a need to further our understanding of the CA-SP1 region of Gag and its role in HIV-1 particle assembly. BVM, PF-46396, and their analogues can be utilized as tools to further explore drug-binding requirements to inform future strategies to improve drug resistance profiles. Development of BVM has provided evidence that small molecules to inhibit HIV-1 replication can target Gag cleavage sites. Four other cleavage sites are present in Gag and a genetic study predicted that a small molecule that blocks MA-CA cleavage maybe a particularly potent inhibitor of HIV-1 replication [119]. However, the intrinsic flexibility in Gag cleavage sites and wide variation in substrate sequence recognition by HIV PR may represent insurmountable problems for the future development of maturation inhibitors.

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Review Article

The Role of TNPO3 in HIV-1 Replication

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TNPO3, transportin-SR2 or Tnp3, a member of the karyopherin β superfamily of proteins, is important for the ability of human immunodeficiency virus (HIV-1) to achieve productive infection, as TNPO3 depletion in human cells leads to a dramatic reduction of infection. Here we describe and discuss recent findings suggesting that TNPO3 assists HIV-1 replication in the nucleus and in fact that TNPO3 may assist PIC maturation in the nucleus. In addition, the viral determinant for the requirement of TNPO3 in HIV-1 infection is discussed. This paper summarizes the most significant recent discoveries about this important host factor and its role in HIV-1 replication.

1. Introduction

The influence of the physiological state of cells on retroviral replication has been known since Temin and Rubin demonstrated that stopping cell division by X-rays or UV light prevents Rous sarcoma virus replication [1]. Subsequent research established the relationship between cell cycle stage and retroviral infection, revealing that retroviruses do not all have the same requirements for productive infection [2, 3]. For example, γ-retroviruses such as murine leukemia virus (MLV) require the host cell to pass through mitosis for efficient infection [4, 5]. The MLV titer decreases at least 10-fold when infecting cells that are arrested in a non-dividing state. By contrast, lentiviruses such as HIV-1 show no difference in productive infection in dividing versus nondividing cells [6]. This evidence suggests that lentiviruses have developed specific mechanisms for the infection of non-dividing cells. The ability of HIV-1 to infect non-dividing cells has been attributed to its capacity to transport the preintegration complex (PIC) to the nucleus [7, 8]. Translocation of the HIV-1 PIC into the nucleus is not a simple process as the PIC is a large complex that contains integrase, matrix, capsid, Vpr, and the viral DNA [7, 9, 10]. Because of its large size, it is unlikely that the PIC enters the nucleus by passive diffusion [11]. On the contrary, HIV-1 PIC translocation into the nucleus must be an active process, possibly making use of nuclear localization signals [12]. Several viral components of the PIC such as matrix, Vpr, integrase, and the central DNA flap have been proposed to be directly involved in PIC transport into the nucleus. However, evidence in the literature both supports and refutes a role for these different components in nuclear translocation [13, 14]. Although only small amounts of capsid can be found in biochemically purified HIV-1 PICs [7, 12, 15, 16], evidence has shown that capsid plays an important role in the ability of HIV-1 to infect non-dividing cells [3, 17–19] The mechanism used by the HIV-1 PIC to enter the nucleus is not completely understood; however, it is widely accepted that nuclear import of the complex is active and energy dependent [8].

In addition to the viral determinants involved in HIV-1 PIC nuclear import, several host factors have been implicated in the process: (1) importin 7 [20–22], (2) importin α3 [23], (3) importin/importin heterodimer [20, 24, 25], (4) NUP153 [19, 26, 27], (5) RanBP2 [28], and (6) TNPO3/transportin-SR2 [29–35].

TNPO3, transportin-SR2 or Tnp3, a member of the karyopherin β superfamily of proteins, is important for the ability of HIV-1 to achieve productive infection, as TNPO3 depletion leads to a reduction of HIV-1 infectivity [29–37]. TNPO3 transports pre-mRNA splicing factors into the nucleus [38] and recognizes them by binding to phosphorylated or nonphosphorylated serine/arginine-rich motifs in

a RanGTP-dependent manner [39, 40]. TNPO3 is also an export factor for certain tRNA species, and its yeast ortholog Mtr10p is an export factor for small ribosomal subunits [36, 41].

2. Role of TNPO3 in Retroviral Infection

The role of TNPO3 in retroviral infection was initially discovered for HIV-1 [30]; however, more recent work has demonstrated that TNPO3 is also important for infection by HIV-2, simian lentiviruses, and, to a lesser extent, equine infectious anemia virus (EIAV) [31, 32, 37, 42] but not MLV or Feline immunodeficiency virus (FIV). Intriguingly, simian immunodeficiency viruses (SIVs) exhibited the strongest dependency on TNPO3 for infection [31, 32, 37, 42].

3. Viral Determinants for the Requirement of TNPO3

3.1. Integrase. A yeast two-hybrid screen identified TNPO3/transportin SR-2 as a host protein that interacts with HIV-1 integrase [29]. These studies confirmed that TNPO3 does, indeed, bind to integrase, suggesting that integrase may be a key viral determinant for the requirement of TNPO3 in productive HIV-1 infection; the same work showed that endogenously expressed TNPO3 in mammalian extracts binds recombinant HIV-1 but not MLV integrase, which agrees with the result that TNPO3 is required for HIV-1 infection but not for MLV [29]. By contrast, the use of recombinant integrases from different retroviruses demonstrated that bacterially purified GST-TNPO3 binds to integrase proteins of HIV-1, MLV, SIVmac, FIV, bovine immunodeficiency virus (BIV), and with less affinity to the integrase of EIAV [31]; this latter result fails to correlate TNPO3 binding to integrase with the requirement for infectivity. We also tested this correlation by using both TNPO3 and viral integrases from mammalian extracts. By pulling-down codon-optimized integrases from different retroviruses expressed in mammalian cells, we demonstrated that endogenous TNPO3 binds HIV-1, HIV-2, and SIVmac integrases, which correlates with the requirement for TNPO3 on infectivity (Figure 1(a)). Similarly, we observed that the FIV integrase binds TNPO3, though somewhat weakly. In contrast, the integrase proteins of EIAV, BIV, and MLV did not bind TNPO3 in this particular assay (Figure 1(a)). As a positive control for binding, we demonstrated that, under similar pull-down conditions, lens epithelium-derived growth factor (LEDGF)/p75 bound HIV-1 integrase (Figure 1(b)). Interestingly, we found a positive correlation between TNPO3 binding and the requirement for TNPO3 in primate lentiviral infection (Figure 2). Although western blot is a semiquantitative assay, it provides a trend. Overall, in the case of lentiviruses, a correlation exists between TNPO3 binding to integrase and the requirement of TNPO3 in infection The fact that the integrase of FIV interacts with TNPO3 and that TNPO3 is not required for FIV infection suggests the existence of two distinct groups of viruses. However, there is no genetic evidence pointing to integrase as the determinant for the requirement of TNPO3 during infection. Indeed, generation of such evidence might not be an easy task, given that integrase mutants affect multiple stages of the viral life cycle and complicate clear interpretation of phenotypes [43].

The differences observed in the interaction of TNPO3 and viral integrases could lie on the origin of the protein used to measure the interactions. However, it is difficult to determine which approach is closer to the interactions that occur inside the cell. In conclusion, TNPO3 binds integrase; however, the role of this interaction during retroviral infection is not understood.

3.2. Capsid. In contrast to integrase, genetic and biochemical evidence exists for capsid as a determinant for the requirement of TNPO3 during HIV-1 infection [31, 36, 37, 44]. By using HIV/MLV chimera viruses on the capsid protein, the Engelman Lab demonstrated that capsid is the genetic determinant for the requirement of TNPO3 during infection [31]. Similarly, by extensive mutagenesis of capsid, the Luban Lab demonstrated that capsid plays a major role in the requirement for TNPO3 during infection [44]. TNPO3 was reported to bind soluble capsid [36], and, more recently, a direct biochemical interaction between TNPO3 and the HIV-1 core has been demonstrated in our laboratory [37]. Interestingly, we found that TNPO3 binds to HIV-1 capsid-nucleocapsid complexes that have been assembled *in vitro*, which recapitulate the surface of the viral core [45]. Altogether, this evidence points out capsid as an important determinant for the requirement of TNPO3 during productive HIV-1 infection.

4. Role of TNPO3 in HIV-1 Nuclear Import

It is believed that TNPO3 is involved in nuclear import of the HIV-1 PIC on the basis of the following evidence [29]: (1) reduction in the number of 2-LTR circles during HIV-1 infection of TNPO3-depleted cells when compared to infection of wild-type cells and (2) observation of decreased nuclear translocation of the PIC in TNPO3-depleted cells by using an HIV-1 virus containing an IN-GFP fusion protein. It should be noted, however, that this interpretation is in question, as more recent work has detected no change in HIV-1 nuclear entry in the face of TNPO3 depletion, implying that the block is subsequent to nuclear import [34, 36, 37, 42, 44]; the different groups who have investigated this issue demonstrated that the number of HIV-1 2-LTR circles in TNPO3-depleted cells was similar when compared to wild-type cells.

It is important to mention that the measurement of 2-LTR circles is indirect evidence of PIC nuclear import. After the viral DNA is imported into the nucleus, it integrates into the genome; however, a fraction of this viral DNA is ligated to produce circular forms by nuclear DNA ligases [46]. These products are known as 2-LTR circles, and they are used as indirect measure of nuclear import. Although the 2-LTR is an indirect measure of PIC nuclear import, this methodology is widely used as a marker of nuclear import [46].

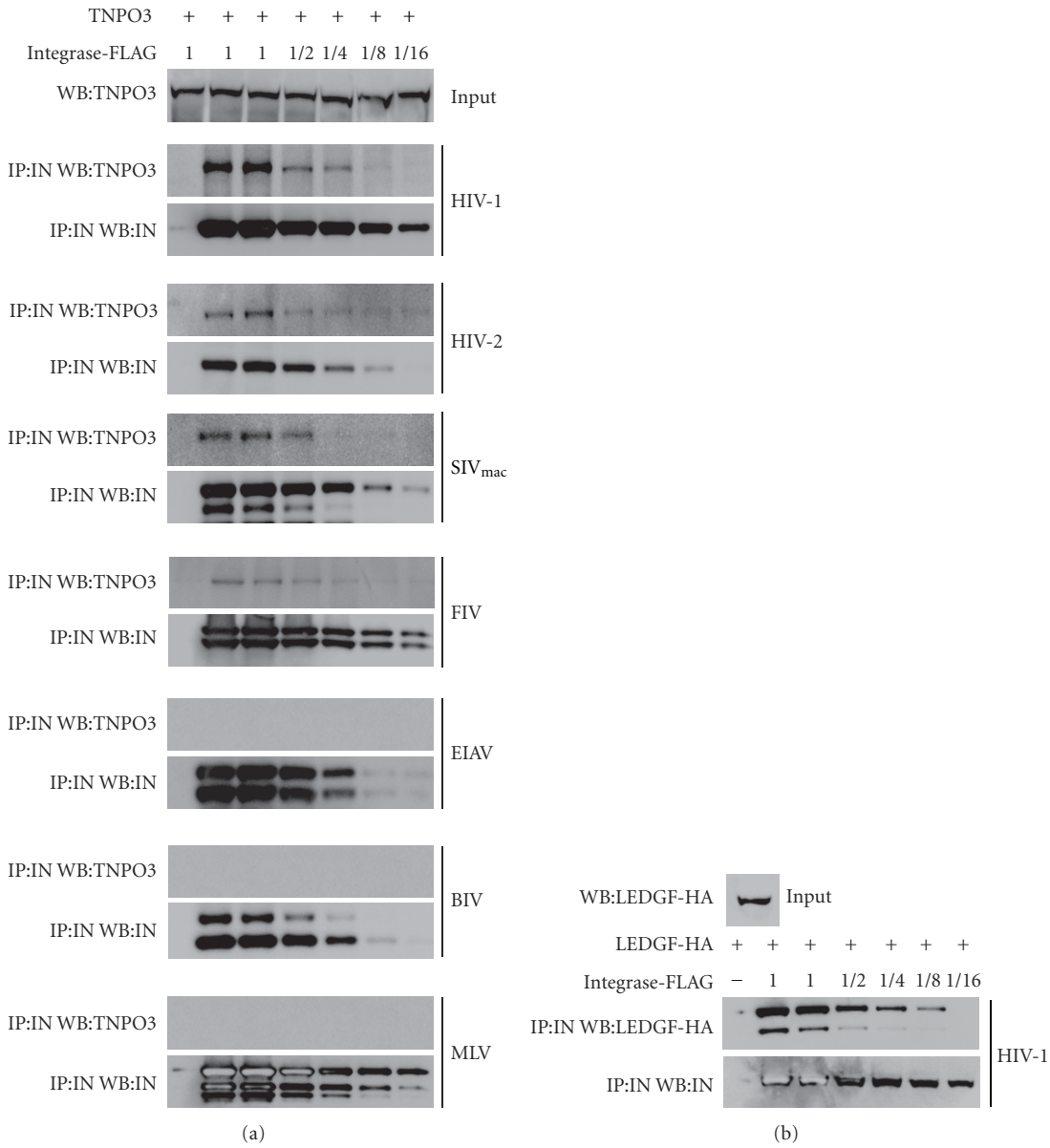

Figure 1: TNPO3 Interaction with retroviral integrases. (a) Human 293T cells, which endogenously express TNPO3, were transfected with different amounts of the indicated mammalian codon-optimized FLAG-tagged retroviral integrases (IN). Twenty-four hours following transfection cells were lysed in extraction buffer (400 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl, pH = 8, 2 mM $MgCl^2$, 5% glycerol and protease inhibitors (Roche)). Subsequently, extracts were treated with DNAase and precleared using protein-A agarose beads (Sigma) at 4°C for 1h. Small aliquot of the initial extract was analyzed by Western blot (WB) using anti-TNPO3 antibodies (INPUT). Subsequently, the extracts were used to immunoprecipitate (IP) the different retroviral integrases using anti-FLAG antibodies. FLAG-peptide eluted complexes were analyzed by WB for the presence of TNPO3 and using anti-TNPO3 and anti-FLAG antibodies, respectively. (b) As a positive control we assayed the known ability of HIV-1 integrase to interact with LEDGF/p75. For this purpose, HA-tagged LEDGF/p75 (LEDGF-HA) was cotransfected together with FLAG-tagged HIV-1 integrase and immunoprecipitated using anti-FLAG beads. Eluted complexes were analyzed for the presence of LEDGF/p75 and HIV-1 integrase by WB using anti-HA and anti-FLAG antibodies, respectively. Similar results were obtained in three independent experiments, and the results of a representative experiment are shown.

Furthermore, no difference was observed in the levels of viral DNA nuclear accumulation in TNPO3-depleted cells relative to control cells following biochemical fractionation, which supports the 2-LTR findings [36]. Altogether, the work from several independent laboratories suggests that TNPO3 is required when the PIC is in the nucleus.

5. Role of TNPO3 in Nuclear Maturation of the PIC

The consensus that TNPO3 assists HIV-1 replication in the nucleus led to testing of the hypothesis that TNPO3 may be promoting a nuclear maturation step [36]. Remarkably, the

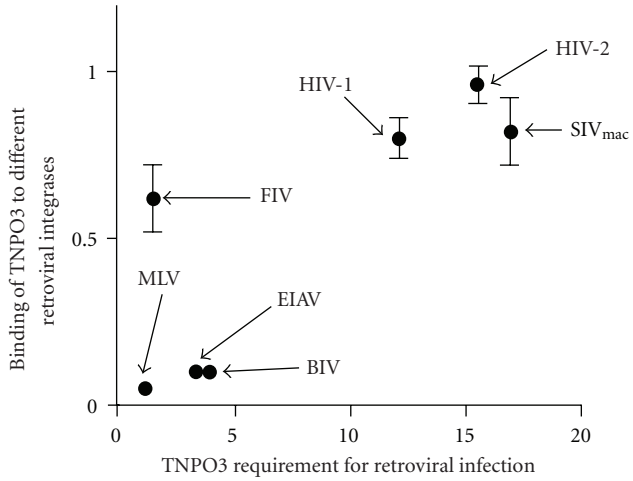

Figure 2: Correlation of the ability of TNPO3 to bind different retroviral integrases with the requirement of TNPO3 in retroviral infection. The ability of TNPO3 to bind to different retroviral integrases was calculated by quantifying the amount of bound TNPO3 relative to the amount of immunoprecipitated integrase specified in Figure 1. The requirement of TNPO3 for the indicated retrovirus was calculated by the fold inhibition in TNPO3-depleted cells when 50% of wild-type cells were infected.

Fassati group demonstrated that more capsid accumulates in the nucleus of TNPO3-depleted cells during HIV-1 infection relative to wild-type cells. These results indicate that the presence of TNPO3 in wild-type cells contributes to the removal of capsid from the nucleus, which may be important for PIC maturation in the nucleus and integration. In agreement with a role of TNPO3 in the nucleus, depletion of TNPO3 altered the selection of chromosomal sites for viral integration [28].

6. Role of CPSF6 in the Ability of TNPO3 to Assist HIV-1 Replication

The cleavage and polyadenylation specificity factor subunit 6 (CPSF6), an SR-protein, is a potential nuclear transport cargo of TNPO3. Interestingly, expression of a CPSF6 fragment (1-358) lacking the nuclear localization signal blocks HIV-1 nuclear import [19]; therefore, it is conceivable that TNPO3 depletion causes accumulation of CPSF6 in the cytosol and that this accumulation impairs HIV-1 replication. Furthermore, a virus containing the capsid mutation N74D is resistant to the replication block imposed by overexpression of the CPSF6 fragment in the cytosol [19]. Intriguingly, infection of the HIV-1 capsid mutant N74D is independent of TNPO3. Altogether, these results imply that the effect of TNPO3-depletion on HIV-1 infection is in part linked to CPSF6.

Analysis of TNPO3-depleted cells revealed minimal changes in the distribution of CPSF6 by cytosolic/nuclear fractionation and immunofluorescence in HeLa cells [37]. These results implied that TNPO3 depletion minimally changes the localization of CPSF6 and suggest that the effect of TNPO3 depletion on HIV-1 infection is independent of a change in CPSF6 localization. However, these results do not exclude the possibility that CPSF6 plays a role in the phenotype observed for HIV-1 in TNPO3-depleted cells.

7. Role of TNPO3 in HIV-1 Infection

TNPO3 is a nuclear importer that is important for HIV-1 replication. Two possible viral determinants of the requirement for TNPO3 in HIV-1 replication have been postulated, integrase and capsid [29, 31, 37, 44]. However, compelling genetic and biochemical evidence has only been found for capsid [31, 37, 44], and thus it remains a question whether integrase is still a player in the ability of TNPO3 to assist HIV-1 replication once infection has taken place. It is possible that capsid and integrase are jointly playing a role in the requirement for TNPO3 in HIV-1 replication. However, this remains to be determined.

Several groups have confirmed the observation that TNPO3-depletion allows formation of 2-LTR circles during HIV-1 infection [36, 37, 42, 44]. Even though formation of 2-LTR circles is an indirect measure of nuclear import, it is one of the most used tools to determine whether the HIV-1 PIC has been transported to the nucleus [46]. These experiments implied that in TNPO3-depleted cells the PIC has been transported to the nucleus; however, HIV-1 integration did not occur [29, 34, 36, 37, 44]. This suggested, in turn, that TNPO3 is assisting some process in the nucleus prior to integration. In agreement with this idea, it has been proposed that TNPO3 plays a role in depleting capsid from the nucleus during infection, which may help PIC maturation in the nucleus [36]. This model suggests that small amounts of HIV-1 capsid that remain bound to the PIC are transported into the nucleus, in agreement with the observation that capsid is the viral determinant for the infection of nondividing cells [17, 18]. However, experiments indicate that biochemically purified PICs contain very little capsid [7, 12, 15, 16], and in fact the presence of capsid in the nucleus during HIV-1 infection has not been clearly established. Future work should attempt to clarify this exciting possibility.

An alternative hypothesis is that TNPO3 binding to the HIV-1 core in the cytoplasm aids the ribonucleoprotein (RNP) complex in a process required only after the complex enters the nucleus [37]. TNPO3 binding to the HIV-1 core may assist the maturation of the PIC in the cytosol; however, assistance provided by TNPO3 to HIV-1 replication in the cytosol will only be noticed when the complex reaches the nucleus. For example, 3′ processing activity of integrase on the HIV long terminal repeats (LTRs) has been suggested to occur in the cytoplasm [7, 47]. It is possible that TNPO3 binding to the HIV-1 core ensures proper 3′-processing of the viral LTRs in the cytoplasm, which will be important for viral integration when the complex reaches the nucleus. Future experiments should test whether TNPO3 depletion can affect 3′-processing of viral LTRs.

The discovery of TNPO3 has pushed the HIV-1 research community to explore in greater depth the mechanism by which HIV-1 crosses the nuclear envelope and integrates into the cellular genome. It is expected that this field will grow steadily in the coming years and bring to light novel mechanistic information and therapeutic opportunities.

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Review Article

Factors Important to the Prioritization and Development of Successful Topical Microbicides for HIV-1

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Significant advancements in topical microbicide development have occurred since the prevention strategy was first described as a means to inhibit the sexual transmission of HIV-1. The lack of clinical efficacy of the first generation microbicide products has focused development attention on specific antiretroviral agents, and these agents have proven partially successful in human clinical trials. With greater understanding of vaginal and rectal virus infection, replication, and dissemination, better microbicide products and delivery strategies should result in products with enhanced potency. However, a variety of development gaps exist which relate to product dosing, formulation and delivery, and pharmacokinetics and pharmacodynamics which must be better understood in order to prioritize microbicide products for clinical development. *In vitro, ex vivo*, and *in vivo* models must be optimized with regard to these development gaps in order to put the right product at the right place, at the right time, and at the right concentration for effective inhibition of virus transmission. As the microbicide field continues to evolve, we must harness the knowledge gained from unsuccessful and successful clinical trials and development programs to continuously enhance our preclinical development algorithms.

1. Introduction

Significant progress has been made in the development of topical anti-HIV microbicides since their initial description and development nearly 20 years ago. The first products developed for microbicide use were nonspecific agents which prevented HIV-1 from entering target cells by disrupting the viral membrane, including nonoxynol-9 (N-9), SAVVY (C31G), and Ushercell (cellulose sulphate) [1–3]. Clinical results with N-9 demonstrated enhanced rates of infection in the treated groups, suggesting the surfactant caused vaginal damage which allowed greater rates of infection [3]. SAVVY was prematurely discontinued due to the HIV incidence being half of the expected rate (one of the characteristics rendering the trial uninformative) [1]; however, it could not be conclusively determined that SAVVY promoted HIV infection as in the case of N-9. Ushercell was also discontinued due to a higher rate of infection compared to the placebo group [2]. Following the failure of the nonspecific surfactants, microbicide development has focused on the identification and development of specific antiretroviral (ARV) agents targeted at preventing early steps in virus replication such as virus attachment and entry and reverse transcription. Most recently, microbicide development has expanded to include the evaluation and development of late-acting products (integrase and protease inhibitors) [4], agents directed at cellular targets important to virus replication and transmission, and agents which boost mucosal and innate immunity to HIV [5]. The first specific antiretroviral compound evaluated was PRO2000, a synthetic naphthalene sulphonate, which specifically targeted CD4 to prevent virus attachment and subsequent entry. PRO2000 was proven safe in Phase I/II human clinical trials but eventually was shown to be ineffective in preventing HIV transmission [6]. In 2010, results from the CAPRISA 004 Phase IIb trial demonstrated that a 1% tenofovir gel reduced HIV transmission by 39% in

the study population overall and by 54% in women with high levels of adherence to the study protocol [7]. The CAPRISA 004 study provided the first positive results which demonstrated that an antiretroviral agent formulated as a vaginal gel could successfully prevent the sexual transmission of HIV, energizing the field of microbicide development. Unfortunately, in the latest clinical trial (VOICE) performed by the Microbicide Trials Network (MTN), equivalent numbers of infections were observed among women in both the placebo and 1% tenofovir gel arms, and the trial was subsequently discontinued [8]. Differences in dosing regimens between the CAPRISA 004 and VOICE trials have been suggested to have contributed to the different results of these trials. The ongoing FACTS trial being performed in South Africa is set to address the reproducibility of pericoital dosing with tenofovir gel. Although the results of VOICE were a disappointment to the microbicide community, the lessons learned and information gained from the tenofovir trials (CAPRISA and VOICE) as well as the other failed trials may prove to be informative and enable the field to better optimize and develop an efficacious microbicide.

Microbicide efficacy clinical trials are very large trials, enrolling anywhere from 800 to over 9,000 women in each trial [9]. Thus, to have multiple failed trials of this magnitude has been extremely costly from both a financial and human life perspective. The risk of trial failure is uniquely high due to the limitations of Phase II trials. Although these trials often provide go-no-go indication, they are very unlikely to have sufficient power to report anything relative to efficacy due to the low seroincidence rates, even in high-risk populations. Since the key to the identification and development of a successful microbicide product likely lies in the performance of better and more informative preclinical evaluations, a greater understanding of the optimal delivery and the pharmacokinetic and pharmacodynamic (PK/PD) profiles of both the active pharmaceutical ingredient (API) and the formulated clinical product is necessary prior to the initiation of human clinical trials. Two decades of research and development and the outcomes of successful and failed human clinical trials have served to define a variety of gaps in the preclinical microbicide development pathway. Thus, as the microbicide development field moves forward to define new products and design informative and successful clinical trials, it is critical to understand how these development gaps might be effectively filled in order to generate the data necessary to understand how to best optimize and prioritize microbicide product development. These critical topical microbicide development gaps may be defined as follows: (1) a better understanding of the environment in which the microbicide must act, including the positive and negative effects of the presence of semen and vaginal fluids, natural and pathogenic organisms, and the physiology of the biological compartments (vagina and rectum); (2) a better understanding of the pharmacokinetic and pharmacodynamic properties of the microbicide product and the use of *in vitro*, *ex vivo,* and *in vivo* models to quantify these critical candidate product properties; (3) a better understanding of means to define appropriate dosing concentrations of a microbicide product and how the dose, formulation, and delivery vehicle impact the pharmacokinetic and pharmacodynamic properties of the product; (4) a better understanding of critical issues in the formulation and delivery of the microbicide products for use in both the vagina and rectum, including the use of dual compartment and oral dosing strategies; (5) the effective implementation of multipurpose prevention technologies, involving broad based anti-infective and contraceptive products. Inherent in each of these development gaps is the overarching goal of developing a product which is acceptable to the population of individuals that will primarily use the products that are developed. Herein we will evaluate each of these development gaps and discuss how information obtained during preclinical development might be improved and better utilized to identify and prioritize microbicide products for development. The most critical requirement of the microbicide development algorithm is the need to have the *right concentration* of the *right microbicide* (or combination of microbicides) present at the *right location* and at the *right time* to prevent HIV infection. Thus, the microbicide development gaps require a more intimate understanding of dosing, formulation, and delivery vehicles, which will result in effective pharmacokinetic and pharmacodynamic properties for candidate products, and allow the right products to be prioritized for development. Since each microbicide is unique, these variables must be independently evaluated in order to develop highly effective microbicide products.

2. The Role of the Complex Biological Environment

The biological environment in which an active microbicide product must act has come to be recognized as a critical variable to microbicide functionality. Microbicide products are now being developed for both vaginal and rectal use and therefore the anatomy and physiology of both compartments needs to be considered as a key feature of the preclinical development algorithm. The complexity of the vaginal environment includes the anatomical features of the cavity, the presence of naturally occurring and pathogenic microorganisms, and the presence of vaginal fluids and mucus. The environment becomes even more complex with the act of coitus and the deposition of semen into the vault. The vagina possesses its own inherent defense mechanisms including the multilayered squamous epithelium which acts as a natural barrier to infection, the hydrogen peroxide producing vaginal flora (*Lactobacillus*) which maintains an acidic environmental pH, mucus which provides a physical barrier to virus transport, and the production of a variety of antimicrobial and innate defense molecules which directly and indirectly inactivate virus or suppress infection and virus replication. It is important that the integrity of this environment be maintained as the first line of defense against HIV infection; the development of all microbicide products involves the early evaluation of the effects of a candidate product on the components of this primary defensive barrier. Microbicide products should be nontoxic to the cellular and tissue structure, should not result in the elimination

of the normal protective populations of H_2O_2 producing *Lactobacillus*, and should remain stable and active at low pH (approximately pH 4.6). All of these properties of a topical microbicide candidate can be accurately assessed in preclinical *in vitro* assays [10, 11]. Maintaining normal vaginal fluids and mucus is also important given that these products typically act as the initial line of defense against infection by microorganisms and HIV [12, 13]. In addition to innate immune responses and microbe-sensing properties, including the production of antimicrobial peptides [14, 15] and proinflammatory cytokines, cell-free and cell-associated viruses are also inactivated at low pH [16–18], and movement to potential target cells is restricted by vaginal mucus. Anti-HIV activity has been directly attributed to components of vaginal fluids, including defensins [19, 20], toll-like receptor (TLR) agonists [21, 22], and secretory leukocyte protease inhibitor (SLPI) [23]. Many studies have been performed to evaluate the antiviral effects of cervicovaginal fluid (CVF). Ghosh et al. showed that CVF incubated with virus prior to the addition of target cells yielded 0 to 100% inhibition of infection, with some samples showing enhancement of virus infection. This study concluded that a wide range of factors that are capable of mediating antimicrobial protection are present in CVF and specifically correlated levels of HBD2, MIP 3α, and HIV-specific IgG antibodies with the protection of target cells from infection with HIV [24]. Other studies have served to confirm and expand these results demonstrating the HIV-inhibitory activity of CVF, and laboratory investigations continue to better understand and harness these protective effects of CVF [25–27]. The results of these experiments suggest pluripotent antiviral effects exerted by a variety of CVF constituents working in concert as opposed to the individual activity of any single product results in the natural inhibitory potential of CVF. Therefore, it is critically important that a microbicide product should not diminish the natural protective effects of vaginal fluid, and all products should be evaluated *in vitro* and *ex vivo* in the presence of CVF to verify that biological activity is maintained.

Another important consideration in the context of vaginal fluid involves the spread, coverage, and dispersion of the microbicide product during sexual intercourse. In studies performed by Keller et al., CVF collected by lavage within an hour following a single dose of 0.5% PRO2000 gel significantly inhibited HIV when evaluated in *in vitro* antiviral assays. This antiviral activity was significantly reduced when the CVF was collected following sexual intercourse, and no significant protective effect was observed in postcoital CVF obtained in the presence compared with the absence of PRO2000 gel application [28]. These results suggest the physical act of sexual intercourse results in mixing and dispersal of the microbicide product resulting in reduced effectiveness of a topical microbicide, and these factors should be evaluated during early product development. Instrumentation and methodology to perform studies to evaluate gel spreading and overall epithelium coverage within the vagina and rectum have been developed and employed to evaluate microbicide products in the context of coitus, and these evaluations will help to determine if a microbicide product is able to be in the right place and right concentration to prevent infection prior to and following coital events [29–31]. Confocal Raman spectroscopy (CRS) has recently been utilized to measure local concentrations of APIs in three dimensions in vaginal or rectal fluids, gels, and tissue explants, and this methodology may yield highly relevant data regarding the penetration of API into vaginal and rectal tissue [32].

Over the past two decades the primary focus of microbicide development has been on preventing vaginal HIV-1 transmission. However, in the developed world unprotective receptive anal intercourse (URAI) is the primary risk factor for HIV acquisition in the MSM population. URAI is now recognized as a significant feature of the sexual practices of women in both the developed and developing countries of the world [33, 34]. The vulnerability of the fragile intestinal mucosa to HIV transmission yields a 20-fold greater infection risk per sex act compared to the infection risk from unprotected vaginal intercourse. Furthermore, the rectum, unlike the vagina, is an open ended, fragile, and poor barrier to pathogens, resulting in an increased risk of infection during URAI. The mucosa accounts for approximately 10% of the colorectal wall thickness and is comprised of single layered epithelium, lamina propria, and muscularis mucosa. As with vaginal virus transmission, it is thought that virus migrates through the epithelial cell layer to the lamina propria where a greater frequency of target cells is present and primed for infection. The gut mucosa comprises the bodies' greatest reservoir of CD4+ cells and other immune competent cells. Ninety percent (90%) of colonic CD4+ cells express the HIV-1 chemokine coreceptor CCR5, rendering this environment a vast reservoir of target cells for HIV-1 infection and transmission. Upon establishment of sites of infection, the presence of an adequate local density of activated target cells for local amplification of the virus and subsequent dissemination to the systemic circulation is required and the gut mucosa appears to provide this susceptible environment to the virus [35]. The fragile nature of the rectum makes it more susceptible to tears and damage during receptive anal intercourse (RAI) which also promotes infection. As with the vagina, infection of the rectum by other opportunistic microorganisms can also increase susceptibility to HIV-1 infection. Sixty percent (60%) of HIV-negative men have been shown to be positive for anal human papilloma virus (HPV), and this number increases to 95% in the HIV-positive male population [36]. Smith et al. reported an increased risk of HIV acquisition among Kenyan men infected with HPV which may derive from the lesions associated with HPV infection, as observed in women with HPV infection [37]. Although some similarities exist between the vaginal and rectal compartments, significant differences in anatomy and physiology exist, and these differences need to be taken into account early in topical microbicide product development.

As mentioned above, the complex environment of the vagina and rectum becomes even more complex upon the deposition of semen. When semen is introduced into the vaginal environment a variety of changes occur. The first and most significant effect is that semen changes the acidic vaginal pH to near neutral pH which alters the balance of normal

flora and provides an environment which facilitates the rise of bacterial vaginosis and yeast infections. Additionally, semen deposited into the reproductive tract promotes an influx of activated inflammatory cells in close proximity to infectious virus and virus-infected cells in the semen [38, 39] and induces changes in the population of leukocytes which are present in the vaginal tract (reviewed in [40]). Semen also can have a "toxic" effect to the vaginal environment that results in recurrent vaginitis that is associated with localized irritation and inflammation [41]. This inflammatory reaction yields additional recruitment and activation of HIV-1 target cells which ultimately facilitates HIV infection and transmission and sometimes results in enhanced HIV-1 infection. The enhanced infection effects can be attributed to the neutralization of the acidic vaginal pH promoting the survival of cell-free and cell-associated virus, the presence of semen-derived enhancer of virus infection (SEVI), and mediation of the electrostatic interaction of spermatozoa with HIV-1 virions (reviewed in [40]). Further, studies performed by Lai et al. have shown that the neutralization of vaginal pH by semen increases the movement of HIV virions in mucus possibly resulting in infectious virus more rapidly reaching the epithelium [42–44]. However, semen has also been shown to possess antiviral properties. The inhibition has been experimentally attributed to the oxidation of SP polyamines by diamine oxidase in the vaginal environment producing radicals that inactivate HIV, cationic polypeptides that are contained in seminal plasma, and the interference of the attachment of HIV-1 to DC-SIGN by a potent inhibitor contained in seminal plasma (reviewed in [40]). In this regard, we have evaluated the antiviral activity of 50 individual semen samples obtained from individual donors, and we have shown both inhibition and enhancement of HIV infection mediated by these diverse semen samples (Figure 1). In the rectum, semen has similar effects as those observed in the vagina. Once a trauma-inducing event occurs, the inflammatory cytokines enable transmission of virus through the epithelial barrier. Thus, current research supports the fact that microbicide candidates must be evaluated in the presence of semen to verify the potency of the candidate and to confirm that there is no antagonism of antiviral efficacy or enhanced toxicity. As was discussed with vaginal fluids, it has also been shown that semen provides a physical barrier to the movement of virus from the semen towards target cells in the epithelium of the vagina or rectum [45, 46].

In both the vagina and the rectum, the form in which infectious virus is presented in these environments must also be carefully considered (cell-free virus versus cell-associated virus, as well as combinations of both forms) for optimal development of a microbicide since cell-associated virus may be less susceptible to some microbicide candidates compared to cell-free virus. Louissaint et al. have recently reported that when using surrogates for cell-free and cell-associated HIV and semen, cell-free and cell-associated surrogate distribution following simulated intercourse coincided within the female reproductive tract [47]. In a small group of gay men (6 total), Butler et al. showed that the virus being transmitted was more closely related to the free virus in seminal plasma [48]. This would, however, need to be confirmed in a larger study. A better understanding of the roles of cell-free and cell-associated virus in establishing infections in the vagina and rectum remains controversial and should continue to be investigated.

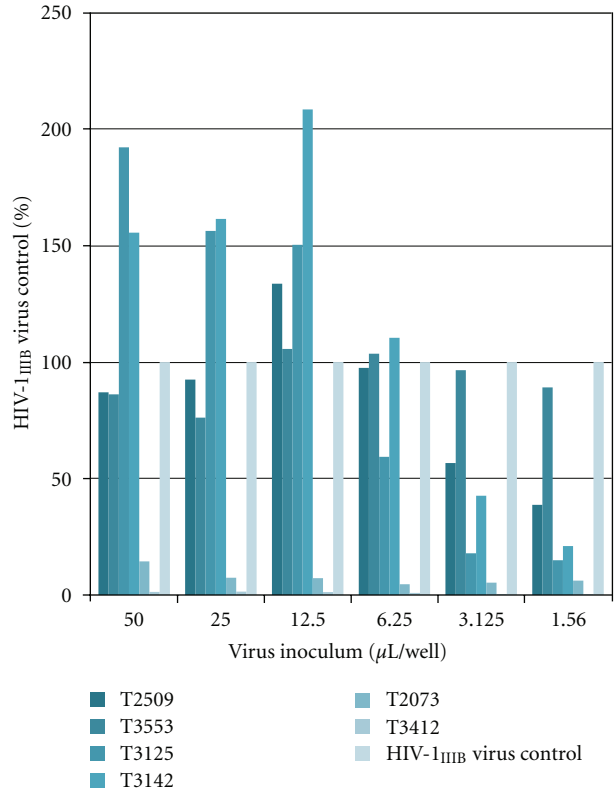

Figure 1: Biological impact of semen on virus infectivity and replication. Fifty samples of whole semen (Lee Biosolutions) were evaluated for biological activity in HeLa-CD4-LTR-β-galactosidase cells infected with varying quantities of infectious HIV-1_{IIIB}. Representative results obtained with six of these samples are presented. Semen was added to the cells in a volume of 50 μL immediately prior to the addition of infectious virus at 6 different virus inoculums ranging from a high inoculum of 50 μL (straight virus) and five additional serial twofold dilutions of virus in tissue culture medium. At 4 hours the virus and semen were washed from the monolayer of cells, and the cultures were incubated for an additional 48 hours at which time virus replication was quantified by β-galactosidase production in the cultures. The results presented demonstrate the three patterns of biological activity observed among the 50 tested samples: (1) enhanced levels of infection (see samples T3142 and T3125 at high virus inoculum), (2) inhibition of infection (T2073 and T3412 as well as T3125 and T3142 at lower viral inoculum), and (3) no effect on infection (T3553).

3. Pharmacokinetics and Pharmacodynamics of Microbicide Products

Over the past several years the importance of pharmacokinetics (measurement of microbicide distribution, absorption, and retention typically measured in tissues and body fluids) and pharmacodynamics (microbicide biological

activity within the compartment) has been increasingly recognized within the microbicide community as a crucial feature in understanding microbicide efficacy and toxicity. *In vitro* and *ex vivo* assays have been designed to better understand these parameters prior to the introduction of products to human clinical trials. Understanding effective API pharmacokinetics also requires an understanding of the mechanisms of HIV infection of vaginal or colorectal tissues and subsequent dissemination of the virus from the initial sites of infection. In nonhuman primate models, 30-to-60 minutes of exposure to an infectious inoculum are sufficient to establish a productive and spreading virus infection [49]. Transmitted or founder viruses target CD4 populations in the mucosa that express high levels of the CCR5 chemokine coreceptor [50]. In explant and NHP models, virus has been demonstrated to penetrate the superficial layers of the stratified epithelium which enables the virus to quickly come into contact with T cells and Langerhans cells contained within these surfaces [35]. Additional NHP studies have demonstrated that the initial infection of cells in the mucosal surfaces occurs within 16 to 72 hours and an established and spreading infection from these sites requires an influx of additional activated T cells [35]. A microbicide will only be effective if the product is able to either prevent the virus from infecting these critical target cell populations or is able to effectively prevent the establishment of a spreading infection from these initially infected cell foci. For this reason pharmacokinetic (PK) and pharmacodynamic (PD) assessments need to be performed and understood in terms of the cells and tissues which must be protected by the microbicide (i.e., epithelium versus stroma, introitus versus cervical os, etc.).

Historically, PK measurements were performed as a component of microbicide safety studies as well as to quantitatively determine if vaginal or rectal delivery resulted in significant API absorption to the systemic circulation. PK/PD evaluations have been routinely performed in animal models which include nonhuman primates, humanized mice, and sheep (reviewed in [51]). Rectal PK studies have been routinely performed in pigtailed macaques [52]. Thus, to date, PK assessments have not been routinely utilized to facilitate determination of the required effective dose of a formulated microbicide product that would be optimized for delivery or to even confirm if sufficient API can be delivered to the critical tissues where infection occurs but have been used to confirm that the dose being used is nontoxic and safe. Based upon the results of recent clinical trials such as CAPRISA 004 and VOICE, it has become apparent that PK/PD studies need to be performed to better understand where API goes in the cells, tissues, and fluids of the vagina and rectum, at what concentrations the API is found in both fluids and relevant infectable tissue, and if sufficient API is present to actually interfere with the infection of HIV.

Current PD evaluations for microbicides involve *ex vivo* infection inhibition studies using tissues from microbicide-treated animals. Multiple biopsy tissues from treated and control untreated animals (or from human volunteers in clinical studies) are exposed to infectious HIV, and the ability of HIV to infect and replicate in the tissue is quantified. Although these studies provide important information and help to bridge the gap between *ex vivo* and *in vivo* evaluations, it is not yet clear if this methodology will accurately quantify the effectiveness of a microbicide in human clinical trials. Similar studies using vaginal lavage to determine if protective concentrations of microbicide products are achieved in vaginal fluids are also being utilized to monitor the attainment of effective microbicide concentration levels [28].

Although nonhuman primate models have been the animal model of choice for PK/PD evaluations and have provided great insight into HIV transmission in the vagina and rectum, they have not proven to be the best predictive model for efficacy of a microbicide product in human clinical trials. For example, whereas complete protection of macaques was achieved with the 6% cellulose sulphate gel, no protection was observed in human volunteers in the clinic [53]. With a 1% tenofovir gel, efficacy studies in nonhuman primates demonstrated protection in all animals whereas in the clinical trial only 39% of women were protected [54]. A topical microbicide development model using mice has also been reported [55, 56] employing humanized bone-marrow-liver-thymus (BLT) mice reconstituted with human CD4+ T and other relevant human cells which are susceptible to intravaginal infection by HIV-1. However, due to the nature of the samples required for evaluations, PK/PD studies are often difficult to perform with the mouse model. Within the microbicide field, consensus has not been achieved on the predictive value of animal models, and many development programs forgo animal efficacy studies in favor of Phase 1 human studies.

For this reason, more predictive and robust and less expensive PK/PD models need to be established for evaluation of products prior to human clinical trials. The *ex vivo* cervical explant model has been extensively used for the evaluation of microbicide safety (toxicity assays) and is currently being utilized for PD evaluations using explant tissue from both human and animal studies. It had been assumed that the evaluation of microbicide efficacy and toxicity in human cervical explant tissue would provide highly relevant data to bridge the gap between *in vitro* results and *in vivo* efficacy evaluations, given the explants are more representative of the tissue and cells being targeted in the vagina (epithelial and immune target cells). In addition, the explant studies are much less expensive than the nonhuman primate models. Unfortunately, data obtained from cervical explant evaluations suggests that the model may not yield conclusive evidence of product efficacy, and there are varying schools of thought on whether these explant studies actually provide added and reliable information beyond that obtained from *in vitro* systems or in animal modeling studies. Besides variability in protocols utilized to perform the explant assay [57–59], there are a variety of limitations to the use of cervical explant cultures, including lack of hormone modulation, lack of recruitment of immune cells, loss of epithelium, and the inability of the explant tissue to regenerate/repair itself [58]. Variability in culture conditions and the relative ability of HIV to grow in the explant cultures, with significant background attributed to bound but nonreplicating virus, confound the interpretation of explant results. Infectious virus

has been shown to replicate in the explant cultures, however the cell population where virus is detected can change. Although significant issues with data interpretation exist, Beer et al. have correlated their explant data to both animal studies [60, 61] and to safety and acceptability trials [61–65] validating the use of cervical explants in microbicide development. As with any of the *in vitro* and *ex vivo* models that have been developed, the use of cervical explants will only be completely validated when the data is correlated to that which was observed in the human clinical trials. Continuing evolution of the explant models will also result in significant enhancement of the utility of the evaluations.

Although cervical explant evaluations may prove to be a needed and predictive component of the microbicide development algorithm, the limitations suggest that additional development must occur to provide more relevant *in vitro* models to understand how a microbicide will function in humans. One of the model systems now being used in the microbicide development community involves *in vitro* evaluation of drug permeability and transport across epithelial cell monolayers. These models evaluate the ability of a microbicide product to transit from the delivery vehicle (gels, rings, films, etc.) and across cell/tissue barriers representative of epithelia cell layers that would be present in the vagina and rectum. These studies are performed in a two-compartment Franz cell apparatus with appropriate tissue culture cells and/or ectocervical tissue [66]. Following incubation of the microbicide product with the cells and/or tissue, sample analysis to quantify microbicide product content is performed utilizing high-pressure liquid chromatography (HPLC). Using these models, Rohan et al. found that tenofovir from a 1% gel permeates into the tissue but the quantity of tenofovir measured after 30 minutes differed among individual ectocervical samples [67]. Mesquita et al. have developed an *in vitro* assay using a transwell assay system that can evaluate both the safety of a microbicide as well as the PD properties of a microbicide [68]. In these assays, a microbicide product must transport through an epithelial cell barrier to the lower tissue culture chamber where the product must protect target cells from *in vitro* infection by infectious HIV-1. Toxicity to the barrier cells can be measured by transepithelial resistance (TER). These *in vitro* assays allow for the evaluation of multiple microbicides at many concentrations. Although not yet proven, these *in vitro* assays may provide relevant information that will help prioritize microbicide candidates for clinical development.

4. Microbicide Dosing and Its Critical Impact on Pharmacokinetics, Pharmacodynamics, and Clinical Efficacy

The initial identification and subsequent development of a successful microbicide are dependent on the robustness of the efficacy and safety testing algorithms that are used to advance products. Preclinical and clinical experiences have driven the natural evolution of these algorithms over time, and it is understood that the algorithms will continue to change in the future [10, 11]. These algorithms should be capable of comparatively evaluating the active pharmaceutical ingredient (API) and the final formulated product with appropriate control compounds, as well as other experimental and approved microbicide products. One of the more difficult parameters to be addressed as a microbicide developer involves the quantitative determination of the clinical dosing of the product. Consensus opinion from nonefficacious and successful development programs and trials suggests that "*more is better*" and to "*dose as high as possible*" to assure that an effective concentration of API is present where and when it is needed to prevent virus transmission. This method of defining the API dosing in the final formulated product may result in a dramatic underestimate or overestimate of the amount of API that is actually required (or achievable), resulting in the extremes of lack of efficacy or potential safety issues. *Ex vivo* and *in vivo* evaluations in monkeys and mice have provided some information on the permeability/uptake of API into tissues in order to better understand the pharmacokinetics and pharmacodynamics properties of the compound and how they may relate to dosing levels; however, the tissue concentrations achieved with high dosing levels of a microbicide are significantly higher than the inhibitory concentrations achieved using cell-based *in vitro* assays. In light of the type of cells used in the *in vitro* evaluations, the lack of robustness of the assays in terms of their quantitative endpoints and timing of endpoint analysis, and the importance of understanding dosing and the prioritization of compounds for clinical use, it is critically important to understand the dosing requirements of an API as early as possible in the development process. Additionally, the differences observed between *in vitro* and *in vivo* effective levels could be attributed to poor distribution, that is, failure to coat all of the folded surfaces. The recently reported microbicide transmission and sterilization assay (MTSA) may provide a quantitative *in vitro* model to predict the tissue API concentration required to prevent virus transmission, and these data may then determine the required dose concentrations of the microbicide product to achieve that tissue API level [69].

The MTSA serves to define the concentration of a microbicide product required to completely suppress the transmission and subsequent replication of transmitted viruses in culture, yielding sterilization of HIV from a culture of cells [69, 70]. In the MTSA, virus is added to the culture in a cell-free or cell-associated form, and the virus infection is allowed to proceed over the course of serial passaging of the infected cells in the presence of various fixed concentrations of the microbicide test compound. The cells are subcultured every three days by adding 20% of the infected culture (cells plus supernatant) to the same original volume of uninfected cells in fresh medium with the same fixed concentration of test agent. At each passage, the cultures are evaluated for virus replication in the culture in order to quantify the timing of virus breakthrough (or frequency of infected cells) at each compound concentration. The concentration at which the compound totally suppresses virus replication in the culture is defined as its sterilizing concentration, and this sterilizing concentration is unique for each microbicide product we have evaluated and in most cases is significantly

Table 1: Comparison of EC_{50} and EC_{99} values determined in the standard transmission inhibition assay to MTSA defined sterilizing concentration.

Compound	EC_{50} in entry transmission assay	EC_{99} in entry transmission assay	Sterilizing concentration determined in MTSA	
			Experiment 1	Experiment 2
IQP-0528 (μM)	0.017	1.0	0.25	1.25
IQP-0410 (μM)	0.059	1.0	>12.5	>12.5
IQP-1187 (μM)	0.053	1.0	0.02	0.1
AZT (μM)	>0.5	>0.5	>31.25	>31.25
UC781 (μM)	0.009	2.98	0.37	1.9
CV-N (μg/mL)	0.001	0.1	12.5	12.5
Efavirenz (μM)	0.03	0.5	0.05	0.05
Tenofovir (μM)	>10	>10	>97.7	>97.7

The dual acting (entry inhibition and NNRTI) pyrimidinediones IQP-0528, IQP-0410, IQP-1187 [69] nonnucleoside RT inhibitors UC781 and efavirenz, nucleoside RT inhibitor AZT, entry inhibitor cyanovirin-N (CVN), and nucleotide RT inhibitor tenofovir (TFV) were evaluated in the MTSA, and the sterilizing concentration was compared to the EC_{50} and EC_{90} determined in a standard virus transmission assay. The concentrations utilized for each compound in the MTSA were derived from their respective EC_{50} concentrations in a cytopathic effect assay and their TIs (EC_{50}/TC_{50}). The concentrations which were utilized are as follows: IQP-0528, IQP-0410, and IQP-1187: 10 through 31,250 times the EC_{50} concentration; AZT and UC781: 10 through 31,250 times the EC_{50} concentration; cyanovirin-N: 10 through 6,250 times the EC_{50} concentration; efavirenz: 10 through 31,250 times the EC_{50} concentration; tenofovir: 2.5 through 97.7 times the EC_{50} concentration. All concentrations evaluated represented 5-fold serial increases in drug concentration with the exception of tenofovir which was in 2.5-fold increments. Passages which were positive for virus production were defined by detection of virus in the cell-free supernatant by RT assay. Cells were passaged for 10 passages in the continuous presence of the fixed compound concentration and for an additional 5 passages in the absence of compound. All tested concentrations were significantly below the defined toxic concentration to CEM-SS cells. Passages which were positive for virus production were defined by detection of virus in the cell-free supernatant by RT assay.

The entry assay results used for comparison to the MTSA results were generated from an assay utilizing HeLa-CD4-LTR-β-Gal Cells with HIV-1_{IIIB}. Compound is added to the preplated cells approximately 15 minutes prior to the addition of virus. Following a 2-hour incubation at 37°/5% CO_2, residual virus and compound are removed through washing. The culture is incubated for an additional 48 hours at which time compound efficacy is determined by evaluating β-galactosidase in the lysate using a chemiluminescent endpoint.

higher than the 50% inhibitory concentrations defined in the shorter term and less robust standard transmission inhibition assays most typically employed for microbicide development (Table 1). With more potent inhibitors, the sterilizing concentration may correlate with the 99% inhibitory concentration of a product in the standard inhibition assays. Thus, the MTSA can be utilized to understand how much of an API will be necessary at the site of infection in order to totally suppress virus infection and replication and to prioritize a panel of APIs for clinical development. The MTSA can be miniaturized and the assay duration minimized by utilization of highly sensitive means to determine the amount of virus present in the culture after infection in the presence or absence of the microbicide product (Q-RT-PCR endpoint) or by quantitatively measuring the number of infected cells in the culture. There are, however, limitations to the MTSA which include the inability of the assay to appropriately mimic the complexity of the *in vivo* situation and the current lack of correlative data with clinical trials that have been successful.

It is possible that dosing determination according to the principles of "*more is better*" and "*go as high as you can go*" will not yield the most effective strategy for defining the dose of a microbicide product for product development. With multiple microbicide trials showing lack of efficacy of the potential microbicide products, it is necessary that we understand if the product failure was a function of lack of potency, if the API was not where it needed to be at the right inhibitory concentration, or if API or excipient toxicities could have led to failure by increasing susceptibility, mediated by reduction barrier effectiveness, or by increasing target cells or receptor density. Critical to all three of these potential explanations is how the microbicide is formulated and delivered. First generation microbicides were developed as coitus-dependent gels. Although this delivery mechanism may be useful in some communities, it may prove impractical in developing countries where a woman might not know when she is going to have sexual intercourse, and societal norms are not accepting of microbicide use. Adherence has been an issue in clinical trials utilizing this dosing strategy [71] and may explain the divergent results of the recent tenofovir trials. Based on the successful use of nevirapine to prevent mother-to-child transmission, the CAPRISA 004 trial was designed so that women would use the tenofovir gel within 12 hours prior to having sex and within 12 hours after having sex (BAT24). Although the relative contribution of each gel application to protection from virus transmission is unclear, it was the first trial to demonstrate marginal protection from infection by HIV [7].

Daily dosing is a third strategy to formulate and administer a microbicide. In the VOICE trial subjects were asked to apply the microbicide once a day independent of sexual intercourse [72]. It was hoped that adherence to the regimen would promote a steady-state drug level and that there would be a high adherence rate since administration of the product would become a daily routine, similar to that of oral contraceptives. The vaginal gel arm of the trial was prematurely discontinued because there was no difference in effect demonstrated between the drug-containing gel and a placebo gel [73]. As of the time of this publication, it is not known if

the lack of effect was due to lack of adherence to the protocol design. Daily dosing may also prove to be an "inconvenience" to women who are having infrequent intercourse which may actually diminish adherence to the microbicide [74].

A fourth dosing strategy is sustained microbicide delivery through an intravaginal ring (IVR). Based on the successful use of hormonal contraception rings, microbicide rings can be worn safely for up to a month and have already proven they can deliver drug over that period of time [75, 76]. Although IVRs are designed to deliver optimal concentrations of drug for protection, they may not release drug equal to the amount of drug being released from daily dosed and coitally dependent gels and films. IVRs do address issues of coitus independence and long-term dosing strategies which have been issues of significant research focus in the microbicide community for the past decade.

With the proven concept that the optimal formulation of a microbicide product will assist and promote the uptake/permeability of an API through the epithelium and into the vaginal and rectal mucosa, the mechanisms by which this API facilitation occurs need to studied and monitored to best take advantage of optimization of formulation design. We have shown that the uptake of pyrimidinedione microbicide products is critically dependent on the appropriate formulation of the API (unpublished data). A better understanding of the role of the formulation and delivery mechanisms thus is critically important with regard to achieving adequate fluid and tissue PK/PD and defining the required dose of the microbicide product to deliver the API at the right concentration to the target cells.

As we continue to understand virus transmission and dissemination through the mucosa, better formulations and delivery vehicles can be developed which will in turn allow us to better evaluate where formulated products are delivering API and at what concentration. This will be important in the design of a product that is deemed acceptable to the end user.

5. Development and Formulation of Microbicides for Dual Compartment Use

As mentioned previously, URAI is one of the highest-risk sexual behaviors for HIV-1 transmission—10 to 20 times riskier than unprotected vaginal sex [34, 77]. In addition, RAI is a component of the sexual practices of both men and women, and among women receptive anal and vaginal intercourse often occur within the same sexual encounter. Additionally, there is increasing evidence that unprotected RAI is being practiced at greater frequencies than previously appreciated by both women and men, in both the developing [33, 78] and developed [79] world. Therefore, there is a real need to develop microbicide products to be delivered rectally as an integral part of the HIV prevention portfolio. Since the practice of URAI is not limited to men who have sex with men (MSM), microbicide products suitable for both rectal and vaginal application are highly needed. Use of a single microbicide product that is safe and efficacious for both vaginal and rectal use would thus be much more convenient (as well as safe and acceptable) than the use of two separate products. Furthermore, use of a single, specifically developed dual compartment product would likely be much more protective than improper utilization of a vaginal microbicide in the rectum, which could potentially increase virus transmission or result in significant safety issues. Finally, rectal microbicides are promising in that little behavior modification would be required to add microbicide protection since lubrication is already a common practice with RAI.

There are some profound differences in the vaginal and rectal compartments that warrant the use of differently formulated products for each. Several safety studies have been performed evaluating the toxicity of vaginal gels in the rectal compartment [80–82]. The results of these studies led to the design of microbicides specifically for rectal administration. One of the key differences in the design of these gels is that vaginal microbicides tend to be hyperosmolar resulting in a gel that is more concentrated than body fluid and ultimately one that will lead to rectal mucosal damage as they will swell with rectal application [78]. Rectal microbicides will need to be isoosmolar to circumvent this potential problem. Additionally the surface area requiring protection by a rectal microbicide is much larger than that of the vagina since it is an open cavity, and the microbicide must be formulated so there is adequate protection in the areas where infectious virus and virus-infected cells in semen migrate [78]. This also impacts the design of the delivery applicators for rectal gel products. pH is another important consideration in the design of vaginal and rectal microbicides. The formulation for each product needs to take into account the differences in the pH of the compartment with vaginal pH of approximately 4.5 and a neutral rectal pH [83]. This pH discordance between the compartments could become inconsequential by use of formulations with minimal buffer capacity causing a shift in pH to match the pH of the local fluids. Since results obtained in the tenofovir trial [82] using the vaginal-optimized gel yielded some adverse reactions when used rectally, the gel was reformulated and is now being evaluated as part of MTN-007. *In vitro* and *ex vivo* data indicated that this gel was more suitable for the rectal environment [84]. Physiologically it appears that prevention success will require different gels for different compartments, although it is evident that the formulation of a product for dual compartment use would be most practical and acceptable given the sexual practices of the users embracing both vaginal and anal intercourse during the same sexual encounter. Development of this type of product will require careful consideration and design so that the issues of pH, osmolarity, volume, and delivery between the two compartments are addressed.

6. Multipurpose Prevention Technologies

Multipurpose prevention technologies have appeared as an important topic of discussion in the microbicide community as an unmet need, but little progress has been made in the development and advancement of such a product. Prevention strategies have mostly focused individually on prevention of unplanned pregnancy, prevention of other reproductive tract infections, and prevention of STIs. However,

accumulating data indicate that these issues are linked, suggesting that a woman at risk for pregnancy is also at risk for contracting an STI or other reproductive tract infection [85]. Additionally, a certain stigma is associated with self-identifying as "high-risk" for HIV and STI, and most women are reluctant to do so, even those that are truly at high risk. Linking pregnancy prevention with disease prevention with a single product could aid in the motivation for women to actively obtain and utilize microbicide products. This substantiates the need for Multipurpose prevention strategies that might include combinations of agents that would target prevention of pregnancy and HIV, pregnancy and other STIs, HIV and other STIs and pregnancy, and HIV and other STIs. These products would need to be affordable, acceptable, and easy to use. Significant research on dual-purpose protection technologies that include vaginal spermicidal anti-infective agents and physical barrier devices has been performed [86, 87]. Anti-infective and contraceptive microbicides could be developed in three ways based on utilizing a single drug with dual activity, a combination of a microbicidal compound with a contraceptive agent or a combination of a drug with a device. Relevant technologies do exist including male and female condoms to prevent pregnancy and STIs but they are not widely accepted [85, 88]. Recent advances in microbicide development have also provided a foundation for the development of other Multipurpose prevention products, including multipurpose IVRs and diaphragms with contraceptives and anti-STI microbicides [85, 89] and probiotics to treat and deliver drugs [85, 90], a successful product will likely be dependent on scientific innovation and persistence and will require a concerted scientific and financial effort between many organizations. A product of this nature could have substantial implications on the well-being of women and men throughout the developed and developing world.

7. Microbicide Acceptability and User Perception

Although a variety of studies have addressed the issue of microbicide acceptability and user perception, product acceptability to the end user remains one of the most critical parameters in developing a successful microbicide product. A product with undesirable characteristics will ultimately result in poor adherence, poor PK, and poor efficacy. Morrow and Hendrix have described linkages between acceptability, PK, and toxicity and how each can greatly impact the other [91]. For this reason evaluation of these microbicide properties is now linked in human clinical trials. Thus, acceptability studies are rather well accepted and utilized in the microbicide field and are not identified as a gap in current product development. However the importance of factoring acceptability into each of the gap evaluations described above remains a critical component of microbicide development algorithms and should be addressed early in development. Our current research has involved a better understanding of the user perceptions of different dosing volumes and delivery vehicles on microbicide product acceptability, as well as understanding user's desire for products which have dual compartment use (manuscripts in press).

8. Summary

Great strides have been made in the development of microbicide products to prevent the sexual transmission of HIV. The microbicide development field has exploited strides in the understanding of mechanisms of HIV transmission, which has resulted in better designed and more predictive assays and models to assess the efficacy and toxicity of candidate products. However, significant gaps in understanding still exist which must be better defined and understood in order for the field to define and prioritize new products for clinical evaluation and eventual use as microbicide products. In order for a microbicide to be successful it will need to be the right product in the right place, at the right time, and at the right concentration. Therefore we need to understand how and where HIV infects target cells in the vagina or rectum, the role of cell-free versus cell-associated virus in initial infectious events, and how and where the virus disseminates after initial infection. The vagina and rectum need to be well understood, and microbicide activity needs to be evaluated in the context of these environments early in preclinical development. Better *in vitro* and *ex vivo* assays need to be developed to address the issues of PK and PD as a means to predict the dosing that will be required for a product in clinical trial. Critical to the dosing requirement are the formulation and delivery of the active pharmaceutical ingredient. Finally, a product will only be successful if it is going to be used, and thus the product needs to be acceptable to the end user. The future of microbicides resides in developing products that will work in both the rectum and vagina and those that are multiprevention agents. As the microbicide field evolves, the preclinical assays and models must adapt to the knowledge obtained from successful and failed clinical trials and development programs. In addition, the limitations of these preclinical *in vitro* and *ex vivo* assays should be recognized and used in conjunction with animal models so that the most thorough characterization of a microbicide can be achieved ultimately resulting in prioritization of the microbicides with the most potential. This will allow for better product discovery and development through better preclinical and clinical testing algorithms ultimately resulting in better prioritization of products for clinical evaluation.

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Review Article

The Continuing Evolution of HIV-1 Therapy: Identification and Development of Novel Antiretroviral Agents Targeting Viral and Cellular Targets

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During the past three decades, over thirty-five anti-HIV-1 therapies have been developed for use in humans and the progression from monotherapeutic treatment regimens to today's highly active combination antiretroviral therapies has had a dramatic impact on disease progression in HIV-1-infected individuals. In spite of the success of AIDS therapies and the existence of inhibitors of HIV-1 reverse transcriptase, protease, entry and fusion, and integrase, HIV-1 therapies still have a variety of problems which require continued development efforts to improve efficacy and reduce toxicity, while making drugs that can be used throughout both the developed and developing world, in pediatric populations, and in pregnant women. Highly active antiretroviral therapies (HAARTs) have significantly delayed the progression to AIDS, and in the developed world HIV-1-infected individuals might be expected to live normal life spans while on lifelong therapies. However, the difficult treatment regimens, the presence of class-specific drug toxicities, and the emergence of drug-resistant virus isolates highlight the fact that improvements in our therapeutic regimens and the identification of new and novel viral and cellular targets for therapy are still necessary. Antiretroviral therapeutic strategies and targets continue to be explored, and the development of increasingly potent molecules within existing classes of drugs and the development of novel strategies are ongoing.

1. Introduction

Since the approval of AZT for the treatment of HIV-1 infection, twenty-three additional therapeutic agents have been approved for use in humans [1]. The first drugs approved in the United States to treat HIV-1 infection inhibit the specific activity of the virally encoded reverse transcriptase, the viral enzyme essential for conversion of the viral RNA genome into a DNA provirus that integrates itself into the host genome. Two classes of reverse transcriptase inhibitors are currently marketed—nonnucleoside reverse transcriptase inhibitors (NNRTIs) and nucleoside/nucleotide reverse transcriptase inhibitors (N(t)RTIs) [2]. Another approved and marketed class of HIV-1 antiviral therapeutics inhibits the HIV-1 protease, a viral enzyme required to process newly synthesized viral polyproteins into the mature viral gene products, enabling the virus to assemble itself into new infectious virus particles [3]. A third class of HIV-1 therapeutics inhibits viral infection by preventing virus attachment to the host cell CCR5 chemokine receptor or prevents the fusion of the viral and cellular membranes [4]. Most recently, compounds which prevent the integration of the HIV-1 proviral precursor into cellular DNA have been successfully developed and utilized. Clinical experience with all HIV-1 agents has clearly demonstrated the ability of HIV-1 to easily evade the antiviral effects of any monotherapeutic drug administration strategy through the rapid accumulation of amino acid changes in the targeted proteins—reverse transcriptase, protease, envelope, and integrase [5]. The high turnover rate of virus replication along with the highly error prone HIV-1 reverse transcriptase, with its lack of proofreading capability, generates significant heterogeneity within the highly related but nonidentical populations (or quasispecies) of viruses circulating in a patient [6]. It is widely

accepted that most drug-resistant viruses preexist within the population of viruses and are selected from within this heterogeneous environment upon application of selective drug pressure [7]. In addition to the high levels of resistance possible to single agents, each of the anti-HIV-1 agents employed to date has had significant dose limiting and long-term toxicities that render successful long term therapy for HIV-1 disease difficult to achieve [8].

In much of the developing world, antiretroviral therapy has successfully suppressed HIV-1 replication in patients, allowing significant delays to the progression of AIDS and in some cases completely normal life spans. However, HIV-1 therapies in general are plagued by patient compliance issues reflective of difficult treatment regimens, involving up to four antiretroviral drugs, significant class-specific toxicity [9], and the emergence and spread of virus isolates selected for resistance to single or multiple antiretroviral agents [10]. In the developing world many of these therapeutic strategies are uniformly unavailable due to the prohibitive cost of the drugs. The absence of an effective vaccine and the lack of effective therapy means that sub-Saharan Africa and Southeast Asia, among other developing regions of the world, remain epicenters for the continued spread of HIV-1, especially among heterosexual women [11]. In these areas of extremely high HIV-1 transmission rates, the opportunities to derail the AIDS pandemic rest on the processes of education and the development of effective topical microbicides, a specific HIV-1 prevention strategy employing HIV-1 drugs to prevent the sexual transmission of HIV-1 [12].

2. Identification and IND-Directed Development of New Antiretroviral Agents

The FDA has published guidance documents that relate to the development of systemic HIV-1 inhibitors [1]. These documents define the preclinical pharmacologic data that must be provided in an IND submission to begin human testing of a new antiretroviral agent. The submitted data package must specifically address the efficacy and toxicity of the test compound in a relevant cell-based assay system. In addition studies should be initiated that adequately address the range and mechanism of action of the test compound. With the wide variety of approved anti-HIV-1 drugs already on the market and the demonstrated efficacy of highly active antiretroviral therapies (HAARTs) [13], the ability of test compounds to be utilized as a component of combination drug therapies with the approved HIV-1 drugs should be evaluated in detail. Finally, drug resistance should be evaluated to define the ease of selection of resistant strains and to define diagnostic resistance-engendering mutations prior to clinical trials. Animal models to evaluate the effectiveness of HIV-1 therapies are available but their predictability for clinical efficacy is still highly debated, and thus most drug development programs bypass these animal models and move directly to Phase I human safety trials.

It is clear that highly active antiretroviral therapy (HAART) has significantly decreased morbidity and mortality among patients infected with HIV-1 and has prolonged the life of infected individuals. HAART has transformed HIV-1 infection from a lethal infection to a chronic disease much like diabetes. However, new anti-HIV-1 agents are still needed to confront the emergence of drug resistance and various adverse effects associated with long-term use of antiretroviral therapy. New antiviral agents that inhibit an increasing number of viral and cellular processes are critical for treating infected patients, as well as for prophylactic use, and all possible targets for countering HIV-1 infection, replication, and persistence need to be considered. Finally, efforts to eradicate HIV-1 from latent reservoirs within the body have gained increasing traction with the success of HAARTs and eradication efforts will require novel drugs and new ways of thinking about antiretroviral therapy of latent and silent HIV-1 infection.

The identification of new antiretroviral agents typically involves either cell-based or biochemical/enzymatic target-based screening programs. The end result of these screening programs is a lead compound which provides a pharmacophore for medicinal chemistry structure-activity relationships (SARs) efforts to enhance the potency (increased efficacy and/or reduced toxicity) of the lead molecule, subsequently yielding candidates which progress through the IND-directed clinical development pathway to human clinical trials. Historically, new drug entities have been highly specific virus-targeted agents which inhibited critical steps in HIV-1 replication. Current development efforts continue to exploit the known targets for antiretroviral intervention, but have been expanded to include agents which target cellular processes that are essential for HIV-1 replication. Genomic, proteomic, and metabolomics approaches have identified large numbers of cellular products and pathways that are positively and negatively impacted by HIV infection, providing a large number of potential novel anti-HIV targets [14]. Herein we provide an overview of current and continuing drug development in the HIV-1 field based on the target of the antiretroviral agent as well as an overview of the methodology utilized to identify and confirm the new molecules as potential drug candidates for clinical development. Methods available to identify and characterize the mechanism of action of new antiretroviral agents are summarized in Table 1.

3. HIV-1 Entry Inhibitors

Though a range of hematopoietic cells, including monocyte-macrophages, B lymphocytes, eosinophils, and dendritic cells, as well as columnar epithelial cells, have been found to be infected by HIV-1, the CD4-positive helper T lymphocyte has been identified as the primary target for HIV-1infection [15]. HIV-1 enters CD4-positive T cells through direct interaction of the viral envelope gp120 with the D1 region of the CD4 receptor on the cell surface of target cells. The interaction of gp120 with CD4 causes a conformational change in the viral envelope gp120, resulting in exposure of the gp41 transmembrane envelope protein which subsequently inserts into and fuses with the target cell membrane. HIV-1 envelope proteins interact with coreceptor molecules on the surface of

Table 1: Anti-HIV-1 screening assays.

Replication event	Assay	Method
Virus attachment	HeLa-CD4-LT4-β-gal cells	HeLa-cell based assay measuring reduction in chemiluminescence of HIV-1-infected cells [16]
	TZM-bl cells	
	gp120/CD4 Ab binding inhibition	Cell based HIV-1 neutralization assay
	gp120 : CD4 ELISA	Biochemical assay with soluble CD4 and monoclonal gp120 antibodies [17]
	gp120/CD4/coreceptor	Cell based, temperature sensitive fusion assay
Fusion and chemokine coreceptor interaction	HL2/3 cells + HeLa-CD4-LTR-β-gal cells	Cell based assay measuring reduction in chemiluminesence [16]
	Coreceptor inhibiton	GHOST-cell based assays measuring reduction in virus replication
	Coreceptor typing	PBMC and Macrophage cell-based assays with tropism-specific clinical HIV-1 isolates [18]
	Compound displacement of chemokine ligands	
	Ca^{++} flux	
Reverse transcription	Homopolymer and heteropolymer RT inhibition	Biochemical assay measuring reduction in dGTP-[P32] incorporation [19]
	E/intermediate/late RT products	PCR amplification
	RNaseH inhibition	Biochemical assay [20]
	RT inhibition assays using enzymes with specific mutations	Biochemical dGTP-[P32] incorporation assay [19]
Nuclear localization	2 LTR product in cell nucleus	PCR detection
Integration	Provirus in genomic DNA	PCR detection [21]
	Integrase Complementation	Cell based IN-mutant and Vpr-IN transfection [22]
	Integrase inhibition	Biochemical SPA assay [23]
	Integrase negative virus	
Protein expression	Northern, Western and flow cytometry	Cell based assays with molecular biology endpoints [24, 25]
	Tat, Rev, and Nef inhibition	Biochemical assays [26, 27]
	Cell-based reporter assay for Rev and Tat function	
	Intracellular p24	CEM-SS cells infected with HIV-1 and lysed to quantitate p24 by ELISA
	LTR-mediated transcriptional activation	
Protease	Intracellular and virion protein processing	Cell based assay with Western analysis [28]
	Polyprotein cleavage	Biochemical FRET assay [29]

CD4-positive cells, typically either the α-chemokine receptor CXCR4 or the β-chemokine receptor CCR5 or both, to trigger the fusion of the viral and cellular membranes. The HIV-1 fusion inhibitor, enfuvirtide, developed by Hoffmann-La Roche and Trimeris, was the first therapeutic in its class to be approved for use in humans by the FDA [30]. Enfuvirtide binds to gp41 and prevents the pore formation required for the capsid of the virus to enter the target cell. Since enfuvirtide is a peptide, the drug is marketed in an injectable form, which has somewhat limited its therapeutic utility. In addition, primary resistance mutations in the HR1 region of gp41 have been identified in 10.5% of enfuvirtide-naïve patients which allow the virus to evade the antiviral effects of the drug [31]. The CCR5 coreceptor antagonist, maraviroc, was developed by Pfizer, and the FDA approved maraviroc for combination therapy in 2007 [32]. By blocking the HIV-1 gp120 protein from associating with the CCR5 coreceptor, maraviroc prevents HIV-1 from entering the target cell. However, since HIV-1 can use other coreceptors for entry, an HIV-1 tropism test must be performed to determine if the drug will be effective in a particular patient. CCR5-tropic HIV-1 strains are more common than CXCR4-tropic strains and have been identified as the strain which is predominantly transmitted, suggesting that maraviroc will be useful for both prevention of virus transmission (topical microbicide use) and treatment. Additionally, the CXCR4 coreceptor is more critical for immune function and cannot be safely blocked, indicating that CXCR4-targeted inhibitors would be immune-toxic in the host. Among individuals found mostly in Northern Europe, there is a polymorphism in CCR5, involving a 32-base pair inactivating deletion known as delta32 (Δ32), which reduces or completely eliminates cell surface expression of CCR5 [33]. Individuals with one CCR5-Δ32 polymorphism exhibit reduced disease progression, while those homozygous for the deletion appear to have natural resistance to HIV-1 infection [33]. There appears to

be no obvious immunologic detriment from the Δ32 deletion, making CCR5 a highly credible antiviral target. Other CCR5 antagonists completed or currently in Phase II clinical development include INCB9471 (Incyte), HGS004 (Human Genome Sciences), PRO140 (Progenics), PF232798 (ViiV Healthcare), cenicriviroc (Tobira Therapeutics), and VCH286 (ViroChem Pharma). CCR5 antagonists in Phase I clinical development include AK602 (Kumamoto University), SCH532706 (Schering), GSK706769 (ViiV Healthcare), and VIR576 (Viro Pharmaceuticals). Other entry inhibitors in development include SP01A (Samaritan Pharmaceuticals) in Phase III clinical trials and ibalizumab (Taimed Biologics), a nonimmunosuppressive monoclonal antibody that binds CD4 [34], in Phase II studies. HIV-1 can enter and bud from lipid rafts of plasma membranes of infected cells. Lipid rafts play a crucial role in colocalizing CD4 and chemokine receptors for entry of HIV-1 into T cells. Depletion of plasma membrane cholesterol relocalizes raft-resident markers to a nonraft environment and inhibits productive infection by HIV-1 [35]. SP01A affects cholesterol synthesis by reducing 3-hydroxy-3-methylglutary coenzyme A (HMG-CoA) reductase mRNA expression. Inhibition of cholesterol synthesis by SP01A modifies the cholesterol content of the host cell membrane lipid rafts and prevents HIV-1 fusion with CD4-positive cells. SP10A is a second generation oral entry inhibitor that is being developed by Samaritan Pharmaceuticals [35].

A number of preclinical assays have been developed to identify potential inhibitors of HIV-1 entry utilizing both replication competent wild type viruses and pseudotype viruses. Compounds may be evaluated for inhibitory activity in CD4-dependent and CD4-independent virus transmission assays. A variety of cell lines have been made which stably express CD4, CCR5, or both coreceptors on the cell surface to evaluate coreceptor inhibitors of HIV-1 infection. Evaluations can be performed to define the specific mechanism of antiviral action of compounds which are directly virucidal, or which inhibit virus attachment, or virus-cell fusion, or virus entry to the target cell using indicator cell lines with reporter gene endpoints (colorimetric, chemiluminescent, and fluorescent) to measure virus replication [16]. Compounds which directly interfere with binding of gp120 to CD4 can be evaluated via ELISA using purified proteins [17]. The effect of inhibitors on the virus gp120/CD4/coreceptor complex that would target gp41 can be evaluated using an indicator cell line, such as HeLa-LTR-CD4-β-galactosidase cells which employ a tat protein-induced transactivation of the reporter gene driven by the HIV-1 long terminal repeat promoter, and manipulating the fusion step with temperature changes. Varying the time of drug addition in high multiplicity of infection (MOI), single round of infection anti-HIV-1 assays is often useful in demonstrating that entry inhibitors must be present prior to 2 hours post-infection of target cells in order to provide antiviral activity.

4. HIV-1 Reverse Transcriptase Inhibitors

Inhibitors of HIV-1 reverse transcriptase can be divided into two classes: nucleoside/nucleotide reverse transcriptase inhibitors (NRTI/NtRTIs) and nonnucleoside reverse transcriptase inhibitors (NNRTIs). NRTIs are analogues of the naturally occurring deoxynucleotides required for synthesis of viral DNA and following phosphorylation to the active triphosphate form by cellular kinases; they compete with the natural deoxynucleotides for incorporation into the growing viral DNA chain. However, unlike the natural deoxynucleotide substrates, NRTIs and NtRTIs lack a 3′-hydroxyl group on the deoxyribose moiety or are pseudosugars unable to be extended. As a result, following incorporation of an NRTI or an NtRTI, the next incoming deoxynucleotide cannot form the 5′-3′ phosphodiester bond needed to extend the DNA chain and thus causing chain termination. Mitochondrial toxicity is recognized as a major adverse effect of nucleoside analogue treatment. Nucleoside analogues are effective in inhibiting HIV-1 replication due to their high affinity for the viral RT enzyme. However, NRTIs can also bind to other human DNA polymerases, like DNA polymerase beta, necessary for repair of nuclear DNA, and mitochondrial DNA polymerase gamma, which is exclusively responsible for the replication of mitochondrial DNA. NRTIs and NtRTIs comprise the first class of antiretroviral drugs developed and approved for use in humans to treat HIV-1 infection. There are a number of FDA-approved nucleoside/nucleotide reverse transcriptase inhibitors. Cytidine analogs include zalcitabine (ddC), which is no longer marketed, emtricitabine (FTC), and lamivudine (3TC). Thymidine analogs include zidovudine (AZT) and stavudine (d4T). Didanosine (ddI) and tenofovir disoproxil fumarate (TDF) are analogs of adenosine, and abacavir sulfate (ABC) is an analog of guanine. HIV-1 can become resistant to NRTIs by two mechanisms. The first resistance mechanism involves the reduced incorporation of the nucleotide analog into DNA over the normal nucleotide. This resistance mechanism results from mutations in the N-terminal polymerase domain of the reverse transcriptase that reduce the enzyme's affinity or ability to bind to the drug. A prime example of this mechanism is the M184V mutation that confers resistance to lamivudine (3TC) and emtricitabine (FTC). Another well-characterized set of mutations is the Q151M complex found in multidrug-resistant HIV-1 which decreases reverse transcriptase's efficiency at incorporating NRTIs but does not affect natural nucleotide incorporation. The complex includes the Q151M mutation along with amino acid changes A62V, V75I, F77L, and F116Y. A virus with Q151M alone is moderately resistant to zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), and slightly resistant to abacavir (ABC). A virus with Q151M in concert with one or more of the other four noted mutations becomes highly resistant to those drugs and is additionally resistant to lamivudine (3TC) and emtricitabine (FTC) [36]. A virus with the Q151M complex in addition to the K70Q mutation significantly enhanced resistance to several approved NRTIs and also resulted in 10-fold resistance to TDF [37]. The K65R mutation emerges in response to treatment with TDF, ABC, ddI, or d4T and has been shown to have an increased frequency in subtype C HIV-1 [38]. The second resistance mechanism involves the ATP-based excision of the incorporated drug by $3' \rightarrow 5'$ exonuclease activity, which allows

the DNA chain to be extended and polymerization to continue [39]. Excision enhancement mutations, typically M41L, D67N, K70R, L210W, T215Y/F, and K219E/Q, are selected by thymidine analogs AZT and D4T and are therefore referred to as thymidine analog mutations (TAMs). The excision-based mutations improve the ability of the RT to bind ATP. ATP-dependent pyrophosphorylation removes the drug and releases a dinucleotide tetraphosphate. The goal of next generation reverse transcriptase inhibitors is to treat patients with multidrug-resistant HIV-1, prolong the time to emergence of drug resistance to the new inhibitors, and to increase drug adherence by minimizing pill burden and side effects. Several NRTIs are in development to treat HIV-1-infected patients. Entecavir (ETV), a guanine analog for HIV-1 infection, is currently in development by Bristol-Myers Squibb and has been FDA approved for treatment of HBV infection since 2005. Apricitabine (ATC), a cytidine analog with antiviral activity against 3TC and AZT-resistant HIV-1 being developed by Avexa Pharmaceuticals, was given fast track approval by the FDA in March 2011. Dexelvucitabine (DFC) and racivir are cytidine analogs in development by Pharmasset. DFC is active against drug-resistant HIV-1 containing the M184V, K65R, L74V and TAMS mutations. However, Incyte discontinued co-development of DFC due to increased incidence of grade 4 hyperlipasemia, a marker of pancreatic inflammation, in a Phase IIb clinical trial. Racivir has completed a Phase II clinical trial in comparison with lamivudine in patients with the M184V lamivudine-resistant virus. Elvucitabine is a cytosine nucleoside analog of stavudine which was evaluated in a Phase II clinical trial by Achillion Pharmaceuticals. Unimpressive clinical results did not provide a rationale for further development of the drug. Another derivative of stavudine, festinavir, is being developed by Bristol-Myers Squibb and has antiviral activity against multidrug-resistant HIV-1 with less toxicity compared to stavudine. Chimerix, Inc. developed a lipid conjugate of tenofovir and unlike tenofovir, disoproxil fumarate and most prodrugs, the CMX157 prodrug is not efficiently cleaved in plasma thus increasing the levels of active tenofovir in target cells. CMX157 is greater than 300 times more potent than tenofovir with increased oral bioavailability [40]. Following a favorable Phase I clinical trial, Chimerix is seeking to outlicense the compound for further development. Another prodrug of tenofovir, GS-7340, is being developed by Gilead Sciences to better target lymphoid tissues and cells [41]. GS-7340 has increased plasma stability compared with tenofovir. A recent Phase I clinical trial resulted in no serious adverse effects. Investigators at the University of Georgia identified 1-(β-D-dioxolane) thymine (DOT) as a potent inhibitor of AZT- and 3TC-resistant HIV-1 strains, and this compound is currently in a Phase I clinical trial [42]. Medivir is developing MIV-210, a nucleoside analog with potent antiviral activity versus drug resistant HIV-1 as well as hepatitis B virus. Following favorable plasma levels of MIV210 and good oral bioavailability in Phase I studies, a Phase IIa clinical trial has been initiated with multidrug-resistant HIV-1 infected patients.

In contrast, to the NRTIs and NtRTIs, NNRTIs have a completely different mode of action. NNRTIs allosterically block reverse transcriptase by binding at a different site on the enzyme as compared to the chain terminating analogs. NNRTIs are not incorporated into the viral DNA but instead inhibit the movement of protein domains of reverse transcriptase essential for DNA synthesis. Since the hydrophobic binding area found in HIV-1 reverse transcriptase does not appear in HIV-2, NNRTIs are specific to inhibition of HIV-1 replication. NNRTIs do not bind to the active site of the polymerase but bind to a less conserved area near the active site in the p66 subdomain. NNRTI binding results in a conformational change in the reverse transcriptase that distorts the positioning of the residues that bind DNA, inhibiting polymerization. NNRTI resistance is conferred by mutations that decrease the binding of the drug to this pocket. Treatment with a regimen including efavirenz (EFV) and nevirapine (NVP) typically results in the appearance of mutations L100I, Y181C/I, K103N, V106A/M, V108I, Y188C/H/L, and G190A/S. Current FDA-approved NNRTIs also include delavirdine (Pfizer) and three diarylpyrimidines developed by Tibotec Therapeutics, dapivirine, etravirine and rilpivirine. The second-generation NNRTIs by Tibotec have better potency, longer half-life, and reduced side effects compared with the older NNRTIs, such as efavirenz. Delavirdine is not recommended for use as part of initial therapy due to its lower efficacy compared to other NNRTIs, interactions with other medications due to its inhibition of CYP3A4, and higher pill burden. As patients live longer on HAART and the pool of NNRTI-resistant virus increases, so does the need for the development of new NNRTIs with antiviral activity against both wild-type and the clinically prevalent NNRTI-resistant HIV-1 strains. Boehringer Ingelheim has presented data on BILR355BS, a dipyridodiazepinone NNRTI compound, with potent antiviral activity ($EC_{50} < 10$ nM) against a wide range of NNRTI-resistant viruses but terminated drug development during the Phase II clinical trial [43]. GSK2248761, belonging to the family of 3-phosphindoles, was developed by ViiV Healthcare and completed Phase II studies, but the FDA put further development on hold due to significant adverse events (seizures). It is unclear if or when development will continue. RDEA806, a new family of triazole NNRTIs, entered Phase IIb clinical trials by Ardea Biosciences in 2009. Lersivirine, developed by Pfizer, belongs to the pyrazole family and completed Phase IIb studies in 2010. The resistance profile for compounds in development is similar to that of other next generation NNRTIs. ImQuest Pharmaceuticals has recently reported a pyrimidinedione NNRTI with highly potent anti-HIV-1 activity and a dual mechanism of action which also involves the inhibition of virus entry [18]. Their lead compound (IQP-0528) is expected to soon enter human clinical trials for both therapeutic and topical microbicide use.

Inhibition of the virus-encoded reverse transcriptase can be evaluated in both cell-based and biochemical assays. High MOI and time of drug addition anti-HIV-1 assays are often useful in demonstrating that RT inhibitors must be present prior to 8 hours postinfection of target cells in order to yield antiviral activity. In cell-based assays, PCR amplification of early, intermediate, and late RT products may be analyzed in treated, HIV-1-infected cells to determine inhibition

of enzymatic activity compared to an untreated, infected cell culture. A biochemical assay utilizing purified, recombinant RT enzymes can also be used to identify inhibitors of wild type and drug-resistant HIV-1 reverse transcriptase [19].

5. HIV-1 RNase H Inhibitors

The ribonuclease H (RNase H) function of the C terminus of reverse transcriptase is required for successful production of a DNA copy of the HIV-1 genome. RNase H is required for processing the tRNA primer used to begin minus-strand DNA synthesis and degradation of the viral RNA during synthesis, followed by preparation of the polypurine tract (PPT) DNA-RNA hybrid, which serves as the primer for positive-strand DNA synthesis. Essential for RNase H activity is a group of three carboxylate-containing amino acid residues, conserved in the class of polynucleotidyl transferases and a fourth conserved in RNase H [44]. RT-RNase H is absolutely essential for HIV-1 replication and is therefore a logical and thus far unexploited target for antiretroviral intervention. Drug discovery efforts focusing on RT-RNase H have lagged behind those for other HIV-1 targets but are ongoing. Nucleoside and nonnucleoside compounds have been reported to inhibit both the polymerase and RNase H activities, though the mechanism of RNase H inhibition is poorly understood. Studies have shown that the NRTI AZT and the NNRTI EFV act in a synergistic fashion (together they inhibit RT function to a greater extent than the sum of their individual inhibitory activities). It has been demonstrated that RT inhibition by EFV may allow the innate RNAse H activity of RT to cleave the RNA template, which, in turn, increases susceptibility to AZT, yielding a synergistic antiviral interaction of the two drugs [45]. AZT incorporates into the growing DNA chain, stopping reverse transcription unless it is excised. In the presence of EFV, RNase H activity of RT is enhanced, leading to destruction of the RNA template before AZT excision can efficiently occur, increasing the apparent activity of AZT [46]. An obstacle to the development of RNase H inhibitors was highlighted in a study of β-thujaplicinol [47] that measured cleavage of RNA by RNAse H. β-thujaplicinol efficiently cleaved RNA strands; however, in the context of reverse transcriptase tightly bound to the RNA substrate, the conformational change during reverse transcription resulted in β-thujaplicinol being unable to inhibit RNase H. This suggested that RNase H inhibitors, such as β-thujaplicinol and dihydroxyl benzoyl naphthyl hydrazine (DHBNH) [47], that bind directly to the RNase H active site within RT might have difficulty accessing this site during transcription when RT is bound to an RNA template. RNase H inhibitors that do not bind in the active site of RNase H within HIV-1 RT, such as the MK3 naphthyridine [48], should be explored, and potential antagonism with other RT inhibitors will need to be addressed. RNase H proteins are native to all forms of life, so building inhibitor specificity toward HIV-1 RNase H without off-target effects will be critical to developing an effective drug.

Inhibitors of RNase H function have been identified using a biochemical polymerase-independent cleavage assay with a 5′-[^{32}P]tC5U/p12 substrate [20]. The radioactive RNA-DNA chimera is hybridized to its DNA complement, which mimics processing of the HIV-1-1 PPT primer from nascent DNA, following initiation of second-strand synthesis. Capillary electrophoresis is used to illustrate RNase H cleavage at the PPT RNA-U3 DNA junction and at two additional positions.

6. HIV-1 NCp7 Inhibitors

HIV-1 p7 nucleocapsid protein (NCp7), which contains two highly conserved zinc fingers with a nonclassical Cys-Xaa2-Cys-Xaa4-His-Xaa4-Cys (CHHC) sequence, is a maturational proteolytic product of the p55 precursor polyprotein [49]. The zinc fingers function in selection and incorporation of viral RNA into budding virions while being a component of the p55 precursor. Zinc fingers of the NCp7 are required for the initial infection of target cells, promote initiation of transcription, and increase the efficiency of template switching during reverse transcription. Due to the essential and pluripotent roles in both early and late stages of HIV-1 replication, as well as the conserved Cys and His chelating residues, the HIV-1 zinc fingers represent attractive antiviral targets and would appear to be multifunctional inhibitors of HIV-1. Disulfide-substituted benzamides (DIBAs) were identified as anti-HIV-1 inhibitors with the ability to chemically modify and eject zinc from the zinc finger of NCp7. Antiviral activity of the DIBAs resulted in the formation of noninfectious virus or in the complete inhibition of virus production *in vitro*, similar to HIV-1 protease inhibitors. Azodicarbonamide (ADA; HPH116) is a nucleocapsid inhibitor that electrophilically attacks the sulfur atoms of the zinc-coordinating cysteine residues of the CCHC domain [50]. ADA is directly virucidal by preventing the initiation of reverse transcription and blocking formation of infectious virus by modification of the CCHC domain within Gag precursors. ADA was evaluated in a Phase II study in 2001, but the status of drug development is unknown. S-acyl-2-mercaptobenzamide thioesters (SAMTs) demonstrate potent antiviral activity *in vitro* as a virucidal agent and in *in vivo* SIV studies in Cynomolgus macaques [51]. NV038, a N,N′-bis(1,2,2-thiadiazol-5-yl)benzene-1,2-diamine, targets NCp7 by reacting with the sulfhydryl group of cysteine residues. NV038 acts via a different mechanism than other reported zinc ejectors, as its structural features do not allow an acyl transfer to Cys or a thiol-sulfide interchange [52]. Studies performed at ImQuest BioSciences have demonstrated a significant inability to select for drug resistant viruses to the zinc finger inhibitors as well as their highly synergistic interaction with all classes of antiretroviral agents.

NCp7-targeted inhibitors have been shown to be virucidal *in vitro*. Cell-free virus is treated with compound then washed away prior to incubation with target cells to demonstrate reduction in virus infectivity [51]. In addition, the zinc finger inhibitors reduce virus production from chronically HIV-1-infected cells. Zinc ejection from purified NCp7 protein can also be assessed biochemically in the presence of inhibitors [52]. Specificity of NCp7 inhibition for the retroviral zinc finger should be addressed by evaluating

the interaction of inhibitors with cellular Sp1, GATA, and PARP zinc fingers.

7. HIV-1 Integrase Inhibitors

Integrase is a viral enzyme that integrates retroviral DNA into the host cell genome. HIV-1 integration occurs through a multistep process that includes two catalytic reactions: 3′ endonucleolytic processing of proviral DNA ends and integration of 3′-processed viral DNA into cellular DNA, referred to as strand transfer. The 3′ processing integrase binds to a short sequence located at either end of the long terminal repeat (LTR) of the viral DNA and catalyzes endonucleotide cleavage, resulting in elimination of a dinucleotide from each of the 3′ ends of the LTR. Cleaved DNA is then used as a substrate for integration. Strand transfer occurs simultaneously at both ends of the viral DNA molecule, with an offset of five base pairs between the two opposite points of insertion. Integration is completed by removal of the unpaired dinucleotides, repair of the single-stranded gaps created between the viral and target DNA, and ligation of the host DNA. Divalent metals, Mg^{2+} or Mn^{2+}, are cofactors required for 3′-processing and strand transfer steps. Raltegravir, the first integrase inhibitor developed by Merck Sharp & Dohme Limited, was FDA approved for use in HIV-1-infected patients in 2007. Other HIV-1 integrase inhibitors currently in Phase III clinical trials include elvitegravir, developed by Japan Tobacco, and dolutegravir, developed jointly by ViiV Healthcare and Shinongi. Raltegravir and elvitegravir possess metal-chelating functions and interact with divalent metals within the active site of HIV-1 integrase. The inhibitors compete directly with viral DNA for binding to the integrase active site at the DDE motif, a highly conserved triad of acidic residues consisting of D64, D116, and E152 which mediate binding of the metal cofactors to the active site, in order to block strand transfer [53]. Two structural components are necessary for integrase binding: a hydrophobic benzyl moiety that buries into a highly hydrophobic pocket near the active site and a chelating triad that binds with two Mg^{2+} ions in a hydrophilic region, anchoring the inhibitor onto the protein surface. Identification of the pharmacophore for inhibition of HIV-1 integrase catalysis has proven to be challenging. For optimal integrase inhibition, the pharmacophore requires a region-specific (N-1) diketoacid of specific length [54]; however, a detailed binding model is lacking, so it has been difficult to develop structure-based design of integrase inhibitors. HIV-1 resistance to raltegravir and elvitegravir has been associated with mutations in the loop of amino acid residues 140–149. Raltegravir has limited intestinal absorption, and thus resistance cannot be overcome by prescribing higher doses. The integrase inhibitor dolutegravir is sensitive to HIV-1 variants resistant to raltegravir or elvitegravir, is bioavailable as a single, oral dose without need of a booster, and has been well tolerated by patients in clinical trials. Clinical trials are underway to support the use of dolutegravir in combination with abacavir and lamivudine, in a new fixed dose combination called 572-Trii. GSK1265744 is in Phase IIa human clinical trials as a new generation candidate to dolutegravir. Merck has developed a second generation integrase inhibitor, MK-2048, with the same mechanism of action as raltegravir with sensitivity to raltegravir-resistant HIV-1 [55]. MK-2048 is being investigated for use as part of preexposure prophylaxis (PrEP) regimen and has been shown to inhibit the integrase enzyme four times longer than raltegravir. BI224436 is in preclinical development by Gilead Sciences following its purchase from Boehringer Ingleheim as a novel noncatalytic site integrase inhibitor that binds to a conserved allosteric pocket of the HIV-1 integrase enzyme [56]. BI224436 has been shown to retain full antiviral activity against viruses encoding resistance mutations to clinically approved drugs targeting HIV-1 integrase. BI224436 has advanced to Phase I clinical trials following ADME evaluations which indicated favorable metabolic stability, low potential for interactions with CYP3A4 and CYP2D6, high permeability, excellent physicochemical properties, and excellent pharmacokinetic profiles in animals.

Structural studies utilizing cocrystallization with prototype foamy virus (PFV) intasome with raltegravir and elvitegravir have been helpful in establishing the binding mode of integrase strand transfer inhibitors. Crystal structures of PFV intasomes containing primary mutations associated with drug resistance, as well secondary amino acid substitutions which may compensate for the impaired viral fitness, revealed conformational rearrangements within the IN active site contributing to raltegravir resistance [21]. Integration of the 2-LTR circular cDNA into the host DNA mediated by the virus-encoded integrase can be evaluated for inhibition in both cell-based and biochemical assays. In a high MOI single-round HIV-1 infection in cells, PCR detection of the provirus in genomic DNA can be assessed. Amersham produces an HIV-1 integrase scintillation proximity assay (SPA) enzyme kit for biochemical evaluation of potential integrase inhibitors [23]. An *in vitro* assay utilizing integrase-mutant HIV-1 molecular clones complemented in trans by Vpr-IN fusion proteins enabled the study of integrase function in replicating viruses [22].

8. HIV-1 Regulatory and Accessory Protein Inhibitors

After integration into the host genome, HIV-1 remains quiescent until basal transcription produces a threshold level of the viral transactivator protein, Tat. Tat increases viral mRNA production several hundredfold by increasing the elongation capacity of RNA polymerase II (Pol II) rather than initiation of transcription. Tat is brought into contact with the transcription machinery after binding the transactivation-responsive (TAR) element, a 59-residue stem loop RNA found at the 5′ end of all HIV-1 transcripts. Tat forms a tight, specific complex with TAR RNA centered on a U-rich region found near the apex of the TAR RNA stem. Interactions between Tat and TAR are absolutely required for the increased processivity of Pol II and the production of full length virus transcripts. Tat binds to the cyclin-dependent kinase 7 (CDK7) and activates the phosphorylation of the carboxy-terminal domain of Pol II by TFIIH and the CDK-activating kinase (CAK) complex [57]. Studies suggest

the interaction between Tat and its cellular counterpart is critical for the function of Tat and the increased processivity of Pol II. Oligonucleotides have been investigated for inhibition of Tat binding to this recognition site in biochemical assays, but they failed to disrupt HIV-1 replication in acute infection of primary lymphocytes [58]. Natural 4-phenylcoumarins isolated from *Marila pluricostata* were identified as Tat antagonists and were able to inhibit HIV-1 replication in cell-based assays [24]. Based on the beta-turn motif present in HIV-1 Tat, a series of novel benzodiazepine analogs were designed as biological mimetics. Preliminary biological evaluation exhibited inhibitory activity on HIV-1 Tat-mediated LTR transcription [59]. BPRHIV001, a coumarin derivative, has been identified as an HIV-1 Tat transactivation inhibitor (EC_{50} of 1.3 nM) with synergistic effects in combination with currently used reverse transcriptase inhibitors [60].

The Rev protein is an essential factor for HIV-1 replication and promotes the export of unspliced or partially spliced mRNA responsible for the production of the viral structural proteins. Within the N-terminal of Rev is the arginine-rich motif (ARM) which comprises both the nuclear localization signal (NLS) to mediate the nuclear and nucleolar localization of Rev and the RNA-binding domain to mediate binding of Rev to the Rev-Responsive Element (RRE), a 240-base region of complex RNA secondary structure. Flanking the ARM are sequences involved in mediating Rev multimerization that appears to be critical for its biological role. Polymerized Rev that interacts with host cellular factors is a prerequisite for RNA binding. The interaction between the HIV-1 Rev protein and the RRE RNA is an attractive target for antiviral therapy due to its role in facilitating the nuclear export of incompletely processed viral transcripts and its necessity for viral replication. For HIV-1, targeting the host cell factors might elicit fewer drug-resistant viruses. Screening for Rev inhibitors is in the early preclinical drug development stage, and various researchers have targeted the nuclear export factor CRM1, interference with the Rev-RRE interaction, Rev protein itself, and other cellular factors involved in HIV-1 transcription [26]. Leptomycin B (LMB), a *Streptomyces* cytotoxin discovered as a potent antifungal antibiotic that blocks the eukaryotic cell cycle, binds CRM1 and disrupts NES-mediated nuclear transport [61]. Variability in LMB production lots in *Streptomyces* cultures that vary greatly in toxicity has hampered the use of LMB. PKF050-638 is also capable of blocking Rev function by binding to CRM1 at position Cys-539 but its cellular toxicity resulted in the failure to pursue its potential as a therapeutic [62]. Neomycin B is capable of interfering with the Rev-RRE interaction, but poor efficacy (EC_{50} of 2.5 mM), toxicity, and poor oral absorption have prevented its development as a useful antiviral drug [63]. Diphenylfuran cations have also been shown to interfere with the Rev-RRE interaction *in vitro* at 0.1 μM concentrations. These aromatic cationic compounds bind tightly to the minor groove of the IIB Rev motif with pronounced selectivity [64]. Antisense oligonucleotides which interact with RRE-IIB have also been investigated and found to bind with specificity and high affinity with apparent dissociation constants in the nanomolar range [65]. Thiabendazole, chlorpropham, and a series of related analogs which inhibit HIV-1 at a late stage, postintegration step of virus replication were identified by The Proctor & Gamble Company and are being investigated by ImQuest BioSciences. The compounds were identified as inhibitors of HIV-1 replication from chronically HIV-1-infected cells with the ability to suppress constitutive virus production in the long term. Mechanistic studies indicate the treatment of infected cells with these compounds results in an accumulation of multiply spliced viral RNA, with a corresponding decrease in the quantity of singly spliced and unspliced viral RNA, suggesting the compounds may inhibit Rev function.

A novel mechanism of antiviral action recently exploited by Trana Discovery involves human transfer RNA (tRNA) as a therapeutic target. The role of $tRNA^{Lys3}_{SUU}$ is essential for the replication and survival of HIV-1 at both reverse transcription as a primer and virus assembly, thereby providing a dual point of intervention by tRNA inhibitors. Efforts to inhibit the $tRNA^{Lys3}_{SUU}$ have centered on mimicking the anticodon stem loop (ASL) of tRNA to prevent binding of viral RNA [66].

Nef is a multifunctional accessory protein of HIV-1 which is critical for high virus replication and disease progression in infected patients. The lack of disease progression in patients infected with *nef*-deleted HIV-1, such as the Sydney Blood Bank Cohort comprised of eight individuals infected with an attenuated, nef/LTR-deleted strain of HIV-1 from a single donor, defines Nef as a pathogenic factor [67]. Developing inhibitors of Nef in order to reduce the severity of HIV-1 disease has been difficult due to the complexity of Nef's multiple functions. Nef is a small protein devoid of enzymatic activity that serves as an adaptor protein to divert host cell proteins to aberrant functions that amplify viral replication. Investigation of Nef function has led to the possibility of developing new anti-HIV-1 drugs targeting Nef's ability to induce CD4 downmodulation, major histocompatibility complex I and II (MHCI/MHCII) downmodulation, Pak2 activation, inhibition of p53 and ASK-1 involved in apoptosis, and enhancement of virion infectivity. Nef-induced CD4 downmodulation involves the internalization of surface CD4 followed by degradation via the endosomal/lysosomal pathway. Inhibition of lysosomal acidification blocks Nef-induced CD4 degradation, without restoring CD4 surface expression. The clathrin-associated adaptor protein 2 (AP2) is a key molecular mediator of Nef-induced CD4 downmodulation, suggesting this interaction is a possible target for antiviral therapy [68]. Another well-conserved property of Nef is its ability to downmodulate MHC class I molecules that enables the infected cell to evade destruction by the immune system during active viral replication. A ternary complex between the cytoplasmic tail of MHC and AP1, with Nef acting as a facilitator, may activate a tyrosine sorting signal in the MHC which diverts newly synthesized MHC molecules from their transit to the plasma membrane to an internal compartment. This ternary complex engages Nef in a novel interaction and could be a potential target for an antiviral compound. Nef may regulate cellular activation through several kinases, such as Pak2 and Hck. Nef binding

with Pak2 has been demonstrated to activate Pak2 in multiple HIV-1 subtypes. However, the structural fluidity of Nef's Pak2 interaction surface could make this Nef interaction difficult to target with antiviral compounds. Structure-function analyses identified an SH3 domain interaction of Hck that interacts with Nef. A series of small Nef interacting proteins composed of an SH3 domain fused to a sequence motif of the CD4 cytoplasmic tail and a prenylation signal for membrane association were investigated [25] and identified two hydrophobic pockets on Nef as potent pharmacophore target sites. Nef augments the infectivity of HIV-1 particles and accounts for the slight delay in replication kinetics observed for *nef*-deficient HIV-1. Triciribine (TCN) is a tricyclic nucleoside that once phosphorylated to its 5′ monophosphate form by intracellular adenosine kinase is active against a wide range of HIV-1 and HIV-2 isolates. TCN was determined to be a late stage inhibitor of HIV-1 replication, and sequencing of TCN-resistant HIV-1 resulted in five-point mutations in the DNA sequence of *nef* [27]. Originally developed as an anticancer therapy, clinical trials indicated severe adverse toxicity with TCN such as hepatic toxicity, hyperglycemia, and thrombocytopenia [69]. Despite the attractiveness of a drug that reduces the inherent infectivity of HIV-1 virions, the prospects for inhibiting Nef-mediated enhancement of infectivity are remote. Overall attempts to develop inhibitors of Nef have demonstrated relatively low binding affinity, high cytotoxicity, and interference with only a subset of Nef interactions and functions.

Viral protein U (Vpu) is a type 1 membrane-associated accessory protein encoded by HIV-1 and functions to form a virus ion channel. Vpu contributes to HIV-1-induced CD4 receptor downregulation by mediating the proteosomal degradation of newly synthesized CD4 in the endoplasmic reticulum. Vpu also enhances the release of progeny virions from infected cells by antagonizing tetherin, an interferon-regulated host restriction factor that directly cross-links virions on the host cell surface [70]. BIT225 was developed by Biotron Limited as a small molecule inhibitor of HIV-1 Vpu to specifically target HIV-1 in the monocyte-macrophage reservoir, similar to tetherin-mediated reduction in infectivity [71]. BIT225 is active against multiple drug-resistant strains of HIV-1, and Phase IIb clinical trials are currently in progress.

Vpr is a multifunctional accessory protein critical for efficient viral infection of CD4-positive T cells and macrophages. Vpr mediates nuclear transport of the HIV-1 preintegration complex (PIC), induces G2 cell cycle arrest, modulates T-cell apoptosis, transcriptionally coactivates viral and host genes, and regulates nuclear factor kappa B (NF-κB) activity [72]. The numerous functions of Vpr in the viral life cycle suggest that Vpr would be an attractive target for HIV-1 therapeutics. Di-tryptophan containing hexameric peptides have been reported to overcome Vpr-mediated cell growth arrest and apoptosis by interfering with nuclear translocation [73]. Damnacanthal (Dam), an anthraquinone derivative isolated from the Tahitian noni fruit, has been identified as an inhibitor of Vpr-induced cell growth cessation [74]. Vipirinin, a 3-phenyl coumarin-based compound in the RIKEN Natural Products Depository, inhibits Vpr-dependent viral infection of human macrophages. The hydrophobic region of residues Glu-25 and Gln-65 was found to be potentially involved in the binding of vipirinin to Vpr [75].

Viral infectivity factor (Vif) is a small, phosphoprotein essential for HIV-1 replication and pathogenesis. Vif neutralizes the host cell antiviral factor, apolipoprotein B mRNA editing enzyme catalytic polypeptide like 3G (APOBEC3G; A3G), which makes the viral particles more infective [76]. RN-18 was identified as an antagonist of Vif function and inhibited HIV-1 replication only in the presence of A3G. RN-18 increases cellular A3G levels in a Vif-dependent manner and increases A3G incorporation into virions without inhibiting general proteasome-mediated protein degradation in order to decrease virus replication [77].

The expression of HIV-1 regulatory proteins occurs early in the infected cell and is critical for appropriate replication of the virus. The ability of an anti-HIV-1 agent to inhibit these regulatory proteins can be evaluated in cell-based reporter assays, analyzed by Northern or Western blot, and by direct biochemical inhibition assays [24–27, 70, 73, 77].

9. Protease Inhibitors

HIV-1 aspartyl protease is a C2-symmetric homodimer that catalyzes the proteolytic cleavage of the polypeptide precursors into mature enzymes and structural proteins. Inhibitors have been designed to mimic the transition state of the protease substrates. A peptide linkage consisting of –NH–CO– is replaced by a hydroxyethylene group, where the protease is unable to cleave. Mutations that confer resistance to HIV-1 protease inhibitors are located primarily in the active site of the enzyme that directly changes the binding of the inhibitor. Nonactive site mutations have been shown to alter dimer stability and conformational flexibility. Over 26 protease inhibitor-specific mutations have been described, of which 15 are primary mutations significant enough to reduce drug efficacy. High-level drug resistance typically requires multiple mutations in the HIV-1 protease. Often, these resistance-associated mutations reduce the catalytic efficiency of the protease, resulting in immature or noninfectious viruses. In addition, mutations develop within Gag cleavage sites, complementing the changes in the resistant protease. Significant associations have been observed between mutations in the nucleocapsid-p1 (NC-p1) and the p1-p6 cleavage sites and various mutations in protease associated with protease inhibitor resistance [78]. Gag A431V or the I437V mutation, within the NC-p1 cleavage site, has been associated with the V82A, I50L, or I84V protease mutations. Gag L449F/P, R452S, P453L mutations within the p1-p6 cleavage site have been associated with I50V or D30N/N88D protease mutations. Cross-resistance is one of the major problems of protease inhibitor treatment. FDA-approved protease inhibitors saquinavir (Hoffman-La Roche), ritonavir (Abbott Laboratories), and indinavir (Merck) are peptidomimetic compounds designed to fit the C2 symmetry in the protease-binding site. Nelfinavir (Agouron Pharmaceuticals) was the first nonpeptidomimetic compound designed to contain

a novel 2-methyl-3-hydroxybenzamide group. Amprenavir (GlaxoSmithKline) is an N,N-disubstituted aminosulfonamide nonpeptide inhibitor with enhanced aqueous solubility compared to previous protease inhibitors and was later replaced on the market with its prodrug, Fosamprenavir, which resulted in lower pill burden. Lopinavir (Abbott Laboratories) is a peptidomimetic protease inhibitor designed for activity against drug-resistant HIV-1 containing mutations at the Val82 residue. Atazanavir (Bristol-Myers Squibb) is an azapeptide protease inhibitor designed to fit the C2 symmetry of the enzyme-binding site and is unique to other PIs as it can only be absorbed in an acidic environment. The resistance profile of atazanavir is also better than previous protease inhibitors. Tipranavir (Boehringer Ingelheim), a nonpeptide inhibitor of protease, was developed from a coumarin template and possesses broad antiviral activity against multiple protease inhibitor-resistant HIV-1. Darunavir (Tibotec, Inc.) is a nonpeptide analog of amprenavir with a critical change at the terminal tetrahydrofuran group, allowing for antiviral activity against amprenavir-resistant HIV-1. Research on new protease inhibitors is directed towards the development of compounds that will not be cross-resistant with other PIs, have a favorable metabolic profile, will not require boosting by RTV, and have a low once-daily pill burden. GlaxoSmithKline discontinued Phase II clinical development of brecanavir due to insurmountable issues regarding formulation. In 2009, GlaxoSmithKline and Concert Pharmaceuticals entered into a collaboration to develop deuterium-containing drugs. CTP518, an analog of atazanavir produced by replacing key hydrogen atoms with deuterium, demonstrated slow hepatic metabolism resulting in an increased half-life and entered Phase I studies in 2010 [79]. CTP518 has the potential to eliminate the need to codose with a boosting agent, such as ritonavir. TM310911, developed by Tibotec Therapeutics, is in Phase II clinical trials with a ritonavir booster. SPI-256, developed by Sequoia Pharmaceuticals in 2008, demonstrated significant potency and a high genetic barrier to resistance *in vitro*. A Phase I study demonstrated safety and tolerability in humans, but SPI-256 development was recently discontinued. SPI-452, a PK enhancer in development by Sequoia Pharmaceuticals, has been shown to increase plasma concentrations of atazanavir and darunavir in Phase I studies without the side effects typically seen with ritonavir as a boosting agent [80]. Cobicistat (GS 9350) by Gilead is a pharmacoenhancer based on CYP3A inhibition, and it represents the PK enhancer in the most advanced development phase. Cobicistat tested against ritonavir with atazanavir plus TDF/FTC and Quad in combination with cobicistat and elvitegravir are all currently in larger Phase III studies.

Cell-based and biochemical assays are available to evaluate the ability of a compound to inhibit the enzymatic cleavage of viral polyproteins by HIV-1 protease. An HIV-1 protease fluorescence resonance energy transfer (FRET) assay kits are commercially available for biochemical evaluation of potential protease inhibitors [29]. HIV-1 protease activity can be monitored in human cells based on expression of a precursor protein harboring the viral protease fused to the reporter protein GFP [28]. Western analysis of intracellular and virion protein processing can be utilized as well to evaluate HIV-1 protease inhibition.

10. Myristoylation Inhibitors

HIV-1 Gag is synthesized in the cytosol as a precursor protein, p55, and is targeted to the plasma membrane where particle assembly and packaging of viral genomic RNA occur. Modification of p55 at the N-terminal glycine residue with myristic acid, a saturated 14-carbon fatty acid, is essential for targeting p55 to the plasma membrane for HIV-1 assembly. Gag myristoylation consists of two reactions: activation of myristic acid to myristoyl-CoA by acyl-CoA synthetase and transfer of the myristoyl group from myristoyl-CoA to the N-terminal glycine of p55 by N-myristoyltransferase (NMT). Several studies have considered NMT as a potential drug target for the inhibition of HIV-1 assembly. NMT inhibitors have been shown to prevent both membrane binding of Gag as well as virus assembly [81]; however, NMT inhibitors are expected to affect a broad spectrum of cellular processes that depend on protein N-myristoylation for membrane binding. Heteroatom-substituted myristic acid analogs, such as 12-methoxydodecanoic acid, can be used by NMT as alternative substrates for covalent attachment to proteins. The hydrophilic nature of these compounds inhibits membrane binding and function of the modified HIV-1 Gag [82]. The biochemical characterization of these compounds in relation to their effect on HIV-1 remains poorly understood. Dinucleoside fatty acyl prodrugs are being explored for the ability to inhibit HIV-1 replication as a topical microbicide by two mechanisms of action including inhibition of reverse transcriptase and inhibition of the cellular N-myristoyl transferase (NMT) [83].

The levels of myristoylation in cells infected with HIV-1 in the presence and absence of compound can be analyzed by labeling infected cells with [^{3}H]myristate and analyzing cell lysates for myristate incorporation into gp41 through immunoprecipitation (IP) with anti-gp41 antibody [81]. Cell-based assays with chronically HIV-1 infected cells can also be used to demonstrate the effects of myristoylation inhibition on proteolytic processing and virus production [84].

11. Maturation Inhibitors

Maturation inhibitors interfere with the final stage of HIV-1 replication, when viral proteins are assembled, packaged, and released from the host cell membrane to form new virus particles. Bevirimat, a betulinic acid-like compound isolated from the Chinese herb *Syzygium claviflorum*, was purchased by Myriad Genetics from Panacos in 2009, as an inhibitor of HIV-1 maturation. Bevirimat binds to the Gag protein and prevents the critical cleavage of p25 (CA-SP1) between Gag codons 363 and 364 to p24 (CA) and p2 (SP1), resulting in virus particles that lack functional capsid protein and have structural defects rendering them incapable of infecting other cells [85]. Clinical trial data reported in 2009 indicated bevirimat was well tolerated and showed good antiviral

activity against HIV-1 with specific Gag protein variations. *In vitro* studies demonstrated the presence of a number of single nucleotide polymorphisms, including H358Y, L363F/M, A364V, and A366T/V, in the CA/SP1 cleavage site that resulted in resistance to bevirimat [86]. Mutations at these sites were not, however, detected in the Phase I and II clinical trials for bevirimat, even in nonresponders. Instead, mutations in the QVT motif of the SP1 peptide (Gag positions 369 to 371) were the primary predictors of failure of response to bevirimat. The comparable potency to other approved HIV-1 drugs, combined with the benefits of oral administration, low probability of drug interactions, and long plasma half-life made bevirimat appear to be a promising new drug candidate. However, Myriad announced in 2010 that it was stopping the development of the maturation inhibitors bevirimat and vivecon.

Maturation inhibitors can be assessed in cell-based assays to evaluate the RNA content and infectivity of virions produced following treatment of infected cells [87]. Electron microscopy can also be used to visualize virions budding from the infected cells following treatment.

12. Cellular Targets

Cells have evolved a number of barriers to resist invading microorganisms. One mechanism that appears to be particularly important in counteracting HIV-1 infection is a group of type 1 interferon-inducible, innate restriction factors that includes tetherin and APOBEC3G. Knowledge of the mechanisms by which restriction factors interfere with HIV-1 replication and how their effects are avoided by HIV-1 in human cells could allow for novel forms of therapeutic intervention. Tetherin is a host protein expressed by many cell types following interferon induction, including CD4-positive T cells, that acts at a late stage of HIV-1 replication to trap mature virions at the plasma membrane by cross-linking to prevent cell-free virus release [88, 89]. Tetherin-retained virions can be reinternalized into the infected cell and targeted to late endosomes where they are destroyed by lysosomal enzymes. However, cell to-cell transmission of HIV-1 is an important mode of dissemination and the possibility of using interferon-based therapy to upregulate the natural antiviral activity of host cells has proven ineffective [90]. APOBEC3G was identified as an inhibitor of HIV-1 replication in cells nonpermissive for replication of HIV-1 mutants lacking a functional Vif gene [91]. APOBEC3G protein can be incorporated into HIV-1 particles through interactions with packaged RNA and the enzyme catalyzes the deamination of deoxycytidines generating minus-strand DNA containing many deoxyuracil nucleotides whose replication results in plus-strand G to A mutations [92]. Hypermutation of HIV-1 DNA can be lethal through deposition of many inactivating missense and nonsense mutations in protein-coding sequences. As previously discussed, RN-18 identified by University of Massachusetts Medical School by high throughput screening of a compound library has been reported to inhibit Vif function by increasing ABOBEC3G concentration within the target cells [93].

Lens epithelial-derived growth factor (LEDGF/p75) is a host protein that binds to HIV-1 integrase and is crucial for viral replication [94]. The mechanism of action is not precisely known but evidence suggests that LEDGF/p75 guides integrase to insert viral DNA into transcriptionally active sites of the host genome. Inhibitors being developed are likely to be highly target specific and less prone to the development of resistance.

Tumor susceptibility gene 101 (TSG101) has been reported to be an essential cellular factor for HIV-1 budding [95]. Inhibiting TSG101 engagement by Gag induces a block of budding virus due to the lipid envelope of nascent particles remaining continuous with the host cell membrane. Monoclonal antibodies and cyclic peptides have been investigated as inhibitors of TSG101 interactions with Gag.

Second generation nonnucleoside rhodanine derivatives have been reported to have improved inhibition of the human DEAD-box RNA helicase DDX3 leading to anti-HIV-1 activity [96]. DEAD-box proteins have nucleic acid-dependent ATPase activity and are involved in ATP-dependent RNA unwinding. DDX3 has been shown to possess relaxed nucleotide substrate specificity, being able to accept ribo- and deoxynucleoside triphosphates as well as nucleoside analogs. DDX3 incorporates into the nucleocapsids and is an essential cofactor for HIV-1 replication. Studies indicate DDX3 is dispensable for host cell metabolism and would therefore provide an excellent antiviral target with predicted low levels of drug resistance without leading to toxicity from interference of a cellular pathway [97].

Antithrombin III has been reported to activate two host cell interactomes dependent on the NFκB transcription factor, extracellular signal-regulated kinases (ERK), mitogen-activated protein kinase (MAPK), and prostaglandin-synthetase 2 (PTGS2) nodules which have anti-HIV-1 effects [98]. Acceleration Biopharmaceuticals is investigating protein interactomes to identify nodules with host cell factors and pathways for viral inhibition.

Antimicrobial peptides derived from cathelicidins, selected on criteria of length, charge, and lack of Cys since defensins have already been reported to demonstrate anti-HIV-1 effects, are being investigated by the University of Nebraska as potential microbicides [98]. A wide variety of other antimicrobial peptides which have been identified to possess anti-HIV-1 activity are cataloged in the Antimicrobial Peptide Database (APD) maintained by The University of Nebraska [99].

The investigation of host cell factors involved in HIV-1 replication involves profiling of well-characterized signal transduction pathways and antiviral immune responses in HIV-1-infected and uninfected cells treated with test material then building an interactome to identify nodules whose blockage might inhibit viral replication. RT-PCR-based gene arrays are used to determine if cellular gene expression is altered by infected cells treated with a potential inhibitor [14, 100, 101]. Data analysis requires a large data base to define potential nodules responsible for the gene expression alterations. Knockout experiments with siRNA can be used in cell-based assays to confirm the inhibition of HIV-1 replication is due to a particular host cell factor.

13. Immunotherapy

Another approach to treating HIV-1 infection is to strengthen the immune response of infected patients. Immune stimulators are designed to improve overall immune function and include preclinical research on Alferon, human leukocyte-derived interferon alfa-n3 developed by Hemisperx Biopharma, that is currently in Phase III clinical trials [102]. Such approaches like Proleukin, developed by Novartis as recombinant human interleukin-2, have failed in the past to demonstrate stimulation of CD4-positive T cell production in HIV-1-infected patients enrolled in the studies [103]. CYT107, recombinant human interleukin-7 developed by Cytheris, is in Phase II clinical trials with raltegravir and maraviroc with the hope of improving T cell counts in patients classified as immunological nonresponders on antiviral therapy [104]. As a growth factor and cytokine physiologically produced by marrow or thymic stromal cells and other epithelia, IL-7 has a crucial stimulating effect on T lymphocyte development and on homeostatic expansion of peripheral T-cells. Tarix Pharmaceuticals is developing TXA127, angiotensin 1–7, to stimulate bone marrow production of progenitor cells [105]. TXA127 is currently in Phase I studies.

Immunomodulatory compounds are designed to signal immune cells to respond to infection in specific ways and may have direct or indirect antiviral activity. Many immunomodulatory molecules have been shown to reduce cell surface antigen expression using flow cytometry, which resulted in inhibition of virus replication through an entry blocking mechanism. Many immunomodulatory compounds will either inhibit or induce cellular proliferation of specific cell types. In many cases, *in vitro* cell-based assays are not possible, and efficacy will need to be demonstrated using relevant animal models.

14. Gene Therapy

Several gene therapy strategies are being studied in order to construct CD4 cells resistant to HIV-1 infection by a population of anti-HIV-1 antisense RNA producing lymphocytes. Enzo Biochem completed a Phase II clinical trial for HGTV43, a retrovirus vector used to deliver three genes encoding U1/anti-HIV-1 antisense RNA targeting TAR and two separate sites of tat/rev region [106], with results indicating antisense RNA was produced from CD4-positive lymphocytes throughout the 24-month observance but no recent news on the status of HGTV43 could be found. A Phase II clinical trial of VRX496, developed by VIRxSYS, was completed in 2010. VRX496 gene therapy is derived from a lentivirus vector and appears to sustain expression of the delivered genes of interest for a longer period of time compared to previous gene therapies and does not appear to elicit an inflammatory immune response. VIRxSYS is attempting to develop a therapy that will allow HIV-1 patients with undetectable viral loads on HAART to discontinue the antiretroviral treatment and still control their viral load. The VRX496 Phase II study demonstrated a decrease in viral load for 88% of the enrolled HIV-1-infected patients with suppression of HIV-1 viremia for more than 14 weeks in some patients in the absence of HAART [107].

Gene therapies are investigated *in vitro* using cell-based anti-HIV-1 assays that measure reduced virus replication, and effects on specific proteins or RNA can be analyzed by Western or Northern blots [108].

15. The Problem of Latent Reservoirs

HIV-1 is known to establish latent reservoirs where the virus is maintained for long periods of time in an essentially quiescent state. Low-rate viral replication also comes from anatomical sites, such as the brain, where drug penetration is limited and only suboptimal drug concentration can be achieved [109]. Studies employing HAART intensification strategies have failed to demonstrate any appreciable reduction in virus load in patients, suggesting the inability to further reduce virus production from these latently infected cells [110]. In recent years considerable interest in the ability to eradicate these latent virus reservoirs and cure HIV-1 infection has evolved. In addition to the HAART intensification studies, efforts have been directed at activating virus production from the latently infected cells to target them for destruction by antiretroviral agents or the host immune system. Compounds developed for this purpose primarily include histone deacetylase (HDAC) inhibitors such as valproic acid, vorinostat, givinostat, and belinostat [111] and nontumor promoting phorbol esters such as prostratin [112]. Compounds which target cellular factors and or regulatory/accessory proteins might also be utilized to target and further reduce virus replication in latently infected cells, such as the transcriptional inhibitors being investigated at ImQuest BioSciences.

The identification and evaluation of compounds which specifically target latently infected cells has primarily utilized the latently infected U1 or ACH-2 cell lines. Primary resting CD4-positive T cells provide the optimal intracellular milieu for establishing latency but are inefficiently infected *in vitro*, since HIV is impaired during reverse transcription and integration. Most primary cell models use one or more rounds of cellular stimulation to remove these blocks, followed by HIV infection during the return to a resting state. Unfortunately, although latently infected nondividing T cells are generated, the process often takes several weeks or months of continuous culture. Investigation into direct infection of resting CD4 T cells by spinoculation has resulted in postintegration latency in these spinoculated cells within 72 h in all CD4 T-cell subsets, including both naive and memory T cells [113]. Cells are sorted by FACS analysis, latent proviruses are activated after additional 72 h of cellular stimulation, and latency can be established and reactivation assessed within 6 days. Using novel reporter viruses, an improved version of this primary CD4 T-cell model has been utilized to study latency in all subsets of CD4 T cells [114]. The ability to target virus in latent reservoirs also requires evaluation in animal models of HIV-1 infection where these reservoirs are established and can be appropriately evaluated.

16. Summary

Three decades of HIV-1 research have greatly contributed to the knowledge scientists and clinicians have available regarding HIV-1 replication, pathogenesis, and therapeutic strategies. Though great strides have been made in the development of anti-HIV-1 inhibitors targeting various viral enzymes and cellular host factors involved in the virus life cycle, we have learned that multi-drug combinations are necessary for the suppression of viremia and the delayed emergence of drug resistance. More drugs targeting essential virus-specific and/or cellular components of the viral replication pathway and virus transmission are needed to treat and prevent HIV-1 infection. The increasing prevalence of drug-resistant virus strains in patient populations, the increasing incidence of transmission of drug-resistant virus during primary HIV-1 infection, the toxicity of the currently approved therapeutic regimens, and the sometimes difficult regimens that must be followed assure that continued HIV-1 drug development will occur in the foreseeable future. Additionally, development issues must include the ability to safely use drugs in pediatric and pregnant individuals and to specifically target virus in latently infected reservoirs. Finally, increasing emphasis on the eradication of HIV from latent reservoirs in infected individuals will require the development of new and novel treatment strategies. The algorithms available for guiding the screening, identification, characterization, and development of these new compounds have been refined over the years as many thousands of compounds have been evaluated and compounds have been approved for use in humans. Current drug development programs must not only prove the efficacy and safety of the new drug candidates but must also show superiority over existing drugs in the same or similar classes. Thus, drug development must be directed at establishing new and novel drug targets, increasing the potency of existing classes of molecules, decreasing the toxicity or pill burden of existing therapies, or adding new drugs to the HAART regimen with superior combination therapy potential or reduced susceptibility to resistant viruses, including drugs designed specifically to attack existing drug-resistant virus strains. Drug development algorithms in the HIV-1 area must be customizable and highly flexible to assure the ability to characterize novel compounds and therapeutic strategies.

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Review Article

Dynamic Association between HIV-1 Gag and Membrane Domains

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HIV-1 particle assembly is driven by the structural protein Gag. Gag binds to and multimerizes on the inner leaflet of the plasma membrane, eventually resulting in formation of spherical particles. During virus spread among T cells, Gag accumulates to the plasma membrane domain that, together with target cell membrane, forms a cell junction known as the virological synapse. While Gag association with plasma membrane microdomains has been implicated in virus assembly and cell-to-cell transmission, recent studies suggest that, rather than merely accumulating to pre-existing microdomains, Gag plays an active role in reorganizing the microdomains via its multimerization activity. In this paper, we will discuss this emerging view of Gag microdomain interactions. Relationships between Gag multimerization and microdomain association will be further discussed in the context of Gag localization to T-cell uropods and virological synapses.

1. Introduction

Microdomain-based compartmentalization of the plasma membrane is implicated in many aspects of the HIV-1 life cycle. In particular, during events in the late phase of the HIV-1 life cycle such as assembly and cell-to-cell transmission, these microdomains have been thought to serve as preformed platforms that facilitate concentration of viral components (e.g., Gag and Env) or delivery of these proteins to specific locations in cells. However, recent studies suggest that Gag is not a simple passenger of microdomains but rather plays an active role in reorganizing microdomains via its membrane-binding and multimerization activities. In this paper, we focus on recent findings on this active role played by Gag during microdomain association. In light of this new view, we will also discuss the implications of plasma membrane microdomains and large-scale domains in cell-to-cell transmission. Microdomains are also thought to affect virion infectivity, attachment of virions to target cells, and virus-cell fusion, in which they modulate distributions and/or activities of Env, Nef, and virus receptors. For these topics, interested readers are referred to more comprehensive papers published in recent years [1–5].

2. HIV-1 Assembly at the Plasma Membrane

The viral structural polyprotein Gag is necessary and sufficient for the assembly of virus-like particles. HIV-1 Gag is synthesized as a 55 kDa polyprotein composed of 4 major structural domains (and 2 spacer polypeptides), as defined by cleavage by the viral protease: matrix (MA), capsid (CA), nucleocapsid (NC), and p6. However, proteolytic cleavage occurs largely after virion assembly and release; thus, its constituents must work together in the context of the full-length Gag polyprotein to drive particle assembly. After its synthesis in the cytosol, Gag traffics to the site of assembly, binds cellular membranes, multimerizes, buds through the membrane, and recruits host factors that mediate membrane scission, releasing an immature particle [6, 7]. It is increasingly apparent that many of these steps occur in a coordinated, interdependent fashion. Among

them, Gag membrane binding and multimerization are implicated in association of virus assembly with membrane microdomains.

Gag membrane binding is mediated by its N-terminal MA domain, containing bipartite membrane binding motifs. The MA domain is cotranslationally myristoylated and contains a highly basic region (HBR) that binds the plasma-membrane-specific acidic phospholipid phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] [8–16] (reviewed in [17]). It has been suggested that exposure of the myristoyl moiety is regulated through a mechanism known as the myristoyl switch [18, 19]. Indeed, NMR studies demonstrated that prior to membrane binding, the myristoyl moiety is sequestered in a hydrophobic cavity of the MA domain. Upon Gag multimerization or $PI(4,5)P_2$ binding, the myristoyl chain is exposed to promote membrane binding [14, 20, 21]. As for MA HBR, RNA appears to competitively regulate its binding to acidic membrane lipids. Studies using *in vitro* assays collectively support a model in which RNA bound to HBR prevents MA from binding to prevalent acidic lipids like phosphatidylserine, but allows MA binding to $PI(4,5)P_2$, thereby enhancing the specificity of Gag binding to $PI(4,5)P_2$-containing membranes, that is, the plasma membrane [10, 22–25].

Two major functional regions that contribute to Gag multimerization are the C-terminal region of the CA domain (CA-CTD) and NC. CA-CTD forms an interface that mediates Gag homodimerization [26–29]. The NC domain is thought to contribute to Gag multimerization via its ability to bind RNA [30–34]. Notably, heterologous leucine zipper dimerization motifs can substitute for NC in Gag multimerization and particle assembly [35–39]. These findings suggest a model in which RNA binding to NC serves a structural role, either as a scaffold or a trigger for CA dimerization. In addition to CA and NC, the Spacer Peptide 1 (SP1) between CA and NC plays an important role in regulating the multimerization process [40].

Higher-order Gag multimerization induces outward curvature of the plasma membrane area where the Gag multimer is bound. This step is likely driven by the inherent curvature of the Gag hexameric lattice, formation of which relies on CA [41]. Consistent with this, a number of CA mutations lead to a budding arrest phenotype, characterized by many electron-dense patches underneath the plasma membrane [29, 42]. Release of nascent particles is driven by the cellular ESCRT (endosomal sorting complexes required for transport) that is recruited to assembling virions through interactions with the NC and p6 domains [43].

HIV-1 has been observed to assemble at the plasma membrane in T cells and some laboratory cell lines such as HeLa cells (see [44] for a review). Assembly in macrophages was originally thought to occur at late endosomes/multivesicular bodies (LE/MVB), based on the apparently intracellular location of assembling Gag and the presence of LE/MVB markers, such as the tetraspanin protein CD63 and ESCRT [45, 46]. However, the sites of assembly in macrophages were found to be actually deep invaginations of the plasma membrane, now known as virus-containing compartments (VCC) (although the architecture of the VCC, in particular whether VCCs are all connected to the plasma membrane, is still under intense investigation) [47–53]. Moreover, markers like CD63 strongly colocalize with Gag, even at assembly sites that are unambiguously on the plasma membrane (e.g., [54, 55]). Therefore, the currently accepted idea is that for most cell types including macrophages, the primary site of HIV-1 assembly is the plasma membrane or its specific domains [56, 57].

3. Plasma Membrane Microdomains Associated with HIV-1 Assembly

The plasma membrane consists of diverse microdomains. This partitioning of membrane components is regulated by lipid-lipid, protein-protein, and protein-lipid interactions and compartmentalizes cellular processes [58]. As with many diverse enveloped viruses, HIV-1 was initially proposed to assemble at lipid rafts, based on sensitivity to cellular cholesterol depletion and cofractionation of viral components with detergent-resistant membranes (DRM). Subsequently, HIV-1 assembly was also proposed to occur at tetraspanin-enriched microdomains based on microscopy.

3.1. Lipid Rafts. Spontaneous partitioning of lipids into an ordered phase and a disordered phase has been observed in chemically defined model membranes and model membranes reconstituted from cellular membrane components [58]. The ordered phase is enriched in cholesterol and saturated lipids, and the disordered phase is enriched in unsaturated lipids. This biophysical phenomenon of lipid phase separation in model membranes has been hypothesized to underlie the phenomenon of lipid rafts in cells. In contrast to model membranes, however, cellular membranes contain a greater diversity of lipids and proteins. The partitioning of these molecules is governed by a much greater complexity of lipid-lipid, protein-lipid, and protein-protein interactions. Thus, the current consensus is that lipid rafts are highly dynamic, submicroscopic membrane domains enriched in sterols and sphingolipids, which can be stabilized to form larger platforms through protein-protein and protein-lipid interactions [58].

To assess the involvement of lipid rafts in HIV-1 assembly processes, biochemical assays that measure either resistance to nonionic detergents or sensitivity to cellular cholesterol depletion have been widely used. Results from these assays generally support lipid raft association of the HIV-1 assembly process [59–71]. Both cholesterol depletion and substitution of the Gag myristoyl moiety with an unsaturated acyl analogue inhibit virus particle production, suggesting a functional role for association between HIV-1 Gag and lipid rafts during virus assembly [66, 68, 69]. While biochemical methods used in these studies require cautious interpretations of data due to their inherent limitations [72–76], studies using different approaches, such as microscopy and virion content analyses described below, also generally support raft association with the HIV-1 assembly process.

Because of the dynamic and submicroscopic nature of lipid rafts, cross-linking of cell-surface proteins, which stabilizes the microdomains they associate with, is often used to observe protein partitioning into microdomains by standard fluorescence microscopy. When two microdomain markers are independently clustered using specific antibodies or toxins, these markers can colocalize within the same patch, or "copatch", indicating propensity of these markers to partition into the same microdomains [77–80]. Consistent with biochemical analysis described above, Gag puncta that represent assembled particles or multimerizing Gag are observed to colocalize or copatch with raft markers, such as the glycosphingolipid GM1 and GPI-anchored proteins [42, 64, 67, 81–83]. However, a recent super resolution microscopy study showed that GM1 does not colocalize with Gag clusters, at least in the particular cell type used [84]. Therefore, while GM1 may have a propensity to associate with lipid rafts, codistribution of this lipid with other raft components may occur only when raft partitioning is stabilized by crosslinking. These new super-resolution microscopy technologies will likely allow us to define the native distribution of each raft component associated with HIV-1 assembly sites.

Finally, analyses of cellular molecules incorporated into HIV-1 particles also support lipid raft involvement during the HIV-1 assembly process. Biochemical, proteomics, and lipidomics studies have shown that the HIV-1 envelope is enriched in many of lipids and proteins that are also components of lipid rafts [85–91]. Of note, the cholesterol content of virions may be upregulated via activities of viral proteins such as HIV-1 Nef [92–94] and MLV glyco-Gag [95]. Importantly, by measuring spectral shift of the lipophilic fluorescent dye laurdan, which is sensitive to ordered packing of its surrounding lipids [96], HIV-1 envelope was shown to contain liquid-ordered domains [97].

3.2. Tetraspanin-Enriched Microdomains. Tetraspanin-enriched microdomains (TEMs) are plasma membrane microdomains organized by the homo- and heterooligomerization of tetraspanins, a family of homologous proteins with four transmembrane domains. Proteomics studies have identified a wide variety of proteins associated with TEMs. Most notably, tetraspanins interact with cell-adhesion molecules, integrins, and cell-signaling proteins, suggesting that TEMs serve as a platform to spatially organize cell-cell and cell-extracellular matrix adhesion and signaling [4, 98, 99]. Tetraspanins CD63 and CD81 have been shown to associate with phosphatidylinositol 4-kinase, a critical enzyme in creating a precursor for $PI(4,5)P_2$ [100]. Importantly, different tetraspanins appear to be at least partially redundant in the cell functions measured in some of these studies.

The first evidence of association between tetraspanin proteins and HIV-1 assembly were early studies that found the tetraspanin protein CD63 enriched in the envelopes of HIV-1 particles. This was taken as evidence that Gag traffics through, or assembles at, an endosomal compartment, such as the LE/MVB. However, it was later shown that Gag associates with CD63 and other tetraspanin proteins at discrete microdomains on the plasma membrane [54, 55].

Tetraspanins, including CD9, CD63, and CD81, are incorporated into virus particles [45, 55, 88, 101–107], coimmunoprecipitate with Gag-laden cellular membranes [108], and strongly colocalize/copatch with Gag by immunofluorescence microscopy assays (e.g., [54, 55, 108]). As for functions, a variety of studies have suggested roles for tetraspanins and TEMs in different phases of the HIV-1 replication cycle such as virus entry (see [4] for a review). However, the role of tetraspanins and TEMs in Gag assembly remains currently unclear. The gross effects of perturbing tetraspanins by siRNA knockdown or overexpression are so far contradictory: some studies report perturbation reduces particle production [108, 109], while others report no effect [107, 110, 111]. In contrast, it is well accepted that tetraspanins incorporated into virus particles have an inhibitory effect on subsequent virus entry [107, 108, 110].

3.3. Gag Determinants for Interactions with Microdomains. While association of Gag with microdomains has been well documented, how this association occurs is only beginning to be elucidated. As saturated acyl chains mediate raft association of many cytoplasmic proteins, it is straightforward to imagine that the N-terminal myristoyl moiety of Gag plays a role. Consistent with this notion, incorporation of an unsaturated myristate analogue in the place of myristate impairs Gag recovery into DRM fractions [66]. Interestingly, an NMR study of MA bound to a soluble $PI(4,5)P_2$ (with short acyl chains, allowing it to remain in aqueous solution) showed that, while $PI(4,5)P_2$ binding induces myristoyl exposure, a hydrophobic cleft of the MA domain sequesters the typically unsaturated sn2 acyl chain of $PI(4,5)P_2$—effectively exchanging an unsaturated acyl chain from $PI(4,5)P_2$ for the saturated myristoyl chain of Gag [14]. This sequestration of the unsaturated sn2 acyl chain of $PI(4,5)P_2$ has been hypothesized to facilitate Gag association with lipid rafts [14]. It remains to be seen if this acyl chain exchange occurs in the more authentic case of Gag binding a lipid bilayer, as opposed to interaction between isolated MA domains and water-soluble lipids.

HIV-1 Gag multimerization has also been observed to enhance microdomain association. Biochemical studies showed that the presence of NC and other Gag regions necessary for multimerization affect the steady-state association of Gag with DRM [61, 65]. The presence of NC is also required for colocalization of Gag with markers for microdomains termed endosome-like domains (ELD), which appear to be a subset of TEMs [54, 112]. ELD association of Gag and other multimeric proteins was reported to be independent of a membrane-binding interface; a variety of plasma membrane targeting motifs were observed to mediate ELD association of a normally-cytosolic oligomeric protein, TyA [113]. Altogether, these results are consistent with a notion that Gag multimerization plays a key role in stable association with specific microdomains at the plasma membrane.

In the context of assembly of many enveloped viruses, membrane microdomains are often regarded as preexisting platforms that accumulate viral structural components, thereby facilitating virus assembly. However, as alluded to

earlier, protein-protein interactions are thought to stabilize or recruit microdomains [114, 115]. Therefore, Gag multimerization was postulated to modulate structure and/or size of Gag-associated microdomains [11, 65, 68, 86]. Consistent with this protein-centric view of microdomains, recent studies suggest that HIV-1 Gag is not just passively accumulated in microdomains but rather actively stabilize, recruit, or reorganize microdomains at the plasma membrane through its multimerization. Fluorescence recovery after photobleaching and single-molecule tracking analyses showed that Gag multimers trap the tetraspanin CD9 and, to a lesser extent, the raft markers GM1 and CD55 and clusters these microdomain components in a Gag-multimerization-dependent manner [82]. Furthermore, copatching and fluorescence resonance energy transfer analyses showed that HIV-1 coalesces TEMs and lipid rafts [42], two microdomains that are otherwise distinct and do not colocalize in cells that do not express Gag [98, 116–120]. Interestingly, correlative fluorescence and scanning electron microscopy showed that copatching between raft and TEM markers does not occur at assembly sites of a Gag mutant that forms multimeric Gag patches but fails to form spherical particles [42]. Therefore, raft-TEM coalescence appears to depend on membrane curvature induced by Gag multimerization. Altogether, Gag is likely to direct the formation of its own microdomains by recruiting and coalescing membrane proteins and microdomains, in a manner dependent on the process of virus assembly.

What determines microdomain recruitment to Gag multimers? Since MA functions as the interface of Gag with lipid bilayer, it is conceivable that MA or MA-interacting molecules drive recruitment of lipid raft and TEM markers. For example, the combination of the N-terminal myristoyl moiety and a saturated acyl chain of $PI(4,5)P_2$, which is postulated to direct Gag to lipid rafts [14], may also direct small lipid rafts to Gag assembly sites. This is also consistent with the enrichment of specific lipids to the viral envelope, relative to the plasma membrane [86, 87]. However, copatching studies suggest that coalescence of lipid rafts and TEMs at assembly sites occur even when MA was replaced with a triple acylation motif or a heterologous lipid-binding domain [42]. Therefore, the MA sequence per se is not essential for reorganization of lipid rafts and TEMs.

As described below, Gag multimerization is also important for Gag localization to larger membrane domains.

4. Large-Scale Membrane Domains Implicated in HIV-1 Spread

In addition to the microdomains described above, larger plasma membrane domains are implicated in HIV-1 spread. One of such domains is the VCC [47–53], which may serve as a virus reservoir that can transfer viruses upon contact with T cells [121–123]. A similar surface-accessible intracellular compartment in dendritic cells also promotes transmission of captured viruses to T cells via cell contacts during trans-infection [124–128]. In this section, however, we focus on membrane domains implicated in T-cell-to-T-cell virus transmission and their relationships with microdomains.

4.1. Virological Synapses. HIV-1 virions released from infected cells may travel in the extracellular space until they come in contact with a target cell by chance (random three-dimensional diffusion and fluid flow). However, this cell-free infection route is much less efficient than cell-to-cell transmission, in which an infected cell physically contacts a target cell and directly transfers the virus. In contrast to cell-free transmission, cell-to-cell transmission of HIV-1 is 10- to several-thousand fold more efficient in cultured T cells [129–132] and is believed to be the major form of transmission for HIV-1 *in vivo*. In addition to HIV-1, direct cell-to-cell transfer is likely to be important for efficient spreading of several other retroviruses such as human T-lymphotropic virus-1 (HTLV-1) [133–136] and murine leukemia virus [137–139] as well as other pathogens (reviewed in [140, 141]). Moreover, a recent study suggested that cell-to-cell transmission enhances resistance of HIV-1 to antiretroviral drugs and therefore potentially constitutes a mechanism by which HIV-1 maintains an active reservoir in infected individuals undergoing combination drug therapy [142].

Cell-to-cell transmission occurs through several distinct plasma membrane structures. These structures include cytonemes [137–139], membrane nanotubes [143], and virological synapses (VSs) [124, 127, 134, 144–146]. Because involvement of membrane microdomains in the first two structures has yet to be described, in this paper we focus on VSs. VSs formed between HIV-1 infected and uninfected T cells are contact structures enriched in Gag, Env, and viral receptors. Stable VS formation between two T cells is primarily mediated by the Env-CD4 interaction [129, 145–148] unlike VS formed by monocyte-derived macrophages [121]. Consistent with this, antibodies that block the Env-CD4 interaction blocks VS formation and cell-to-cell virus transfer [129, 130, 145, 147, 149] (although neutralization by patient-derived antibodies is ineffective perhaps due to the delayed virion maturation during transfer at the VS [129, 144, 150]). The VS is also enriched in adhesion molecules such as LFA-1, although the significance of these adhesion molecules in VS formation and virus transfer/transmission varies depending on the experimental systems [146–148, 151].

VSs were first described for HTLV-1 [134]. Early studies including this HTLV-1 study and subsequent studies on HIV-1 VS have pointed to the importance of cytoskeleton in VS stability and formation [134, 145, 152–154]. Recent studies further suggest that polarization of HIV-1 Env is dependent on the microtubules and microtubule-dependent trafficking of secretory lysosomes that bear Env [155]. Consistent with this finding, Zap70, which regulates cell polarization in the immunological synapses by controlling localization of the microtubule organizing center (MTOC) [156], facilitates formation of VSs and cell-to-cell transmission [157]. The actin cytoskeleton is also important for VS formation, as evident from the impact of actin depolymerizing agents and a myosin light chain kinase inhibitor on VS formation, cell-to-cell virus transfer and transmission [145, 152, 158].

In addition to cytoskeleton, lipid rafts and TEMs are implicated in VS formation as well. Markers for both microdomains accumulate to VS [110, 146, 159, 160]. Consistent

with a role for lipid rafts in VS formation, cholesterol depletion was observed to diminish formation of VS, as defined by the accumulation of CD4 (on the target cell) and HIV antigens (in the donor cell) at the cell-cell interface [159]. However, whether this impact was due to disruption of lipid rafts or inhibition of other cholesterol-dependent processes is unknown. If the former is the case, what particular role lipid rafts play in VS formation also remains to be determined.

As for the role of TEMs, multiple and potentially opposing roles played by tetraspanins (for a review, see Thali [4]) make it difficult to assess the contribution of TEMs to VS formation. Anti-tetraspanin antibodies were observed to reduce VS formation albeit modestly [160]. Consistent with the inhibitory effect of tetraspanins on infectivity of virions [107, 108, 110], tetraspanins also prevent Env-mediated cell-cell fusion [161], inhibition of which was suggested to help preserve productive VSs [110]. Moreover, the presence of CD81, but not other tetraspanins, was shown to facilitate polarized localization of Gag [108]. On the other hand, CD81 was observed to decrease cell-to-cell virus transmission, perhaps via inhibition of virion infectivity [110]. Therefore, it remains to be determined whether and in what context TEMs or tetraspanins play a positive or negative role in cell-to-cell transmission of HIV-1 via VSs.

4.2. Uropods. A majority of T cells in lymph nodes where cell-to-cell transmission likely occurs frequently are highly motile and adopt a polarized morphology [162–166]. The front end of a polarized T cell is called the leading edge, and the protrusion at the rear is called a uropod [167–169]. Functionally, uropods seem to promote T-cell migration by facilitating deadhesion of integrins such as LFA-1 that mediates substrate adhesion at the leading edge [169]. During T-cell migration, uropods also mediate contact with other T cells [170] and recruit bystander T cells to sites of inflammation [171]. Interestingly, Gag accumulates to the plasma membrane area constituting the uropod surface in polarized T cells [67, 129, 158] (Figure 1(a)). Moreover, upon contact with uninfected T cells, this plasma membrane domain participates in the VS, as supported by the observation that Gag and uropod markers on the infected cell and CD4 on the uninfected cell accumulate at the site of cell-cell contact [158]. These findings suggest a model in which the uropod surface of polarized HIV-1-infected T cells serves as a preformed platform that participates in VS formation.

Which part of an HIV-1-infected cell mediates the initial contact with a target cell remains to be determined. It is possible that uropods, where the virus is concentrated, establish the initial contacts, and these contacts eventually develop into VSs without large-scale shift in cell polarity (as shown in Figure 1(a)). Consistent with this possibility, uropods are enriched in various adhesion molecules that help promote contact with other cells. However, it is also possible that initial contacts may be established at other regions of cells such as the leading edge. Under this scenario, after initial contact, viral proteins and VS components that are preaccumulated at the uropod would subsequently move laterally to the cell-cell junction. In support of this latter possibility, patches containing HIV-1 Gag have been observed to move laterally over the cell surface to the VS [144, 146]. Regardless of the pathways taken by Gag to the uropod and cell junctions, this preaccumulation of Gag at the uropod may constitute an important early step in VS formation.

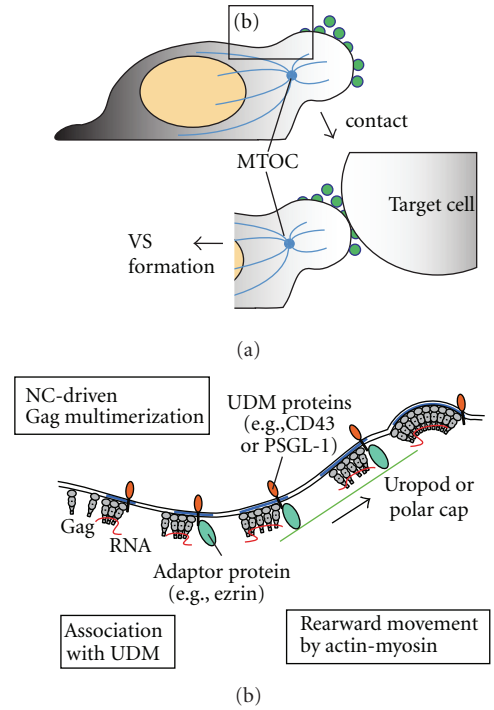

Figure 1: (a) Gag accumulates at the uropod surface. While it remains to be determined whether the first contact between virus-producing and target cells occurs right at the uropod or elsewhere during VS formation, virus-laden uropods do participate in VS formation as determined by concentration of uropod markers at the VS. (b) A working model for a mechanism by which Gag multimers associate with rearward actin flow that directs Gag to the uropod. NC-dependent Gag multimerization underneath the plasma membrane promotes association between Gag multimer and UDM. Of note, in contrast to lipid raft and TEM markers, UDM proteins appear to accumulate at assembly sites of wild-type Gag as well as those of a Gag mutant that multimerizes but fails to bud (GNL, unpublished data).

The molecular mechanisms of Gag localization to the uropod also remain to be determined. Notably, Gag accumulation to uropods requires higher-order multimerization driven by NC [158, 172], while the dimerization function of CA-CTD is neither sufficient nor necessary [158]. In this regard, it is important to note that crosslinking of cell surface proteins with antibodies induces polarized localization of these proteins in leukocytes and other cell types [173]. Such "capping" has also been observed for lipids cross-linked by pentavalent cholera toxin [174, 175]. During T-cell polarization, similar polar cap formation occurs spontaneously at the cell surface from which a uropod originates [169, 176].

These capping phenomena depend on myosin II-driven rearward actin flow [177–181]. Thus, in a manner similar to capping, higher-order Gag multimerization might trigger Gag association with the actin flow, which in turn drives accumulation of Gag in uropods. In support of this model (Figure 1(b)), a myosin light chain kinase inhibitor ML7, which inhibits myosin II, dispersed Gag all over the cell surface [145, 158].

The nature of the link between Gag multimers and retrograde actin flow is currently unknown. While NC has been implicated in interaction with actin [182, 183], this does not account for uropod localization of Gag-LZ in which NC was replaced with a heterologous leucine zipper [158, 172]. As Gag multimerization recruits and stabilizes lipid rafts and TEMs at assembly sites (discussed earlier), it is conceivable that reorganization of these microdomains, as well as cellular proteins associated with these microdomains, is involved in polarized localization of Gag multimers to uropods and subsequently to the VS. In support of this hypothesis, markers for both microdomains are found to accumulate at uropods [169] and VSs [110, 146, 159, 160]. Indeed, in HIV-1-expressing T cells, both a raft marker CD59 and a tetraspanin CD81 copolarize with Gag to uropods. However, using a T-cell line that polarizes spontaneously, we observed that Gag copatches with CD59 only to a very low extent prior to cell polarization (GNL unpublished data). Even within uropods, copatching between Gag and CD59 was poor (GNL unpublished data). Copatching between Gag and CD81 was shown to be higher but still at a modest level [158]. In contrast, uropod markers PSGL-1, CD43, and CD44 strongly copatch with Gag both before and after polarization [158] (GNL unpublished data). Therefore, at least in these T cells, Gag appears to associate predominantly with a specific microdomain enriched in uropod-directed proteins (uropod-directed microdomain or UDM), which is likely to be distinct from CD59-positive lipid rafts.

One can postulate that in T cells Gag multimerization induces recruitment of UDMs more efficiently than that of lipid rafts or perhaps TEMs. UDM association may in turn promote association between Gag multimers and actin flow and thereby facilitate Gag localization to the uropod (Figure 1(b)). In support of this possibility, PSGL-1 comigrates with Gag toward the uropod as T cells polarize [158]. This possibility is further supported by the observation that actin-binding proteins such as ezrin and moesin, which are found in HIV-1 virions [90], bind cytoplasmic domains of several uropod-specific transmembrane proteins and promote localization of these proteins to uropods [169]. Alternatively, it is possible that while PSGL-1 and other UDM proteins are recruited to Gag multimers, Gag might not require UDM association for localization to the uropod. In such case, recruited UDM proteins may serve other functions in cell-to-cell transmission. Elucidating the mechanism by which Gag multimers associate with UDMs will likely allow us to determine the potential role of this association in Gag localization and cell-to-cell HIV-1 transmission.

5. Future Perspectives

The plasma membrane microdomains that constitute virus assembly sites have been frequently depicted as stable preformed platforms. However, a more nuanced view of plasma membrane compartmentalization is that they exist along a continuum of size and stability. On one end, large domains such as the Gag-laden uropod surface may serve as a preformed stable structure poised to form cell-cell junctions or VSs. On the other extreme, microdomains are submicroscopic, dynamic, and unstable unless protein-protein and protein-lipid interactions drive their stabilization. At least for HIV-1, it is increasingly clear that Gag multimerization and/or membrane curvature reorganizes plasma membrane microdomains at assembly sites. With this new view, a number of new questions arise: What are the characteristics specific to virus-reorganized microdomains compared to those of original individual microdomains? What is the nature of association (or lack thereof) between monomeric Gag and microdomains? Do other enveloped viruses alter microdomain organization at their assembly sites, and if so, what are the differences in composition and function among these virus-reorganized microdomains?

Cellular proteins and lipids that specifically associate with membrane microdomains of virus assembly sites affect HIV-1 particle production and infectivity, either positively (e.g., cholesterol, see [5] for a review; sphingolipids [184]) or negatively (e.g., tetraspanins; see above). Incorporation of viral proteins such as Env into virus particles may also be modulated by microdomains [1, 2]. To fully understand incorporation of these molecules into virus particles, it is crucial to elucidate the mechanisms by which Gag multimerization reorganizes microdomains. Although even Gag derivatives with heterologous membrane-binding domains can induce coalescence of lipid rafts and TEMs, membrane-binding domains of Gag may still modulate compositions of reorganized microdomains via molecular interactions. In this regard, it is interesting to note that a highly basic protein can induce formation of a microdomain enriched in acidic lipids, which in turn attract other basic proteins [185, 186]. As Gag and other viral structural proteins contain highly basic regions, it is conceivable that multimerization of these viral proteins may induce acidic lipid clustering and thereby trigger association of basic-region-containing proteins to assembly sites. Whether such indirect mechanism, in addition to direct protein-protein interactions, modulates microdomain compositions will potentially be of functional significance.

Less well-characterized functions for reorganized microdomains include contribution to polarized localization and cell-to-cell transmission. On this front, future studies need to be directed to understanding (1) the relationships among UDMs, lipid rafts, and TEMs, (2) the mechanism by which Gag multimerization facilitates association of Gag with the retrograde actin flow, and (3) the role for UDM proteins in polarized localization and VS functions. These studies will help us further understand molecular mechanisms that facilitate VS formation and cell-to-cell transmission.

Authors' Contribution

I. B. Hogue and G. N. Llewellyn have contributed to this work equally.

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Review Article

Retroviral Env Glycoprotein Trafficking and Incorporation into Virions

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Together with the Gag protein, the Env glycoprotein is a major retroviral structural protein and is essential for forming infectious virus particles. Env is synthesized, processed, and transported to certain microdomains at the plasma membrane and takes advantage of the same host machinery for its trafficking as that used by cellular glycoproteins. Incorporation of Env into progeny virions is probably mediated by the interaction between Env and Gag, in some cases with the additional involvement of certain host factors. Although several general models have been proposed to explain the incorporation of retroviral Env glycoproteins into virions, the actual mechanism for this process is still unclear, partly because structural data on the Env protein cytoplasmic tail is lacking. This paper presents the current understanding of the synthesis, trafficking, and virion incorporation of retroviral Env proteins.

1. Introduction

All replication-competent retroviruses encode genes for three major proteins: Gag, Pol, and Env. Complex retroviruses, such as human immunodeficiency virus type 1 (HIV-1), encode additional regulatory and accessory proteins required for efficient replication in host cell or the infected host organism. Gag, an essential retroviral protein, is necessary and sufficient for the assembly, budding, and release of virus-like particles (VLPs) in all types of retroviruses except the spumaviruses. Gag is synthesized on cytosolic ribosomes and is assembled as a polyprotein precursor. During and/or shortly after budding and release, the polyprotein is cleaved into several domains by the viral protease (Figure 1) as reviewed in [1–3]. The major domains of the precursor Gag are the matrix (MA), capsid (CA), and nucleocapsid (NC). The primary role of the N-terminal MA domain is targeting of the Gag precursor protein to the site of assembly, typically the plasma membrane (PM). In general, electrostatic interactions between basic amino acid residues in MA and the acidic inner leaflet of the PM are important for Gag-membrane targeting [4, 5]. In the case of HIV-1, the N-terminal myristate group and a cluster of basic residues in the MA domain of the HIV-1 Gag that interacts with phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) together target the Gag precursor $Pr55^{Gag}$ to the PM [6, 7]. Although the Gag-membrane targeting of both murine leukemia virus (MLV) and Mason-Pfizer monkey virus (MPMV) is also affected by $PI(4,5)P_2$ modulation [8, 9], it has been reported that the membrane targeting of Rous sarcoma virus (RSV) and human T-lymphotropic virus type 1 (HTLV-1) is largely independent of $PI(4,5)P_2$ [10, 11]. The MA domain also plays a role in the incorporation of the Env glycoprotein into virions. The CA domain is important for Gag-Gag interactions during virus assembly and constitutes the outer part of the viral core after Gag processing by the viral protease [12–14]. NC is the primary nucleic acid binding domain of Gag. This small, basic domain is responsible for the binding and incorporation of the viral RNA genome into virions, which is mediated by Gag interactions with genomic RNA.

Gag proteins are synthesized and transported to the PM. Many studies demonstrate that the major site of HIV-1 assembly is the PM [15–18], although late endosomes could be a platform for virus assembly under specific conditions [19]. In primary macrophages, HIV-1 has been shown to assemble in endosomal vesicles. However, studies

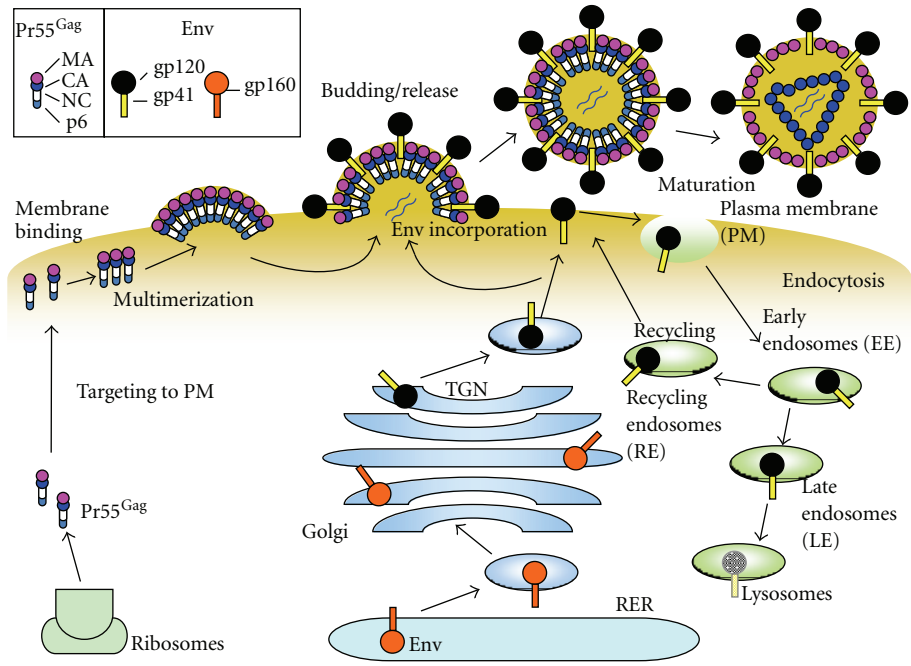


Figure 1: Synthesis and trafficking of HIV-1 Gag and Env proteins. Precursor Gag ($Pr55^{Gag}$) (left) is synthesized on cytosolic ribosomes and traffics to the plasma membrane (PM), where it forms multimers (middle). Env is synthesized as the gp160 precursor, and undergoes glycosylation and oligomerization in the RER. Oligomerized gp160 is transported to the Golgi and the TGN, where it is processed into the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 by cellular enzymes. The gp120/gp41 complexes are transported through the secretory pathway to the PM and are incorporated into virus particles (middle). At the PM, most of the Env protein is endocytosed into early endosomes (EE), which mature into late endosomes (LE) and then into lysosomes for Env degradation (right). However, some Env proteins are recycled to the PM through recycling endosomes (RE). During and after virus release, processing of $Pr55^{Gag}$ by virus proteases yields mature virions. The protein domains of $Pr55^{Gag}$ and Env are illustrated in the insert at the top left. The illustration was adapted from Checkley et al. with permission from Elsevier [23].

have recently suggested that the above vesicles are not late endosomes but rather membrane invaginations connected to the PM [20–22].

In addition to Gag, the other major structural retroviral protein is the Env glycoprotein. Env proteins are required for virus entry into target cells and are thus essential for forming infectious retroviral particles. In this paper, we discuss current knowledge about the biosynthesis, intracellular trafficking, and virion incorporation of retroviral Env proteins, as well as the membrane microdomains involved in virus assembly and/or transfer. Most of this information was obtained from studies on HIV-1.

2. Env Biosynthesis and Trafficking to the Plasma Membrane

Retroviral Env glycoproteins are synthesized from a spliced form of the viral genomic RNA as reviewed in [23–25] (Figure 1). Translation of the Env protein occurs on ribosomes bound to the endoplasmic reticulum (ER) and starts with the leader sequence, which contains a small, N-terminal hydrophobic signal peptide. The Env protein is cotranslationally inserted into the lumen of the rough ER. In the ER, the leader sequence is removed by cellular signal peptidases. In addition, Env polypeptides are N- and O-glycosylated and subsequently trimmed [26, 27]. The number and location of glycosylated residues varies broadly among retroviruses. The hydrophobic transmembrane (TM) domain prevents Env proteins from being fully released into the lumen of the ER [28, 29]. The amino acid sequence following the TM is referred to as the cytoplasmic tail (CT), which varies from 30 to around 150 residues, depending on the virus. Env proteins are folded and assembled into oligomers in the RER. Retroviral Env proteins form trimers [30–33]. The HIV-1 accessory protein Vpu binds to the CD4 receptor through its cytoplasmic domain and downregulates the receptor by transporting it to the proteasome for degradation, thereby preventing premature interactions between Env and its receptor [34–36].

In the Golgi, cleavage of the retroviral Env precursor occurs at a polybasic (e.g., K/R-X-K/R-R) motif by cellular proteases such as furin or closely related enzymes probably within or near the trans-Golgi network (TGN) [37–43]. For HIV-1, the surface glycoprotein gp120 and the TM glycoprotein gp41, which bind together noncovalently, are both formed from the same precursor protein, gp160. Gp160 processing is essential for the activation of Env fusogenicity and virus infectivity [38, 42, 44–46]. Similarly, cleavage of Env is also essential for membrane fusion and virus infectivity in MLV [39, 47–50], in RSV [51, 52], and in mouse mammary tumor virus (MMTV) [53]. A recent

report showed that cleavage of MLV Env by furin also plays an important role in Env intracellular trafficking and incorporation [54]. Although most retroviral Env proteins including that of HIV-1 are associated with intracellular membranes [55–57], at least part of the gp120/gp41 trimer complex traffics through the secretory pathway to the PM. It has been suggested that AP-1, one of adaptor proteins for clathrin-coated vesicle formation, is involved in the correct sorting of HIV-1 Env from the TGN to the PM, [58, 59]. It has been reported that intracellular CTLA-4-containing secretory granules are involved in the trafficking of HIV-1 Env to the PM although the subsequent trafficking of Env after the Golgi is not well understood [60].

After reaching the PM, like those of other lentiviruses, HIV-1 Env undergoes rapid endocytosis, which is mediated by the interaction between the $\mu 2$ subunit of the clathrin adaptor AP-2 and a membrane-proximal, Tyr-based motif (YxxL) in the gp41 CT [58, 61, 62]. Although some of the endocytosed Env is recycled back to the PM, most retroviral Env is associated with intracellular membranes [63, 64]. The level of gp120-gp41 oligomers on HIV-1 virions is relatively low [33]. Maintaining low levels of Env at the cell surface allows the infected cells to evade the host immune response and to avoid induction of Env-mediated apoptosis. Gammaretroviruses such as MLV and MPMV also have dileucine- and Tyr-based motifs in their Env CT. These motifs are important to regulate intracellular trafficking of Env of both retroviruses via interactions with clathrin adaptors [65, 66].

As for pseudotyping of gammaretroviruses, it has been reported that the feline endogenous retrovirus RD114 Env does not allow pseudotyping with viral cores from lentiviruses such as SIV, whereas the RD114 Env is incorporated into MLV virions [67–69]. Intracellular trafficking of Gag and Env was examined using a set of chimeric viruses between MLV and RD114 [57]. Interestingly, it was found that the RD114 Env was mainly localized along the secretory pathway, whereas the MLV Env was mostly localized in endosomes, and that intracellular localization was dependent on specific motifs in the Env CT [57]. In addition, subsequent work revealed that an acidic cluster in the RD114 Env CT regulates assembly of not only the RD114 Env but also the MLV Env through the interaction with a host factor, phosphofurin acidic-cluster-sorting protein 1 [66].

3. Env Incorporation into Virions

Several models have been proposed for the incorporation of retroviral Env glycoprotein into virions as reviewed in [23, 70] (Figure 2).

3.1. Passive Incorporation. Passive incorporation is the simplest model for the incorporation of Env proteins into virus particles (Figure 2(a)). There are several lines of evidence supporting this model.

First, viral pseudotyping with a foreign glycoprotein can occur easily in many cases although there are some exceptions, one of which is the exclusion of HIV-1 or SIV Env with the long CT from most retrovirus cores [70]. With respect to HIV-1, the virus can be pseudotyped with Env glycoproteins not only from several other retroviruses but also with those from other virus families such as ortho (para) myxoviruses and flaviviruses [71–84].

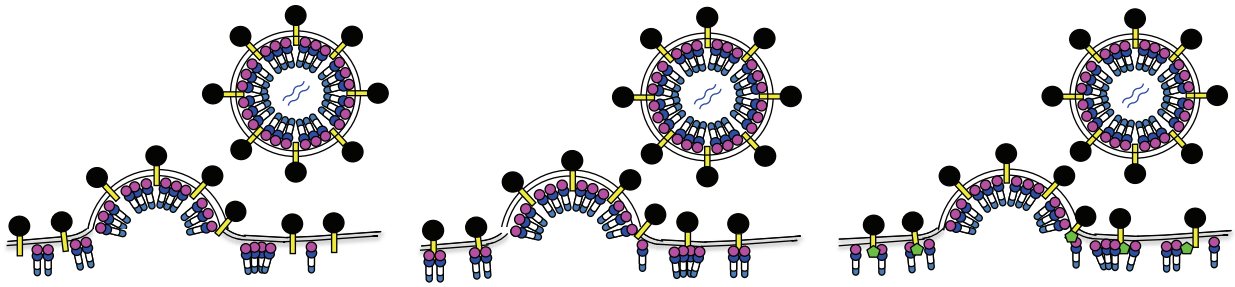
Second, retroviruses allow passive incorporation of host membrane proteins into virus particles [85–87]. Most cellular proteins are incorporated into the retrovirus envelope without significant sorting [88, 89].

Finally, in the case of HIV-1, several studies have demonstrated that the gp41 CT can be removed without affecting incorporation of the Env into virions, although this has been shown to occur only for some laboratory cell lines such as HeLa or 293T [90–94].

3.2. Regulated Incorporation through Direct Gag-Env Interactions. Although several lines of evidence support the passive incorporation model for retroviral Env, there is much evidence indicating that Env incorporation into virions is regulated by direct interactions between Gag and Env proteins (Figure 2(b)). Although removal of the gp41 CT sequence of HIV-1 has little effect on Env incorporation in some cell types, as described above, smaller deletions in CT regions cause severe defects in Env incorporation [95–100]. The MA domain of Gag has been shown to be important for Env incorporation into virions [91, 92, 101, 102]. The defect in Env incorporation caused by deletion of the gp41 CT is reversed by several MA mutations, indicating that an interaction between Env and the MA domain of Gag is required for incorporation of full-length Env into virions, at least in the case of HIV-1 [93, 98].

More evidence for direct Gag-Env interaction comes from the finding that HIV-1 Env directs Gag budding to the basolateral surface of polarized epithelial Madin-Darby canine kidney (MDCK) cells through the CT of HIV-1 Env, whereas Gag alone buds in a nonpolarized fashion [103–106]. The Tyr-based motif in the gp41 CT is also utilized in polarized budding of HIV-1 in lymphocytes [107]. Surprisingly, the polarized budding of HIV-1 in MDCK cells could also be promoted by MLV and HTLV-1 Env through their CT [108]. It also has been reported that coexpression of $Pr55^{Gag}$ inhibits endocytosis of HIV-1 Env through its interaction with the gp41 CT [63]. Another example of the specific Gag-Env interactions was demonstrated using Gag and Env proteins of MLV and HIV-1 in rat neurons [109]. Similarly, MLV Env is preferentially recruited onto MLV Gag through its CT domain in the presence of both MLV and HIV-1 cores although the authors also show an alternative mechanism by which the recruitment to HIV-1 budding sites is independent of the CT domain of MLV Env [110]. Furthermore, RSV Env is exclusively recruited to RSV budding sites through its CT, suggesting that the interaction between Env and Gag is direct in the case of this avian retrovirus [111].

In addition to the circumstantial evidence discussed above, some biochemical data suggest a direct interaction between Gag and Env. *In vitro* binding between MA and a gp41 CT-GST fusion protein has been reported for both



(a) Passive Incorporation

(b) Regulated Incorporation-1 (Direct Gag-Env Interaction)

(c) Regulated Incorporation-2 (Indirect Gag-Env Interaction)

Figure 2: Proposed models for Env incorporation. (a) The passive incorporation model assumes no interaction between Gag and Env. (b) In the first regulated incorporation model, a direct interaction between the MA domain of Gag and the CT domain of Env occurs during Env incorporation. (c) In the second regulated incorporation model, Gag and Env interact indirectly through a bridging protein (green pentagon) that binds to both proteins. The color scheme for Gag and Env is the same as that in Figure 1. The illustration was adapted from Checkley et al. with permission from Elsevier [23].

HIV-1 and SIV [112, 113]. Peptides corresponding to a large central domain of gp41 CT inhibited the capture of membrane-free $Pr55^{Gag}$ with an anti-p24 antibody [114]. In addition, a stable, detergent-resistant gp41-$Pr55^{Gag}$ interaction was detected in immature HIV-1 virions. The retention of gp41 in detergent-treated virions is dependent on the CT region, suggesting a direct or indirect interaction between $Pr55^{Gag}$ and gp41 [115, 116].

3.3. Regulated Incorporation through Indirect Gag-Env Interactions. In the third model, it is assumed that host cellular factors (mostly proteins) play a role in bridging Gag and Env in virus-infected cells (Figure 2(c)). Several host factors have been reported to bind to Gag and/or Env of HIV-1 or SIV however, only a couple of host factors were shown to be required for Env incorporation and/or viral replication.

The 47-kDa tail-interacting protein (TIP47) has been reported to bridge Gag and Env, allowing efficient Env incorporation in HIV-1 [117, 118]. The same group also showed that both the WE motif near the N-terminus of the MA domain and the YW motif in the gp41 CT domain are important for interactions between Gag or Env and TIP47 [118]. In a subsequent paper, the same group showed that mutations in either the WE motif of MA or the YW motif in the gp41 CT caused defects in virus replication in primary monocyte-derived macrophages [119]. Although this finding of an important role for TIP47 in Env incorporation in HIV-1 has received much attention from retrovirologists, no confirmatory data have been published by other researchers in this field.

Human discs large protein (hDlg1) has been reported to interact with the CT of HTLV-1 Env and to colocalize with both Env and Gag in virus-infected cells [120]. Subsequent work demonstrated that Dlg1 also binds HIV-1 Gag and that the expression level of Dlg1 is inversely correlated with HIV-1 Env expression and incorporation levels of the Env proteins, although the mechanism behind this phenomena needs to be investigated [121].

Prenylated Rab acceptor 1 (PRA1), which was identified as a Rab regulatory protein, was reported to be a binding partner for the SIV gp41 CT in a mammalian yeast two-hybrid (Y2H) assay [122]. Although colocalization of PRA1 and SIV Env was observed, changes in the endogenous levels of PRA1 did not affect virus production, Env incorporation, or infectivity of SIV or HIV-1 [123].

A Prohibitin 1/Prohibitin 2 (Phb1/Phb2) heterodimer was identified as a binding partner of the gp41 CT of HIV-1 using human T-cell lines and tandem affinity chromatography [124]. Phb1 and Phb2 are members of the prohibitin superfamily of proteins, which are localized to several cellular compartments such as the mitochondria, nucleus, and the PM [125, 126]. Gp41 CT mutants, in which binding to Phb1/Phb2 is disrupted, could replicate well in permissive cell types such as MT-4, but could not replicate efficiently in nonpermissive H9 cells [124]. Further analysis is necessary to elucidate the mechanism by which these proteins regulate virus replication through interactions with Env.

Luman, a transcription factor that is mainly localized to the ER, was found to interact with the gp41 CT of HIV-1 in a Y2H screen using a cDNA library from human peripheral blood lymphocytes (PBL) [127]. Overexpression of a constitutively active form of this protein reduced the intracellular levels of Gag and Env, leading to a decrease in virus release. The mechanism for this negative effect on virus assembly involves Luman binding to Tat, which decreases Tat-medicated transcription [127].

By using a Y2H screen with human cDNA libraries, p115-RhoGEF, an activator of Rho GTPase, was found to interact with the gp41 CT through its C-terminal regulatory domain [128]. The gp41 mutants that lost the ability to bind p115 showed impaired replication kinetics in T-cell lines such as SupT1, H9, and Jurkat, suggesting that the gp41 CT could

modulate the activity of p115-RhoGEF to support virus replication [128].

In addition to the host factors described above, calmodulin [129–132] and α-catenin [133–135] have been reported to interact with HIV-1 and/or SIV. However, their roles in virus replication, especially with respect to the Env functions of both proteins, have not been clearly elucidated.

4. Membrane Microdomains

Regardless of whether direct or indirect interactions between retroviral Gag and Env proteins are required for Env incorporation into virions, a great deal of experimental evidence suggests that retroviruses assemble and bud from "membrane microdomains." The most well-known microdomains are "lipid raft(s)," which are enriched in cholesterol and sphingolipids [136, 137]. Lipid rafts are widely thought to function as a platform for the assembly of protein complexes and to allow various biological processes such as cellular transport and signal transduction to proceed efficiently [138, 139]. Lipid rafts are reportedly used as assembly platforms or entry scaffolds in the replication of enveloped viruses such as retroviruses [140–146]. The association of Gag/Env with lipid rafts is important for the regulation of Env incorporation and pseudotyping [143, 144, 147, 148]. Evidence that both the HIV-1 $Pr55^{Gag}$ and Env proteins are preferentially localized to lipid rafts comes from biochemical studies as well as direct observations by microscopy [142, 149, 150].

Another membrane microdomain for retrovirus assembly is the "tetraspanin-enriched microdomain (TEM)" [151–154]. Tetraspanins are a superfamily of cell surface proteins that are ubiquitously expressed in mammalian cells. TEMs also act as platforms for signal transduction and immune responses. TEMs have been reported to be involved in the assembly and release of not only HIV-1, but also HTLV-1 and HCV [155]. When both HIV-1 and influenza virus were produced in the same cell, only HIV-1 colocalized with the TEM marker, and its release was inhibited by an anti-CD9 Ab, which led to extensive aggregation of tetraspanins [156]. Analysis of dynamics of both lipid rafts and TEMs by quantitative microscopy has revealed that components of both lipid rafts and TEMs are recruited during viral assembly to create a new microdomain that is different from preexisting membrane microdomains [153, 157].

There have been three recent reports in which both pseudotyping and microdomain issues were discussed. In the first paper, the authors examined HIV-1 assembly under conditions where the Env proteins of HIV-1 and Ebola virus were coexpressed with HIV-1 Gag in the same cell [158]. They found that infectious HIV-1 virions were released with both types of Env proteins. Interestingly, however, the virions contained either HIV-1 Env or Ebola virus glycoprotein (GP), but not both Env proteins within a single virion. These results suggest that HIV-1 Env and Ebola virus GP localized to distinct microdomains on the surface of the same cell [158]. In the second paper, the subcellular localization of Gag and Env proteins was investigated using a combination of three different retroviral Env proteins (RSV Env, MLV Env, or vesicular stomatitis virus (VSV) G) and two different Gag proteins (RSV or HIV-1) [111]. Both VSV-G and MLV Env were redistributed to the virus budding sites when coexpressed with HIV-1 or RSV Gag. In contrast, RSV Env was mostly transported to RSV budding sites. A subsequent paper from the same group showed that the CT of MLV is not required for recruitment of MLV Env to HIV-1 budding sites, suggesting that there are no specific interactions between MLV Env and HIV-1 Gag [110]. Collectively, these results also suggest that retroviral Env glycoproteins are not recruited to preexisting membrane platforms but rather that they are actively recruited to newly formed microdomains on the cell surface [111].

Human retroviruses such as HIV-1 and HTLV-1 spread more efficiently between target T cells by cell-cell infection than by cell-free infection [159, 160]. Sattentau et al. proposed, in analogy to the "immunological synapse", the "virological synapse (VS)" as a point of contact between virus-infected cells and uninfected cells [161, 162]. The molecular mechanisms of retroviral VS formation are as follows. (1) With respect to HIV-1 T-cell VS, initial contact between virus-infected cells and uninfected cells occurs through gp120-CD4 binding. Subsequent interactions between integrins and ICAMs enforce and maintain the stability of these junctions. (2) The gp120-CD4 interaction recruits CD4, coreceptors such as CXCR or CCR5, adhesion molecules, and filamentous actin into the synaptic area. (3) The cellular secretory machinery and microtubule organizing centers (MTOC) are polarized towards the HIV-1 assembly sites at the PM to form the VS. It has been reported that a so-called microsynapse formed by nanotubes between virus-infected cells and uninfected cells is also involved in cell-cell infection of HIV-1 [84, 163]. In cell-cell transfer of HTLV-1-infected cells, an extracellular matrix structure referred to as the "viral biofilm" was proposed as an alternative to the VS [164]. In addition to HIV-1 and HTLV-1, the spread of MLV between fibroblasts also occurs via the VS [165, 166]. It is noteworthy that assembly of MLV is directed towards cell-cell contact sites through the interaction of the CT of MLV Env with Gag [167, 168]. Although the concept of cell-cell infection through the VS is now well appreciated, the detailed molecular mechanism of VS assembly and its relevance to viral spread *in vivo* will require further elucidation through the use of more advanced techniques.

5. Conclusions and Perspectives

Incorporation of Env glycoproteins into virions is crucial for producing infectious retroviral particles. Although this paper has introduced several experimental models for retroviral Env trafficking and/or incorporation, the correct mechanism for this process is still unclear. The following questions must be clearly addressed to not only gain a better understanding of this complex biological process, but also to develop new antiretroviral compounds that target Env incorporation.

(1) What are the structures of the CTs of retroviral Env proteins? The answers for this question will give

useful information on elucidating a role of the Env CTs in the Env trafficking and/or incorporation in virus-infected cells.

(2) What host factor(s) are necessary for the retroviral Env trafficking and/or incorporation into virions?

(3) Where and how Env and Gag proteins of retroviruses are recruited to the assembly sites in order to form infectious virus particles?

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Review Article

Restriction of Retroviral Replication by Tetherin/BST-2

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Tetherin/BST-2 is an important host restriction factor that limits the replication of HIV and other enveloped viruses. Tetherin is a type II membrane glycoprotein with a very unusual domain structure that allows it to engage budding virions and retain them on the plasma membrane of infected cells. Following the initial report identifying tetherin as the host cell factor targeted by the HIV-1 Vpu gene, knowledge of the molecular, structural, and cellular biology of tetherin has rapidly advanced. This paper summarizes the discovery and impact of tetherin biology on the HIV field, with a focus on recent advances in understanding its structure and function. The relevance of tetherin to replication and spread of other retroviruses is also reviewed. Tetherin is a unique host restriction factor that is likely to continue to provide new insights into host-virus interactions and illustrates well the varied ways by which host organisms defend against viral pathogens.

1. Introduction

Viruses and their host organisms engage in a series of conflicts in which viruses can be thought of as leading the offense, placing the host on defense. Host defenses against retroviral replication have arisen in a wide variety of forms. Classical cellular and humoral immune responses may limit retroviral replication and may be sufficient to prevent adverse outcomes in some host-virus interactions. However, throughout the evolution of mammals a series of alternative host defense factors have arisen whose apparent primary function is to counteract retroviruses in ways that lie outside of classical innate or adaptive immunity. These intrinsic host defense mechanisms against viruses have come to light largely through comparative studies of inhibition or "restriction" of replication of HIV or SIV in cells from different origins and are collectively referred to as host restriction factors. APOBEC3G, TRIM5alpha, and tetherin are the most prominent of a series of host restriction factors to be identified in recent years that limit HIV replication. This paper focuses on the discovery and subsequent characterization of tetherin, with an emphasis on recent work aimed at elucidating how its structure leads to retention of particles on the plasma membrane and on how Vpu acts to overcome tetherin-mediated restriction.

2. Identification of Tetherin as an Antiviral Host Restriction Factor

The discovery of tetherin is intimately linked to studies of the effects of the HIV accessory gene Vpu. Vpu is a small integral membrane protein encoded by HIV-1 and a limited subset of SIV species. Early studies utilizing HIV proviruses deficient for Vpu expression revealed that fewer particles were released from infected cells despite apparently normal production of all other viral proteins [1, 2]. Furthermore, electron microscopic analysis revealed striking accumulations of particles at the cell surface and within intracellular compartments of infected cells, revealing a defect at a late stage of particle release [3]. Subsequent work revealed that one of two important functions of Vpu was the downregulation of CD4 through interactions with cellular proteasomal degradation pathways [4–9]. Vpu was found to bind both CD4 and the human beta transducing-repeat containing protein (β-TrCP) [10, 11], connecting CD4 to the ubiquitin-proteasome machinery and inducing its degradation in the endoplasmic reticulum. Casein kinase phosphorylation sites on the Vpu cytoplasmic tail at residues 52 and 56 were found to be critical for β-TrCP interactions and for CD4 downregulation [10, 12]. This line of investigation along with other investigations into Vpu function prior to the discovery

of tetherin is reviewed in [13]. However, the ability of Vpu to enhance particle release in human cells was not explained by downregulation of CD4 and remained a mystery for many years.

Experiments leading to the discovery of the function of the HIV Vif protein and its host restriction factor APOBEC3G [14, 15] provided a potential clue to the particle release function of Vpu. Like the infectivity conferred by Vif, the particle release function of Vpu proved to be cell type specific, suggesting that it might be overcoming a cellular factor involved in limiting particle release [16, 17]. A key experiment demonstrated that heterokaryons between restrictive, Vpu-responsive HeLa cells and permissive, Vpu-unresponsive Cos-7 cells were restricted in particle release, suggesting that a negative (restricting) factor was dominant [18]. Vpu was able to enhance particle release in the heterokaryons, demonstrating that the factor from human cells restricting particle release could be overcome by Vpu [18].

Several cellular factors were described as potential targets of Vpu prior to or concomitant with the identification of tetherin, including TASK-1 [19] and CAML [20]. However, neither of these factors has subsequently proven to be the restriction factor targeted by Vpu. Instead, a series of key findings led by Stuart Neil in the Bieniasz laboratory resulted in the ultimate identification of tetherin as the restriction factor targeted by Vpu. First, these investigators demonstrated clearly that the effect of Vpu was on particle release rather than other steps in virus assembly, while retention of virions and subsequent endocytosis occurred in the absence of Vpu [21]. The specific particle retention activity was found to be prominent in HeLa cells as before, while a subset of human cells such as HOS or 293T cells lacked this activity. The next key observation was that the restricting activity could be induced by type I interferons. Neil and colleagues demonstrated that retention of Vpu-deficient HIV-1 particles at the plasma membrane could be induced in 293T or HOS cells and that treatment with the protease subtilisin released the particles from the cell surface [22]. Furthermore, the restricting activity extended to additional virus genera, as Ebola VP40 release was similarly deficient in an IFN-induced manner and its release could be enhanced by Vpu. These results suggested that an interferon-inducible, proteinaceous tether was responsible for retaining enveloped viruses at the cell surface. In 2008 this factor was identified by the same group as BST-2/CD317 and renamed tetherin because of this prominent biological function [23].

BST-2 had first been cloned as a membrane antigen present on bone marrow stromal cells and synovial cells that was thought to be involved in pre-B-cell growth [24]. The same protein had been identified as a membrane antigen termed HM1.24, present on terminally differentiated B cells, and was thought to be a potential anticancer target for multiple myeloma [25]. The terminology for the HM1.24 antigen was later changed to CD317 [26]. BST-2 was later shown to be an interferon-inducible antigen and identical to plasmacytoid dendritic cell antigen-1 (PDCA1) in mice [27]. CD317/BST-2 is a highly unusual type II integral membrane protein, with a transmembrane domain near its N-terminus and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor (Figure 1). The protein localizes to lipid rafts on the plasma membrane and to the trans-Golgi network (TGN) and is endocytosed from the plasma membrane through a clathrin-dependent pathway [28]. Remarkably, a membrane proteomic screen examining the effects of the K5 protein of KSHV revealed a marked downregulation of CD317/BST-2 and even showed almost as an afterthought that HIV-1 Vpu downregulated the protein [29]. This published observation led the Guatelli group to examine CD317/BST-2 as a candidate restriction factor targeted by Vpu, and their findings were published soon after the identification of tetherin by the Bieniasz group [30]. For the purpose of this paper, BST-2/CD317/tetherin will be hereafter referred to simply as tetherin.

3. Structural Biology of Tetherin and Functional Implications

One of the most fascinating aspects of tetherin biology is how its structure allows for retention of enveloped virions through protein-lipid and protein-protein interactions occurring at the particle budding site. As already mentioned, tetherin's basic domain structure is highly unusual. Tetherin is a type II membrane protein bearing a small N-terminal cytoplasmic domain, a transmembrane region, an ectodomain forming a coiled-coil in tetherin dimers, and a C-terminal GPI anchor (Figure 1) [31]. The double-membrane anchor plays a key role in the ability of tetherin to restrict enveloped virus particle release, presumably because one anchor is present on the plasma membrane of the cell and the second is inserted into the viral membrane [23] (Figure 2). Three cysteines in the N-terminal ectodomain of tetherin (C53, C63, C91) are capable of forming disulfide-linked dimers [32, 33], and mutation of all three abolished dimer formation and greatly reduced the ability of tetherin to restrict Vpu-deficient HIV release [34]. Two N-linked glycosylation sites (N65 and N92) lead to some variability of migration on SDS-PAGE analysis and appear to play a role in correct folding and transport of tetherin to the cell surface in one report [34], while another group found that alteration of N-linked glycosylation sites had no effect on virus restriction or cell surface levels [33].

Four reports of the tetherin ectodomain structure have been published [35–38]. The ectodomain forms a long extended rod-like conformation in a loose or imperfect coiled-coil parallel dimer [35, 38], suggesting that there is some conformational flexibility in the C-terminal portion of the ectodomain that may be required to accommodate dynamic changes in membrane deformation at the particle budding site. Disulfide bonds stabilize the dimeric N-terminal region, which cannot stably dimerize in their absence [38]. Unexpectedly, tetrameric forms of tetherin were also detected in crystallization studies [36, 38]. The biological function of tetherin tetramers remains uncertain and mutations designed to disrupt the tetramer did not prevent tetherin-mediated particle restriction [36, 38]. The crystal structure of murine BST-2/tetherin ectodomain

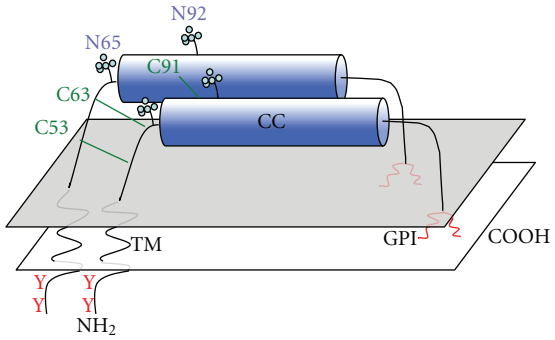

Figure 1: Schematic representation of tetherin domain structure. Tetherin is depicted as a parallel dimer with both transmembrane (TM) and glycophosphatidylinositol (GPI) membrane anchors in the same membrane. Disulfide linkages are depicted in green, and N-linked glycosylation sites pictured. CC: coiled coil; Y: tyrosine residues critical for endocytic motif.

revealed similar ectodomain architecture, and suggested that tetrameric assemblies may form a curved assembly that functions as a sensor of membrane curvature, analogous to BAR domains [37]. The authors of this paper suggest that tetrameric assemblies may facilitate the clustering of tetherin around the neck of a budding virus as has been seen in immunoelectron microscopic analysis [39, 40]. At the current time, the significance of the tetrameric assemblies remains unclear but quite intriguing.

While tetherin is thought to be a raft-associated protein through its C-terminal GPI anchor, a recent report questioned this and suggested that instead the C-terminus of tetherin acts as a second transmembrane domain [41]. This unexpected result is intriguing and awaits further verification.

4. Tetherin Clustering in Membrane Microdomains and Role of the Actin Cytoskeleton

The functional significance of tetherin's unusual structure and topology to its mechanism of restriction of viral budding have not yet been entirely delineated. However, there is significant biochemical and microscopic evidence that tetherin functions as a physical tether connecting virions to the plasma membrane. Immunoelectron microscopic analysis has shown clear evidence of clustering of tetherin on discrete cell surface microdomains and sometimes on filopodia or at the location of coated pits, in the absence of viral infection [39, 40]. In infected cells, immunogold beads are most often observed at the neck of the budding particle and at the site of connections between particle membranes [39, 40] (Figure 2(a)) Tetherin is enriched on the particle membrane itself [39, 40, 42], as well as on filamentous connections that sometimes are present linking particles to one another [40]. Microdomain clustering of tetherin can also be readily observed by superresolution light microscopic techniques [43, 44]. We recently described a tetherin ectodomain mutant with four substitutions in the coiled-coil region (4S)

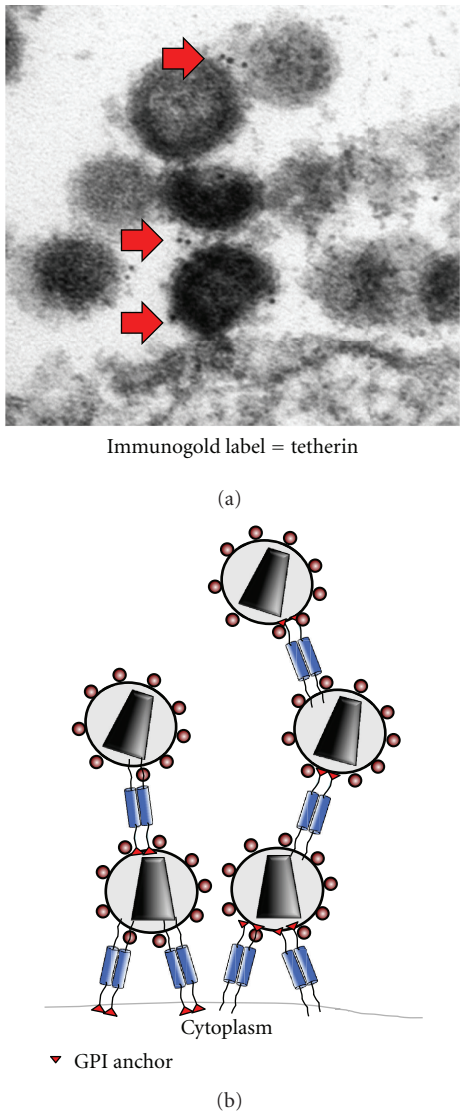

Figure 2: (a) Tetherin on the cell surface of A3.01 T cells infected with NLUdel virus, treated with indinavir to preserve particle morphology for preparation. Arrows indicate immunogold beads; primary antibody was rabbit anti-tetherin polyclonal antisera. (b) Schematic depiction of parallel homodimers of tetherin retaining HIV particles on the plasma membrane; tetherin is not to scale in this diagram.

that was expressed well on the cell surface, yet lost the ability to cluster in plasma membrane microdomains and was unable to restrict release of viral particles [43]. The loss of discrete puncta formation of the 4S mutant was associated with an increase in lateral mobility as measured by fluorescence recovery after photobleaching (FRAP), while wild-type, restrictive tetherin was constrained in lateral mobility when compared with classical GPI-anchored proteins [43]. These findings imply that tetherin's restriction of particle release requires localization in discrete microdomains that help to form or are in the immediate vicinity of the developing particle bud. In other words, tetherin's presence

on the plasma membrane globally may not be as important as its discrete localization at the site of particle budding. While clustering appears to be associated with restriction, relief of restriction by Vpu is not achieved through removal of tetherin from lipid rafts as measured by partitioning into detergent-resistant membranes [45, 46]. The lack of mobility of tetherin in clustered plasma membrane sites is potentially regulated through interactions not only with lipid microdomains but also with the underlying cytoskeleton.

The potential for regulation of tetherin clustering through interactions with the underlying actin cytoskeleton is supported by the report from Rollason and colleagues of a direct interaction between tetherin and the RhoGAP protein RICH2 [47]. RICH2 contains both an N-terminal BAR domain and a Rho/Rac/cdc42 GAP domain [48, 49]. The presence of a BAR domain capable of inducing membrane tubulation is curious, given the previously mentioned modeling of tetherin tetramers as a BAR domain [37]. The potential for tetherin to act as a link to the regulation of Rac and Rho through the GAP activity of RICH2 is also intriguing. Perhaps more directly relevant to peripheral clustering of tetherin is the known interaction of RICH2 with EBP50 (ERM-binding phosphoprotein 50) through its C-terminal ESTAL domain [50, 51]. EBP50 acts as a linker between ERM proteins and the cytoplasmic tails of integral membrane proteins, in this case tetherin. This suggests that tetherin is connected indirectly to the underlying cortical actin cytoskeleton through a RICH2-EBP50-ezrin complex. Because RICH2 interacts with the same region of the tetherin cytoplasmic tail that binds μ1 and μ2 and directs its clathrin-mediated endocytosis [28], the interaction with RICH2 and the actin cytoskeleton might be predicted to stabilize tetherin on the plasma membrane and prevent its endocytosis. Much remains to be learned about the functional role of tetherin's interaction with RICH2 and connection to actin, as well as with the potential modulation of Rho family GTPases. One pressing question that has not yet been addressed is whether this cytoskeletal anchoring plays a role in restriction of particle release and in the punctate clustering of tetherin on the cell surface.

A counterargument against the role of additional cellular factors in tetherin-mediated restriction may be made in light of evidence from the Bieniasz laboratory demonstrating that an artificial tetherin-like molecule pieced together from domains of three distinct proteins (art-tetherin) can restrict particle release [34]. This strategy employed stitching together the cytoplasmic tail and transmembrane domain of the transferrin receptor, the helical coiled-coil domain of DMPK (dystrophia myotonica protein kinase), and the C-terminus of uPAR that includes a GPI anchor. The investigators in effect recreated the domain architecture of tetherin from sequence-unrelated proteins and quite strikingly were able to inhibit HIV particle release through overexpression of art-tetherin [34]. Despite the ability of this artificial construct to restrict particle release, cellular interactors of wild-type tetherin in relevant human cells clearly play a role in its endocytosis and recycling, and the potential for functional significance of the RICH2-EBP50-ezrin-actin linkage remains.

5. Counteraction of Tetherin-Mediated Restriction of Particle Release by Vpu

Following the identification of tetherin as the restriction factor responsible for retention of HIV particles, attention turned to understanding the molecular and cellular mechanisms underlying the relief of tetherin-mediated restriction by Vpu. Comparison of the effects of Vpu on tetherin molecules from nonhuman primates helped to identify critical domains involved in tetherin-Vpu interactions and provided important clues to the evolution of tetherin and of viral countermeasures designed to overcome restriction. Counteraction of tetherin-mediated restriction was mapped to specific interactions between the transmembrane domain of Vpu and the transmembrane domain of tetherin [34, 52–55]. Coimmunoprecipitation studies performed by several groups confirmed a physical interaction between tetherin and Vpu, and the interaction required residues within the TM domains of both Vpu and tetherin as suggested by genetic studies [54, 56–58]. A single-residue alteration in human tetherin to one found in tetherin from the Tantalus monkey (T45I) rendered it Vpu insensitive, yet still able to restrict HIV-1 [55]. Tetherin variants from rhesus macaques and mice were similarly able to restrict HIV-1 release and yet were insensitive to Vpu, and transfer of the corresponding TM region between tetherin molecules from different species conferred sensitivity or resistance [52]. Furthermore, there is strong evidence of positive selection among primate tetherin molecules, and the selected changes were enriched in the N-terminal and TM regions of tetherin, suggesting frequent episodes of evolution under selection pressure to evade viral countermeasures [52, 55]. The discovery that SIV Nef proteins downregulate tetherin from rhesus macaque, sooty mangabey, and African green monkey but are inactive against human tetherin provided evidence that primate lentiviruses have targeted tetherin in different ways over evolutionary history [56, 59]. The Vpu proteins from SIVgsn, SIVmus, and SIVmon are able to downregulate both CD4 and tetherin in cells from their cognate primate species, while Vpu from SIVcpz, the precursor virus of HIV-1, is unable to downregulate chimpanzee tetherin and instead utilizes Nef for this function [60]. The Vpu protein of HIV-1 group M, but not group O or group N, is able to downregulate both tetherin and CD4, and the presence of this fully functional Vpu has been proposed as a reason for the worldwide spread of group M versus the nonpandemic HIV-1 strains [60, 61]. Thus, species-specific differences in tetherin and in lentiviral countermeasures against tetherin have played a major role in cross-species transmission and subsequent spread of lentiviruses and have likely been an important contributor to the current HIV-1 pandemic. While these species-specific differences are the rule, there are exceptions. Shingai and colleagues demonstrated that some HIV-1 Vpu proteins are able to antagonize rhesus tetherin, indicating that some HIV-1 isolates encode a Vpu protein with a broader host range [62].

Tetherin cell surface levels are downregulated by Vpu, and degradation of tetherin by Vpu has been observed in a

wide variety of cell types [30, 54, 63, 64]. The logical hypothesis suggested by this association was that Vpu overcomes restriction by removing tetherin from plasma membrane viral assembly sites and targeting tetherin for degradation, as has been well established for CD4. The downregulation of CD4 by Vpu requires the phosphorylation of serines 52 and 56 on the Vpu cytoplasmic tail, interaction with β-TrCP, and degradation of CD4 through the ubiquitin-proteasome pathway [10–12, 65]. The mechanism and importance of downregulation of tetherin by Vpu, however, have not yet been as clearly worked out. Several groups have reported that relief of tetherin-mediated restriction of particle release can occur in the absence of degradation of tetherin [57, 66, 67], indicating that degradation is not the essential step in the action of Vpu that leads to relief of restriction. Goffinet and colleagues generated a series of tetherin cytoplasmic tail mutants including lysine mutants that were not degraded upon expression of Vpu. The mutants remained competent for restriction of particle release, and despite their lack of degradation Vpu potently relieved the restriction to particle release [66]. The involvement of β-TrCP in Vpu-mediated targeted degradation of tetherin has been supported by a number of investigators [54, 63, 64, 68], which would seem to suggest that a proteasomal pathway of degradation similar to that involved in the Vpu- β-TrCP-CD4 pathway is essential. Proteasomal degradation of tetherin has indeed been supported in some studies [63, 64] but is not universally accepted as the major pathway. Instead, a β-TrCP-dependent endolysosomal pathway for tetherin degradation has been reported [54, 58, 68]. According to this model, Vpu still acts as an adaptor molecule linking tetherin to β-TrCP, but does not connect tetherin to the ER-associated protein degradation (ERAD) pathway. Instead, interactions in the TGN or early endosome compartments direct tetherin to degradation in lysosomal compartments. There still is work to be done to clarify this pathway and to derive a clearer understanding of the role of β-TrCP and of the degradation of tetherin that is initiated or facilitated by Vpu.

The site of interaction of Vpu with tetherin is not known with certainty. Expression of Vpu alters the intracellular pattern of tetherin, with decreased cell surface of tetherin and prominent colocalization of tetherin and Vpu in the TGN [23, 43, 57, 68]. Mutants of Vpu that are unable to interact with tetherin fail to redistribute tetherin to the TGN, suggesting that tetherin may be retained in the TGN through TM-TM interactions with Vpu [57]. The rate of tetherin endocytosis from the plasma membrane is not significantly altered by Vpu [43, 57, 69]. These data suggest that Vpu may alter delivery of newly synthesized tetherin to the plasma membrane and/or disrupt outward tetherin recycling from the endosomal recycling compartment. Taken together with the data described above regarding endolysosomal degradation, a consistent model would posit that Vpu interacts with and traps tetherin in the TGN or other post-ER compartments, thereafter shunting tetherin to degradation in lysosomal compartments and preventing newly synthesized tetherin from trafficking to the plasma membrane. Alternatively, Vpu may disrupt outward trafficking of tetherin to the particle assembly microdomain on the plasma membrane through additional effects on host trafficking factors.

6. Counteraction of Tetherin by Other Viruses

The significance of tetherin as a bona fide host restriction factor is convincingly demonstrated by the fact that diverse families of enveloped viruses have developed distinct mechanisms to overcome its inhibitory effects. One of the earliest factors identified that enhanced the release of *vpu*-deficient HIV-1 and produced efficient release of HIV-2 in restrictive cell types was the envelope glycoprotein of certain strains of HIV-2, in particular ROD10 Env [70–72]. Although the effect of HIV-2 Env on particle release was described well before the identification of tetherin as the target of Vpu, it is now clear that it does so through acting as a tetherin antagonist. HIV-2 Env appears to exclude tetherin from the site of viral budding through direct interaction with tetherin leading to sequestration within the TGN [73]. Determinants of tetherin antagonism by HIV-2 Env include a highly conserved endocytic-sorting motif (GYXXθ) in the cytoplasmic tail of gp41 [73, 74]. This sorting motif binds clathrin in an AP-2-dependent manner and is responsible for the redistribution of tetherin from the plasma membrane and concentration within endosomal compartments, in particular the TGN [73, 75, 76]. Interestingly, the gp41 ectodomain of HIV-2 Env has also been implicated in tetherin antagonism [73, 77]. The exact region required for physical tetherin interaction remains unclear due to the inability to differentiate those areas responsible for interaction and those residues involved in maintenance of tertiary Env structure. Additionally, proteolytic Env cleavage into gp120/gp41 subunits is required, as the unprocessed form is incompetent for virion egress and tetherin sequestration [5, 64]. It is interesting to note that, while Vpu expression leads to reduced cellular levels of tetherin, HIV-2 Env reduces cell surface levels but not total cellular levels of tetherin [73]. Finally, the ability of HIV-2 Env to counteract restriction is dependent on conservation of the tetherin ectodomain sequence [78]. Together, these data strongly suggest an interaction between the tetherin and mature HIV-2 Env ectodomains that leads to intracellular trapping of tetherin and abrogates restriction of particle release.

The K5 protein of KSHV (Human Herpesvirus 8; HHV-8) was the first viral component shown to specifically target tetherin prior to its identification as a viral restriction factor [29]. The K5 protein is a RICH-CH (MARCH) family of cellular transmembrane E3 ubiquitin ligases. This family of proteins facilitates the ubiquitination and subsequent degradation of transmembrane proteins. K5 exhibits potent immunomodulatory function resulting in the degradation of major histocompatibility complex (MHC) proteins (MHC), adhesion molecules, and NK receptor ligands while also promoting the degradation of tetherin through ubiquitination of lysine residues in the tetherin cytoplasmic tail [79, 80]. K5-mediated tetherin degradation is ESCRT-dependent, and ubiquitination of K18 in the CT of tetherin by K5 is critical for the efficient release of KSHV [79, 80]. In the case of K5, it is clear that ubiquitination in a post-ER compartment

targets tetherin for degradation via ubiquitin-dependent endolysosomal pathways [80].

Ebola virus overcomes tetherin-mediated restriction through the activity of its surface glycoprotein (GP) [81]. The Ebola virus GP has a broad species specificity comprising an ability to antagonize both human and murine tetherin. The Ebola GP mechanism of action appears to be novel, as it relieves restriction without reducing tetherin cell surface concentration and can even relieve the restriction conferred by a wholly artificial tetherin molecule [82]. It was recently reported that the GP2 subunit of Ebola interacts with tetherin, and another filovirus GP (Marburg virus GP) was shown to have anti-tetherin activity [83]. The mechanism of action of Ebola GP is perhaps the least clear of the tetherin antagonists that have been described to date.

7. *In Vivo* Significance of Tetherin for Viral Spread and Pathogenesis

The importance of tetherin for restricting viral replication is strongly supported by the multiple mechanisms described above by which viruses can overcome its tethering function and by the evidence of positive selection of tetherin in the primate lineage. The assumption would logically be that tetherin inhibits release of free virus, preventing infection of additional cells and limiting overall replication (and potentially pathogenesis) within an organism. However, whether or not tetherin restricts cell-cell spread remains to be definitively established. Casartelli and coworkers demonstrated that the formation of virologic synapses was not prevented by tetherin, but that tetherin did limit cell-cell transmission of virus [84]. Another group found similarly that cell-cell transmission was inhibited by tetherin in a flow-cytometry-based assay [85]. In contrast, Jolly and colleagues demonstrated that depletion of tetherin diminished virologic synapse formation and cell-cell spread and suggested that under some circumstances tetherin may actually enhance cell-cell transmission [86]. Depletion of tetherin in mature dendritic cells was not associated with a significant enhancement of transmission to CD4+ T cells in another report, although modest enhancement or inhibition of cell-cell transmission was seen that differed with the stimulus utilized for maturation of dendritic cells [87]. Currently there is a need for further investigation into this question, as there is not a clear consensus in the field.

Tetherin knockout mice have provided additional weight to the argument that this protein has evolved as an interferon-induced host defense mechanism to limit viral replication *in vivo*. Liberatore and Bieniasz used poly(I:C) to enhance tetherin expression in wild-type mice and found that replication of Moloney murine leukemia virus (Mo-MLV) in these mice was significantly attenuated as compared with tetherin-deficient mice [88]. Using a murine leukemia virus strain that induces a strong interferon response, they then demonstrated that tetherin-deficient mice developed both higher levels of MLV viremia and enhanced pathology [88]. A different strategy utilizing a naturally occurring polymorphism in tetherin in NZW mice allowed Barrett and colleagues to study Friend virus replication in mice homozygous for enhanced versus normal tetherin cell surface expression. These investigators demonstrated that enhanced cell surface tetherin *in vivo* correlated with diminished replication of Friend virus and improved outcomes [89]. Together these reports provide solid evidence that tetherin acts as an antiretroviral host restriction factor *in vivo*. A modest inhibitory effect of tetherin on Mo-MLV replication was also reported by Swiecki and colleagues, consistent with the effects seen by Liberatore and Bieniasz in the absence of IFN induction [90]. Surprisingly, however, these authors observed lower viral titers and enhanced virus-specific CD8+ T-cell responses in tetherin-deficient mice infected with vesicular stomatitis virus or influenza virus. Thus, while tetherin's antiretroviral effects are clear, there may be more complexity in how tetherin alters antigen processing and affects the replication of other enveloped viruses *in vivo*.

8. Summary

Tetherin is an unusual host protein that restricts enveloped particle release at the very latest stage of the viral life-cycle through physically tethering virions to the plasma membrane. A number of unrelated viruses have developed the means to overcome restriction by tetherin and have done so through different mechanisms. The acquisition of Vpu by primate lentiviruses and its ability to counteract restriction by human tetherin is thought to be an important factor in cross-species transmission and potentially in the magnitude of the HIV-1 pandemic itself. The flurry of recent studies examining tetherin and its antagonists emphasizes the significance of this potent antiviral host restriction factor. Future studies should shed light not only on the mechanism of action of Vpu, but will likely identify additional enveloped viruses that have developed the means to antagonize tetherin. Studies examining the cellular interactions of tetherin are also poised to provide new insights into the nature of the particle assembly site, trafficking of membrane glycoproteins to the particle assembly site, and the role of the cortical actin cytoskeleton in particle release.

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Review Article

The Impact of Macrophage Nucleotide Pools on HIV-1 Reverse Transcription, Viral Replication, and the Development of Novel Antiviral Agents

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Macrophages are ubiquitous and represent a significant viral reservoir for HIV-1. Macrophages are nondividing, terminally differentiated cells, which have a unique cellular microenvironment relative to actively dividing T lymphocytes, all of which can impact HIV-1 infection/replication, design of inhibitors targeting viral replication in these cells, emergence of mutations within the HIV-1 genome, and disease progression. Scarce dNTPs drive rNTP incorporation into the proviral DNA in macrophages but not lymphocytes. Furthermore, the efficacy of a ribose-based inhibitor that potently inhibits HIV-1 replication in macrophages, has prompted a reconsideration of the previously accepted dogma that 2′-deoxy-based inhibitors demonstrate effective inhibition of HIV-1 replication. Additionally, higher levels of dUTP and rNTP incorporation in macrophages, and lack of repair mechanisms relative to lymphocytes, provide a further mechanistic understanding required to develop targeted inhibition of viral replication in macrophages. Together, the concentrations of dNTPs and rNTPs within macrophages comprise a distinctive cellular environment that directly impacts HIV-1 replication in macrophages and provides unique insight into novel therapeutic mechanisms that could be exploited to eliminate virus from these cells.

1. Introduction

Macrophages are a key reservoir for HIV-1, and their ubiquitous nature, multiple, and often independent microenvironments in which they are contained, coupled with their susceptibility to HIV-1 infection [1–3], dictate that further understanding must be garnered about the distinctive characteristics of macrophages and the subsequent impact on the dynamics of HIV-1 infection in these cells. Despite these factors, most of the attention on reservoirs for latent HIV-1 has focused on cells of lymphoid origin, most notably $CD4^+/CD45RO^+$ memory lymphocytes [4]. Consequently, the interplay between HIV-1 infection in macrophages and macrophage-like cells is markedly less defined. Additionally, the relationship between *in vitro* observations and *in vivo* dynamics is not fully elucidated. Much evidence exists to support the existence of HIV-1 replication in macrophage/macrophage-like cells *in vivo* [5–11], including a recent report from Deleage et al., and confirmed the presence of HIV-1 in macrophages within seminal vesicles of patients on effective highly active antiretroviral therapy (HAART) [12]. Correspondingly, a variety of studies have presented evidence that monocytes harbor productive viral replication in patients receiving HAART [13, 14], with other reports demonstrating that $CD16^+$ monocytes, a subset of monocytes, are a source of HIV-1 permissive cells that preferentially harbor HIV-1 *in vivo* [15]. Complementary to these findings, a recent report by Spivak and colleagues demonstrated that circulating monocytes do not harbor latent HIV-1 in elite controllers [16], and an additional finding from

Ortiz et al. demonstrated the presence of SIV originating from nonlymphocytic compartments in CD3-depleted rhesus macaques [17]. Despite these findings, they did report the presence of HIV-1 in $CD4^+$ T cells in some patients receiving HAART. Together, these studies correlate *in vitro* hypotheses with *in vivo* evidence implicating macrophages as key modulators in viral persistence and warrant further studies designed to fully elucidate this relationship.

As macrophages are found in diverse tissues that are often independent microenvironments, systemically, and function largely in innate immunity and subsequent antigen presentation to $CD4^+$ T lymphocytes in adaptive immunity, their cell cycle and metabolism are clearly distinct from that observed in the activated, proliferating $CD4^+$ T lymphocyte.

Significantly lower levels of dNTP in macrophages than observed in T lymphocytes (Table 1) [18, 19] present a macrophage cellular environment that harbors extremely limited dNTPs, but still high rNTPs (Table 2). This extreme disparity between dNTP and rNTP pools in macrophages can promote preferential incorporation of rNTP into the growing viral DNA strand [19]. Furthermore, understanding which nucleotides present with the highest concentrations in macrophages, which is often distinct and independent from that observed in lymphocytes, serves to facilitate a more robust mechanistic understanding of nucleotide incorporation to be drawn upon in nucleoside analogue drug design. It is now known that the meager macrophage nucleotide dNTP pool is shaped by the macrophages/monocyte restriction factor, SAMHD1, whose triphosphohydrolase activity reduces intracellular dNTP to concentrations that are suboptimal for HIV-1 RT-mediated viral DNA synthesis [20, 21].

Although levels of dNTP and rNTP and ratios have been elucidated in macrophages, the impact of preferential rNMP incorporation in macrophages has only recently been explored. Recent reports demonstrate that a concomitant lack of monoribonucleotide repair machinery in these cells, and pausing during DNA synthesis (which is a known correlate of mutagenesis), may point to viral mutagenesis [22].

The cellular milieu of macrophages presents with multiple facets that are specific to these cells, all of which comprise a unique microenvironment wherein concentrations of dNTPs and rNTPs orchestrate a complex relationship between HIV-1 and individual or distinct populations of macrophages. Much of these data have been compiled with the use of *in vitro* monocyte-derived macrophages, which represent an excellent tool to model potential *in vivo* dynamics of macrophages found in various microenvironments, although differences between an *in vitro* system and that observed in humans could exist. Nonetheless, compiling a detailed understanding of this interplay can provide a foundation from which to exploit macrophage-specific factors to achieve targeted elimination of HIV-1 from these cells.

2. dNTP Levels in Macrophages: Affecting HIV-1 Reverse Transcription

Lentiviruses possess the unique ability to replicate in nondividing and terminally differentiated cells, unlike many other viruses including oncoretroviruses [23]. The manner in which this is accomplished, and the complex and multifaceted mechanisms that are employed to achieve productive viral replication in nondividing cells, is unique from that observed in activated dividing cells, such as T lymphocytes.

It is well established that activated, proliferating cells possess significantly higher levels of endogenous dNTPs, which are required for ongoing cellular chromosomal replication in an activated and dividing cell. It follows that dNTP concentrations in T cells are 6–133-fold higher in lymphocytes compared to macrophages, independent of the T cell or macrophage activation state (Table 1) [18, 19, 21, 24], as macrophages are terminally differentiated nondividing cells.

HIV-1 replication requires a basal level of dNTP to be present to facilitate efficient production of proviral DNA, and without sufficient dNTP levels, productive viral replication occurs suboptimally [24]. Despite significantly lower levels of dNTP in macrophages versus lymphocytes (Table 1), HIV-1 replication is able to proceed due to the uniquely high affinity of HIV-1 RT for its substrate, which facilitates its function.

Viral replication kinetics are delayed in macrophages versus $CD4^+$ T cells, and is thought to be a direct function, at least in part, of lower levels of dNTPs available in these cells. Addition of deoxynucleosides (dNs) to the extracellular culture medium, which elevates cellular dNTP concentrations, significantly increases the rate of viral reverse transcription in HIV-infected primary human macrophages, indicating that low levels of dNTPs are a rate-limiting step in the production of HIV-1 [24, 26, 27]. Additionally, the Michaelis constant [28] for dNTPs is low, allowing for efficient binding despite lower overall levels of dNTPs in macrophages (Table 1). This low K_m is thought to be a result of enzymatic adaptation of viral RT to infect macrophages, allowing for efficient catalysis of viral DNA synthesis despite the low dNTP levels present in these cells [24, 26].

3. Levels of dNTPs and Impact on Relative Rate of Incorporation in Macrophages

dNTPs are significantly lower in macrophages compared to T lymphocytes. Despite the low levels of dNTPs in macrophages, the growing viral DNA strand maintains the ability to incorporate selective dNTPs in a concentration responsive manner. For example, noncanonical dUTP concentrations in macrophages is approximately 60-fold higher than that of TTP in macrophages, but is similar to TTP in lymphocytes. Biochemical simulation studies revealed that dUTP is efficiently incorporated into the growing viral DNA strand in the macrophage but not T-cell dNTP environment, suggesting that levels of dNTPs may in part effect which dNTP is incorporated [29].

Although increased levels of one dNTP relative to another (e.g., higher levels of dUTP versus lower levels of another dTTP) could confer preferential incorporation, increased levels could also mask differences in K_m, which could also represent a contributing factor in incorporation of dNTP into the growing viral DNA strand. Analysis of pre-steady state and steady state kinetics of dUTP incorporation demonstrated that there is minimal selectivity of HIV-1 RT for TTP compared to dUTP, eliminating the potential for

Table 1: Concentrations of dCTP, dGTP, dATP, TTP, and dUTP in activated or resting primary human macrophages versus lymphocytes. Concentrations of dNTPs are 6–133-fold lower in macrophages versus lymphocytes, independent of activation state [18, 25]. ±indicates standard deviation. Data represents at least five independent experiments performed with pooled cells from six independent donors.

	dCTP	dGTP	dATP	TTP	dUTP
			μM		
Activated lymphocytes	3.7 ± 2.7	1.52 ± 1.01	9.2 ± 4.5	16.0 ± 5.3	12.0 ± 1.8
Activated macrophages	0.15 ± 0.10	0.05 ± 0.03	0.10 ± 0.07	0.15 ± 0.10	2.0 ± 9.5
Fold difference between activated lymphocytes versus activated macrophages	25	30	92	107	6
Resting lymphocytes	4.5 ± 2.9	0.91 ± 0.35	5.3 ± 2.2	2.9 ± 2.0	21.6 ± 0.5
Resting macrophages	0.07 ± 0.05	0.07 ± 0.05	0.04 ± 0.03	0.05 ± 0.04	2.9 ± 1.3
Fold difference between resting lymphocytes versus resting macrophages	64	13	133	58	8

Table 2: Concentrations of CTP, GTP, ATP, UTP in activated or resting primary human macrophages versus lymphocytes. Concentrations of rNTPs are only 4–7-fold lower in macrophages versus lymphocytes independent of activation state [18, 25]. ±indicates standard deviation. Data represents at least five independent experiments performed with pooled cells from six independent donors.

	CTP	GTP	ATP	UTP
		μM		
Activated lymphocytes	182 ± 24	1,745 ± 128	6,719 ± 560	690 ± 100
Activated macrophages	27 ± 8	303 ± 60	1,011 ± 247	141 ± 17
Fold difference between activated lymphocytes versus activated macrophages	7	6	7	5
Resting lymphocytes	111 ± 30	923 ± 234	4,753 ± 896	453 ± 174
Resting macrophages	25 ± 8	323 ± 95	1,124 ± 339	173 ± 47
Fold difference between resting lymphocytes versus resting macrophages	4	3	4	3

selectivity for substrates as a key factor in frequency of dUTP or TTP incorporation into the HIV-1 proviral genome. It was also demonstrated that 2,3-dideoxyuridine, a specific inhibitor of dUTP incorporation, confers anti-HIV activity in macrophages, but not T lymphocytes, further underscoring the hypothesis that higher levels of dUTP result in preferential incorporation of dUTP as opposed to other dNTPs in HIV-infected macrophages but not in lymphocytes [29]. Overall, dNTP levels and the lack of dUTP/dTTP discrimination are what determine incorporation frequency of dUTP into HIV-1 proviral DNA. The observed antiviral potency of 2′,3′-dideoxyuridine in macrophages provides a proof of principle concept wherein nucleoside analogues could be designed to target inhibition of specific nucleotides in a cell-specific manner, especially with respect to targeting of macrophage-derived viral sanctuaries.

4. Cellular Factors and Regulation of dNTP Levels: Host Defense to HIV-1 Infection in Macrophages

Although lower levels of dNTPs in macrophages and macrophage-like cells, including dendritic cells, are thought to be a key modulator of inefficient viral replication in these cells, the distinct variation between cellular milieus in macrophages in contrast tolymphocytes has led to speculation that a cellular factor unique to macrophages could also contribute to differences in replication kinetics in macrophages versus lymphocytes.

Recent reports have identified the sterile alpha motif (SAM) domain and HD domain-containing protein 1 (SAMHD1) protein, which is encoded by the SAMHD1 gene, as a cellular factor that regulates cell-specific restriction of HIV-1 replication in cells of the myeloid lineage [20, 30, 31]. Recent work has shown that reducing the level of dNTPs results in inefficient HIV-1 replication in monocytes/macrophages, and SAMHD1 was identified circuitously by further analysis of the Vpx-mediated enhancement of SIV infection in its natural hosts, and the observed enhanced SIV infection rates in myeloid cells [20]. These data led to the hypothesis that a cellular factor unique to monocyte/macrophage cells could exist and may be modulated by Vpx resulting in the observed enhancement of SIV infection in macrophages but not lymphocytes [31]. SAMHD1 functions as a host restriction factor, to efficiently block viral replication in macrophages and dendritic cells by hydrolyzing cellular dNTPs to a nucleoside and a triphosphate further limiting the pool of available dNTPs for incorporation into the proviral genome (Figure 1). When comparing HIV-1 replication efficiency, the rank order is lymphocytes > macrophages > dendritic cells. It follows that SAMHD1 levels are inversely proportional, wherein SAMHD1 levels are dendritic cells > macrophages > lymphocytes, demonstrating

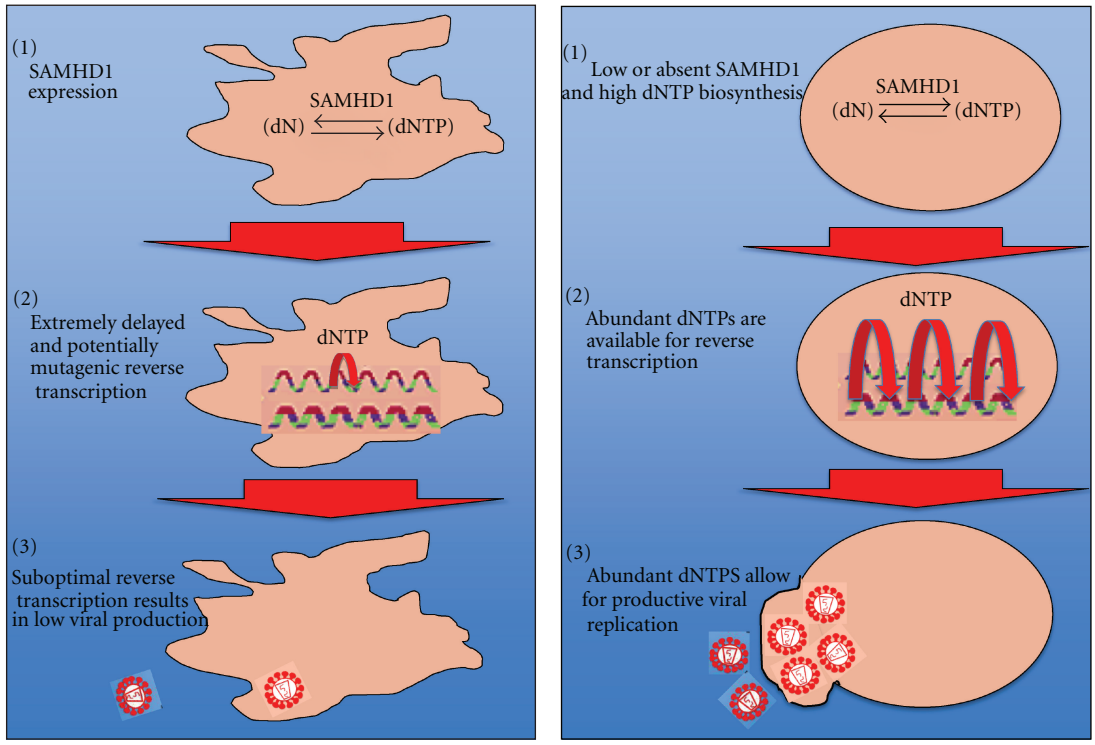

Figure 1: SAMHD1 (SAM domain and HD domain-containing protein 1) and its regulatory mechanism of dNTPs as a host restriction mechanism to prevent HIV-1 infection in macrophages/macrophage-like cells. SAMHD1 cleaves dNTPs into a nucleoside and a triphosphate, rendering levels of intact dNTPs suboptimal to facilitate HIV-1 RT mediated DNA synthesis (a), but low SAMHD1 expression in lymphocytes prevents SAMHD1-mediated restriction in dividing cells such as activated CD4 T cells (b) [20].

a correlation between SAMHD1 levels and inefficient viral replication [31]. In SIV_{sm} and HIV-2 infections of their natural hosts, the cellular restriction of SAMHD1 is counteracted, as Vpx prevents the SAMHD1-mediated hydrolysis of dNTPs in macrophages, allowing for more efficient viral replication in these cells [30]. The identification of SAMHD1 as a myeloid-specific restriction factor that could provide host-derived protection against infection provides an exciting foundation from which to launch further studies not only about the role of SAMHD1 in modulation of infection in macrophages, but about how controlled interference of imbalanced and scarce dNTPs in HIV-1 target cells could provide a protective measure against infection.

5. dNTP/rNTP Levels in Macrophages: Novel Mechanism for Viral Replication in Macrophages

It is not an unexpected finding that levels of dNTPs are lower in macrophages, which are nondividing terminally differentiated cells, however recent reports elucidated a previously unknown milieu in macrophages relative to ratios and levels of dNTP : rNTP versus that observed in lymphocytes [18, 19]. With respect to delineation of function between dNTPs and rNTPs, dNTPs are primarily a component of chromosomal replication and DNA damage repair, whereas rNTPs perform other functions including substrates for RNA polymerases, metabolic energy carriers, and substrates for a variety of enzymes involved in signal transduction cascades [21, 32]. Therefore, it follows that the levels of rNTP may not be lower in macrophages strictly as a function of the fact that they are nondividing cells, or because dNTP levels are lower in this cell type.

Recent reports confirmed that although dNTPs are 6–133-fold lower in macrophages versus lymphocytes, independent of activation state, levels of rNTP are only 4–7-fold lower in macrophages (Tables 1 and 2) [18, 19]. These reports were complemented by the finding that rNTP are preferentially incorporated into proviral DNA in the macrophage but not the lymphocyte dNTP : rNTP simulated microenvironment in a biochemical simulation assay [19], a finding that is distinct from the previously accepted dogma that dNTP are incorporated into the growing viral DNA strand exclusively. This report demonstrates that rNTPs are incorporated into the proviral DNA strand in macrophages and also predicts that ribonucleoside chain terminators could be specific inhibitors of HIV-1 replication in macrophages, wherein their mechanism of action would be dictated by the unique landscape of dNTP : rNTP found in macrophages. These data do not exclude the fact that dNTPs are incorporated into HIV-1 proviral DNA, as dNTP-based inhibitors demonstrate anti-HIV activity in macrophages,

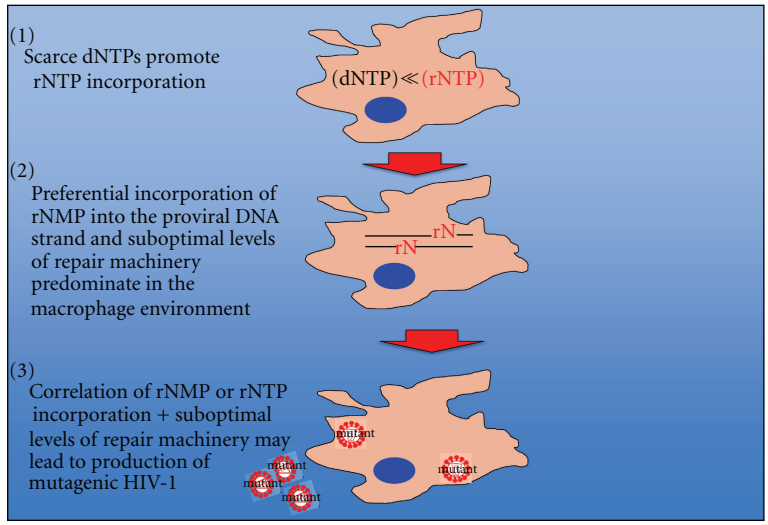

Figure 2: Potential impact of levels/ratios of dNTP : rNTP in macrophages upon emergence of mutagenic HIV-1. Similar ratios of dNTP : rNTP (point 1) confer preferential incorporation of rNTP and rNMP into the growing viral DNA strand (point 2). Together, with suboptimal levels of repair machinery found in macrophages, these incorporations are a known correlate for production of mutagenic HIV-1 (point 3).

although potency is diminished for most nucleoside reverse transcriptase inhibitors (NRTI), versus lymphocytes [2, 33, 34]. The fact that rNTP are preferentially incorporated into the growing viral DNA strand in the biochemical simulation of the macrophage cellular environment could in part be responsible for the fact that deoxy-based NRTI are not as potent in macrophages compared to lymphocytes, where dNTPs are preferentially incorporated, especially with respect to chronic infection [2, 33, 34].

Last, these data imply that ribonucleoside inhibitors could demonstrate potency against HIV-1 in macrophages, and a recent report confirmed this hypothesis, demonstrating that two ribonucleoside inhibitors, determined to be chain terminators, inhibit HIV-1 RT-mediated DNA synthesis in a dose dependent manner [25]. Together, these data underscore the importance of differences in the macrophage landscape versus lymphocytes, and define for the first time that ribonucleoside inhibitors represent a novel class of antiretroviral therapy that can specifically target HIV-1 replication in macrophages.

6. rNMP Incorporation and Implications Relative to Emergence of Mutagenic HIV-1 from Macrophages

Although dNTPs are frequently incorporated into DNA, two phosphate groups are cleaved, with the resulting energy used to create the phosphodiester bond that functions to attach the single remaining phosphate to the growing DNA strand. Therefore, upon discovery that rNTP are preferentially incorporated into the growing viral DNA strand in macrophages, it follows that the incorporated rNTP may undergo the same biphosphate cleavage, potentially resulting in incorporation of rNMP into the growing viral DNA strand in macrophages.

Recent reports demonstrate that rNMP is incorporated into HIV-1 proviral DNA, as determined by the presence of 2 LTR circles with quantitative real-time PCR, at a rate of 1/146 nucleotides in macrophages. Additionally, macrophages possess significantly diminished capacity for repairing monoribonucleotides versus that observed in activated lymphocytes, and rNTP incorporation in the template strand preceding the 3′ terminus causes pausing during DNA synthesis, which is a known correlate of mutagenesis. Taken together, the presence of rNMP in the HIV-1 proviral genome, suboptimal levels of repair machinery to remove rNMP in macrophages versus activated lymphocytes, and the established correlation between site-specific incorporation and pausing in DNA synthesis, provide an environment in macrophages that could be a source for increased production of mutagenic HIV-1 [35, 36]. Additionally, it has been previously determined that intracellular levels of the active, triphosphorylated form of nucleoside analogues, NRTI-TP, is significantly lower in macrophages versus lymphocytes, and is often not delivered at adequate levels to inhibit viral replication [37]. Suboptimal levels of drug delivered to macrophages could provide selective pressure for emergence of drug resistant HIV-1, and together with the established environment in macrophages that correlates with increased production of mutagenic HIV-1, point to macrophages as a cell-specific microenvironment that could in theory result in emergence of drug resistant HIV-1 (Figure 2).

rNMP incorporation occurs at a rate of 1/146 nucleotides in the HIV-1 proviral genome in macrophages, and dNTPs are clearly still incorporated, raising questions about the relative impact of rNMP incorporation in macrophages and its systemic implications *in vivo*. Macrophages are found in every tissue and organ, and due to high CCR5 expression, presence in mucosal sites that often confer primary infection,

and rapid localization to the site of infection, all represent significant rationale for *in vivo* relevance of rNMP incorporation into the growing viral DNA strand in macrophages.

7. Relationship between Small dNTP/rNTP, Inflammation, and HIV-1 Disease Progression

dNTPs perform a variety of cellular functions, and levels can be increased as a function of chronic activation of the cell, as is the case in activated versus resting lymphocytes, most notably for TTP and dUTP (Table 1). $CD4^+$ T lymphocytes are activated by a variety of stimuli, including paracrine and autocrine cytokine stimulation by proinflammatory cytokines, often as a function of interaction with macrophages/macrophage-like cells in the context of MHCII antigen presentation [38, 39]. In a state of chronic hyperactivation, as is hallmarked by chronic HIV-1 infection that orchestrates increased markers of circulating pro-inflammatory cytokines [40, 41], levels of dNTPs in lymphocytes *in vivo* could, in theory be higher than that of a systemic milieu wherein macrophages are not persistently mediating $CD4^+$ T cell activation via antigen presentation and crosstalk within tissue specific microenvironments, including the lymph nodes. As the mechanism of action of NRTI is competition with endogenous nucleotides for incorporation into the growing viral DNA strand, an hypothesized macrophage-mediated increase in dNTPs in $CD4^+$ T cells could decrease the potency of NRTI in chronically infected patients. Although this interaction has not yet been proven *in vivo*, better understanding of this relationship, and events governing it, could ultimately elucidate key information that could be used to discover immune-based therapies designed to circumvent hyperactivation of HIV-1 target cells.

8. Conclusions

Macrophages are ubiquitous, are infected early in HIV-1 infection, express high levels of CCR5 to garner permissivity to infection, and are sites for establishment and maintenance of latent HIV-1 [4]. These attributes, all of which define macrophages as critical to systemic HIV-1 infection, merit exploration and definition of the dynamics between HIV-1 infection and macrophages, and the corresponding relationship to systemic viremia and disease progression.

Recent work has begun to establish that various cell-specific attributes of macrophages, including levels and ratios of dNTP, rNTP, and the presence of newly discovered cellular factors unique to macrophages/macrophage-like cells, significantly alter the manner in which HIV-1 replicates in these cells. Recent reports have demonstrated that dUTP is preferentially incorporated relative toother dNTPs in macrophages, and that rNTPs in general are preferentially incorporated into the growing viral DNA strand in macrophages, but not lymphocytes. Additionally, the discovery that rNMPs are incorporated at a rate of 1/146 nucleotides in macrophages, coupled with the established markedly diminished repair capability in macrophages, and the correlation with DNA pausing and production of mutagenic DNA provides a complex landscape wherein HIV-1 replication is altered as a function of the target cell in which replication was facilitated.

These data afford novel insight into previously unknown mechanisms of HIV-1 replication in macrophages, which are currently being used to design inhibitors targeting incorporation of rNTP, rNMP, or dUTP. As current HAART has not been able to eliminate virus from all tissues and reservoirs, it is unlikely that inhibitors of rNTP, rNMP, or dUTP could solely eliminate virus from macrophage-derived reservoirs. However, together this knowledge about dNTP and rNTP incorporation into proviral DNA, and their impact upon HIV-1 infection in macrophages defines a complex landscape and provide a springboard from which to launch a multipronged approach to eliminate virus from macrophage-derived viral sanctuaries.

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Review Article

The Impact of HIV Genetic Polymorphisms and Subtype Differences on the Occurrence of Resistance to Antiretroviral Drugs

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The vast majority of reports on drug resistance deal with subtype B infections in developed countries, and this is largely due to historical delays in access to antiretroviral therapy (ART) on a worldwide basis. This notwithstanding the concept that naturally occurring polymorphisms among different non-B subtypes can affect HIV-1 susceptibility to antiretroviral drugs (ARVs) is supported by both enzymatic and virological data. These findings suggest that such polymorphisms can affect both the magnitude of resistance conferred by some major mutations as well as the propensity to acquire certain resistance mutations, even though such differences are sometimes difficult to demonstrate in phenotypic assays. It is mandatory that tools are optimized to assure accurate measurements of drug susceptibility in non-B subtypes and to recognize that each subtype may have a distinct resistance profile and that differences in resistance pathways may also impact on cross-resistance and the choice of regimens to be used in second-line therapy. Although responsiveness to first-line therapy should not theoretically be affected by considerations of viral subtype and drug resistance, well-designed long-term longitudinal studies involving patients infected by viruses of different subtypes should be carried out.

1. Introduction

Nonsubtype B infections are responsible for most HIV cases worldwide [1]. HIV-1 group M has been classified into subtypes, circulating and unique recombinant forms (CRF and URF, resp.), due to its significant natural genetic variation; this includes subtypes A–D, F–H, and J–K and many CRFs and URFs. Although subtype B is the most prevalent in the Western World (Western Europe, the Americas, Japan, and Australia), non-B subtypes predominate in the rest of the world: that is, subtype C in sub-Saharan Africa, India, and Brazil, CRF01_AE in South East Asia, CRF02_AG in West Africa, and subtype A in Eastern Europe and Northern Asia [1–3]. The proportion of non-B subtypes in North and South America and Western Europe is increasing [4–7]. Combination antiretroviral therapy (ART) is now used in many areas of the world, and HIV resistance to antiretroviral drugs (ARVs) has widely emerged. Thus, non-B subtypes will presumably become even more common in western countries.

Reduced sensitivity to ARVs in non-B subtypes has been less well studied than in subtype B, mainly because of the predominance of subtype B in those countries in which ARVs first became available, coupled with the availability of genotypic and phenotypic antiretroviral drug resistance testing in such countries [8]. This notwithstanding there is a potential for genetic differences among subtypes to yield differential patterns of resistance-conferring mutations in response to ARVs and this possibility is supported by the fact that HIV-1 naturally varies in genetic content by as much as 35% among subtypes. Indeed, variation is higher in some areas of the genome (40% in the env gene) and lower in others (8–10% in the *pol*, *gag,* and *IN* genes) [8]. Since differences in codon sequences at positions associated with drug resistance mutations might predispose viral isolates from different subtypes to encode different

amino acid substitutions, it is possible that HIV-1 genetic diversity may influence the types of resistance mutations that might eventually emerge upon drug exposure as well as the rate of emergence of such mutations and phenotypic resistance [8, 9]. Such diversity may also affect the degree of cross-resistance to ARVs of the same class, with the potential to impact on virologic failure, clinical outcomes, and preservation of immunological responsiveness [8].

For example, studies of single dose nevirapine (sdNVP) for prevention of mother-to-child transmission (PMTCT) showed a disparity in overall resistance among subtypes, with frequencies of 69, 36, 19, and 21% against NVP in women with subtypes C, D, A, and CRF02_AG infections, respectively. Often, this result occurred prior to treatment and despite the absence of resistance mutations [10–13]. Very sensitive PCR detection procedures, which reveal resistance due to minority species, have revealed a higher incidence of NVP resistance (K103N, Y181C) in 70–87% of individuals with subtype C compared with 42% of individuals with subtype A [14–16].

Evaluations of virological and biochemical data also suggest that natural amino acid background can affect the magnitude of resistance conferred by many mutations responsible for antiretroviral drug resistance [17], as is best illustrated by HIV-2 and group O viruses that show high-level innate resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs) through the presence of natural polymorphisms that can confer drug resistance (Table 1) [18, 19]. However, many studies on antiretroviral drug resistance in non-B subtypes exposed to chronic suppressive therapy have yielded less definitive results with respect to the importance of natural HIV-1 diversity as a factor leading to differences in types of drug resistance mutations and the propensity to develop drug resistance in the first place [8, 17].

Although genotypic ARV resistance testing is of proven benefit in deciding on best choice of ARVs for individual treatment and serves as a repository of information on HIV resistance mutations, several factors underscore the difficulties in defining intersubtype differences. For example, genotyping can classify the major viral subtypes, but significant proportions (~15%) of infections remain unassigned or differentially assigned using different subtyping algorithms [8, 20, 21]. Certainly, HIV resistance databases make efforts to incorporate newer subtype data into pools of data, but the availability of HIV genotypes from areas of the world with non-B subtype predominance is still comparatively low [22]. The factors responsible include lesser availability of ARV therapy, the high cost of drug resistance testing, and limited opportunities for research in resource-limited areas. In some cases, resistance tests may often be performed only on participants enrolled in study cohorts or trials but not in general practice.

2. Resistance to Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

As an example of disparity, subtype C patients in Botswana treated with ZDV/ddI developed an atypical thymidine analogue mutation (TAM) resistance pathway (67N/70R/215Y) compared to subtype B (the TAM 1 and TAM 2 pathways) [23]. This distinction was not observed in patients with subtype C in Malawi, India, or South Africa [24–27]. Results from Botswana also reported a high incidence of K65R (30%) in subtype C patients who received d4T/ddI plus NVP or efavirenz (EFV) [28]. A much larger study from Malawi detected K65R or K70E in 23% of patients failing first-line therapy with d4T/3TC/NVP [26], while K65R was detected in 7% and 15% of patients in South Africa failing first- or second-line regimens, respectively, whose nucleoside backbones included d4T/3TC or ddI/ZDV [29, 30]. A study from Israel also reported a high frequency of K65R in subtype C viruses from Ethiopian immigrants [31], and a report from India showed that K65R was present in about 10–12% of patients who had received d4T/3TC/NVP in first-line therapy [32]. Such differences in K65R and thymidine analogue mutations (TAMs) might be attributed to treatment regimen and disease stage [24–27].

Access to viral load testing lead in India was also associated with early detection of NRTI-treatment failure, leading to use of new, second-line regimens and preventing acquisition of TAMs and K65R [24]. Additional studies support regional differences among subtype C subepidemics from Ethiopia, Brazil, and sub-Saharan Africa, that impact on NRTI resistance rates as a result of different NRTI-based regimens [8, 33, 34].

Higher rates of the K65R mutation in subtype C [26, 28, 29] suggest that these viruses may have a particular predisposition toward acquiring this mutation [35]. A subtype C RNA template mechanism has been proposed to explain this phenomenon that involves higher rates of K65R mutagenesis in subtype C viruses than in other subtypes (Figure 1) [36, 37], and this mechanism seems to be template dependent and is independent of the source of the reverse transcriptase (RT) employed [36]. Subtype C viruses apparently have an intrinsic difficulty in synthesizing stretches of adenine homopolymeric runs that leads to template pausing at codon 65, facilitating the acquisition of K65R under selective drug pressure [37, 38], whereas the subtype B template favors pausing at codon 67 that may facilitate the generation of D67N and TAMs rather than K65R [37–39]. In addition, the introduction of codons from positions 64 and 65 in the RT of subtype C into a subtype B backbone was sufficient to lead to selection of K65R by multiple NRTIs [37–39]. Figure 1 provides a pictorial representation of the preferential development of K65R in subtype C viruses.

Ultrasensitive pyrosequencing has also been used to detect the spread of K65R as transmitted and/or minority species in treatment-naïve populations [40, 41]. Patients harboring subtype C infections showed a higher frequency of K65R than subtype B variants (1.04% versus 0.25%) by this method but these differences were not duplicated using limiting dilution clonal sequencing approaches [40]. While these findings are consistent with PCR-induced pausing, leading to low-level spontaneous generation of K65R in subtype C, they do not negate the higher rates of development of K65R in subtype C populations failing regimens containing d4T, ddI, or tenofovir (TDV) [32]. The occurrence of K65R in

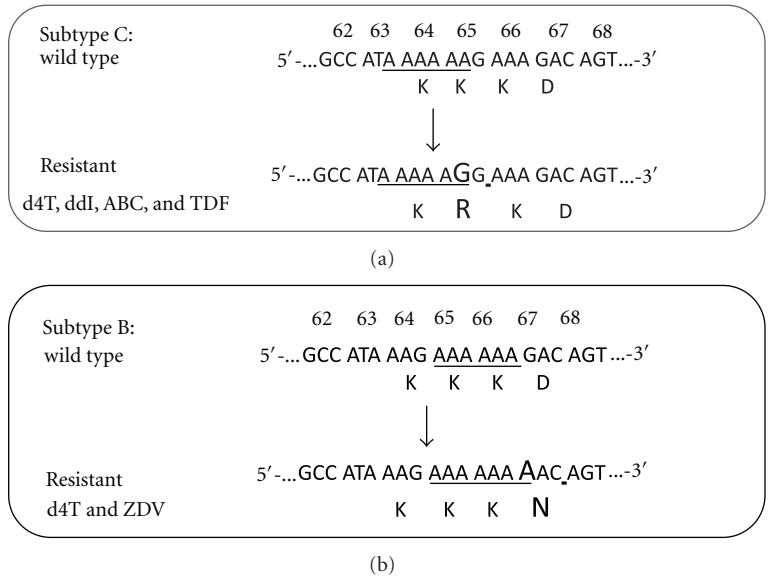

Figure 1: Subtype-specific poly-A nucleotide motifs lead to template pausing under pressure with thymidine analogues that favor K65R selection in subtype C and D67N selection in subtype B. Depiction of the template-based propensity of subtype C versus B viruses to develop the K65R mutation that is associated with broad cross-resistance among multiple members of the NRTI family of drugs. The codons located at positions 63, 64, and 65 in subtype C RT seem to be critically involved in the preferential development of K65R in subtype C. d4T: stavudine, ddI: didanosine, ABC: abacavir, TDF: tenofovir. It should be noted that the use of stavudine in particular has been shown to yeild K65R in subtype C infections with high frequency. Regimens that are based on the use of TDF and ABC, among other drugs, can help mitigate the development of the K65R mutation.

subtype C and CRF01_AE is also associated with the Y181C NVP mutation within the viral backbone [30, 42].

Subtype C selected the K65R mutation in drug resistance selection studies faster than subtype B under TFV pressure [35]. However, K65R may be less frequent in subtype A than other subtypes [43]. And a higher propensity to acquire TAMs was reported in patients carrying CRF_06 (AGK recombinants) as compared to patients carrying CRF02_AG from Burkina Faso [44].

The differential selection of K65R pathways in subtype C seems related to template differences, ddI and d4T-containing regimens, as well as to the presence of Y181C. Further genotypic studies will be required to ascertain subtype differences in acquisition of resistance to NRTIs.

3. Resistance to Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Selection studies in culture have shown that a V106M mutation commonly develops in subtype C viruses following drug pressure with NVP or EFV, whereas a V106A mutation is more commonly selected in subtype B. This difference is due to a nucleotide polymorphism at codon 106 in RT [45, 46], and the clinical importance of V106M in non-B subtypes has been confirmed in multiple studies showing that V106M is frequently seen in non-B subtypes (C and CRF01_AE) after therapy with NVP or EFV [23, 25, 27, 47–50].

The G190A substitution was also relatively more frequent among subtype C infected patients failing NNRTI-based therapy in Israel and India, and G190A/S was seen in the Israeli study as a natural polymorphism in subtype C from Ethiopian immigrants [25, 49]. The frequencies of these mutations among treated patients in both studies were higher than in subtype B and C drug-naïve individuals.

Although the overall prevalence of V106M in subtype C is higher than in subtype B (12% versus, 0%) in individuals failing NNRTI-based regimens, K103N (29% versus 40%) and Y181C (12% versus 23%) remain important pathways for both subtype C and B, respectively (http://hivdb.stanford.edu/). Only minor differences in HIV resistance pathways seem to occur among subtypes A, B, and C with the second generation NNRTI etravirine (ETR) [50].

4. PR Mutations

The results of work with protease inhibitors PIs indicate that the D30N mutation was not observed in CRF02_AG and CRF02_AE isolates in patients failing nelfinavir (NFV) therapy but rather that the N88S mutation emerged after NFV use in CRF01_AE and after indinavir [51] use in subtype B [52, 53]. Although another study reported an absence of the D30N mutation in CRF01_AE, no information on the specific type of PIs received by the patients was available [54]. A lower frequency of D30N was seen in subtype C isolates from Ethiopian immigrants to Israel after NFV usage than in subtype C viruses from Botswana [55, 56], suggesting that subtype C viruses from Ethiopia (the origin of the samples

Table 1: Examples of polymorphisms and mutations in reverse transcriptase (RT), protease (PR), and integrase (IN) of different subtypes that may impact on emergent resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs), protease inhibitors (PIs), and integrase strand transfer inhibitors (INSTIs).

Drug class	Type/group/ subtype	Polymorphism or mutation associated with drug resistance	Drug(s) affected	Mutation(s) and their consequences	Reference
		Reverse transcriptase			
NRTI	C	64-65-66 KKK motif	ddI, d4T, TDF	K65R	[30]
NRTI	HIV-2	T69N, V75I, V118I, L210N, T215S, K219N	NRTIs	TAMs/K65R	[66]
NNRTI	C	V106V	EFV, NVP	V106M	[45]
NNRTI	G	A98S	NNRTIs		[66]
NNRTI	HIV-2	Y181I,Y188L, G190A K101A, V106I, V179I	All NNRTIs	Cross- NNRTI resistance	[68]
NNRTI	O	Y181C, A98S, K103R, V179E	All NNRTIs	Cross- NNRTI resistance	[18]
		Protease			
PI	Non-B	M36I	PIs		[59]
PI	G, AE	K20I	PIs		[63]
PI	G	V82I	PIs	I82M/T/S	[63]
PI	A, C, F, G, AE, AG	L89M	PIs	L89I	[71]
PI	HIV-2	L10I/V, K 20V, V32I, M36I, M46I, I47V, L63E/K, A71V, G73A, V77T, V82I/L,	PIs	APV and other PIs	[68]
		Integrase			
INSTIs	B	R263	MK-2048, DTG	R263K	[85]
	C	G118	MK-2048, DTG	G118R	[82]

ddI: didanosine; d4T: stavudine; TFV, tenofovir: EFV, efavirenz: NVP, nevirapine: DTG, dolutegravir.

identified in Israel) and southern Africa might behave in different fashion. M89I/V mutations were observed in F, G, and C subtypes but not in other subtypes [26], and the V82I natural polymorphism in subtype G led to the emergence of I82M/T/S in treatment failure [57]. The L90M mutation is rare in subtype F but common in subtype B from Brazil [58], and a recent paper suggests that polymorphisms at position 36 in PR may be important in determining the emergence of specific patterns of resistance mutations among viruses of different subtypes [59].

To gain an understanding of the underlying mechanisms leading to the overall higher preponderance of D30N in subtype B relative to other subtypes, molecular dynamic simulations were performed. D30N appeared to selectively confer resistance to NFV in subtype B by increasing the flexibility of the protease (PR) flap region and destabilizing the PR inhibitor complex [60]. In subtype C, D30N required the accessory N83T mutation to confer resistance and rescue fitness [61].

Two comprehensive surveys reported differences in natural protease polymorphisms among non-B subtypes [62, 63] and positions less frequently mutated in non-B subtypes than in subtype B after exposure to ARVs. Residues of importance in subtype A in PR were at positions 10, 20, and 63, whereas, in subtype C, they were at residues 20, 53, 63, 74, and 82. Other differences were at residues 13 and 20 in subtype D, residues 10, 14, 20, and 77 in subtype F, residues 20, 67, 73, 82, and 88 in subtype G, residues 20, 63, 82, and 89 in CRF01_AE, and residue 20 in CRF02_AG [63].

Higher rates of accumulation of NRTI and PI resistance mutations and equal rates of emergence of NNRTI mutations were also found in subtype B compared to C [64]. A study from southern Brazil also showed a lower frequency of primary resistance to PIs in subtype C compared to subtype B, suggesting that PI mutations may be less well tolerated at the structural level in subtype C [65].

However, HIV-1 subtype diversity has not limited the overall benefit of ART (Table 1). This notwithstanding there are subtype differences in the type and preference of pathways of resistance with some mutations emerging almost exclusively in some non-B subtypes, for example, the protease mutation 82 M in subtype G versus 82A/F/S in the others, 88D in subtype B versus 88S in subtypes C and CRF02_AG [66]. Furthermore, HIV-2 has major mutations in regard to NRTIs, NNRTIs, and PIs, which contribute to innate NNRTI resistance and rapid development of multiclass drug resistance (Table 1) [67, 68]. The V106M RT mutation in subtypes C and A versus V106A in subtype B is observed with resistance against NVP and EFV. Polymorphisms at RT residue 98, common in subtype G, are associated with NNRTI resistance in subtype B and may lower the resistance barrier and duration of efficacy of some NNRTIs [69]. The frequency of some resistance mutations shared by B and non-B subtypes can vary after failure of

first-line therapeutic regimens, as in the case of the K65R mutation. Differences in type and frequency of resistance mutations should not be underestimated. However, the TAM pathway 67N/70R/215Y found in subtype C in Botswana will probably be adequately detected by most resistance algorithms, since it does not involve new mutations.

A lower risk for accumulation of major (primary) resistance mutations in subtype C than B has been reported [64]. The major mutations that emerged in both subtypes were the same. Since both subtypes B and C patients had similar profiles of virological failure after use of the same ART regimens, this rules out ancillary factors responsible for these differences. Minor mutations in subtype B PR may appear as frequent natural polymorphisms in several non-B subtypes (e.g., M36I, L89M) [58, 59]. The fact that the L89M polymorphism can lead to the M89I mutation that confers resistance to PIs suggests that there might be a lower accumulation of major mutations in C subtypes, if natural polymorphisms act similarly in subtype C as they do when present as secondary resistance mutations in subtype B.

The majority of non-B HIV-1 subtype isolates possess wild-type susceptibilities similar to those of subtype B wild-type isolates. Compared to B subtypes, diminished susceptibilities among wild-type isolates have been found for CRF02_AG recombinant viruses in three different studies in regard to ATV and NFV [63, 69, 70]. No study has yet assigned statistical significance of drug susceptibility levels due to polymorphisms and small sample size. One analysis performed molecular modeling and suggested that distortions in the K26 pocket of A/G proteases appear to be responsible for a lower binding energy of NFV and hence lower susceptibility of A/G viruses to this drug [70]. A/G isolates with lower susceptibilities to certain PIs (NFV and atazanavir (ATV)) have also been found. One study has detected an important proportion of WT isolates with lower susceptibilities to ATV [71]. In most cases, phenotypes have been determined by commercial or in-house assays that were developed primarily to measure B-subtype drug susceptibilities based on the laboratory adapted strains NL4-3 or HXB2, through use of a modified clone of a laboratory strain that lacks both the terminal part of Gag and most of Pol. It should be recognized that most commercial assays do not monitor polymorphisms, and indeed sequences that lie within particular regions, such as the substrates of PR within gag or the RNaseH and connection domains within pol, can influence drug resistance in both B and non-B subtypes but may not be easily recognized. Although some work has been carried out in this field, it is clear that other studies are required [72–75].

There are few data on the potential for cross-resistance to PIs among non-B subtypes in regard to NFV, although there is a tendency to select for the L90M pathway instead of D30N in subtype C. Competition fitness assays support the notion that subtype C viruses bearing D30N are impaired in replicative fitness, a finding that may explain the above results [61].

Thermodynamic studies performed on target-inhibitor interactions in PR have specifically described a lower affinity of non-B subtype proteases for PIs and amplification of primary resistance mutations on the basis of polymorphisms that are present in background.

In addition to the foregoing, interesting results on polymorphisms that confer hypersusceptibility to some PIs have been recently reported [76]. Some of these polymorphisms can potentially delay acquisition of drug resistance and may therefore enhance the long-term effectiveness of relevant drugs.

5. Integrase Inhibitors and Drug Resistance

New data are emerging that subtype differences are also present in regard to integrase strand transfer inhibitors (INSTIs) despite the fact that HIV-1 subtype B and C wild-type integrase (IN) enzymes are similarly susceptible to clinically approved INSTIs [77–81]. This notwithstanding there are now data to indicate that the presence of resistance mutations may differentially affect susceptibility to specific INSTIs in viruses of different subtypes [77]. Moreover, such data have been obtained both in tissue culture using recombinant viruses of different subtypes that contain specific IN mutations as well as in biochemical integrase strand transfer and integrase 3′synthetase assays, in which specific drug resistance mutations have been introduced into recombinant purified integrase enzymes derived from either subtype B or subtype C viruses [77].

Of particular interest may be that a novel next-generation INSTI termed MK-2078 with a higher genetic barrier for selection of resistance than either raltegravir (RAL) or elvitegravir (EGV) was able to differentially select for a novel G118R substitution in IN in subtype C compared with subtype B viruses [82]. This mutation conferred only slight resistance to MK-2048 but gave rise to 25-fold resistance against RAL when it was present together with a polymorphic substitution at position L74M in CRF02-AG cloned patient isolates [83]. It is also well known that INSTI Q148RHK resistance mutations that affect susceptibility to a novel INSTI, dolutegravir (DTG) in HIV-1 subtype B may not affect susceptibility of subtype C viruses or HIV-2 viruses and IN enzymes to the latter compound [84].

Finally, tissue culture selection with DTG has identified a novel R263K resistance mutation in subtype B but not subtype C viruses [85]. In contrast, the same series of selections with DTG in subtype C viruses yielded the same G118R mutation that had previously been obtained with MK-2048, also in subtype C. This raises the possibility that G118R may have the potential to be an important resistance mutation for next-generation INSTIs in subtype C viruses but that this role may be played by R263K in the context of subtype B viruses. Of course, definitive information on this topic may have to await the widespread clinical use of DTG and the characterization of mutations within IN that may arise in the event of rising viral loads and treatment failure.

6. Clinical Practice

HIV resistance in non-B subtypes has rarely been reported on the basis of single drugs or NRTI backbones but, rather, mutations have been reported for specific drug classes.

Cross-resistance can be estimated only for some NRTIs and NNRTIs but not for most PIs that are the only drugs eligible as part of second-line regimens in most regions of the world. The potential for cross-resistance to NFV in viruses of CRF01_AE and CRF02_AG origin could be higher than has been observed in subtype B, due to the preferential selection of the N88S and L90M substitutions, although such data are not yet available for most PIs in the context of non-subtype B viruses. NRTI backbones may also vary in the mutation profile they select for according to drug combinations that are used. Newer compounds (e.g., TFV and ATV/r) are now preferred both in resource-rich countries and non-B subtype prevalent areas. Although HIV resistance databases continue to enter HIV genotype data from nonB subtype variants, few data sets are available to date (stanford HIV resistance database, Agence Nationale pour la Recherche sur le SIDA-France (ANRS), etc.) for drugs that have become part of first-line therapy in developed countries, for example, TDF, ATV, darunavir, ETR, and RAL. In this context as well, it is relevant that some studies have attempted to address the clinical impact of HIV diversity on treatment response as well as the limitations of such approaches [86, 87].

7. Future Considerations

The preferential emergence of some mutations and changes in the frequency of these mutations in select non-B subtypes needs greater attention and research on the role of polymorphisms in nonsubtype B viruses that increase in frequency after drug exposure and that may contribute to drug resistance (e.g., A98G/S in RT and M36I and K20I in PR) [88] should be priorized, particularly in parts of Africa in which treatment failure has been reported in as many as 40% of patients after two years [89] and in India where resistance rates of 80% to two drug classes have been reported after failure of first-line regimens that employed various NRTI/NNRTI combinations [90]. To date, no study has tested the degree of resistance or cross-resistance that certain mutational combinations (67N/70R/215Y) may confer in tissue culture. Newer studies should assess pre- and posttreatment genotypes in order to determine associations of certain polymorphisms with drug resistance, including variations of polymorphisms in variants of the same subtype that are located in different geographical regions. This would improve the appropriateness of use of certain drugs over others in the context of second- or third-line therapeutic regimens.

The different studies conducted in populations affected by nonsubtype B viruses are too heterogeneous to permit pooling of data [8]. Such studies have addressed different research questions and used nonequivalent NRTI backbones (e.g., ZVD/ddI and ZDV/3TC) and have also grouped mutations by drug class without providing information on the nature of the regimen at virologic failure. Resistance has also been reported in different ways (e.g., different algorithms or resistance lists), making it difficult to relate resistance mutations to a specific drug or combination of drugs. More longitudinal studies on response to first-line ARV combinations are needed to better recognize intersubtype differences. Pre- and posttherapy genotype resistance testing is also desirable.

8. Conclusions

Virological and biochemical data provide compelling evidence on the differential effect of genetic background on both the type and degree of HIV-1 antiretroviral drug resistance. Genetic background can affect the degree of protein binding caused by primary mutations and restore the function of PR to a differential degree in different subtypes based on background polymorphisms, although this effect was not discernible in the absence of typical major resistance mutations but rather when particular backgrounds of combinations of major resistance mutations and background polymorphisms were represented. Clearly, some background polymorphisms can act as secondary resistance substitutions.

Phenotypic assays have failed to find differences of large magnitude in the susceptibilities of HIV B versus non-B subtypes, consistent with what has been learned at a molecular level. Unfortunately, only few datasets exist on relative susceptibility levels among subtypes carrying specific major resistance mutations, and more information is required, particularly because many polymorphisms in non-B viruses are considered to be secondary resistance mutations since they can emerge after drug exposure in subtype B viruses. The effect of such polymorphisms within different genetic backgrounds cannot always be extrapolated to non-B subtypes and might sometimes contribute to higher levels of resistance depending on genetic backbone. They could also have either a neutral effect or hypersensitize HIV to ARVs, and I93L is an example of a secondary resistance mutation in subtype B that in subtype C causes hypersusceptibility to PIs [61].

Novel NNRTI resistance mutations in subtype C were not recognized in subtype B. In tissue culture, subtype C can acquire a V106M mutation under NNRTI drug pressure compared to V106A in subtype B. V106M can confer broad cross-resistance to an extent that supersedes that conferred by V106A.

The acquisition of resistance could have important implications in regard to durability of therapy. In culture, the emergence of the K65R mutation is quicker in subtype C than in B [30, 35], and several biochemical mechanisms have been proposed to explain this observation, based on subtype C templates [36–38, 91]. K65R has been seen in approximately 70% of patients failing ddI-containing nucleoside backbones in Botswana [28] but does not appear to emerge frequently in subtype C patients who have received either TDF or TDF/FTC as part of triple therapy [30], a possible reflection of the use of well-tolerated effective drugs that have long mutually reinforcing intracellular half-lives that act in combination to suppress viral replication and prevent the emergence of resistance mutations. Higher numbers of patients and longer followup will be required to determine if there is a consistent impact of subtype C in the emergence of K65R in the clinic.

Multiple *in vitro* and clinical studies have confirmed that PR and Gag can act as a functional unit and coevolve

when HIV is subject to drug pressure. Both genes can clearly mutate under PI pressure, and Gag mutations can act as compensatory substitutions that may increase levels of viral replication capacity and resistance. The recombinant phenotyping systems used for clinical samples do not now adequately monitor Gag. While differences among Gag may vary between only −2 to 2.5-fold between subtypes, different subtypes might develop compensatory Gag mutations at different rates, establishing a need to take Gag into account in determining a phenotype. One study reported that a recombinant construct included Gag of clinical origin but did not test the same subtypes as were reported in other work [57].

Although various mutations can impact on drug sensitivity to differential extent, such information cannot yet be generated with regard to non-B subtypes due to a paucity of paired phenotypic and genotypic data. Three studies analyzed genotypes and phenotypes of non-B subtypes in clinical trials: one on use of single dose NVP for prevention of mother-to-child transmission and two on double and triple NRTI combinations that are no longer used [8].

Cross-resistance acquires importance in settings with limited access to antiretroviral therapy, and few *in vitro* comparative data are available for PIs in non-B subtypes. However, such data may be crucial to understanding cross-resistance to specific drugs [58, 59], since some PIs may be the only potentially accessible option for drug sequencing in salvage therapy in many resource-limited settings. The fact that resistance to PIs commonly requires that large numbers of resistance mutations be present may yield a situation in which the individual contribution of any single mutation to drug resistance, with some exceptions, will be limited, a definite advantage of using drugs with a high genetic barrier toward the development of drug resistance. Thus, differences among subtypes with regard to development of drug resistance are more likely to be important for NRTIs and NNRTIs than for PIs. Clearly, large numbers of paired samples need to be systematically collected from naïve and treated patients infected with subtypes C, AE, AG, A, and G, in order for genotypic and phenotypic analysis to be conducted for both established drug classes as well as for newer classes of drugs such as inhibitors of integrase.

Finally, this paper has focused on classes of HIV drugs for which significant datasets are available in regard to subtypes and differential drug resistance. Limitations of both space and available datasets have precluded us from discussing the topic of entry inhibitors. However, most available data suggest that the only two approved entry inhibitors, that is, the fusion inhibitor, enfuvirtide, and the CCR5 entry antagonist, maraviroc, are both active against HIV isolates of multiple subtypes.

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Review Article

HIV-1 Reverse Transcriptase Still Remains a New Drug Target: Structure, Function, Classical Inhibitors, and New Inhibitors with Innovative Mechanisms of Actions

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During the retrotranscription process, characteristic of all retroviruses, the viral ssRNA genome is converted into integration-competent dsDNA. This process is accomplished by the virus-coded reverse transcriptase (RT) protein, which is a primary target in the current treatments for HIV-1 infection. In particular, in the approved therapeutic regimens two classes of drugs target RT, namely, nucleoside RT inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). Both classes inhibit the RT-associated polymerase activity: the NRTIs compete with the natural dNTP substrate and act as chain terminators, while the NNRTIs bind to an allosteric pocket and inhibit polymerization noncompetitively. In addition to these two classes, other RT inhibitors (RTIs) that target RT by distinct mechanisms have been identified and are currently under development. These include translocation-defective RTIs, delayed chain terminators RTIs, lethal mutagenesis RTIs, dinucleotide tetraphosphates, nucleotide-competing RTIs, pyrophosphate analogs, RT-associated RNase H function inhibitors, and dual activities inhibitors. This paper describes the HIV-1 RT function and molecular structure, illustrates the currently approved RTIs, and focuses on the mechanisms of action of the newer classes of RTIs.

1. Introduction

Since the human immunodeficiency virus (HIV) has been established to be the etiological agent of the acquired immunodeficiency syndrome (AIDS) [1, 2], an originally unpredicted number of drugs have been approved for the treatment of the HIV-infected patients [3]. This success in effective drugs identification, certainly unique in the treatment of viral infections, together with the use of such armamentarium in different combination therapeutic regimens, has transformed a highly lethal syndrome into a chronic disease [4]. The management of this disease, however, is still complex and worrisome due to problems such as monitoring of therapy efficacy, chronic administration drug toxicity, poor tolerability, drug resistance development, or therapy adjustment after treatment failures [4]. For all these reasons, the search for new inhibitors, possibly acting with molecular mechanisms different from the ones of the already approved drugs or anyway showing different patterns of drug resistance and, possibly, with diverse drug-associated chronic toxicity, is still a worldwide health care issue.

The success in HIV infection therapy is certainly related to the fact that the HIV life cycle has been intensely dissected; several of its steps have been validated as drug targets, and, subsequently, a number of viral inhibitors have been identified and developed against many of them [3, 4]. Among the HIV proteins which have been deeply characterized as major drug targets is the reverse transcriptase (RT), the virus coded enzyme that converts the ssRNA viral genome into the dsDNA provirus which is consequently imported into the cell host nucleus and integrated into the host chromosome by another virus-coded protein, integrase (IN). The present paper focuses on the RT function within the virus cycle, its molecular structure, the mechanism of action of the

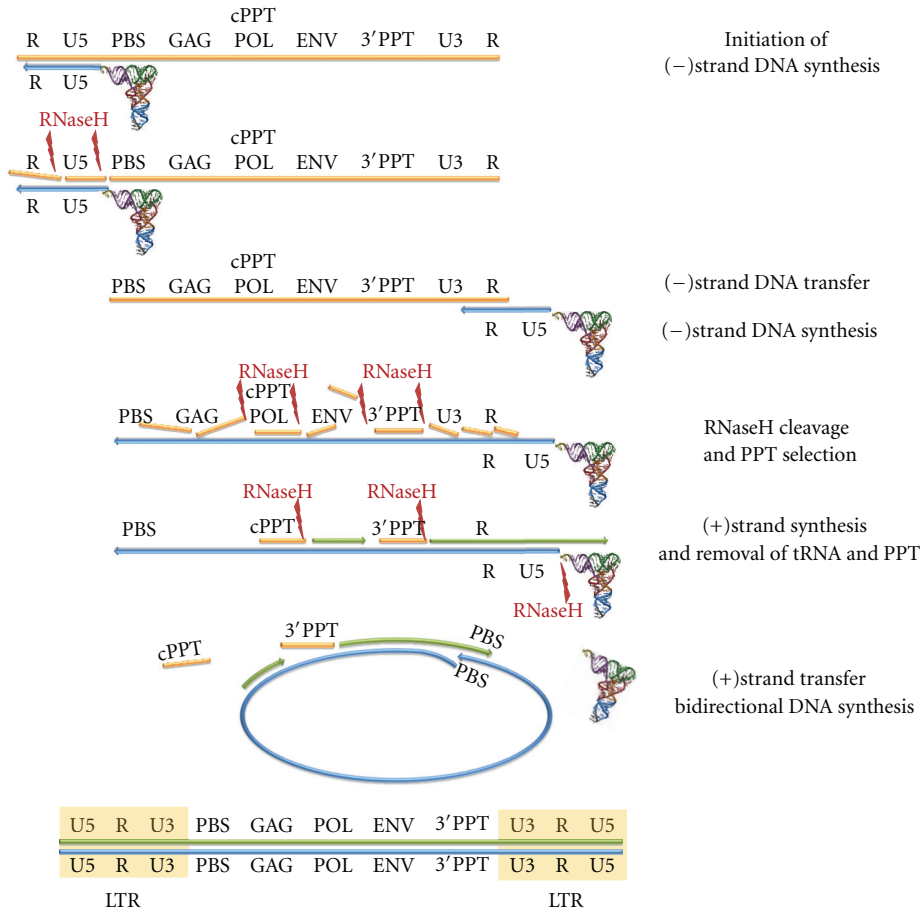


Figure 1: HIV-1 reverse transcription process. Step 1: host cell tRNALys3 hybridizes to the PBS near the 5′-end of the (+)strand RNA genome (orange). (−)strand DNA (blue) synthesis starts using host tRNALys3 as a primer. DNA synthesis proceeds up to the 5′-end of the RNA genome. Step 2: RNase H hydrolysis of the RNA portion of the RNA:DNA hybrid product exposes the ssDNA product determining the (−)strand strong stop DNA. Step 3: strand transfer of the (−)strand DNA through its hybridization with the R region at the 3′-end of the ssRNA genome and further elongation of the (−)strand DNA. Step 4: DNA synthesis proceeds, and the RNase H function cleaves the RNA strand of the RNA:DNA at numerous points leaving intact two specific sequences (cPPT, 3′PPT) resistant to the RNase H cleavage. Step 5: (−)strand DNA synthesis (green) initiation using PPTs as primers. Step 6: RNase H hydrolysis of the PPT segments and the junction of the tRNA:DNA hybrid, freeing the PBS sequence of the (+)strand DNA. Step 8: strand transfer of the PBS sequence of the (+)strand DNA that anneals to the PBS on the (−)strand DNA. DNA synthesis then continues with strand displacement synthesis. Step 9: the product is a linear dsDNA with long terminal repeats (LTRs) at both ends.

currently approved RT inhibitors (RTIs), and the newer classes of RTIs and their modes of action.

2. Retrotranscription Process

After the HIV particle fuses with the host cell surface, the viral particle content is released within the host cell cytoplasm where the viral ssRNA genome serves as template to obtain a proviral dsDNA that is integrated into the host genome, becoming a source of mRNAs coding for viral proteins and ssRNA genomes that, together, will form the new viral particles. The conversion of the viral ssRNA genome into integration-competent dsDNA, termed retrotranscription (Figure 1), is characteristic of all retroviruses, and its accomplishment requires viral as well as cellular elements, among which the most important is the virus-coded RT protein.

Each HIV particle contains two copies of (+)ssRNA genome sequence of 9,7 kb [5] coding for structural and nonstructural proteins and having, in the 5′- and 3′-ends, two identical sequences. Near the 5′-end of the viral genome there is an 18-nucleotides-long segment, termed primer binding site (PBS), which is complementary to the 3′-end 18 nucleotides of the human tRNALys3. When the cellular tRNA is hybridized to the PBS, it serves as an RNA primer, and the RT-associated DNA polymerase function can initiate the first (−)strand DNA synthesis using the viral RNA genome as a template (Figure 1). After tRNA elongation until the ssRNA 5′-end, there is a first (−)strand strong-stop DNA. In fact, the (−)strand DNA synthesis generates an RNA:DNA hybrid that is a substrate for the

RT-associated ribonuclease H (RNase H) function which selectively degrades the RNA strand of the RNA:DNA hybrid [6], leaving the nascent (−)strand DNA free to hybridize with the complementary sequence at the 3′-end of one of the two viral genomic ssRNAs. A strand transfer, therefore, occurs from the R region at the 5′-end of the genome to the equivalent R region at the 3′-end (see Figure 1). After this step, termed (−)strand transfer, (−)strand synthesis can continue along the viral RNA starting from its 3′-end. Whilst DNA synthesis proceeds, the RNase H function cleaves the RNA strand of the RNA:DNA at numerous points. Although most of the RNase H cleavages do not appear to be sequence specific, there are two specific purine-rich sequences, known as the polypurine tracts (PPTs), that are resistant to the RNase H cleavage and remain annealed with the nascent (−)strand DNA. These two well-defined sites are located in the central part of the HIV-1 genome. In particular, the 3′-end PPT defines the 5′-end of the viral coding (+)strand DNA synthesis since this PPT serves as primer [7, 8]. The (+)strand DNA synthesis continues to the 5′-end of the (−)strand DNA and uses also the 18-nucleotides PBS sequence of the tRNA as a template. Importantly, the 19th base from the 3′-end of tRNALys3 is a methyl A, and the presence of this modified base blocks the RT, generating a (+)strand strong-stop DNA. Subsequently, the RNase H function cleaves the RNA segment of the tRNA:DNA hybrid, freeing the PBS sequence of the (+)strand DNA and allowing it to anneal to the complementary site near the 3′-end of the extended (−)strand DNA [9]. Then, a bidirectional synthesis occurs to complete a viral dsDNA that has a 90-nucleotides single-stranded flap at the center. This unusual situation is probably solved by host mechanisms, and one candidate for flap removal is the flap endonuclease-1 (FEN-1) [8]. Finally, a specific cleavage removes the PPT primers and exposes the integration sequence to facilitate the insertion of the viral dsDNA into the host chromosome.

3. RT Structure and Functions

As a major target for anti-HIV therapy, RT has been the subject of extensive research through crystal structure determinations, biochemical assays, and single-molecule analyses. RT derives from a virus-coded polyprotein that is processed by the viral protease to give rise to two related subunits of different length, the p66 and the p51, that share a common amino terminus and combine in a stable asymmetric heterodimer [10]. Analysis of the crystal structure of RT reveals that p66 is composed of two spatially distinct domains, polymerase and RNase H domains (Figure 2). The polymerase domain shows a characteristic highly conserved structure that resembles a right hand, consisting of fingers (residues 1–85 and 118–155), palm (residues 86–117 and 156–237), and thumb (residues 238–318) subdomains. The p66 subunit also comprises the connection subdomain (residues 319–426) and RNase H domain (residues 427–560) [11, 12]. The p51 subunit lacks the RNase H domain and has the same four subdomains of the p66 polymerase domain whose relative positions, however, are different. For this reason, the p51 subunit folds differently from p66; it does not have enzymatic activities while it serves to anchor the proper folding of the p66 subunit that performs all the catalytic functions.

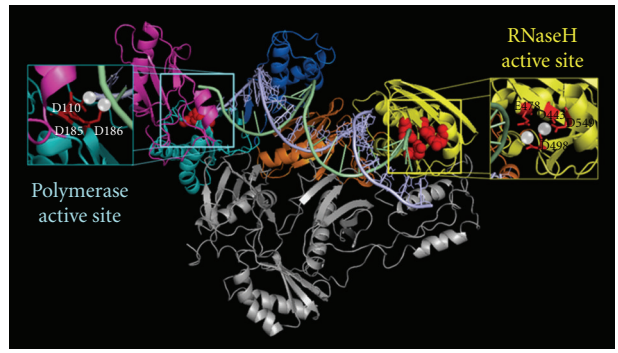


Figure 2: Structure of HIV-1 RT. The enzyme has two domains: the p66 (colored) and the p51 (gray). The polymerase domain shows a characteristic highly conserved structure that resembles a right hand, consisting of fingers domain (magenta), palm domain (cyan), thumb domain (blue). The p66 subunit also comprises the connection domain (orange) and RNase H domain (yellow). The polymerase active site is located in the middle of palm, fingers, and thumb subdomains. The three catalytic aspartic acid residues (D110, D185 and D186) located in the palm subdomain of p66 that bind the cofactor divalent ions (Mg^{2+}) are shown (red). The RNase H domain is located at C-terminus of the p66 subunit, 60 Å far from polymerase active site. The RNase H active site contains a DDE motif comprising the carboxylates residues D443, E478, D498, and D549 that can coordinate two divalent Mg^{2+}.

RT is primarily responsible for several distinct activities that are all indispensable for the retrotranscription process: RNA- and DNA-dependent DNA synthesis, RNase H activity, strand transfer, and strand displacement synthesis [13]. The presence of all these functions in a single protein is facilitated by the highly dynamic RT nature which allows RT to spontaneously slide over long distances of RNA:DNA and DNA:DNA duplexes, to easily target the primer terminus for DNA polymerization, to rapidly access multiple sites, and, hence, to make up for its low processivity [13]. RT sliding does not require energy from nucleotide hydrolysis, and it is supposed to be a thermally driven diffusion process [13]. Noteworthy, it has been recently shown that RT can bind to the nucleic acid substrates in two different orientations, termed "RNase H cleavage competent orientation" and "polymerase competent orientation," and that each of them allows to catalyze one of the two RT-associated enzymatic activities [14]. These two binding modes are in a dynamic equilibrium, and it has been demonstrated that RT can spontaneously and rapidly switch between these orientations without dissociating from the substrate. This flipping can be influenced by the presence of small molecules as nucleotides that stabilize the polymerase competent orientation or inhibitors that, conversely, destabilize it [8]. Together, shuttling and switching give rise to a very complex series of conformational changes that increase enormously the replication efficiency, combining DNA polymerization and RNA cleavage.

3.1. RNA- and DNA-Dependent DNA Synthesis. The DNA synthesis, catalyzed by both RT-associated RNA- and DNA-dependent DNA polymerase activities (RDDP and DDDP, resp.), occurs with a mechanism that is similar to other DNA polymerases [15]. The polymerase active site is located in the middle of the palm, fingers, and thumb subdomains. In particular, the palm subdomain is very important for positioning of the primer terminus in the correct orientation for nucleophilic attack on an incoming dNTP [16]. Three aspartic acids residues (D110, D185, and D186) located in the palm subdomain of p66 bind the divalent ion cofactor (Mg^{2+}) through their catalytic carboxylates group, and are essential for catalysis (Figure 2) [17]. DNA synthesis requires that RT binds to the template:primer on the priming binding site; this interaction is stabilized by a change of the conformation of the p66 thumb (from close to open). Then, the dNTP binds at the nucleotide binding site to form an RT:DNA:dNTP ternary complex [18]. Afterwards, a conformational change of the fingers traps the dNTP, precisely aligning the α-phosphate of the dNTP and the 3′-OH of the primer inside of polymerase active site (this is actually the rate limiting step). Under these conditions, the enzyme catalyzes the formation of a phosphodiester bond between the primer 3′-OH and the dNMP with the release of a pyrophosphate. Then, the pyrophosphate is free to go out of the catalytic site. Finally, translocation of the elongated DNA primer frees the nucleotide-binding site for the next incoming dNTP or, alternatively, RT can dissociate from the complex. Compared to cellular DNA polymerases, RT exhibits a very low processivity, typically dissociating from the substrate after synthesizing only a few to a few hundred nucleotides. This may contribute to the fidelity of RT and results in the accumulation of mutations during reverse transcription.

Importantly, during its DNA polymerase activity RT can run up against several template secondary structures. Particularly, the RNA template can form stable RNA:RNA interactions that can occlude the polymerization site and/or displace the primer terminus. In this case, RT has been shown to realize a strand displacement synthesis, in which the sliding movement can contribute to the reannealing of the primer, displacing the RNA [17].

3.2. DNA-Directed RNA Cleavage. RT is able to degrade selectively the RNA portion of an RNA:DNA hybrid and to remove the priming tRNA and PPT. This RNase H function is essential for virus replication since RNase H-deficient viruses are noninfectious [19]. The RNase H domain is located at C-terminus of the p66 subunit, 60 Å far from polymerase active site (Figure 2) equivalent to 17 nucleotides of a DNA:DNA hybrid and/or 18 nucleotides of a RNA:DNA hybrid [20]. The RNase H active site contains a highly conserved, essential, DDE motif comprising the carboxylates residues D443, E478, D498, and D549, that can coordinate two divalent Mg^{2+} cations, consistently with the proposed phosphoryl transfer geometry [21]. Mutations in any of the D443, D498, and E478 residues abolish enzyme activity [22, 23]. The RNase H domain can catalyze a phosphoryl transfer through nucleophilic substitution reactions on phosphate ester. This action occurs through the deprotonation of a water molecule, with the production of a nucleophilic hydroxide group that attacks the scissile phosphate group on the RNA previously activated by coordination with the Mg^{2+} cofactor [24]. The reason for the RNase H cleavage specificity for the RNA portion of the RNA:DNA hybrid mainly relies on its particular minor groove width and its interaction with the "primer grip" (an extensive network of contacts between the hybrid phosphate backbone and several residues far ~4–9 bp from the RNase H active site) [16]. The RNA:DNA hybrid has a minor groove width of ~9-10 Å, that is intermediate between the A- and B-form of other double-stranded nucleic acids (dsNA). The HIV-1 RNase H hydrolyzes much less efficiently hybrids with lower widths, such as the PPTs that show a width of 7 Å probably due to the presence of A-tracts [17, 25]. This fact allows the PPT recognition as RNA primers for DNA synthesis and may also represent a further specific viral target.

The RNase H catalysis can occur in a polymerase-dependent or polymerase-independent mode, and it is possible to distinguish three different cleavage types: "DNA 3′-end-directed cleavage," "RNA 5′-end-directed cleavage," and "internal cleavage" [26]. The former acts during (−)strand DNA synthesis, when the RNase H active site cleaves the RNA in a position based on the binding of the polymerase active site to the 3′-end of the new (−)DNA [27]. The second one acts when RT binds to a recessed RNA 5′-end annealed to a longer DNA strand, and the RNase H function cleaves the RNA strand 13–19 nucleotides away from its 5′-end. The internal cleavage occurs since the RNA cleavage is slower than DNA synthesis, and, given that a viral particle contains 50–100 RTs molecules and only two copies of (+)RNA, all the nonpolymerizing RTs can bind to the hybrid and degrade the RNA segment by a polymerase-independent mode [16].

3.3. Strand Transfer. The strand transfer is a critical step during the reverse transcription process in which two complementary ssNAs have to anneal to allow the pursuance of DNA synthesis (Figure 1). In both (−) and (+)strand transfers the ssNA develops secondary structures: the R region consists of a strong-structured motif TAR hairpin and a poly(A) hairpin [28]. Also the PBS sequence at the 3′-end of the (−)strand DNA can form a stable hairpin structure. Therefore, RT is helped in performing this step by the presence of the viral-coded nucleocapsid (NC) protein [29, 30]. The strand transfer process, together with the RT fidelity and the presence of other host factors such as APOBEC [31], helps to explain the high rate of recombination events to allow HIV to evolve rapidly and develop resistance to drugs.

3.4. Pyrophosphorolysis. As most DNA polymerases, RT can catalyze the reversal of the dNTP incorporation that is termed pyrophosphorolysis. RT has the ability to carry out this reverse reaction using a pyrophosphate (PPi) molecule or an NTP, such as ATP, as the acceptor substrate [32–34] giving rise to a dinucleotide tetraphosphate (formed by the excised dNMP and the acceptor ATP substrate) and

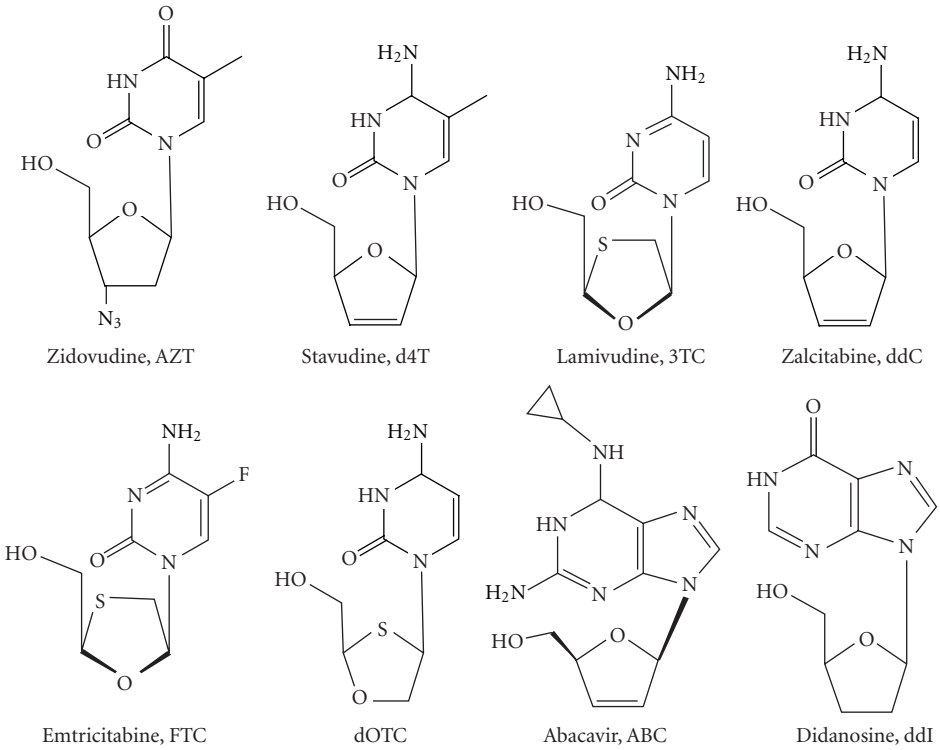


Figure 3: Chemical structures of approved NRTIs.

a free 3′-OH end as reaction products. This RT function is particularly important, as discussed later, in some drug resistance mechanisms.

4. Current RTIs: Structure, Mode of Action, and Resistance

The approved combination treatments used for HIV-1 include two classes of RTIs that target the viral enzyme with two different mechanism of action. The first class comprises compounds known as nucleoside/nucleotide RT inhibitors (NRTIs/NtRTIs), while the second class comprises compounds known as nonnucleoside RT inhibitors (NNRTIs).

4.1. Nucleoside RT Inhibitors. There are currently eight NRTIs clinically available, structurally resembling both pyrimidine and purine analogues [3]. Pyrimidine nucleoside analogues include thymidine analogues such as 3′-azido-2′,3′-dideoxythymidine (zidovudine, AZT), and 2′,3′-didehydro-2′,3′-dideoxythymidine (stavudine, d4T) and cytosine analogues such as (−)-2′,3′-dideoxy-3′-thiacytidine (lamivudine, 3TC), 2′,3′-dideoxycytidine (zalcitabine, ddC) which, however, is no longer recommended due to peripheral neuropathy [35], (−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine (emtricitabine, FTC), and [(−)-2′-deoxy-3′-oxa-4′-thiacytidine) (dOTC). Purine nucleoside analogues include (IS-4R)-4-[2-amino-6(cyclopropylamino)-9H-purin-9yl]-2-cyclopentane-I-methanol (abacavir, ABC) and 2′,3′-dideoxyinosine (didanosine, ddI) as guanosine and adenine analogues, respectively (Figure 3) [3]. These agents, in order to inhibit reverse transcription, have to be phosphorylated by cellular kinases to their triphosphate derivatives. All NRTIs follow the same mechanism of RT inhibition: once activated to their triphosphate form, they are incorporated by RT into the growing primer (Figure 4), competing with the natural dNTPs and terminating DNA synthesis due to their lack of the 3′-hydroxyl group (Figure 5). Therefore, once incorporated into dsDNA they prevent the incorporation of the incoming nucleotide. Importantly, while HIV-1 RT uses these NRTIs as substrates, the cellular DNA polymerases do not recognize them with the same affinity.

Under selective drug pressure, drug resistant viral mutants can gain a competitive advantage over wt virus and become the dominant quasispecies. HIV-1 resistance to NRTIs usually involves two general mechanisms: NRTI discrimination, that reduces the NRTI incorporation rate, and NRTI excision that unblocks NRTI-terminated primers. A simple example of discrimination is steric hindrance in which there is a selective alteration of the NRTI binding and/or incorporation rate such as in the case of the M184V mutation and 3TC [36, 37], where the valine substitution makes steric contacts with the sulfur of the oxathiolane ring of 3TC triphosphate, preventing its proper positioning for catalysis [38]. Even though the discrimination mechanism is less obvious for other NRTIs, in which structurally poorer compounds (e.g., the ones just lacking the 3′-OH group) should be differentially recognized, mutations in the nucleoside-binding site such as K65R, T69D, L74V, V75T, located in the β3-β4 loop of the p66 fingers subdomain, have

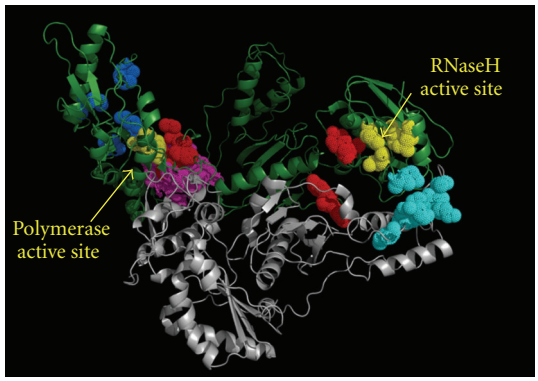


Figure 4: Amino acid residues involved in RTI binding. RT two subunits are in green (p66) and in gray (p51). The catalytic residues of the polymerase active site and the RNase H active site are colored in yellow. NRTIs and NtRTIs interact with residues close to the polymerase active site (blue). NNRTIs bind in a hydrophobic pocket next to the polymerase active site (magenta). RHRTIs such as DKAs, N-hydroxyimides, N-hydroxy quinazolinediones and naphthyridine derivatives bind in the RNase H active site (in yellow on the right). Vinylogous ureas bind to a hydrophobic pocket at the interface between the RNase H domain and the p51 subunit (cyan). Hydrazone derivatives have been proposed to bind two different sites (red). One located between the polymerase active site and the NNRTI-binding pocket (sharing a few residues with it) and the second one located between the RNase H and the connection domain. Anthraquinone derivatives have been proposed to bind to the first hydrazone pocket next to the NNRTI-binding site.

been reported to allow a better RT discrimination between NRTI triphosphates and natural dNTPs, since they are involved in the RT interaction with the incoming dNTP [39, 40]. Differently, M41L, D67N, D70R, L210W, T215F/Y, and K219Q mutations, located around the dNTP-binding pocket and also termed thymidine analogs mutations (TAMs), increase NRTI excision. In particular, D67N and K70R are the most important in the excision of 3′-end NRTI-terminated DNA while T215F/Y may increase the RT affinity for the excision substrate ATP so that the NRTI excision is reasonably efficient at ATP physiological concentrations [32, 40, 41]. Other TAMs such as M41L and L210W may stabilize the 215F/Y interaction with the dNTP-binding pocket [42], whereas the K219Q mutation may increase the RT processivity to compensate the higher rate of 3′-nucleotide removal [32, 34]. Recently, mutations in the connection and RNase H domains have also been shown to confer NRTI resistance [43–47]. In particular, connection mutations such as E312Q, G335C/D, N348I, A360I/V, V365I, and A376S have been shown to increase AZT resistance up to 500-fold in the context of TAMs by reducing RNase H activity [43]. This RNase H-dependent mechanism of NRTI resistance has been proposed to be due to an increase in NRTI excision determined by a reduction of RNase H activity [44]. In contrast, the connection mutation G333D, in the context of TAMs and M184V mutation, increases discrimination against 3TC-MP incorporation [48], suggesting an RNase H-independent mechanism of NRTI resistance probably due to long-range interactions and conformational changes in the connection domain [49].

4.2. Nucleotide RT Inhibitors. NtRTIs, such as (R)-9-(2phosphonylmethoxypropyl)-adenine (tenofovir, PMPA) (Figure 6), are compounds that already have a phosphonate group resistant to hydrolysis [3]. Therefore, they only need two phosphorylation steps to be converted to their active diphosphate derivatives, abbreviating the intracellular activation pathway and allowing a more rapid and complete conversion to the active agent [50, 51]. Similarly to NRTIs, NtRTIs are phosphorylated to the corresponding diphosphates by cellular enzymes and serve as alternative substrates (competitive inhibitors); once incorporated into the growing viral DNA, they act as obligatory chain terminators [50]. NtRTIs such as tenofovir are taken as prodrugs to facilitate penetration of target cell membranes. Subsequently, endogenous chemolytic enzymes release the original nucleoside monophosphate analogue that exerts its action [51].

4.3. Nonnucleoside RT Inhibitors. NNRTIs are structurally and chemically dissimilar compounds that bind in noncompetitive manner to a hydrophobic RT pocket close to the polymerase active site (Figure 4), distorting the protein and inhibiting the chemical step of polymerization [3, 52]. In fact, NNRTIs binding to RT induces rotamer conformational changes in some residues (Y181 and Y188) and makes the thumb region more rigid, blocking DNA synthesis. Importantly, unlike NRTIs, NNRTIs do not require intracellular metabolism to exert their activity. More than thirty different classes of compounds could be considered to be NNRTIs [3]. The currently approved NNRTIs are 11-cyclopropyl-4-methyl-5H-dipyrido[3,2-b:2′,3′-e][1,4]diazepin-6(11H)-one (nevirapine), (S)-6-chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1H-benzo[d][1,3]oxazin-2(4H)-one (efavirenz), N-(2-(4-(3-(isopropylamino)pyridin-2-yl) piperazine-1-carbonyl)indolin-5-yl)methanesulfonamide (delavirdine) and 4-((6-amino-5-bromo-2-((4-cyanophenyl)amino)pyrimidine-4-yl)oxy)-3,5-dimethylbenzonitrile (etravirine) and 4-((4-((4-(cyanomethyl)-2,6-dimethylphenyl)amino)pyrimidin-2-yl)amino)benzonitrile (rilpivirine) (Figure 7).

Crystallography, molecular modeling and docking studies have revealed that first generation NNRTIs assume a butterfly-like conformation [53–57]. The stabilization of the NNRTI binding in the allosteric site is accomplished through (i) stacking interactions between the NNRTIs aromatic rings and the side chains of Y181, Y188, W229, and Y318 residues in the RT lipophilic pocket; (ii) electrostatic forces (particularly significant for K101, K103, and E138 residues); (iii) van der Waals interactions with L100, V106, V179, Y181, G190, W229, L234, and Y318 residues; (iv) hydrogen bonds between NNRTI and the main chain (carbonyl/amino) peptide bonds of RT [53, 54, 58, 59]. Larger first-generation inhibitors, such as delavirdine, extend towards the flexible loop containing the P236 residue, while maintaining stacking interactions with the tyrosine residues 181 and 188 and hydrogen bonding with K103 [60]. Stacking interactions

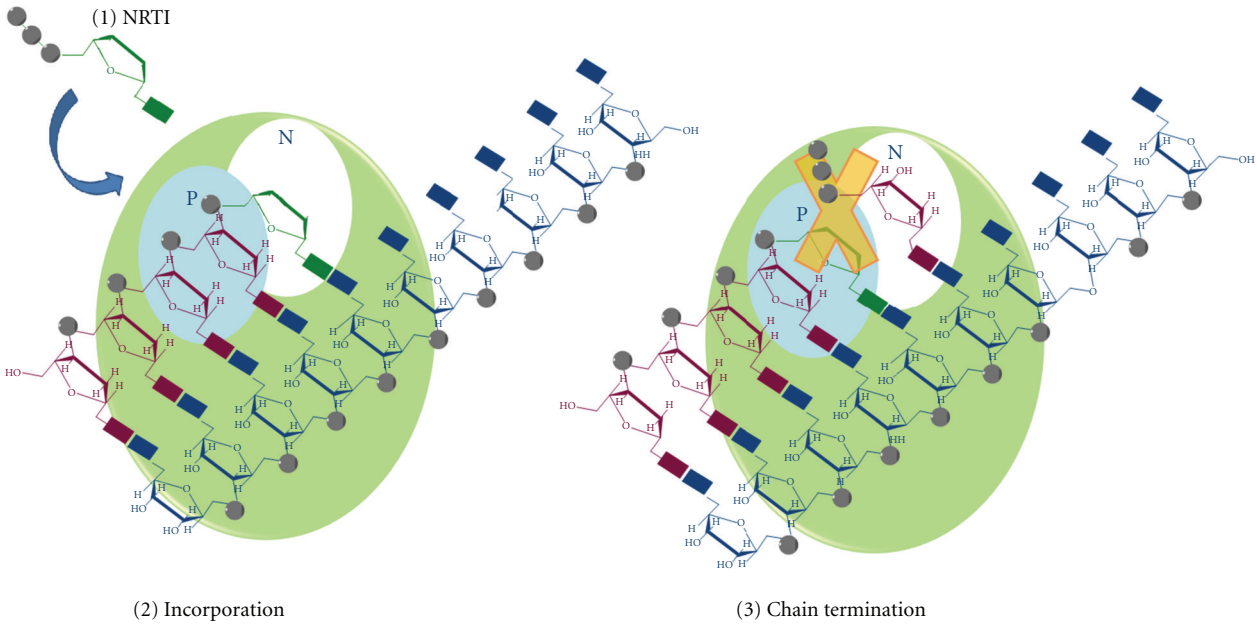
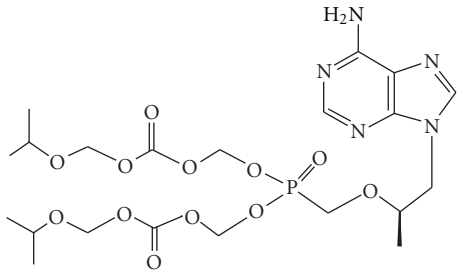


Figure 5: Mechanism of action of RT inhibitors acting as chain terminators. The RT is represented as a pale green circle with the priming binding site in cyan (P) and the nucleotide binding site in white (N). The RNA template is showed in blue and the (−)strand DNA in purple. The NRTI triphosphate (strong green) (1) competes for the binding with the natural dNTPs, it is incorporated into the growing DNA (2) and it blocks the further DNA elongation because it lacks the 3′-hydroxyl group (3).



Tenofovir, PMPA

Figure 6: Chemical structure of approved NtRTI.

are less important in the case of efavirenz binding, while hydrogen bonds between the inhibitor and the protein backbone of K101 and K103 residues are critical [61].

First-generation NNRTIs, such as nevirapine and delavirdine, easily select resistant RTs that contain single amino acid mutations such as Y181C, K103N, and Y188C [62, 63], that change their key hydrophobic interactions at the NNRTI binding site. Second-generation NNRTIs, such as efavirenz and dapivirine, usually require two or more mutations in the HIV-1 RT before significantly decreasing their antiviral potency. In general, two or more HIV-1 RT mutations are clustered in the NNRTI pocket, suggesting a direct stereochemical mode of reduction of NNRTI binding, even though other mechanisms may also be present such as the one shown by V108I mutation that induces resistance by perturbing the Y181 and Y188 residues [61] or the one proposed for K103N mutation that should stabilize the apo-RT conformation and, hence, create an energy barrier to NNRTIs binding, reducing their potency [61]. Interestingly, NRTI-resistant mutant virus strains keep full sensitivity to the inhibitory effects of NNRTIs, and vice versa. Recently, however, mutations in the connection and RNase H domains such as N384I, T369I, and E399D have been shown to confer resistance to both NRTIs and NNRTIs probably by altering the template:primer positioning [44, 47, 64].

5. New Nucleoside RT Inhibitors

The NRTIs therapeutic use is limited by several factors [65]. Firstly, drug-drug interactions with other NRTIs used in combination treatments such as the one observed between AZT and D4T, that share the same phosphorylation pathway and show a less than additive effect when used in combination [66], or between ddI and tenofovir which determine an increase in single drugs toxicity [65]. Secondly, drug-drug interactions with other molecules such as the one observed when ABC or tenofovir is administered with some protease inhibitors [65, 67], or when ABC is administered with ethanol [68]. Thirdly, several adverse events such as mitochondrial toxicity (linked to myopathy, cardiomyopathy, anemia, lipoatrophy), drug hypersensitivity reactions, and renal dysfunctions have been associated with NRTI treatment [65]. Fourthly, as described above, the selection of NRTI-resistant strains, which is still the main limitation in view

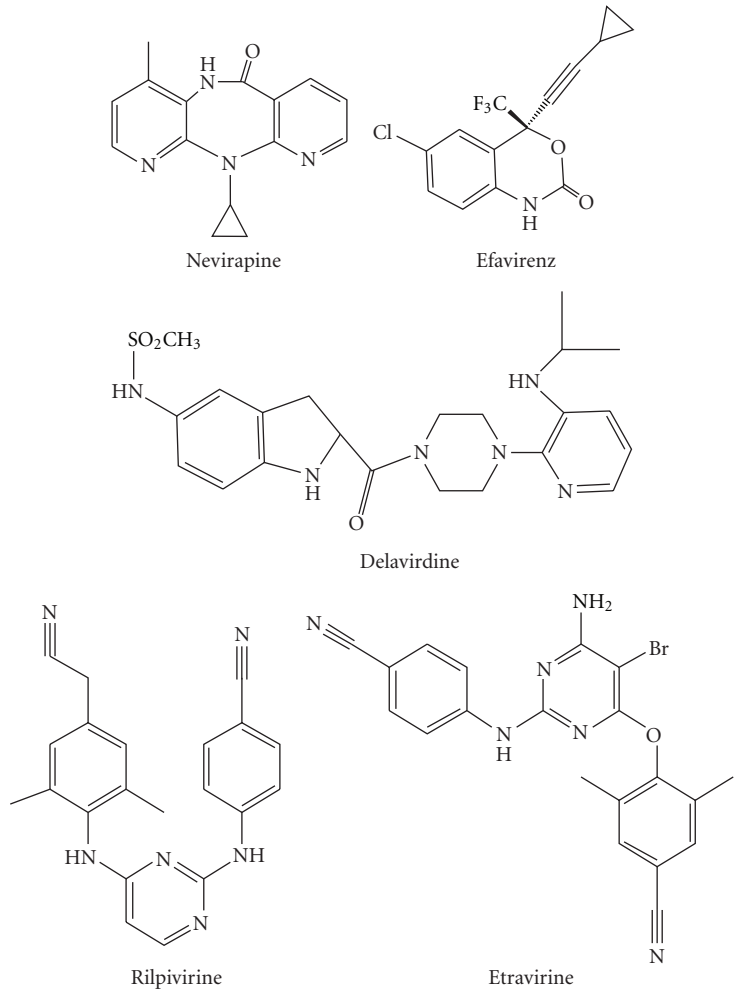


Figure 7: Chemical structures of approved NNRTIs.

of the need for life-long antiviral treatments. Particularly, it has been reported that almost 50% of the viremic patients actually harbor M184V RT mutant strains and that 6–16% of the patients have been infected with viruses resistant to at least one drug and, hence, have a poorer response to therapy and a lower barrier to select further drug-resistant strains [65, 69]. Given this scenario, the new NRTIs which are currently under investigation are sought to have a favorable resistance profile, reduced adverse effects, and/or a novel mechanism of action.

5.1. Nucleoside RT Inhibitors in Development Acting as Chain Terminators. (−)-2′-deoxy-3′-oxa-4′-thiocytidine (Apricitabine, ATC) (Figure 8) is a (−)enantiomer deoxycytidine analog with a favorable resistance profile. In fact, ATC shows only a 2-fold potency reduction on TAM strains, with or without the M184V mutation, and on K65R mutant strain, while it shows a 10-fold potency reduction on Q151M mutant strains [70–72]. ATC has a favorable toxic profile with little effects on mitochondrial DNA levels [73], while it shows negative drug-drug interactions when administered with 3TC or FTC [74]. Overall, ATC seems to be a good candidate in NRTI-experienced patients including individuals who have experienced virological failure on 3TC and FTC containing regimens or harboring M184V mutant strains. In fact, ATC has successfully completed the primary endpoint of a phase IIb trial in drug-resistant HIV patients with the M184V mutation.

L-β-2′,3′-didehydro-2′,3′-dideoxy-5-fluorocytidine (Elvucitabine, L-d4TC) (Figure 8) is an L-cytidine analog under investigation in phase I/II clinical trials that is more potent than 3TC and that shows no mitochondrial toxicity [75] and an interesting protecting effect on the mitochondrial toxicity due to other NRTIs [76]. L-d4TC resistance profile shows that it selects for M14V RT mutants [77] and has a 10-fold potency reduction on K65R mutant strains [78].

1-β-D-2,6-diaminopurine dioxolane (Amdoxovir, DAPD) (Figure 8) is a prodrug under investigation in phase II clinical trials which is deaminated to 1-β-D-dioxolane guanosine (DXG) that, upon triphosphorylation, is the active drug. DAPD has a favorable resistance profile since it shows minimal resistance to TAM- and M184V-resistant strains

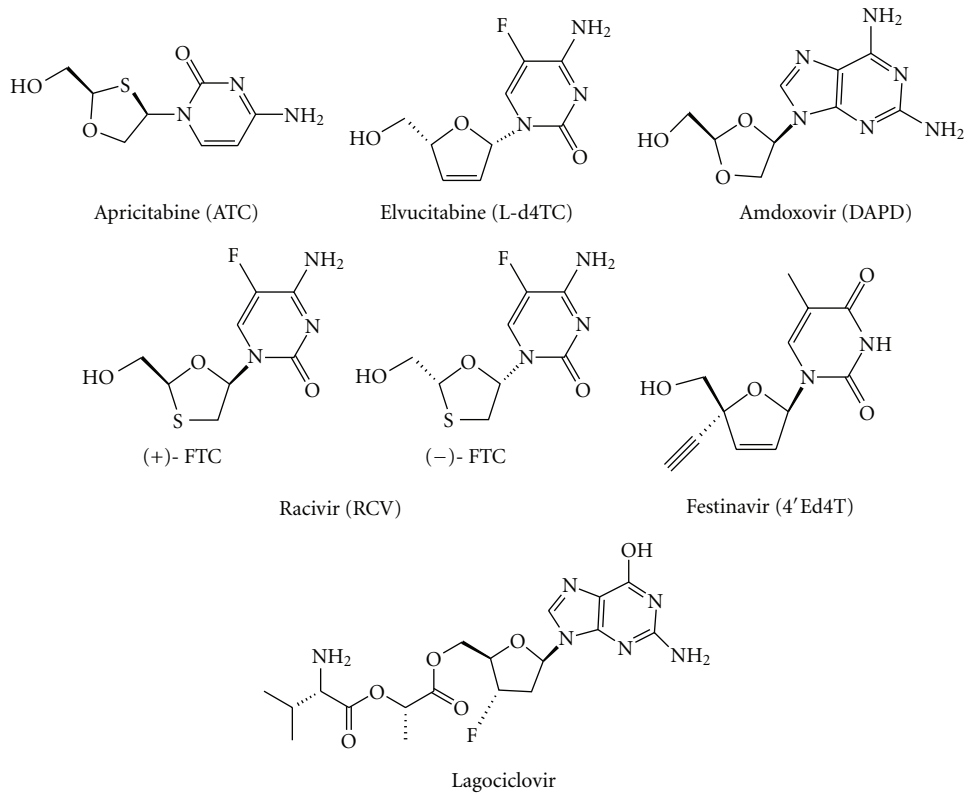


Figure 8: Chemical structures of new NRTIs acting as chain terminators.

[79, 80], while it shows a >10-fold potency reduction on K65R and Q151M strains [81]. While DAPD, *in vitro*, reduces the mitochondrial DNA content, DXG does not affect it [82].

(±)-β-2′,3′-dideoxy-3′-thia-5-fluorocytosine (Racivir, RCV) (Figure 8) is a racemic mixture of (+) and (−)FTC currently under evaluation in phase II/III clinical trials as part of a combination therapy. While both molecules inhibit RT [83], (−)FTC is better phosphorylated than (+)FTC in cells [84], and, therefore, it shows a higher potency in virus inhibition [85]. The RCV resistance profile is interesting; in fact, (−)FTC selects for M184V-resistant strains, while (+)FTC selects for T215Y-resistant strains [86]. Since the simultaneous selection of these two amino acid mutations is incompatible, such racemic mixture orthogonal resistance profile determines a delay in the onset of the drug resistance selection [87]. The long-term mitochondrial toxicity, however, is still to be fully assessed since (+)FTC triphosphate is only 36-fold selective for RT versus DNA polymerase γ [88].

In addition, the chain terminator NRTIs Festinavir (4′-Ed4T) [89] and Lagociclovir [90] (Figure 8) are currently under development.

5.2. Nucleoside RT Inhibitors with Innovative Mode of Action. The RT inhibition by NRTIs can also be achieved by mechanisms different from the classical chain termination due to the lack of a 3′-hydroxyl group. In particular, new classes of inhibitors with new modes of action are the translocation-defective RT inhibitors (TDRTI), the delayed chain terminators RT inhibitors (DCTRTI), the lethal mutagenesis RT inhibitors (LMRTI), and the dinucleotide tetraphosphates ($N_{p4}Ns$).

5.2.1. Translocation-Defective RT Inhibitors. TDRTIs are NRTIs with modifications of the sugar moiety that block the RT translocation after the NRTI incorporation. 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) (Figure 9) is the most potent derivative of a series of 4′-substituted nucleoside analogs which, differently from the other NRTIs, have a 3′-hydroxyl group [91]. EFdA is able to inhibit many drug-resistant strains several orders of magnitude more potently than the other approved NRTIs. For instance, it inhibits the M184V mutant strain with an EC_{50} value of 8 nM, while some other drug-resistant strains are even hypersensitive to EFdA [92]. Importantly, RT can use EFdA triphosphate (EFdA-TP) as substrate but, despite the presence of the 3′-hydroxyl group, the incorporated EFdA monophosphate (EFdA-MP) blocks further DNA synthesis since the enzyme is not able to efficiently translocate on a RNA:DNA or a DNA:DNA hybrid containing a 3′-terminal EFdA-MP [93] (Figure 10). In fact, on the one hand, the North (C2′-exo/C3′-endo) EFdA sugar ring conformation (which is the proper 3′-terminus position for in-line nucleophilic attack on the α-phosphate of the incoming dNTP) has been shown to be required for efficient binding at the primer-binding and

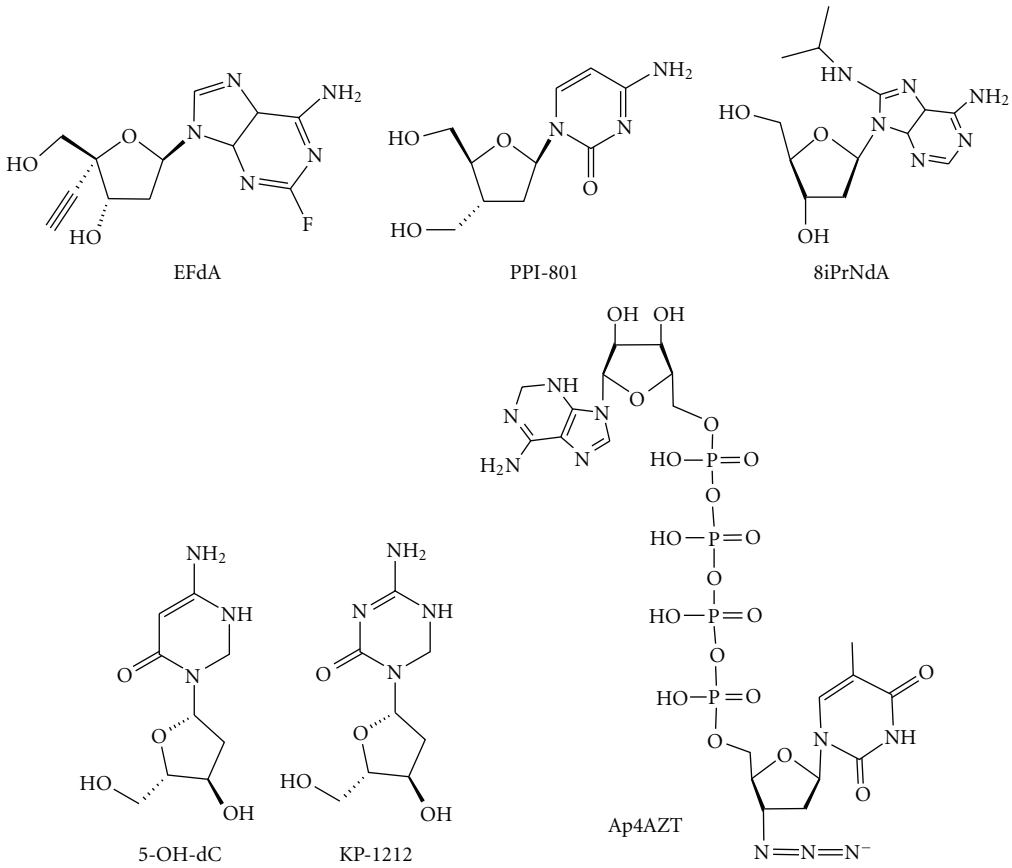


Figure 9: Chemical structures of NRTIs with new mechanisms of action.

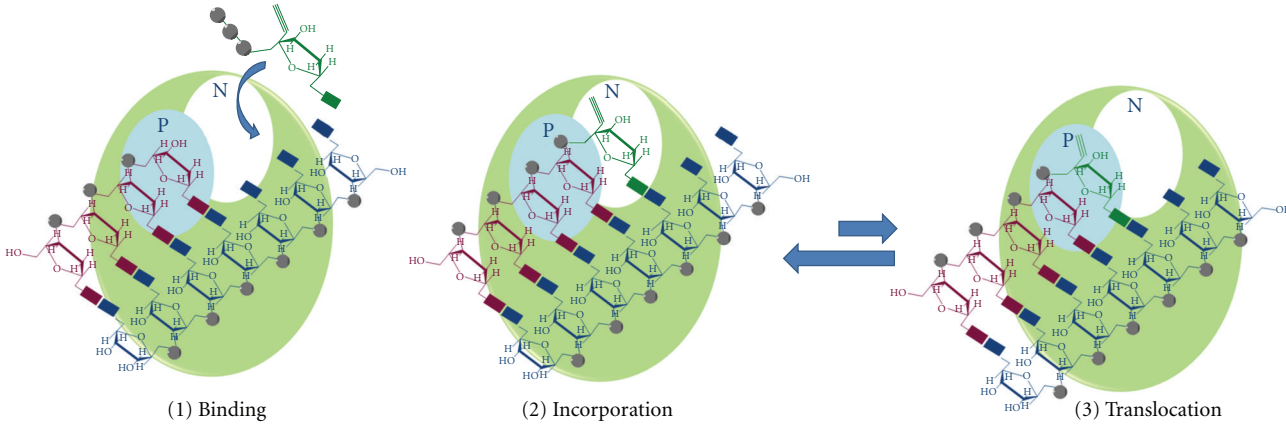


Figure 10: Mechanism of action of TDRTIs. The RT is represented as a pale green circle with the priming binding site in cyan (P) and the nucleotide binding site in white (N). The RNA template is shown in blue and the (−)strand DNA in purple. The TDRTI triphosphate (strong green) can be used as RT substrate (1) and is incorporated in the nucleic acid (2). The incorporated TDRTI blocks the further DNA synthesis since the enzyme is not able to efficiently translocate (3).

RT polymerase active sites suggesting that, once incorporated into the DNA, the EFdA 3′-hydroxyl group is not likely to prevent by itself additional nucleotides incorporation, and, thus, it does not contribute to the mechanism of chain termination [94]. On the other hand, molecular modeling studies suggested that the 4′-ethynyl of EFdA may fit into a hydrophobic pocket defined by residues A114, Y215, F160, M184 and the aliphatic D185 chain [93]. Hence, it has been proposed that the presence of a 4′-ethynyl substitution on the ribose ring possibly hampers RT to translocate the 3′-EFdA-MP terminus DNA. Under these circumstances, RT is stabilized in a pretranslocation state which antagonizes the further nucleotide addition, since the dNTP-binding site is not accessible and the incorporation of the next

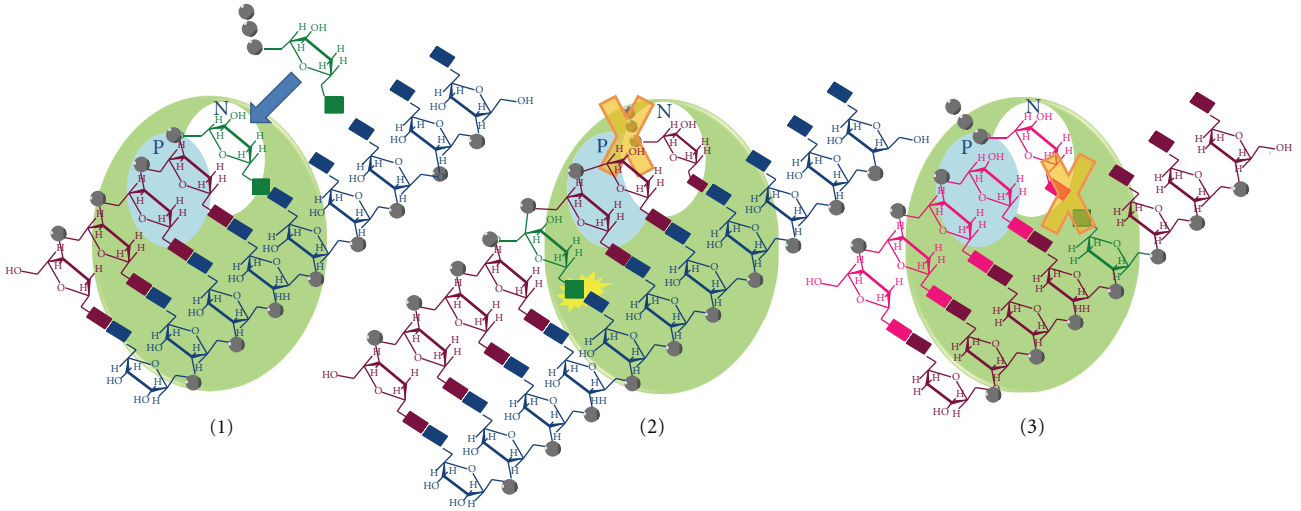


Figure 11: Mechanism of action of DCTRTIs. The RT is represented as a pale green circle with the priming binding site in cyan (P) and the nucleotide binding site in white (N). The RNA template is shown in blue and the (−)strand DNA in purple. DCTRTI triphosphate (strong green) is incorporated into the growing DNA chain (1). After further nucleotides addition, its presence blocks DNA elongation, probably through steric hindrance interference (yellow) between the RNA:DNA hybrid and the RT nucleic-acid-binding cleft (2). In addition, their incorporation can also block the synthesis of the (+)strand DNA affecting the base pairing (3).

complementary nucleotide is prevented [93]. Notably, in spite of the fact that the diminished translocation makes the 3′-EFdA-MP terminus DNA an excellent substrate for NRTI excision, the net excision process has been reported to be not very efficient, apparently because once the nucleotide is excised through pyrophosphorolysis to form EFdA-TP, the latter is rapidly reincorporated [93]. Moreover, it has been recently reported that EFdA is a poor substrate for DNA polymerase γ (it is incorporated 4,300-fold less than dATP), suggesting minimal mitochondrial toxicity [95].

5.2.2. Delayed Chain Terminators RT Inhibitors. DCTRTIs are NRTIs that allow further incorporation of dNTPs into the growing DNA chain since they have a 3′-hydroxyl group. However, after further nucleotide addition, their presence blocks DNA elongation, probably through steric hindrance interference between the RNA:DNA hybrid and the RT nucleic acid binding cleft, close to the polymerase active site (Figure 11). They can also block the synthesis of the (+)strand DNA affecting the base pairing.

2′,3′-dideoxy-3′C-hydroxymethyl cytidine (PPI-801) (Figure 9) has been reported to allow the incorporation of one additional dNTP prior to mediating chain termination [65]. Interestingly, the incorporated PPI-801 is not accessible for nucleotide excision, and, therefore, this class of compounds is proposed to be attractive because it should be active also on NRTI-resistant strains with enhanced 3′-end nucleotide excision.

8-isopropyl-amino-2′-deoxyadenosine (8iPrNdA) (Figure 9) is a recently reported molecule belonging to a series of nucleoside analogs with a natural deoxyribose moiety and modifications at position 8 of the adenine base [96]. These modifications may induce a steric clash with helix αH in the thumb domain of the p66 subunit, causing delayed chain termination. In fact, once incorporated into the elongated DNA, 8iPrNdA stops the further DNA synthesis after the incorporation of three additional dNTPs [96]. Even though the potency and selectivity of 8iPrNdA are not very high, it is an interesting example of an NRTI with modifications on the adenine base and not on the sugar moiety.

5.2.3. Lethal Mutagenesis RT Inhibitors. LMRTIs are NRTIs that allow further incorporation of dNTPs into the growing DNA chain. However, their incorporation causes a significant increase of nucleotide mismatches that determines a high mutation rate that eventually leads to viral replication suppression.

5-hydroxydeoxycytidine (5-OH-dC) (Figure 9) is a deoxycytidine analog that can efficiently base pair with both guanosine and adenosine nucleotides [97]. Viral growth in the presence of 5-OH-dC determines a 2.5-fold increase in G to A substitutions and a decline in viral infectivity over serial passages [97]. The fact that a relatively small increase in the HIV mutation frequency has a large effect on viral lethality substantiates the concept that the HIV mutation frequency is close to the error threshold for the viability of the quasispecies and that NRTIs that may significantly increase mutation frequency can act almost analogously to the cellular cytidine deaminase APOBEC3G [97].

5-aza-5,6-dihydro-2′-deoxycytidine (KP-1212) (Figure 9) is a deoxycytidine analog with a modified base and a natural sugar moiety that can also base pair with both guanosine and adenosine nucleotides [98]. The virus grown in the presence of KP-1212 accumulates a number of mutations that, eventually, stops its replication [98]. KP-1212 has been reported to interact also with DNA polymerase γ [99], suggesting a possible mitochondrial toxicity that, however, has not been observed in cells [98].

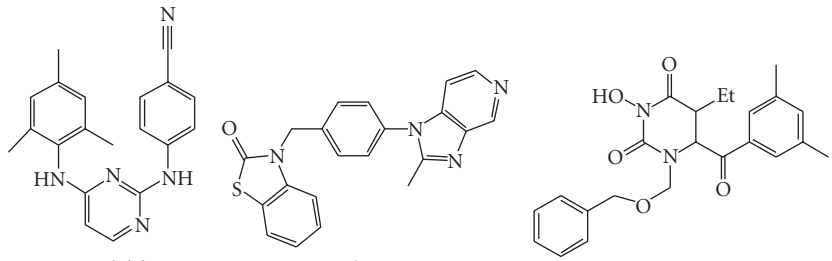


Figure 12: Chemical structures of new NNRTIs.

5.2.4. Dinucleotide Tetraphosphates. As described above, nucleotides excision is a major mechanism of NRTI resistance. During this mechanism RT catalyzes the pyrophosphorolysis of, for instance, a 3′-AZT-MP terminated DNA. In fact, in the presence of the PPi donor ATP, RT catalyzes the excision reaction which results in the production of a dinucleoside tetraphosphate (i.i. $A_{p4}AZT$) freeing the 3′-end for further DNA elongation. Notably, X-ray crystal studies have shown that the AMP part of the $A_{p4}AZT$ dinucleotide (Figure 9) binds differently to wt and drug-resistant mutant RTs [100]. These observations demonstrate that (i) RT can catalyze the reverse reaction and (ii) drug resistance mutations create a high-affinity ATP-binding site and open the possibility of designing drugs that can inhibit the enzyme mimicking the $N_{p4}N$ excision product that may be particularly active on NRTI-resistant strains. Up to now, a few $N_{p4}Ns$ have been synthesized that are able to inhibit wt and AZT-resistant RTs in the low micromolar range [101]. Notably, while the tetraphosphate linker, that avoids the intracellular phosphorylation step, is a potential advantage of these molecules, it is also an obstacle for their stability and cellular permeability. More studies dedicated to a further exploration of the ATP-binding site may lead to potent and innovative drugs.

6. New Nonnucleoside RT Inhibitors

The NNRTIs therapeutic use is limited mainly by the selection of NNRTI resistant virus, even though drug hypersensitivity and/or serious central nervous system dysfunctions are also toxicity issues for some NNRTIs. For this reason, there is still an active focus on the development of new NNRTIs, especially for compounds with high potency against K103N, Y181C, and Y188V mutant viruses. Besides the fact that more than 30 different conformational classes of NNRTIs have been reported to date [102, 103], the recent development of new NNRTIs has been focused on the identification of molecules that retain high conformation flexibility and positional adaptability in order to adjust the inhibitor conformation to the NNRTI-binding pocket, whose shape is different according to the presence of the diverse amino acid residues involved in NNRTI resistance. In fact, while first-generation NNRTIs, such as nevirapine, delavirdine, or efavirenz, bind to RT in "two-wing" (or "butterfly-like") conformation, the most recently developed NNRTIs show a "U" (or "horseshoe") conformation which gives an increased plasticity to these derivatives [104, 105]. Success stories of such an approach are the latest approved NNRTIs, etravirine and rilpivirine (Figure 7), and another compound under clinical investigation in phase I/II clinical trials, dapivirine (Figure 12) [104, 105].

Another complementary strategy used to improve the NNRTIs performance is to design derivatives that make strong interactions with highly conserved amino acid residues in the NNRTI- binding pocket such as F227, W229, L234, and Y318 [105, 106]. In fact, these first three residues are part of the primer grip region that maintains the primer terminus in an appropriate orientation for the nucleophilic attack on the incoming dNTP. Specifically, the W229 residue is the prime candidate residue for drug design, and, in fact, among others, the above-mentioned rilpivirine has been reported to make strong interactions with the indole ring of W229.

Another reported interesting NNRTI is 3-(4-(2-methyl-1H-imidazo[4,5-c]pyridin-1-yl)benzyl)benzo[d]thiazol-2(3H)-one (CP94707) (Figure 12) that inhibits, even though not very potently, wt and mutant Y181C and Y188C RTs at the same concentrations and shows only a 2-fold reduction in potency of inhibition on K103N RT [107]. CP94707 makes little contact with Y181 and Y188 residues, while it makes aromatic ring stacking interactions with W229 amino acid [107]. In addition, CP94707 binding to RT results in rearrangement of the distally positioned Y115 side chain, 15 Å away, to a conformation that is incompatible with binding of dNTPs. Y115, in fact, can act as a gatekeeper residue that discriminates between deoxynucleotides and ribonucleotides. Therefore, it has been proposed that CP94707 may have a nonconventional mode of action [108].

An NNRTIs series of N-hydroxyimide derivatives, such as compound 1-((benzyloxy)methyl)-6-(3,5-dimethylbenzoyl)-5-ethyl-3-hydroxydihydropyrimidine-2,4(1H,3H)-dione (HDIP) (Figure 12), have been developed as dual RT and IN inhibitors (DRT-INI). In fact, they have been reported to inhibit both the RT-associated RDDP function and the IN activity [109, 110] and have been proposed to bind to the NNRTI-binding site and also chelate the magnesium ion in the IN active site [109, 110].

7. Nucleotide Competing RT Inhibitors

A series of indolopyridones, therefore belonging to the NNRTIs, have been shown to inhibit RT interacting differently

from the classic NNRTIs. In particular, 5-methyl-1-(4-nitrophenyl)-2-oxo-2,5-dihydro-1H pyrido[3,2-b]indole-3-carbonitrile (INDOPY-1) (Figure 13) (i) inhibits also HIV-2 RT [111], while the other NNRTIs are inactive against this enzyme; (ii) it is active against K103N, Y181C, and Y188C mutant RTs as potently as on wt RT, while it is 3.6-fold less active against the K103N/L100I double-mutant RT [112]; (iii) it is active on TAM viruses, while it is 3- to 8-fold less effective on M184V or Y115F mutant viruses, it is more than 100-fold less potent on the M184V/Y115F double-mutant virus, and it is slightly more effective on K65R mutant virus [111–113]. In addition, the INDOPY-1 analog 1-(4-nitrophenyl)-2-oxo-2,5-dihydro-1H-pyrido[3,2-b] indole-3-carbonitrile (VRX329747) (Figure 13) selected HIV-1 RT mutated at the amino acid residues M41L, A62V, S68N, G112S, V118I, and M184V, which are all located around the incoming nucleotide-binding site [112]. Further, binding and biochemical studies revealed that (i) the M184V mutation reduces the affinity to INDOPY-1, while the Y115F mutation facilitates the dNTP binding, and their combined effects enhance the ability of the enzyme to discriminate against the inhibitor [113]; (ii) RT complexed with INDOPY-1 is trapped in the posttranslocational state [113]; (iii) the INDOPY-1 has preference with respect to substrate primer identity since its binding to RT is higher on a DNA:DNA versus a RNA:DNA primer:template [114]; (iv) when assayed by steady-state kinetic analysis with homopolymeric template primers, INDOPY-1 inhibits RT-catalyzed DNA polymerization with a competitive [111] or mixed-type [112] mode with respect to dNTPs. Overall, these observations suggest that the binding site of the indolopyridones and nucleotide substrates can at least partially overlap and they are therefore proposed as Nucleotide competing RT inhibitors (NcRTIs).

4-dimethylamino-6-vinylpyrimidines (DAVPs) is another class of compounds that have been reported to compete with the incoming dNTP and therefore can be considered NcRTI [115, 116]. However, differently from INDOPY-1, DAVP1 (Figure 13) is 4000- and 5000-fold less potent on mutant K103N and Y181C RTs, respectively [115], and binds also to unligated RT (while INDOPY-1 binds only to the RT:template:primer complex) [116]. X-ray crystal studies have confirmed that DAVP1 binds to an RT site that is distinct from the NNRTI-binding pocket, and it is close to the RT polymerase catalytic site [117]. This site is located in a hinge region, at the interface between the p66 thumb and p66 palm subdomains, that comprises the amino acid residues M230 and G231 (participating to the primer grip region and helping in the correct positioning of the 3′-OH end of the DNA primer), G262, K263 and W266 (involved in the template primer recognition), M184 and D186 (the first is involved in DNA synthesis fidelity, while the second is part of the catalytically essential YXDD motif) [117]. Hence, the DAVP1 binding site is located in a region critical for the correct positioning of the 3′-OH primer for the in-line nucleophilic attack by the incoming dNTP and the subsequent chemical bond formation with its α-phosphate. Notably, the X-ray study also revealed that in the RT/DAVP-1 complex the RT conformation is analogous to the "closed"

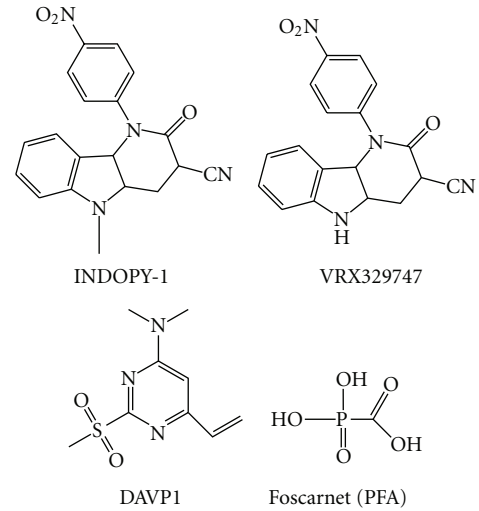


Figure 13: Chemical structures of NcRTIs.

conformation observed in unliganded RTs (with the p66 thumb subdomain folded into the DNA-binding cleft) and differs from that observed in RT/NNRTI complexes that has a hyperextended "open" conformation [117]. However, considering the proposed binding site, the reason for the loss of DAVP1 activity against K103N and Y181C mutant RTs remains unclear. While it has been hypothesized that DAVP1, owing to its small size, could travel between the NNRTI and nucleoside-binding pockets [117], more studies are needed to understand the DAVP1 mode of action.

8. PPi Analogs Inhibitors

Foscarnet (phosphonoformate, PFA) (Figure 13) is a PPi analogue that targets the DNA polymerase of herpes viruses as well as the RT of retroviruses [118]. Foscarnet is used intravenously to treat opportunistic viral infections, particularly CMV retinitis in patients with AIDS, but its pharmacokinetic profile is complicated by nephrotoxicity [119]. When assayed against HIV-1 RT, it competitively blocks pyrophosphorolysis and PPi exchange reactions, suggesting that foscarnet and PPi share overlapping binding sites [120]. It has been shown that foscarnet traps the RT pretranslocated complex preventing the binding of the next nucleotide, and, thus, the pretranslocated complex has been proposed as a target for drug discovery [121]. *In vivo* and *in vitro* foscarnet-resistant HIV-1 variants have been shown to carry mutations in the RT gene at several positions, including W88G/S, E89K/G, L92I, A114S, S156A, Q161L, and H208Y [122–125]. Notably, most of the mutations that reduce the susceptibility to PFA also confer hypersensitivity to AZT and it has been suggested that foscarnet analogs may inhibit the phosphorolytic rescue of NRTI-terminated primers and be used to prevent the excision-based mode of NRTI resistance [126].

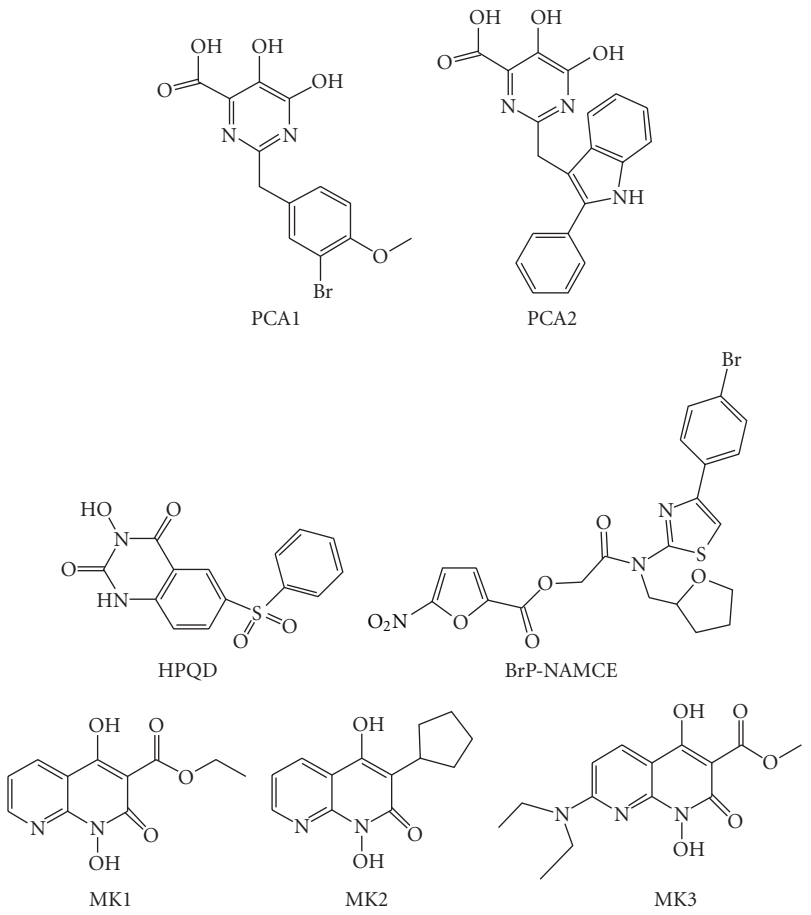


Figure 14: Chemical structures of metal chelating RHRTIs.

9. RNase H Inhibitors

Despite the fact that the RT-associated RNase H function is essential for the reverse transcription process as well as the RT-associated DNA polymerase function, no effective RNase H RTIs (RHRTIs) have been developed yet. In the last few years, however, a few classes of RHRTI that are specifically targeted to the RNase H active site (Figure 4) have been identified [19, 127]. Most of them are able to chelate the divalent magnesium ion within the RNase H active site, but they also exert a high cellular toxicity, possibly due to an unspecific metal chelation, since the RNase H active site is an open pocket and offers, at least so far, little elements for selective small-molecule optimization.

9.1. Metal Chelating RHRTI. Pyrimidinol carboxylic acids 2-(3-bromo-4-methoxybenzyl)-5,6-dihydroxypyrimidine-4-carboxylic acid (PCA1), 5,6-dihydroxy-2-((2-phenyl-1H-indol-3-yl)methyl)pyrimidine-4-carboxylic acid (PCA2) and *N*-hydroxy quinazolinedione inhibitors 3-hydroxy-6-(phenylsulfonyl)quinazoline-2,4(1H,3H)-dione) (HPQD) (Figure 14) were designed to coordinate the two metal ions in the active site of RNase H and showed no interactions with the polymerase metal-binding site [128]. However, so far they have not been further developed.

Similarly, Nitrofuran-2-carboxylic acids derivatives such as the 5-nitro-furan-2-carboxylic acid [[4-(4-bromophenyl)-thiazol-2-yl]-(tetrahydro-furan-2-ylmethyl)-carbamoyl]-methyl ester (BrP-NAMCE) (Figure 14) were identified to inhibit the RNase H function by chelating the magnesium ion [129], and other analogs were also reported [130], but more derivatization studies are needed in order to develop effective inhibitors.

Naphthyridine derivatives ethyl 1,4-dihydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylate (MK1), 3-cyclopentyl-1,4-dihydroxy-1,8-naphthyridin-2(1H)-one (MK2) and methyl 7-(diethylamino)-1,4-dihydroxy-2-oxo-1,2-dihydro-1,8-naphthyridine-3-carboxylate (MK3) (Figure 14) have been reported to bind to the RNase H active site by coordinating the two metal ions, engaging the conserved catalytic DDE motif [131]. Interestingly, they were reported to be sandwiched by a loop containing residues A538 and H539 residues on the one side and N474 on the opposite side. In addition, MK3 was also shown to bind to a site adjacent to the NNRTI including amino acid residues L100, V108, Y181, Y183, D186, L187, K223, F227, L228, W229, and L234 [131]. Unlike the binding to the RNase H active site, the binding to this alternate site appears to be predominantly mediated via the hydrophobic interactions with the diethylaminophenoxy group unique to MK3. The rilevance of the MK3 binding

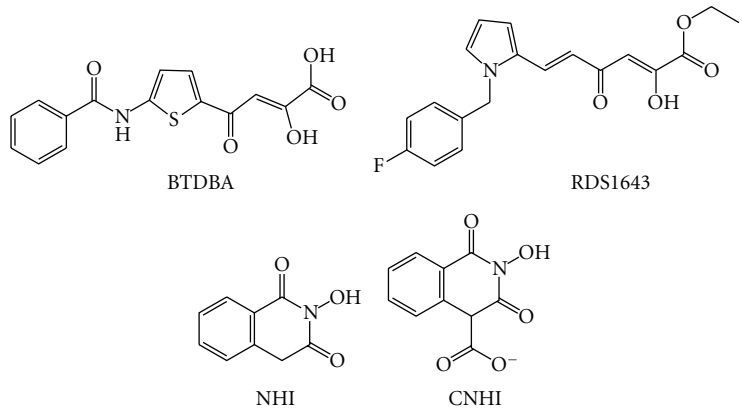


Figure 15: Chemical structures of dual RHRTI-INIs.

to this site is not clear; however, the site is similar to the binding site for DHBNH (see later).

9.2. Dual RHRTI and IN Inhibitors. The first recently discovered RHRTIs were the diketo acid (DKA) derivatives 4-[5-(benzoylamino)thien-2-yl]-2,4-dioxobutanoic acid (BTDBA) (Figure 15) [132] and 6-[1-(4-fluorophenyl)methyl-1*H*pyrrol-2-yl)]-2,4-dioxo-5-hexenoic acid ethyl ester (RDS1643) (Figure 15) [133], that were independently developed against the HIV-1 IN. Due to similarities between RNase H and IN active sites, they were explored as RHRTIs and found to be active. Both of them are able to chelate Mg^{2+} in the RNase H catalytic site and are inactive on the DNA polymerase function [132, 133]. For this reason DKAs are currently under development as dual RNase H and INIs (DRH-INI) [19, 134–136].

Other derivatives that have also been developed as DRH-INIs are *N*-hydroxyimide. The prototype of these inhibitors was the 2-hydroxyisoquinoline-1,3(2*H*,4*H*)-diones (NHI) (Figure 15) [137, 138] that was shown, by crystal structures with the isolated RNase H domain, to bind to RT in a strictly metal dependent manner, confirming the metal-ion-mediated mode of action. More recently, other *N*-hydroxyimide derivatives were synthesized such as DRH-INIs [139, 140]. Interestingly, the methyl 2-Hydroxy-1,3-dioxo-1,2,3,4-tetrahydroisoquinoline-4-carboxylate analog (CNHI) (Figure 15) has also been shown to inhibit the replication of the double-mutant G140S/Q148H, which is the most resistant strain to the INI raltegravir [140], indicating that it is possible to design compounds with the same scaffold that may (i) inhibit both RNase H and IN and (ii) inhibit specifically one of the two enzymes. Further studies will be needed to dissect the specifics of the two active sites.

9.3. Nonmetal Chelator RHRTI. Unlike the above-mentioned compounds, vinylogous ureas compounds 2-amino-5,6,7,8-tetrahydro-4*H*-cyclohepta[*b*]thiophene-3-carboxamide (NSC727447) and *N*-[3-(aminocarbonyl)-4,5-dimethyl-2-thienyl]-2-furancarboxamide (NSC727448) (Figure 16) that inhibit the RNase H function are ineffective on the DNA polymerase function, but they do not chelate the magnesium

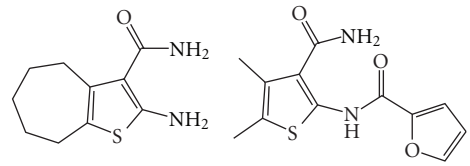


Figure 16: Chemical structures of nonmetal chelating RHRTIs.

ion [141]. These two derivatives were further developed into more potent analogs that, however, were devoid of antiviral activity in cell culture [142]. Molecular modeling studies showed that they bind to an hydrophobic pocket comprising residues V276, C280, K281, K275, R277, and R284 of the p51 thumb and residues G541 and H539 of the RNase H domain (Figure 4) [142]. Further studies are certainly warranted since this new pocket is highly attractive for RHRTIs development.

10. Dual RNase H and Polymerase Inhibitors

An interesting class of RHRTIs is the hydrazone derivatives, whose first reported analog was *N*-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) (Figure 17). Unlike other NNRTIs or RHRTIs, BBNH inhibits both the polymerase and the RNase H activities of HIV-1 RT [143] and therefore can be classified as dual NNRTI (DNNRTI). In addition, BBNH inhibits both RT-associated RNase H and RDDP activities of K103N, Y181I, Y188H, and Y188L mutant RTs with potency similar to wt RT, while, when assayed on Y181C mutant RT, it inhibits only the RDDP function and is inactive on the RNase H function [144]. This information, together with the data on other hydrazone derivatives that chelate the metal ion cofactor in the RNase H site [145], led to propose that two BBNH molecules could bind RT in two different sites, the first one in the polymerase domain, possibly near the NNRTI-binding site, and the second one possibly located in the RNase H domain. Subsequently, another

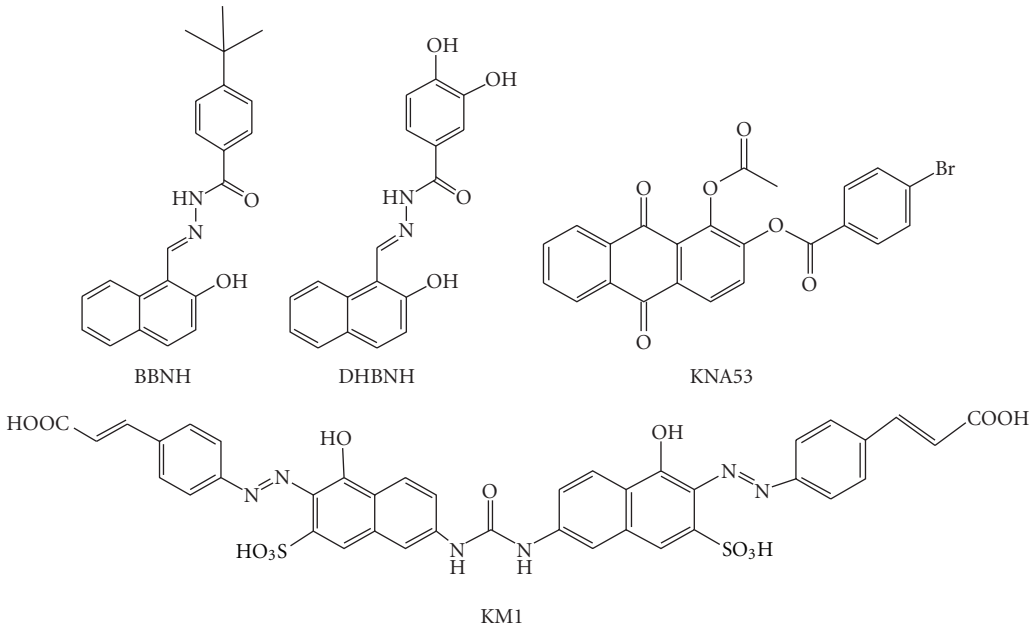


Figure 17: Chemical structures of dual RNase H and polymerase inhibitors.

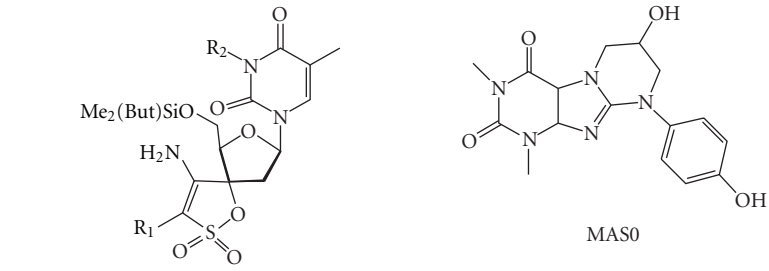


Figure 18: Chemical structures of DimRTIs.

derivative, (E)-3,4-dihydroxy-N′-((2-hydroxynaphthalen-1-yl)methylene)benzohydrazide (DHBNH) (Figure 17), has been reported to bind near the polymerase active site in a pocket different from the NNRTI-binding site and also >50 Å away from the RNase H active site (Figure 4) [146]. Hence, it was hypothesized that DHBNH may either perturb the trajectory of the template primer, so that RNase H cannot operate on its substrate, or that it may also bind to a second site, in or near the RNase H domain, that was not seen in the crystal. More recently, molecular docking studies on a series of hydrazone analogs proposed that they bind to a pocket that includes residues Y405, W406, Q500, and Y501 of p66 subunit, and, hence, they form hydrophobic interactions with RT and with base pairs in the groove of the RNA:DNA substrate [147]. In fact, residues D499 and A502, adjacent to Q500, which were perturbed by the hydrazone derivatives presence [147], are part of the primer grip of the RNase H domain and play a role in aligning the DNA:RNA substrate with the active site. Therefore, the hydrazones binding to Q500 may disrupt the primer grip's role in the activity of RNase H.

A second class of DNNRTI is a series of emodin [148] and alizarine anthraquinone derivatives [149, 150] such as 1-acetoxy-9,10-dioxo-9,10-dihydroanthracen-2-yl 4-bromobenzoate (KNA-53) (Figure 17), that inhibits both RT-associated functions of wt and K103N RTs and only the RNase H function of Y181C RT. Mode of action studies and molecular dynamic simulation led to proposing that the anthraquinone derivatives bind to the site adjacent to the NNRTI pocket, which was originally reported [146] for the hydrazones derivatives (Figure 4) [149]. Accordingly, it has been suggested that the anthraquinone inhibition of the RNase H function may be due to a change in the RNA:DNA hybrid RT accommodation, induced by their binding, which results in a possible variation in the nucleic acid trajectory toward the RNase H catalytic site [149].

A third class of DNNRTI is the naphthalenesulfonic acid derivatives that were originally reported to have a selective

activity on the RT-associated RDDP function [151] and were further developed by structure-based design, molecular similarity, and combinatorial medicinal chemistry to obtain compound 2-Naphthalenesulfonic acid (4-hydroxy-7-[[[[5-hydroxy-6-[(4 cinnamylphenyl)azo]-7-sulfo-2-naphthalenyl]amino]-carbonyl]amino]-3-[(4-cinnamylphenyl)]azo (KM-1) (Figure 17), that inhibits both RT functions in the nanomolar range [152]. Subsequently, KM-1 was shown to weaken the RT DNA-binding affinity and to displace DNA from the enzyme [153]. Hence, it has been proposed to preclude the proper alignment of DNA at the polymerase active site, depleting the active DNA-bound RT complex required for nucleotide incorporation [153].

It is important to note that questions have been raised regarding the use of combinations between RHRTIs and NRTIs. In fact, RHRTIs have been proposed to lead to an increase in NRTIs resistance by mimicking the RNase H-dependent mechanism of NRTI resistance of some connection domain mutations [43]. Recently, however, studies on the effects of some RHRTIs on the HIV-1 susceptibility to AZT and 3TC have shown that none of the tested RHRTIs decreased NRTI susceptibility, while only one DNNRTI decreased AZT susceptibility by 5-fold [154]. More studies are needed to fully understand the interplay between RNase H inhibition and NRTIs susceptibility as well as its clinical relevance.

11. RT Dimerization Inhibitors

RT dimerization is an absolute requirement for all enzymatic activities, and, accordingly, the development of inhibitors targeting the dimerization of RT represents a promising alternative antiviral strategy [155]. Up to now only a series of small molecules have been found which are able to inhibit RT dimerization. Among them are the above-mentioned BBNH derivative [143, 145] and the [2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-beta-D-ribofuranose]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (TSAO) (Figure 18) derivatives [156], that make extensive contact with the $\beta 7/\beta 8$ loop of the p51 subunit, that forms the "floor" of the NNRTI binding pocket and fits in a groove-like structure that constitutes the template:primer binding site in the p66 subunit. More recently, a structure-based ligand study has identified compounds 7-hydroxy-9-(4-hydroxyphenyl)-1,3-dimethyl-1,6,7,8,9,10a-hexahydropyrimido [2,1-f]purine-2,4(3H,4aH)-dione MAS0 as potent dimerization RT inhibitors (DimRTIs) (Figure 18) [157].

12. Other Potential Targets in RT

The increase in knowledge regarding HIV life cycle and specifically the function of the HIV RT and its essential interactions with other proteins will reveal potential drug targets. Even though no inhibitors have been identified yet, to the best of our knowledge, the DNA synthesis initiation (with an RNA:RNA primer), the PPT hydrolysis, the strand transfer, and pyrophosphorolysis RT functions are all potential aspects of the RT activities that may be targeted by small molecules. In addition, RT makes contact with other viral proteins such as NC and IN. These binding surfaces might be potential targets since their disruption may alter viral protein efficiency. Furthermore, some cellular factors have been described to interact with RT (and with the RT:IN complex) during reverse transcription and may have a role in its function [158]. Therefore, a better understanding of these interactions may offer other new target sites. Finally, intracellular immunity approaches may also involve proteins that affect RT functions and may thus offer additional target possibilities [31]. In conclusion, although RT has been the very first targeted HIV protein and is probably the most explored one, it still presents uninvestigated (or under investigation) functions and aspects that still make it a new fascinating target for innovative drug development.

Acknowledgments

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Review Article

HIV Assembly and Budding: Ca^{2+} Signaling and Non-ESCRT Proteins Set the Stage

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More than a decade has elapsed since the link between the endosomal sorting complex required for transport (ESCRT) machinery and HIV-1 protein trafficking and budding was first identified. L domains in HIV-1 Gag mediate recruitment of ESCRT which function in bud abscission releasing the viral particle from the host cell. Beyond virus budding, the ESCRT machinery is also involved in the endocytic pathway, cytokinesis, and autophagy. In the past few years, the number of non-ESCRT host proteins shown to be required in the assembly process has also grown. In this paper, we highlight the role of recently identified cellular factors that link ESCRT machinery to calcium signaling machinery and we suggest that this liaison contributes to setting the stage for productive ESCRT recruitment and mediation of abscission. Parallel paradigms for non-ESCRT roles in virus budding and cytokinesis will be discussed.

1. Focus of This Paper

Determinants intrinsic to the structural precursor polyprotein (Gag) that is encoded by the Human Immunodeficiency Virus-type 1 (HIV-1) and other retroviruses direct targeting of Gag to the plasma membrane, membrane and genome RNA binding, Gag multimerization, and budding of the assemblage into the extracellular space as virus particles (reviewed in [1–4]). Through a proteomic search aimed at identification of cellular factors that might participate with Gag and ESCRT, we identified the inositol 1,4,5-triphosphate receptor (IP3R) as a protein enriched in an endosome- and plasma-membrane-enriched fraction [5] only when Gag was expressed (unpublished observation). IP3R protein forms a transmembrane calcium ion (Ca^{2+}) channel that is mostly found on the membrane of the endoplasmic reticulum (ER), the major intracellular Ca^{2+} store in the cell. IP3R has also been detected on the plasma membrane, late endosome/multivesicular bodies (LE/MVBs), and the nucleus (reviewed in [6–8]). Efficient HIV-1 Gag trafficking and viral particle release were shown to require activation of IP3R [9]. IP3R activation requires phospholipase-C- (PLC-) catalyzed hydrolysis of $PI(4,5)P_2$ to generate inositol 1,4,5-triphosphate (IP3), the activating ligand for the receptor (reviewed in [6–8]). Binding of IP3 initiates conformational changes leading to channel opening and release of Ca^{2+} into the cytosol [10]. Earlier studies on HIV particle production had demonstrated that induction of a transient rise in the cytosolic Ca^{2+} concentration resulted in a dramatic rise in viral particle release, suggesting that Ca^{2+} is a limiting factor in late-stage replication [11, 12]. Taken together, these observations collectively suggested that IP3R is the physiological provider of the required Ca^{2+}. The proteomic search also identified several additional proteins that function in regulation of Ca^{2+} signaling, including Sprouty2 (Spry2), a modulator of Ca^{2+} signaling [13] and other modes of signaling [14, 15]. We demonstrated that Spry2 is also required for productive HIV egress [16, 17]. Proteins such as IP3R and Spry2 have been shown to function with the same elements of cytoskeletal and vesicular transport that are integral to ESCRT machinery [18–20]. Over the past few years, a number of other non-ESCRT host proteins have been shown to be required for Gag assembly. Some of these have been discussed in recent reviews [2, 21, 22]. We will discuss

how these host proteins set the stage for ESCRT recruitment and ESCRT-mediated abscission events. We apologize to those investigators whose studies may be pertinent but were not explicitly cited.

2. Introduction

Enveloped viruses, like HIV-1, exit the host cell by budding. The segment of the plasma membrane that serves as assembly platform evaginates to form the budded particle and becomes the viral envelope. Since the Gag precursor is the viral gene product that plays the key role in recruiting other viral components to the assembly site [23, 24], the assembly process must necessarily include a mechanism for stable localization of Gag at the plasma membrane (PM). Once on the PM, Gag has intrinsic assembly capability that is attributed to functions of its four domains (matrix-capsid-nucleocapsid-p6). The N-terminal matrix (MA) domain mediates membrane binding ([25–29] and references in [1]). The capsid (CA) domain provides Gag with capability for self-assembly into higher-order multimers ([30–35] and references in [36]). The nucleocapsid domain (NC) mediates binding to viral RNA and nonspecific RNAs as well as promoting Gag association [37–39] and references in [40]. The C-terminally located p6 region mediates the untethering of the assembled Gag particle from the host [41, 42]. Orderly cleavage of Gag at interdomain junctions within the structural precursor polyprotein by a virus-encoded proteinase [43–47] occurring concurrently with budding results in mature proteins whose rearrangement transforms the bud to a mature, infectious particle [48, 49]. The final step of the virus assembly process, which results in the pinching off of the particle from the host cell, is mediated by ESCRT proteins that have been recruited to the bud neck by motifs in p6 that are designated as "late" or L domains (reviewed in and references in [50, 51]). Thus, Gag is both necessary and sufficient for viral particle assembly [52].

3. Plasma Membrane Targeting: Role of PI(4,5)P_2

As a cytosolic protein, the synthesis of Gag takes place on soluble polysomes in the cell interior [53]. A myristoylation reaction occurs cotranslationally during which Gag acquires a myristoyl moiety on the N-terminal glycine which plays a role in assembly [28, 54, 55]. At the earliest experimentally feasible time points, Gag has been demonstrated to have a cytosolic distribution when examined by confocal microscopy [11], biochemical fractionation [56], and immunogold electron microscopy [57]. Eventually, the entire Gag population becomes membrane associated with the PM as the preferred site at steady state (references in [23]). This is consistent with the results of *in vitro* binding studies wherein MA, which is highly basic ([25–29] and references in [1]), mediates binding to membranes reconstituted with acidic phospholipids ([26, 27] and references in [1]). It is also consistent with observations that the cytoplasmic leaflet of the PM is unique among cell membranes in having a net negative charge due to high levels of acidic phospholipids [58]. The targeting phospholipid was identified as the complex acidic phospholipid, phosphatidylinositol 4,5 bisphosphate (PI(4,5)P_2) [59]. Depletion of PI(4,5)P_2, using plasmamembrane-targeted lipid phosphatases, caused Gag to be localized to LE/MVBs and prevented Gag localization to the PM [59]. PI(4,5)P_2 is mostly found on the PM where it represents a minor plasma membrane lipid component [60]. Structural analysis of PI(4,5)P_2 binding to HIV-1 MA shows contacts made by the head group (i.e., phosphates and inositol ring) with basic residues and the nestling of adjacent acyl groups into a hydrophobic cleft [61] while studies with full-length Gag underscored the importance of the phosphoinositide acyl chain [62]. These *in vitro* studies also predict initiation of Gag structural changes following PI(4,5)P_2 binding. Studies with the matrix protein show that PI(4,5)P_2 binding results in exposure of the N-terminal myristate [61]. Studies with Gag in the presence of nucleic acid reveal an interplay between binding to PI(4,5)P_2, binding to nucleic acid, and capsid (CA) domain-mediated self-association [63]. The model of Gag membrane association founded on Gag interaction with PI(4,5)P_2 is supported by the inhibitory effect on Gag particle release of depletion of plasma membrane PI(4,5)P_2 [59, 64, 65]. It should be noted that as important as PI(4,5)P_2 is to HIV-1 Gag membrane targeting, the importance of PI(4,5)P_2 to targeting and release of other retroviral Gags varies. Mo-MLV exhibits a preference and a requirement for PI(4,5)P_2 [66]. Equine infectious anemia virus (EIAV) budding is less impacted by depletion of PI(4,5)P_2 due to preferential binding to PI(3,5)P_2 [65]. PI(3,5)P_2 is a phospholipid that is predominantly associated with endosomal compartments at steady state [67] implying endosomal targeting of EAIV Gag in the cell. EIAV Gag trafficking requires such targeting as inactivation of the PI(3)P_2 5-kinase, which is responsible for the endosomal placement of PI(3,5)P_2 [67], inhibits EIAV Gag VLP production [65]. ASV budding appears to rely on electrostatic interaction with acidic phospholipids and exhibits no specific reliance on phosphoinositide components of the PM [68]. Thus, HIV-1 Gag membrane association is mediated by a specific bipartite determinant in the MA domain comprised of myristate and basic amino acid clusters [1] with Gag-PI(4,5)P_2 binding serving as the basis for targeted membrane association. Gag's preferential association with the plasma membrane is due to two inherent features of PI(4,5)P_2: (i) the PM is where most of cellular PI(4,5)P_2 is located [60] and (ii) PI(4,5)P_2 molecules are products of *in situ* synthesis (i.e., PM-localized molecules are produced at the PM; [69]). Thus, PI(4,5)P_2 targeting provides a mechanism to direct Gag from its site of synthesis in the cell interior to the plasma membrane.

Detection of assembled HIV-1 Gag inside membrane compartments with the characteristics of LE/MVBs has been documented [70, 71], and altered Gag residency in LE/MVBs following stimulatory or inhibitory effects on virus production has been demonstrated [11, 72, 73]. Additionally, the virus particle has components that are typical exosome markers [74]. However, for macrophages, at least, those apparently intracellular membrane compartments

with LE/MVB features were demonstrated to be actually extracellular space delineated by intracytoplasmic plasma membrane [75, 76]. Moreover, Gag particle production has been shown to be insensitive to interference with LE/MVB function [77]. The role of the LE/MVB in Gag assembly and release thus remains controversial. We suspect that at the root of this controversy is the complex nature of the LE/MVB itself. It cannot be precluded that the endosomal machinery can interact with Gag in the traditional manner, wherein ESCRT machinery facilitates sorting of cargo proteins into MVBs for ultimate delivery to degradative compartments. However, the handling of sorted proteins by the MVB is not always unidirectional. Though targeted to the LE/MVB in both HeLa and Jurkat cells, the 29KE/31KE Gag mutant is released at near wild-type levels from Jurkat cells but is trapped inside HeLa cells [78] which shows that trafficking within the MVB can be influenced by its environment (i.e., cell dependence). EIAV Gag is another interesting case since, despite its endosomal targeting, EIAV Gag VLPs are released from cells such as COS-1 and HeLa [65]. It would be interesting to know if EIAV Gag induces any alteration in the MVB and, if so, whether this facilitates productive infection. Direct delivery of Gag to the site of release on the plasma membrane circumvents the potentially nonproductive outcome of Gag association with endosomal machinery. A Gag assembly model that incorporates Gag-$PI(4,5)P_2$-based targeting of Gag to assembly sites on the PM permits a more productive path from Gag synthesis to release of an assembled Gag particle.

4. Late Domains in Gag Recruit ESCRT Machinery

Budding structures accumulate on the plasma membrane if the C-terminal p6 region is missing from Gag [41, 42]. The p6 region bearing the L domain has counterparts in other retroviruses and is functionally exchangeable with these within and outside the genera; for example, the PTAP motif from the p6 region of HIV-1 Gag was shown to substitute for the PY motif in the L domain-bearing region (p2b) of the avian sarcoma virus (ASV) and vice versa [79–83] and references in [50, 51, 84, 85]. Functional exchangeability demonstrates that there are multiple, though not necessarily equally effective, ways for Gag to access the ESCRT machinery. Accordingly, Tsg101 as binding partner of the HIV PTAP motif and Nedd4 family members as binding partner of the ASV PY motif facilitate release of HIV-1 and ASV, respectively, through functionally exchangeable but independent routes (i.e., Tsg101 can replace Nedd4 function in facilitating ASV budding [86, 87]). Members of the Nedd4 family of ubiquitin ligases can also replace Tsg101 in facilitating HIV-1 release under certain circumstances [88–91]. The binding of the ESCRT adaptor, Alix, to the secondary L domain in Gag serves this purpose as well (reviewed in [92]). The ESCRT machinery is now known to comprise >25 proteins, organized into four complexes (ESCRT-0, -I, -II, and -III) that function sequentially along with several additional associated factors (reviewed in [93–95]). Irrespective of how Gag is linked to the ESCRT machinery, in all cases ESCRT-III and Vps4 must be recruited to the bud neck at the membrane site to execute the final bud scission event and to release the ESCRT factors from the assemblage for recycling back to the cytosolic pool for participation in future events [96, 97]. A feature of retroviral utilization of the ESCRT machinery is the selective use of the ESCRT complexes. HIV-1 viral particle production requires ESCRT-I and ESCRT-III but not ESCRT-II [98] while ASV requires ESCRT-II but not ESCRT-1 [99]. These observations, along with recognition that ESCRTs, which normally function in transport of some cellular proteins to degradative cellular compartments, are required for exit of assembled Gag from the cell, suggests that non-ESCRT host proteins may play a key role in allowing the ESCRT machinery to be utilized differentially by the virus compared to the host. Thus, non-ESCRT proteins may permit HIV to exploit ESCRT machinery by preventing the Gag-ESCRT complex from participating in interactions with ESCRT partners that are nonproductive for the virus.

5. Parallels between HIV-1 Budding, Cytokinesis, and Autophagy

"All organisms do things the same way except that it is completely different in every detail" J. Haber

The abscission event in virus budding results in separation of the enveloped virus from the host cell. Another process where the abscission event results in separation of two membrane-enclosed cellular entities is cytokinesis. Cytokinesis, itself a multistep process, is the terminal stage in cell division [100]. Abscission of the intercellular bridge/midbody results in separation of the mitotic daughter cells. Recruitment of ESCRT and mediation of the abscission event by ESCRT is the basis for the parallel between HIV-1 budding and cytokinesis [101, 102]. The parallel may extend to events occurring before ESCRT recruitment and participation, (i.e., in a pre-ESCRT stage). Paradigms that govern the pre-ESCRT stage of cytokinesis, which has been an active area of research long before discovery of HIV, may likewise apply to the pre-ESCRT stage of viral budding.

A theme that is emerging as a cell prepares for cytokinesis is the reshaping of calcium signaling [103]. Local and global elevations in cytosolic Ca^{2+} level are achieved by ion release from the ER (the cell's major intracellular Ca^{2+} store) and by influx from the extracellular environment [104]. Decrease in Ca^{2+} content of the ER triggers activation of Ca^{2+} influx channels on the plasma membrane and refilling of the ER store in a process called store-operated-calcium-entry (SOCE) [105, 106]. A major cellular change that occurs during cell division prior to cytokinesis is the uncoupling of Ca^{2+} store depletion and SOCE [107, 108]. Why this is necessary is presently not known but the effect is to render the pre-ESCRT events in cytokinesis independent of SOCE and reliant on the internal stores as the Ca^{2+} source. Independence from SOCE and reshaping of calcium signaling as a pre-ESCRT stage paradigm also appear to be the case for HIV-1 budding. Blockade of SOCE with 2-aminoethoxydiphenylborate (2-APB), a small molecule

inhibitor of store refilling through SOCE [109], had no effect on release of the HIV-1 Gag particle [110]. Blockade of a G protein-coupled receptor cascade [111] triggered by Ca^{2+} entry through receptor-operated calcium entry (ROCE; [112]) also had no effect on Gag particle release [110]. Additionally, cells where productive Gag budding is occurring (i.e., expression of wild-type Gag) exhibit higher cytosolic Ca^{2+} compared to mock-transfected cells or cells expressing a budding-impaired PTAP Gag mutant [110]. Possibly, insulating the calcium machinery from external Ca^{2+} sources allows both virus budding and cytokinesis to proceed more efficiently. Figure 1 shows the elements of the Ca^{2+} signaling machinery implicated in HIV-1 release.

Cytokinesis and viral budding share several general features (Figure 2). The first step in both processes is the targeting of the requisite components to the eventual scission site, that is, the plasma membrane. Formation of the cleavage furrow is a visual marker of initiation of cytokinesis and aspects of this event that appear similar to the budding process are furrow ingression, that is, a progressive narrowing of the eventual scission region to form a bud neck. In cytokinesis, the separating bodies are of comparable volumes; in viral budding, they are of unequal volumes. IP3R, intact $PI(4,5)P_2$, $PI(4,5)P_2$ hydrolysis, and Ca^{2+} are all required for the normal progression of cytokinesis in cellular systems where cell division has been well studied, for example, spermatocyte and oocytes [113–116]. There is a requirement for Ca^{2+} to maintain furrow or neck stability, necessitating constant PLC-mediated hydrolysis of $PI(4,5)P_2$ [117, 118]. Components involved in Ca^{2+} mobilization and cytoskeleton remodeling are recruited to the furrow [117–119]. Similarly, in addition to intact $PI(4,5)P_2$ [59], HIV budding requires IP3R and PLC activity [9, 110]. Analogous to IP3R recruitment to the furrow in cytokinesis, there is also recruitment of IP3R to Gag budding sites on the plasma membrane [110].

In cytokinesis, the non-ESCRT protein mediating recruitment of ESCRTs is Cep55. Cep55 recruits Tsg101, a component of ESCRT-I, and Alix, an ESCRT adaptor protein that binds both ESCRT-1 and ESCRT-III, to the eventual scission site once furrow ingression is completed [101, 102, 122–124]. These ESCRT factors, in turn, recruit the ESCRT-III complex required to carry out the terminal step in cytokinesis, abscission, that is, the severing of the thin intercellular bridge that connects the two daughter cells [125–127]. The counterpart of the Cep55-ESCRT link in viral budding is the targeting of Gag to the eventual scission site on the plasma membrane and recruitment of Tsg101 and/or Alix through the L domains and eventually ESCRT-III.

Autophagy, the process involved in the breakdown of intracellular proteins and organelles, is now appreciated as a mechanism of great importance in both cell survival and cell death [128]. It is the latest cellular process linked to ESCRT function. Indeed, autophagy is a necessary postabscission step in cytokinesis [129]. Following cytokinesis, the dividing cells are connected by an intracellular bridge that contains the midbody. This structure persists long after division as a midbody derivative that is inherited asymmetrically by the daughter cell with the older centrosome. Recent findings in

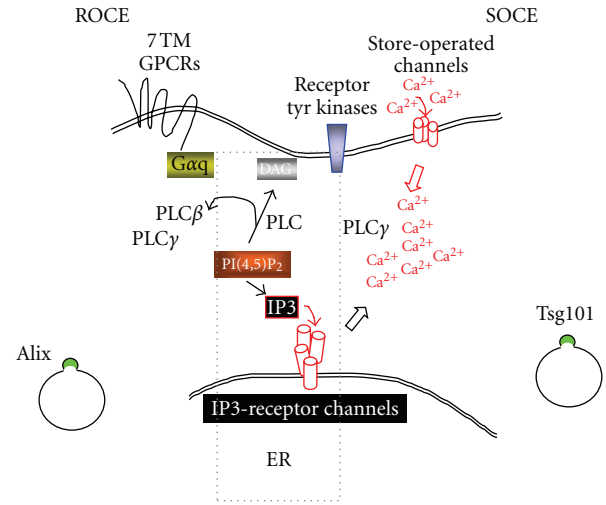

Figure 1: Elements of Ca^{2+} signaling machinery implicated in HIV-1 release. Tsg101-mediated release requires the core elements, IP3R, $PI(4,5)P_2$, and PLC. Alix-mediated release requires these, SOCE and ROCE. It is not known whether SOCE and ROCE are controlled by distinct Ca^{2+} channels [120] or if the same channel complexes mediate SOCE when recruited to lipid rafts and ROCE when they are outside of lipid rafts [121].

mammalian cells and in *Drosophila melanogaster* indicate that ESCRTs are required for efficient trafficking through the endolysosomal system where the autophagic cargo is degraded [130–132]. As with cytokinesis and viral budding, IP3R-mediated Ca^{2+} signaling is emerging as critical for the pre-ESCRT stage in autophagy [133]. *De novo* synthesis of phospholipids is coupled with autophagosome formation [134]. Pairing phosphoinositides with Ca^{2+} ions in endolysosomes has been suggested to control the direction and specificity of membrane trafficking [135]. All three processes, cytokinesis [136], viral budding [137], and autophagy [138, 139], require or involve SNAREs to conduct some of the critical events. The participation of calcium machinery components in all three processes suggests that the requirement for and reshaping of calcium signaling is a common feature governing their pre-ESCRT stages.

6. Non-ESCRT Proteins and Other Factors Engaged in the Pre-ESCRT Stages of HIV-1 Assembly

For a number of non-ESCRT host proteins shown to be important for release of the Gag particle [2, 4, 22, 140], disruption of the protein function does not result in the canonical L domain phenotype (i.e., arrested budding structures at the periphery of cells examined by EM). Rather, Gag is found in the cell interior. We and others [2] interpret this to indicate participation of these proteins in assembly step(s) preceding ESCRT-mediated budding. Some of these proteins have regulatory links to each other. Among these are the human vacuolar protein sorting (hVps) protein 18 (Vps18), a class C Vps complex component, and Mon2. Both have been shown to be required for Gag PM localization

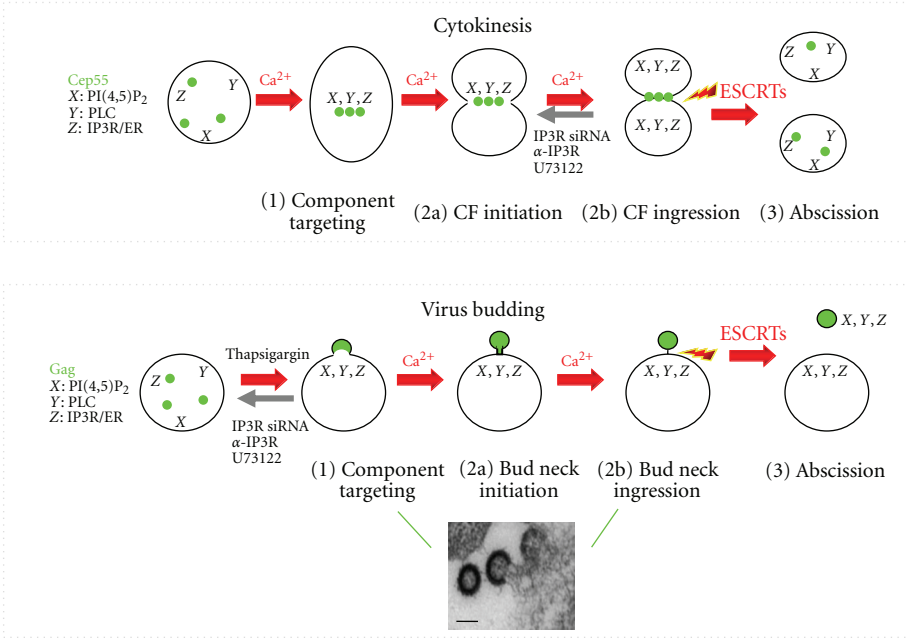

FIGURE 2: Similarities between cytokinesis (top) and viral particle production (bottom). CF: cleavage furrow. EM image shows HIV-1 VLPs in the process of budding. Bars indicate 100 nm.

and virus production [141]. In yeast, class C Vps proteins have been shown to regulate PM localization of at least one protein [142] and to assume roles antagonistic to ESCRT in the recycling of membrane proteins [143]. The human orthologue of Mon2 (hMon2) can bind and regulate the subcellular localization of adaptor proteins such as AP-1, AP-3, and Arf1 which have previously been shown to be required for Gag PM localization and Gag particle production [72, 144, 145]. The notion of non-ESCRT proteins regulating the activity of other non-ESCRT proteins in the pre-ESCRT stage has a parallel in cytokinesis as illustrated by the host protein, TEX14. This non-ESCRT protein binds Cep55 at the same motif used to recruit Tsg101 or Alix and negatively regulates ESCRT recruitment [146]. Through protein-protein interactions, non-ESCRT proteins could thus impose temporal and spatial control of the recruitment of participating proteins, including Gag itself, to assembly sites on the PM during the pre-ESCRT stage.

Another pre-ESCRT event is alteration of the lipid composition of the assembly site. Quantitative analyses indicate that the viral envelope differs from the PM of its host cell in having higher levels of cholesterol and $PI(4,5)P_2$ [58, 147]. Since the viral envelope is derived from the PM microdomain serving as the Gag assembly site, reorganization of the lipid bilayer in this location may occur as part of the assembly process. A feature of PM $PI(4,5)P_2$ is that the greater majority is sequestered by electrostatic interaction with basic proteins that are resident at the PM (e.g., myristylated alanine-rich C kinase substrate (MARCKS; growth-associated protein (GAP)43; N-methyl-D-aspartate (NMDA) receptor, and the epidermal growth factor receptor (EGFR)) and is only released by a local rise in Ca^{2+} [148]. Another property of $PI(4,5)P_2$ is that it does not have a natural inclination for clustering due to the energy barrier posed by repulsion of the large polar head groups when they are in proximity. It has been shown that Ca^{2+} can reduce this barrier and induce $PI(4,5)P_2$ clustering in lipid monolayers [149]. Recruitment of IP3R machinery to the cell periphery and release of Ca^{2+} may function to increase the portion of PM $PI(4,5)P_2$ available for interaction with Gag and to permit the clustering of $PI(4,5)P_2$ molecules upon Gag multimerization. This model is summarized in Figure 3 and may explain how the budding requirement for both intact and hydrolyzed $PI(4,5)P_2$ could be simultaneously resolved.

That budding structures are still formed by Gag mutants with disrupted PTAP motifs despite their impairment in recruitment of Tsg101 or in cells where Tsg101 has been depleted [50, 51] indicates that assembly site membrane deformation is a pre-ESCRT stage event. Although not required for initiation [116], Ca^{2+} is required for furrow ingression and for stability of the intercellular bridge in cytokinesis [113–116]. Furrow ingression in the presence of Ca^{2+} leads to a productive ESCRT recruitment stage as indicated by completion of cytokinesis. Analogous to furrow ingression is the formation of the virus bud neck where the ESCRT scission complex is recruited. The fact that the budding structures of Gag mutants with disrupted PTAP motifs accumulate on the plasma membrane indicates a failure in ESCRT recruitment even though the mutant has been demonstrated to be capable of employing alternative modes of linking to ESCRT (i.e., via Nedd4 or Alix). Our study [110] shows that, in cells expressing HIV-1 Gag, IP3R was translocated from the cell interior to the periphery and colocalized with Gag on the plasma membrane. Interestingly, IP3R redistribution is not induced in cells expressing the PTAP Gag mutant even though release of the mutant, albeit

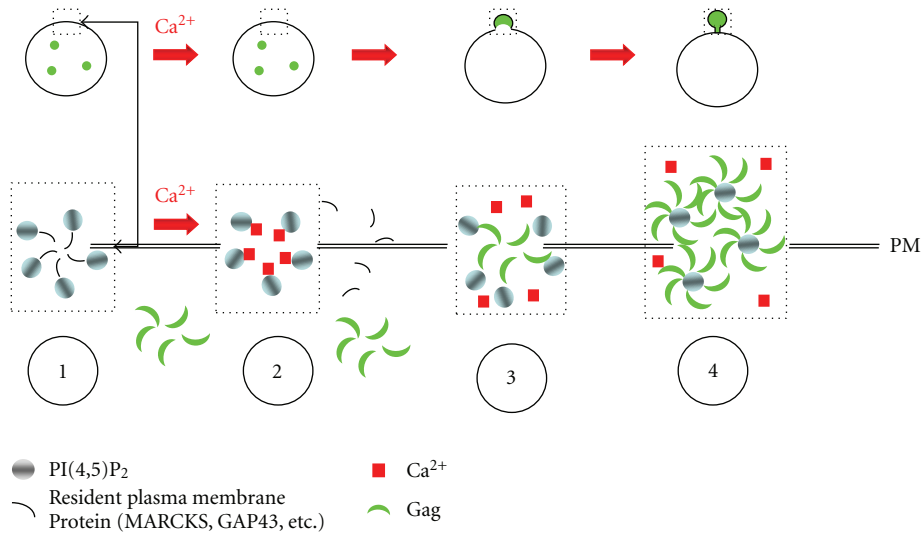

FIGURE 3: Ca^{2+} facilitates Gag-$PI(4,5)P_2$ interaction and stabilization on the plasma membrane. Top, the squares highlight the top-down view of the plasma membrane shown below. Bottom, (1) most of the $PI(4,5)P_2$ on the plasma-membrane is sequestered with plasma membrane-resident proteins that are highly basic and therefore unavailable to Gag. (2) A local rise in Ca^{2+} permits the cation to replace the resident proteins, freeing the $PI(4,5)P_2$ from these proteins. (3) $PI(4,5)P_2$, made available by Ca^{2+}, recruits Gag to the plasma membrane. (4) Gag multimerization forms local PIP2 clusters that stabilize Gag association with the membrane, preventing loss of Gag from the narrowing bud neck in preparation for ESCRT recruitment.

inefficient, also requires IP3R-regulated machinery. The lack of Ca^{2+} store recruitment, which IP3R recruitment signifies, to the cell periphery of cells expressing such mutants indicates that, as is the case for furrow ingression, competency in linking to ESCRT is a property of bud necks formed in the presence of Ca^{2+}.

The ability of the endoplasmic reticulum to form tubules and small vesicles is what permits the stores to be recruited [150]. Movement of IP3R-contaning ER vesicles along microtubules has been shown to be facilitated by a kinesin [151]. Kinesins are a large family of cellular protein motors that use the energy of ATP hydrolysis to induce movement along the microtubule [152]. Kinesins have been identified as being involved in an intracellular process required for Gag release: (i) Kinesin KIF4 was reported to bind Gag directly through the MA domain [153] and was later found to regulate intracellular trafficking and stability of Gag [154]; (ii) Kinesin KIF3, a binding partner of AP-3 shown to be required for release of the viral particles assembled by Gag [72], has also been reported to be involved in Gag release [155]. Which particular kinesin is involved in IP3R transport is unknown. Kinesin-mediated translocation of IP3R along microtubules would allow for directed delivery of Ca^{2+} stores to the budding site and, thereby, establish a localized region where Ca^{2+} would be elevated. Thus, for Ca^{2+} provision, utilization of the internal Ca^{2+} stores may provide a major advantage over Ca^{2+} influx which is mediated by channels that are homogenously distributed on the plasma membrane.

The notion that intact $PI(4,5)P_2$ is required for targeting Gag to the plasma membrane and that PLC-hydrolyzed $PI(4,5)P_2$ is required for ESCRT-recruitment-competent bud neck ingression suggests the need for regulatory mechanisms that would ensure availability of the right form of the phospholipid for the right event in the pre-ESCRT stage. The "hydrolysis stimulates synthesis" model proposes that hydrolysis and synthesis of $PI(4,5)P_2$ are tightly coupled events such that synthesis stimulates hydrolysis while $PI(4,5)P_2$ hydrolysis signals its production [69]. Ca^{2+} might be a key regulator: Ca^{2+} is an activator of the lipid kinase that is critical for $PI(4,5)P_2$ synthesis [156] and of the PLC that catalyzes $PI(4,5)P_2$ hydrolysis [157]. However, Gag PM targeting appears to require a more nuanced intact $PI(4,5)P_2$ population. Although it has been clearly demonstrated that depletion of $PI(4,5)P_2$ with plasmamembrane-targeted lipid phosphatases prevents Gag localization to the PM [59], other experimental approaches give different results. For example, increased Gag PM targeting and VLP release were not observed following a clear increase in PM $PI(4,5)P_2$ in cells treated with a PLC inhibitor [9]. Also, a loss of Gag PM targeting was reported in cells that did not exhibit a detectable change in $PI(4,5)P_2$ level or subcellular distribution [145]. There is growing recognition that PM $PI(4,5)P_2$ exists in multiple pools and that the dynamic nature of these pools is important for cellular processes mediated by $PI(4,5)P_2$ [148, 156]. Perhaps this conundrum, that is, the lack of a clear correlation between Gag PM targeting and the $PI(4,5)P_2$ level, reflects a requirement for a $PI(4,5)P_2$ pool that is specifically made available for Gag. The non-ESCRT proteins, Spry2 and ADP-ribosylation factor-1 (ARF1), have activities that make them potential participants in such regulatory mechanisms. Spry2 is required for Gag particle budding [16, 17] and for production of infectious virus (Ehrlich, Khan, Powell and Carter, unpublished observations). It has several activities that can affect $PI(4,5)P_2$ metabolism; namely, binding of phospholipase C [13] and of $PI(4,5)P_2$ [13, 17] and it can inhibit receptor-mediated

activation of PLCγ [13]. Binding to PI(4,5)P_2 exerted the greatest influence on Gag particle production [17]. Involvement of ARF-1 in Gag assembly was demonstrated by Joshi et al. [145]. Although this protein is best known for its role in post-Golgi trafficking, ARF1 is also a stimulator of PI(4,5)P_2 synthesis by directly activating PI(4)P 5-kinase and by inducing formation of an enhancer of the kinase [158]. Thus, together with local Ca^{2+}, Sprouty and ARF1 proteins have the potential to ensure the dynamic existence of PI(4,5)P_2 pools specifically made available for interaction with Gag.

Several other non-ESCRT proteins whose dysfunction inhibited transport of Gag from the cell interior to the plasma membrane may also be involved in Gag assembly as pre-ESCRT stage participants. Admittedly, further studies will be needed to elucidate their exact contribution; however, interestingly, these proteins also have links to cytokinesis and autophagy. In addition to the aforementioned SNARES [145], these include citron kinase, a Rho effector [159]; Rab9 [160] and other GTPases [161]; POSH [162]; AP-1 [144]; NPC-1 [73]; and Filamin A [163]. Direct participation in cytokinesis is documented for citron kinase, AP-1, and Filamin A [164–166]. NPC-1 and POSH both affect the metabolism of two important factors in cytokinesis, cholesterol [167], and calcium [168], respectively. Rab9 and other small GTPases have been implicated in cytokinesis and autophagy [118, 169].

7. Non-ESCRT Proteins in the ESCRT Recruitment Stage

The formation of a Gag-Tsg101 complex occurs as part of the Gag assembly process as long as L domain-1 is intact. Although the precise stage at which Tsg101 docks on the PTAP motif is not known, association after stable bud neck formation might be more favorable as it precludes nonproductive interactions with ESCRT-II that would signal internalization of the Gag assemblage or premature ESCRT-III scission. Spry2 forms complexes with components of ESCRT-II [16]. Thus, Spry2 facilitates release driven by both the primary and the secondary HIV-1 Gag L domains, possibly due to its ability to compete with ESCRT-I factors for interaction with ESCRT-II components [16]. This notion is consistent with the fact that HIV-1 budding does not require ESCRT-II [98, 99]. Not surprisingly since the interaction of ESCRT-I with ESCRT-II leads to cargo internalization, it has been suggested that association with Tsg101 increases susceptibility to internalization [170]. Delaying the recruitment of ESCRT machinery to the budding site may provide a means of maximizing viral budding efficiency. A parallel to this as a regulation possibility in cytokinesis may be the aforementioned function of TEX14, a protein believed to control premature progression to the abscission stage by competing with Tsg101 and Alix for binding to Cep55 [146].

8. Concluding Remarks

In this paper, we have focused on proteins involved in steps in HIV-1 trafficking and budding that take place prior to Gag recruitment of ESCRT machinery. As described here, proteins that function in PI(4,5)P_2 binding, synthesis or hydrolysis, Ca^{2+} store recruitment, IP3R-mediated Ca^{2+} store release, and vesicular biogenesis or transport appear to comprise the major classes of participants in the pre-ESCRT stages. Cellular activities in almost all cells are regulated by common signaling systems and Ca^{2+} is a ubiquitous intracellular messenger that is known to control a diverse range of processes. The discovery of Ca^{2+} signaling as a cofactor in HIV-1 protein trafficking and release, its potential link to exploitation of the ESCRT machinery by the virus for viral particle production, and the general similarity of this coupling to other cellular activities in which ESCRTs participate, that is, cytokinesis and autophagy, may provide new therapeutic avenues for HIV treatment strategies.

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Review Article

APOBEC3 versus Retroviruses, Immunity versus Invasion: Clash of the Titans

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Since the identification of APOBEC3G (A3G) as a potent restriction factor of HIV-1, a tremendous amount of effort has led to a broadened understanding of both A3G and the APOBEC3 (A3) family to which it belongs. In spite of the fine-tuned viral counterattack to A3 activity, in the form of the HIV-1 Vif protein, enthusiasm for leveraging the Vif : A3G axis as a point of clinical intervention remains high. In an impressive explosion of information over the last decade, additional A3 family members have been identified as antiviral proteins, mechanistic details of the restrictive capacity of these proteins have been elucidated, structure-function studies have revealed important molecular details of the Vif : A3G interaction, and clinical cohorts have been scrutinized for correlations between *A3* expression and function and viral pathogenesis. In the last year, novel and unexpected findings regarding the role of A3G in immunity have refocused efforts on exploring the potential of harnessing the natural power of this immune defense. These most recent reports allude to functions of the A3 proteins that extend beyond their well-characterized designation as restriction factors. The emerging story implicates the A3 family as not only defense proteins, but also as participants in the broader innate immune response.

1. Introduction

In 2002, the cloning of *APOBEC3G* (*A3G*; then called *CEM15*) and the identification of the protein product of this gene as the first cellular protein capable of restricting HIV-1 infection revealed a novel direction for chemotherapeutic intervention and ignited the search for additional defense proteins capable of counteracting viral invasion [1]. The report of this cloning solved a long-standing enigma in the field of HIV-1 pathogenesis. Early work examining and comparing the pathogenesis of wild-type and Vif-deficient HIV-1 had yielded conflicting results with some laboratories concluding that Vif was dispensable for productive infection while other groups maintained that Vif expression was essential [2–4]. Ultimately, it was decisively shown that the requirement for Vif was cell-type dependent; permissive cells supported the growth of HIV-1Δ*vif* while nonpermissive cells limited such viral replication [5, 6]. Most interesting and relevant was the inability of Vif-deficient HIV to productively infect primary CD4+ T cells, one of the critical natural targets of HIV-1 infection [2, 3, 5, 7, 8]. The molecular explanation for the "Vif phenotype" remained unexplained for the subsequent decade. Proffered in this early work was the idea that permissive cells expressed a cellular factor that compensated for Vif. An equally valid suggestion was that nonpermissive cells harbored an inhibitory activity of HIV-1 that was itself overcome by the Vif protein. It was subsequently established, in a pair of elegant experiments utilizing heterokaryons formed from fusion of nonpermissive and permissive cell lines that, in fact, nonpermissive cells expressed an activity that suppressed HIV-1Δ*vif* replication [9, 10]. The genetic relatedness of two T lymphocyte lines, one nonpermissive and the other permissive, was exploited in a classical subtractive hybridization experiment; *A3G* was identified as this described suppressive activity. It was found to be almost exclusively expressed by nonpermissive cells and its stable expression in permissive cells conveyed the ability to resist an HIV-1 challenge [1].

It was quickly appreciated that *A3G* was but one family member of a previously identified gene locus [11]. Subsequent investigation also revealed that A3G exhibited a potent DNA-mutating ability [12]. In humans, seven family members within the locus have been identified; rhesus macaques, the nonhuman primate that serves as the most important animal model for HIV treatment and vaccine testing, also have seven *APOBEC3* genes, while the murine genome contains a single *A3* gene [13–15]. In each of these organisms, the role the *A3* genes play in counteracting viral invasion is critical. All seven *A3* family members identified in humans exhibit powerful suppressive activity against a range of viruses while the homologous proteins in mice and primates appear to perform similar functions [16–18]. While A3 inhibitory activity is relatively broad, the most well-characterized and studied function is their striking ability to restrict retroviral infection [19]. In an evolutionary response to this restriction, the retroviruses have countered with a battery of genes exquisitely fine-tuned to overcome these endogenous defense proteins.

2. The Laboratory Setting

With one exception (A3C), each of the seven A3 family members in humans has been observed to be capable of combating HIV-1 [1, 17, 20–27]. Whether the antiviral activity observed is relevant during the course of a natural HIV-1 infection has not been unequivocally established for any of the family members and there are valid concerns raised in the interpretation of various data regarding levels of protein expression and potency. However, it is becoming increasingly clear that understanding the battle that is waged between the innate immune system and HIV-1 during acute infection is imperative and the A3 proteins are critical players in this initial encounter.

While the relative potencies of individual A3 family members in the setting of a natural infection have been difficult to assess, it has been convincingly established that, in the tissue culture setting, A3G exhibits the most potent activity against HIV-1. In a variety of cell types, both primary cells and established cells lines, and under varying experimental conditions, including both single-round infectivity assays and multiple-round replication assays, A3G suppresses the infectivity of HIV-1. HIV-1 Vif has evolved to counteract this impressive activity of A3G by preventing virion encapsidation of this host factor [28–35]. Vif acts as an adapter protein bridging A3G and a Cullin5-elongin B/C-Rbx ubiquitin ligase [36]. Within this complex A3G is ubiquitinylated and subsequently degraded in the 26S proteasome [36, 37]. Other modalities involving Vif prevention of A3G encapsidation have also been documented [28, 32, 34]. Interestingly, dominance of A3G over Vif has been noted under conditions of elevated and/or stabilized expression [1, 34, 36]. This ability to suppress HIV-1 even in the presence of Vif is noteworthy as it has distinct implications for the development of chemotherapeutics designed to interfere with the A3G : Vif axis.

The anti-HIV-1 functionality of A3G is multifaceted. Its most extensively characterized anti-HIV-1 function is its ability to catalyze cytidine deamination of HIV-1 DNA on the minus strand resulting in the detection of guanosine-to-adenosine transition mutations in reverse transcripts; upwards of 10% of guanosines may be mutated leading to the labeling of this A3G-mediated process as *hypermutation* [34, 38, 39]. The fate of such hypermutated transcripts is not well understood, but certainly this dramatic mutational burden effectively short-circuits viral infection.

Work from multiple groups has also uncovered deamination-independent anti-HIV effects of A3G that are seen during viral infection [22, 40–49]. The characterization of this editing-independent antiviral function has suggested a block to viral replication that occurs after entry but before integration. While the molecular details of this deaminase-independent function of A3G remain unclear, defective reverse transcription products are commonly observed, indicating that A3G likely acts during the process of reverse transcription. A more comprehensive understanding of this inhibition will be important. All members of the A3 family contain at least one conserved cytidine deaminase active site (CDA; family members A3B, A3D, A3F, and A3G contain two such domains) composed of the signature sequence His/Cys-X-Glu-$X_{23–28}$-Pro-Cys-X_2-Cys [11, 15]. Early structure-function analysis of A3G was performed by disrupting these suspected catalytic domains with site-directed mutagenesis [41]. The conserved histidine, glutamic acid, and cysteine resides in both the N-terminal and C-terminal domains of A3G were individually mutated and the resulting proteins were independently examined for their catalytic function as well as their ability to suppress HIV-1Δ*vif* infection. The data clearly indicated that the C-terminal CDA domain was responsible for A3G enzymatic function. Unexpectedly the data also suggested that, under specific experimental conditions, significant anti-HIV-1 inhibition could be imparted in the absence of the characteristic mutagenic activity. Subsequent work in a range of experimental systems has supported these original observations. Controversy over these observations primarily stems from claims that these data have most often been cited in experimental settings using mutant A3G exhibiting elevated expression levels [41, 42, 50, 51]. In attempts to clarify the role of A3G expression levels a number of groups have compared A3G protein expression in transiently transfected cell lines and primary CD4+ T cells/macrophages, reporting that expression levels achieved during transient transfection exceed levels observed in primary cells. However, a few cautionary notes are warranted. A3G that is mutated, for instance, at the critical glutamic acid at residue 259 of the protein, has also been shown to have a more limited ability to block the process of reverse transcription thereby suggesting that distinguishing deamination-dependent and -independent activities may be challenging [16]. Additional support for a pleiotropic antiviral function of A3G is provided by observations in which the A3G phenotype is unaffected in cells that do not express uracil DNA glycosylase 2 or SMUG, enzymes responsible for the removal of uracils from single- or double-stranded DNA [52, 53]. As a significant suppressor of HIV-1,

a multipronged ability of A3G to inhibit HIV-1 would have notable benefits to the invaded host.

Using a variety of cell lines and experimental conditions, the anti-HIV-1 activity of A3B, A3D, A3F, and A3H (haplotypes I, II, V, and VII) has also been conclusively demonstrated [17, 20, 21, 23–25, 27, 54, 55]. Hypermutation is often recorded as coincident with antiviral activity, although, in the case of A3B and A3F, as with A3G, there are observations of HIV-1 suppression in the absence of hypermutation [24, 42, 43]. Sensitivity to Vif regulation has been observed for A3D, A3F, A3G and A3H while A3B and A3H/Haplotype I resist Vif-mediated virion exclusion and thus exhibit detectable activity against wild-type HIV-1 virus. However, not all of these family members are equally likely to contribute to HIV-1 resistance during a natural infection; A3B is primarily expressed in B cells and makes it unlikely that this protein contributes appreciably to inhibition of HIV-1 [17, 20, 21, 23, 24, 56–58]. Similarly, the expression of the A3H/Haplotype I restricts wildtype HIV-1, but the protein is inherently unstable [20, 56]. A question with important clinical implications is whether this intrinsic instability may be overcome while harnessing the natural power to combat wild-type viral infection [58, 59].

Until recently, the role of A3A in HIV-1 inhibition was unappreciated outside of two significant observations: the first being a correlation between its expression in monocytes and the susceptibility of these cells to HIV-1 infection, and the second was that expression of A3A was confined to cells of the myeloid lineage and this expression was positively regulated by INF-α [60–62]. Berger et al. have now described a novel and critical role A3A plays in the early phase of HIV infection, specifically in myeloid cells [22]. When primary myeloid cells were infected with HIV-1 and the induction of expression at the A3 locus was examined, it was shown that these cells preferentially induced *A3A*, on both the mRNA and protein levels; induction of other A3 family members in these cells was not detected and A3A induction in peripheral blood lymphocytes was negligible. The induced A3A was protective upon HIV-1 challenge and depletion of A3A in primary macrophages and dendritic cells increased viral replication in both single-round infectivity assays and a spreading infection. Similar to other A3 proteins this viral restriction was primarily observed as a profound suppression in the accumulation of viral DNA suggesting interference with an early step of reverse transcription; limited editing of viral reverse transcripts was detectable, but the evidence suggested that enzymatic function was not the sole antiviral function. Notwithstanding its common role as an *A3* family member involved in HIV-1 control, A3A exerts its antiviral function uniquely. It is not producer cell-derived A3A that impacts virus replication, but rather it is the pool of A3A present in the actual *target* cell itself that inhibits incoming HIV-1 particles. Data from independent laboratories strongly support these conclusions for this role of A3A in target myeloid cells [63–65].

Within cells of the myeloid lineage, A3A appears to be the critical suppressor, exerting its effect independently of its editing ability. In CD4+ T cells in the tissue culture model of infection, A3G activity dominates, and its inhibitory function is exerted utilizing both editing-dependent and -independent mechanisms. A3A functions in the target cell while A3G functions in the producer cell. Recent observations, however, have now suggested an unexpected and intricate antiviral role played by the A3G expressed in target cells [66]. Expression of either A3A or A3G activate the cellular DNA damage response (DDR) [67]. In the case of A3A, a G_1/S-phase cell cycle arrest is also induced and its catalytic domain is implicated in the effect. While the relevance of these interesting observations in regard to HIV-1 infection is not immediately obvious (the A3A experiments were performed in human osteosarcoma cells) the role that the DDR response pathway plays in the innate immune response has only recently been explored and appreciated [68, 69]. Experimental observations support the idea that triggering the DDR pathway acts as an alerting mechanism for the innate immune system [66, 68, 70, 71]. In the emerging A3G story this certainly seems to be the case (Figure 1). Norman et al. examined expression of the critical Natural killer (NK) cell-activating ligand, NKG2D-L, in HIV-1-infected primary CD4+ T cells [66]. They compared expression of NKG2D-L under conditions of wildtype HIV-1 infection and HIV-1Δ*vif* infection and found a surprising discrepancy: the combination of Vpr and A3G in the HIV-1Δ*vif* infections activated the DDR ultimately leading to the upregulation of both A3G and *NKG2D-L*. Increased expression of NKG2D-L sensitized the HIV-1-infected cell to NK-mediated killing. In the presence of Vif this NK-mediated killing was blunted. The role of target cell-expressed A3G was further verified using shRNA's targeting A3G mRNA; loss of A3G in an HIV-1Δ*vif* setting resulted in diminished NK-killing and increased (infected) cell survival. The authors suggest that, in a natural infection, the A3G-dependent sensitization of HIV-1-infected cells to NK-mediated killing is hindered by the loss of A3G through Vif-mediated degradation. It bears mentioning that infection of murine primary B cells with the transforming retrovirus Abelson murine leukemia virus (Ab-MuLV) also leads to the induction of activation-induced deaminase (AID) expression [72]. AID is a member of the larger APOBEC-AID family of cytidine deaminases (this grouping includes the founding member, APOBEC1, APOBEC2, APOBEC3A-H and AID). This induction of AID also results in the upregulation of an NKG2D ligand, rendering the infected cells susceptible to NK-mediated lysis. The *in vivo* effect is the profound containment of Ab-MuLV replication and the ability of the host animals to restrict the virus and survive this pathogenic encounter. This indirect effect of AID is also linked to the DDR-stimulated signaling pathways. Details on the mechanistic details of these antiviral functions have not yet been fully characterized. Particularly intriguing is whether the catalytic function of A3G and/or AID is necessary for these effects, and, if so, how is this enzymatic capacity utilized. With the description of the involvement of the DDR, it is suspected that the signature cytidine deaminase modality would be important but confirmation of such speculation is warranted. Based on these observations, therapeutic approaches that interfere with the process of Vif-regulated degradation of A3G could potentially strengthen

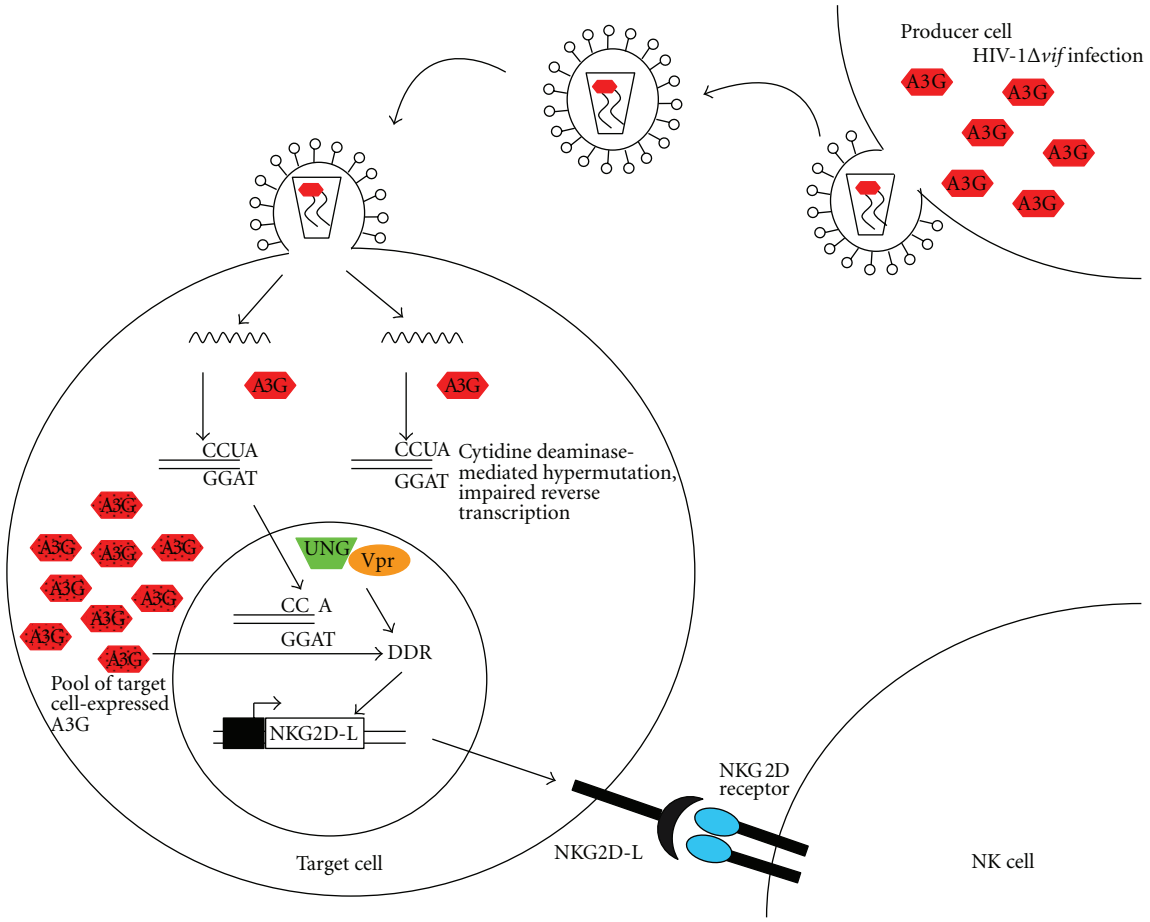

Figure 1: A3G can exert multiple antiviral effects against HIV-1 infection. Virion-packaged A3G restricts HIV-1Δ*vif* replication via cytidine deaminase-mediated hypermutation as well as interfering with efficient reverse transcription. Additionally, the introduction of the uridines into the minus-strand DNA during reverse transcription triggers the DNA damage response (DDR). This induction of DDR involves other proteins, including the host protein, UNG, and the HIV-1 Vpr protein. Among other downstream effects, the DDR stimulates the transcriptional synthesis of NKG2D ligands. The subsequent expression of these proteins on the surface of the HIV-infected cell sensitizes it to NK cell lysis. It should also be noted that A3G expression *within* the target cell (designated as dotted symbols to distinguish it from the virion-packaged A3G). Also critically participates in the DDR activation.

not only a potent intracellular defense, but also impact the ability of NK cells to attack infected cells.

3. The Picture in the Clinic

As astounding as our progress has been in understanding the molecular and mechanistic details of A3 proteins and their interaction with HIV-1, providing data for the *in vivo* relevance of A3 activity has been significantly more challenging. Experiments manipulating A3G in the laboratory have supported the proposition that elevated expression levels of this restriction factor can and do alter wildtype HIV-1 infectivity; clinical correlates of this *in vitro* observation have been more difficult to gather. With few exceptions, the clinical work to date has principally focused on A3G and the effect its fluctuating expression levels and catalytic activity can have on HIV-1 infection and progression. Clinical analyses do not often lend themselves to large sample sets, and the confounding combinatorial effects of host genetics and environment strain efforts of reproducibility. With these openly acknowledged limitations recognized, there remains an increasing amount of suggestive evidence that corroborates the idea that A3G expression and/or activity can modulate natural HIV-1 infection [59, 75, 77, 79–81] (Table 1).

In infected individuals, hypermutated HIV-1 proviral genomes and elevated A3G expression levels have been correlated with both lower viral loads and increased CD4+ T cells counts [75, 80–83]. In a relatively large study, Land et al. noted the significant association between proviral hypermutation and increased peripheral blood CD4+ T cell count. A3G expression was not directly quantified and the detected hypermutation was used as a surrogate for catalytic function of A3G.

More direct analysis of *A3G* expression in the setting of a natural HIV-1 infection has also yielded tantalizing hints of A3G control. Working with a small cohort of women, one group recently reported an interesting correlation between

Table 1: Clinical studies correlating A3 family members and HIV-1 pathogenesis.

A3 Family member	Correlation reported	Identification of cohort	Reference
A3B	Homozygous deletion of gene associated with higher: rates of HIV infection after exposure, viral set point, and rate of disease progression	4216 HIV+ patients pooled from 5 longitudinal cohorts: ALIVE, MACS, SFCC, HGDS and MHCS [73] (US-based studies)	An et al. [74]
A3F and A3G	Level of detectable proviral hypermutations that exhibited A3F/A3G cytidine deaminase signatures associated with higher CD4+ cell count	215 HIV+ female commercial sex workers plus 25 HIV+ women who were infected perinatally (Nairobi, Kenya)	Land et al. [75]
A3F and A3G	Elevated expression of A3F and A3G in PBMCs associated with establishment of lower viral set point	30 women from a well-established [76] cohort of female commercial sex workers (Dakar, Senegal)	Ulenga et al. [77]
A3G	186R polymorphism in African Americans associated with rapid progression to AIDS	2430 HIV+ patients pooled from 5 longitudinal cohorts: ALIVE, MACS, SFCC, HGDS and MHCS [73] (US-based studies)	An et al. [78]
A3G	Elevated expression of A3G in CD14+ cells associated with resistance to HIV-1 infection after exposure	30 HESN individuals (Florence, Italy)	Biasin et al. [79]
A3G	Elevated expression levels inversely associated with viral load in LTNPs	6 uninfected volunteers; 17 HIV+ progressors; 8 HIV+ LTNPs	Jin et al. [80]
A3G	C40693T polymorphism, located within intronic sequences, associated with increased risk of infection	122 HIV-exposed individuals; 69 sero converted after exposure, 53 retained seronegative status (Montreal, Canada)	Valcke et al. [73]
A3G	HESN individuals expressed elevated levels of A3G when compared to healthy controls; elevated levels of A3G associated with higher CD4+ cell count in HIV+ patients	26 healthy controls, 37 HESN individuals, 45 HIV+ patients (Mexico City, Mexico)	Vázquez-Pérez et al. [81]
A3H	Haplotype I associated with protection from HIV-1 infection	70 serodiscordant couples (Florence, Italy)	Cagliani et al. [59]

HESN: highly exposed seronegative; LTNP: long-term nonprogressors.

individuals expressing higher levels of A3G before HIV-1 infection with the establishment of a lower viral set point after infection [77]. Perhaps the most interesting cohorts in which to examine A3G expression levels and the importance of these levels during viral infection *in vivo* are long-term nonprogressors (LTNPs), elite suppressors (ESs), and highly exposed seronegative (HESN) individuals. To date, there has been no reporting of A3G expression (or activity) as an explanation for the innate ability of an ES to completely control the HIV-1 virus. However, there has been an observation that elevated A3G levels do correlate with higher CD4+ T cell counts and lower viremia within a group of identified LTNPs, suggesting that, under certain conditions, overexpression of A3G may be protective [80]. Two independent studies, examining approximately 67 individuals who have been repeatedly exposed to HIV-1, yet retain their seronegative status, have also presented evidence that elevated A3G expression levels correlate with viral restriction [79, 81]. Amongst these two cohorts a variety of cell types were studied, including PBMCs, CD4+ T cells, CD8+ T cells, CD14+ monocytes, and cervical cells. These cells were assayed for the level of A3G expression primarily determined at the transcriptional level; in a small number of instances, protein expression was also determined. Calculated levels of mRNA and protein in HESN individuals were then compared to both HIV+ individuals and healthy controls and, in both experimental groups, HESN expressed statistically higher levels of A3G expression. One study carried the results further and was also able to show that PBMCs isolated from HESN individuals were able to more effectively limit a wildtype HIV-1 challenge [79]. Interestingly, both PBMCs and CD14+ cells, isolated from these HESN individuals, appeared to exhibit higher responsiveness to IFN-α treatment as measured by the induction of A3G expression.

Finally, a recent experiment utilizing the SIV/macaque model for HIV-1 infection also suggests that investigating and understanding the consequences of increased A3G (and A3F, in this case) expression levels may elucidate the protective role these defense proteins can play *in vivo* [84]. Infected macaques were separated by clinical stage (chronic versus AIDS stage of infection) and compared to uninfected controls. In isolated PBMCs, CD4+ T cells, and peripheral lymph nodes there was a demonstrated negative correlation between *A3F/G* mRNA expression and viral loads. In addition, the difference in A3F/G expression between control and infected animals was even more pronounced when individuals whose disease course mimicked that of HIV-1/LTNPs were specifically compared. One of the novel aspects of this reporting was the kinetic observation of the *in vivo*

regulation of *A3G* gene expression after SIV challenge. Seven days after infection *A3G* expression levels began to rise and this expression induction peaked on Day 10 after infection. Peak viremia was measured on Day 14. The concomitant rise of A3G levels, leading the rise of replicating virus levels, suggests that the struggle for control between this intracellular restriction factor and the invading pathogen occurs early, during acute infection. This supports previous reports noting the HIV-1-induction of A3G expression and the critical importance this early encounter may play on establishing viral set point [22, 79, 81, 84–86].

In spite of the meticulous analyses and important work accomplished, the current clinical understanding of how and whether A3 family members modulate HIV-1 infection is limited and somewhat unsatisfactory. A consensus has not yet emerged and such agreement will likely require a more collaborative and coordinated effort, across cohorts and experimental approaches. The details of designing such experiments are themselves still fraught with unknown parameters; for instance, which cell types and/or tissues should be examined? Is an examination of proviral hypermutation or viral genome editing enough to serve as a marker for A3G antiviral function? Is a measurement of A3G mRNA sufficient to draw conclusions regarding expression of the protein and resultant antiviral activity? At least two groups have noted a disconcerting disconnect between A3G mRNA and protein expression in PBMCs [60, 79]. Do other A3 family members play distinct roles at discrete stages of viral infection? In spite of this minefield of questions and the intrinsic limits placed on a data set as soon as a cohort of study is chosen, ventures into the clinical realm are paramount and it is only this data that can ultimately reveal the role of the A3 family in potentially containing HIV-1 infection.

4. The Murine Story

In contrast to the undetermined impact human A3 proteins have in limiting natural HIV-1 infection, systematic and directed experiments in mice have conclusively shown that murine A3 (mA3) is essential in containing and restricting several murine retroviruses: MMTV, a betaretrovirus (mouse mammary tumor virus), F-MuLV (Friend murine leukemia virus), a gammaretrovirus, as well as FV (Friend virus) [86–88]. Other murine gammaretroviruses, such as MLV (murine leukemia virus), are resistant to mA3 restriction [89–91]. Unlike the complex *APOBEC3* locus in humans, which contains a tandem array of seven genes, the murine genome encodes a single *APOBEC3* gene, *mA3* [11, 92]. The knockout of *mA3* was achieved quickly after the identification and cloning of A3G [93]. While a preliminary examination of the mice was relatively uninteresting, detailed characterization of the response of these animals to specific viral challenge was both illuminating and exciting.

In a series of informative and elegant *in vivo* experiments, it was shown that MMTV spreads more rapidly and is disseminated more extensively in mice lacking a functional *mA3* gene as compared to wildtype mice. The *mA3* knockout mice exhibited higher initial viral loads and a shorter time to the development of mammary tumors [86]. It was interesting to note that the protection afforded by mA3 was not absolute; mA3 blunted, but did not completely inhibit, MMTV infection, suggesting even partial protection has a significant role in *in vivo* pathogenesis. The molecular mechanism of this mA3-dependent repression of infection remains unidentified, although it does appear that this antiviral function is exerted independently of any detectable hypermutation or viral genome editing. In this setting mA3-mediated containment of MMTV bears a striking resemblance to A3A-dependent control of HIV-1 in myeloid cells: neither inhibition requires a detectable hypermutation function, although the block to viral infection traces to an early post-entry step, and antiviral function is exerted by protein expressed in target cells [22, 86]. In the case of mA3, antiviral function was a combinatorial effect of both virion-packaged and endogenously expressed protein. In terms of potentially harnessing the innate power of the A3 proteins, the most intriguing observation was the reporting that pre-treatment of wildtype mice with either LPS or INF-α upregulated mA3 expression in dendritic cells, the first cells infected during MMTV exposure. This early elevation of mA3 expression directly correlated with increased resistance to MMTV. Mice lacking *mA3* were unable to restrict viral infection despite either treatment [94]. This result speaks directly to some of the underlying concerns regarding the detrimental consequences of manipulating the expression of A3G and certainly bolsters the hypothesis that increased expression of this protein could ameliorate restriction of HIV-1 infection.

Finally, it is interesting to note that in addition to reducing MMTV replication, virion-incorporated mA3 has also been shown to be able to markedly reduce the *transmission* of virus [95]. MMTV, as a number of other retroviruses, including HIV-1, is transmitted vertically through breastfeeding. In an investigation examining the route of transmission, Okeoma et al. report that not only was *mA3* mRNA readily detectable in mammary epithelial tissue but that this packaged mA3 significantly decreased MMTV transmission. In an effort to extend these observations to HIV-1 infection, this group examined expression of the *A3* genes in primary human mammary tissue and found significant levels of both *A3F* and *A3G* mRNA. Whether this expression translates into protection from the vertical transmission of HIV-1 is not yet clear. However, the trajectory of this study is interesting taken in the context of HIV-1 infection in which breastfeeding accounts for approximately 40% of vertical transmission [96].

FV causes immunosuppression and leukemia in mice. Interestingly, mice strains are differentially susceptible to FV, and a number of genes have been implicated in the resistance to this disease [97]. Both cell-mediated and humoral responses appear necessary for recovery and, naturally enough, the major histocompatibility complex (MHC) locus has been identified as important. However, an essential non-MHC gene, *Recovery from Friend virus gene 3* (*Rfv3*), has also been implicated [98]. Mice strains resistant to FV

(e.g., C57BL/6), possess *Rfv3* resistance alleles, develop high concentrations of protective neutralizing antibodies, and recover from viremia. Mice strains susceptible to FV infection (e.g., BALB/c) fail to mount the protective humoral response, develop splenomegaly and erythroleukemia, and succumb to viral infection. In a revealing study, passive immunization of susceptible mice decreased mortality dramatically, suggesting that the *Rfv3* locus critically influences the production of the protective neutralizing antibodies [99].

The first reporting of the genetic region encompassing *Rfv3* was in 1979 [98]. It was to be 30 years before two groups simultaneously identified *Rfv3* as *mA3* [87, 88]. Using a range of both *in vivo* and *in vitro* experiments they convincingly showed that *mA3* expression was critical to the restriction of FV infection and resulted in the suppression of virus particle infectivity. This inhibition to viral replication occurred after entry, but before integration, presumably affecting an early stage of FV infection (potentially reverse transcription). The description of the restrictive capacity of *mA3* was reminiscent of the extensive data characterizing the A3G anti-HIV-1 function. It should also be noted that, in the FV system, mA3 function was exerted independently of any detectable cytidine deamination activity. While the observations supported the identity of mA3 as the suppressive factor, a consensus on what distinguished a resistant *mA3* allele from a susceptible allele was less discernable. Preliminary data implicated the influence of *mA3* polymorphisms on expression level, essentially suggesting the resistant *mA3* alleles were more highly expressed [87, 88, 100]. In addition, there was also suggestion of an important role for a coinherited B-cell-activating factor receptor (*BAFF-R*) allele [101].

Recent work probing the resistant versus susceptible *mA3* alleles has supported previous suggestion that an *mA3* splice variant lacking exon 5 may be more potent than a full-length isoform [89, 102]. This latest work suggests that the *mA3*Δexon 5 variant is more efficiently translated and the overall combinatorial effect of elevated mRNA levels and preferential translation of the *mA3*Δexon 5 account for significantly higher levels of mA3 protein capable of potently restricting FV infection [102]. A small number of genetic variants within the *A3* family and their respective relationship to HIV-1 disease acquisition and progression have been described: the H186R variant of A3G is associated with rapid progression in African American populations, the C40693T variant of *A3G*, as well as the homozygous loss of *A3B*, may be associated with increased infection susceptibility, and Haplotype I of A3H may provide resistance to infection [59, 73, 74, 78]. To date, a molecular understanding of how these variants modify (or fail to modify) HIV-1 disease is sorely lacking. Details of the defining characteristics of the resistant *mA3* alleles are of significant interest upon contemplation that such differences, when identified, could be thoroughly dissected in a relevant *in vivo* setting, perhaps providing valuable insight into mechanistic detail. Such details may expand our understanding of the human versions of the *A3* family and the critical polymorphisms.

What is also underscored in these reports is the importance of characterizing *both* expression levels and allelic differences of individual *A3* genes within this family. Fluctuations of *A3G* mRNA levels, in which *A3G* gene expression is upregulated, have been reported across the immature-to-mature differentiation transition in dendritic cells (DCs) [65, 103]. The ability of mature DC's to resist HIV-1 infection is well documented, and this correlative observation is intriguing [104, 105]. An observation reported in the MMTV system is also suggestive: the DC's of mice stimulated with LPS 24 hours prior to a viral challenge exhibited a modest (3-4-fold) increase in *mA3* mRNA levels, but displayed a significantly increased restriction of MMTV [94]. Finally, a recent paper examining a novel role for A3G in the sensitization of infected cells to NK-mediated lysis suggests that small fluctuations in *A3G* expression levels may have profound functional consequences [66]. These studies are interesting for their suggestion that modest elevations of *mA3* and *A3G* gene expression can lead to impressive increases in viral restriction.

5. Concluding Remarks

The unfolding story of the multifunctional characteristics of the A3 family is fascinating. When the identification and characterization of A3G as a potent restriction factor emerged, the field raised numerous important questions and formulated strategies for capitalizing on this natural innate defense. Over several years, the identity of the entire A3 family of proteins as important innate restriction factors has been established. The ability of A3G to inhibit HIV-1Δ*vif* infection has been analyzed by a significant number of laboratories, but the full complement of molecular details on how it exerts its antiviral function has not yet been gathered. Cytidine deamination undoubtedly occurs in the setting of a natural viral infection, but it is not entirely clear whether this enzymatic function is the only modality through which A3G can obstruct HIV-1 *in vivo*. An improved understanding of the details of how antiviral functions are exerted is needed. In addition, the important, and likely critical contribution of additional A3 family members *in vivo*, remains largely uncharacterized, although recent work using a tissue-culture model would suggest that a collaborative effort amongst family members is essential [17]. Utilization of both the MMTV/mA3 and FV/mA3 murine systems may be particularly useful. They are the only *in vivo* models of A3 restriction that currently exist. Alteration of the murine genome is relatively tractable and there is a single *A3* gene in the rodent genome; potentially, *mA3* genetic variants may be assessed in this setting. Other outstanding questions include the determination of whether any of the A3 proteins require cofactors or post-translational modifications to function effectively. An important co-factor for APOBEC1 has been delineated and while there is a preliminary suggestion that A3F/3G antiviral activity requires a co-factor, no specific proteins have been identified to date [106, 107].

Manipulation of the Vif : A3G interaction is also a viable point of chemotherapeutic intervention. To date only one compound specifically targeting Vif and thereby liberating functional A3G from Vif regulation has been reported;

rapidly evolving technology may soon identify others [108]. A more comprehensive understanding of the interface involved in this viral and cellular protein association could identify new target sequences. For instance, recent identification of the transcription factor CBF-β as a member of the ubiquitin-ligase complex recruited by Vif to degrade A3G may prove interesting when considering novel drug targets [109]. Liberating A3G from Vif-mediated control has been shown to impact HIV-1 replication *in vitro* and suggests elevated levels of A3G can have a significant impact on the kinetics of viral replication, but whether expression levels of *A3* genes can be modulated *in vivo* remains to be determined. A better fundamental understanding of gene regulation and the important regulatory elements within this family is also essential. To date only one of the promoters within the *A3* family has been identified and characterized [85].

A more collaborative and concerted effort in the examination of various cohorts is more likely to reveal whether there exist meaningful associations between *A3* genes and the ability to completely resist or partially restrict HIV-1. In light of the recent data being produced in the murine systems, an examination of rapid progressors and various *A3* genetic variants is warranted. Additionally, data sets analyzing *A3G* genetic variants, while relevant and useful, may have missed important information about other family members; the recent findings involving *A3A* would suggest that this gene would also be important to examine in a number of cohorts.

Expanded roles for members of the A3 family have also been reported. These reports attribute an importance to A3 proteins that extends beyond the relatively simple arena of restriction factor. A3G's participation in marking cells for NK-mediated lysis would expand the reach of the A3 family into induced innate immunity, a series of cellular interactions important in bridging the innate and adaptive responses. Further describing and characterizing this observation will be important as it has potentially important implications for treatment during acute infection and vaccine design. In ten years the field has exploded, from the recognition of a single potential restriction factor (A3G) to an impressive understanding of a family of proteins that influence, modulate, and enhance the innate immune response. It begs the tantalizing question: what will the next decade bring?

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Review Article

Mechanisms of HIV Transcriptional Regulation and Their Contribution to Latency

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Long-lived latent HIV-infected cells lead to the rebound of virus replication following antiretroviral treatment interruption and present a major barrier to eliminating HIV infection. These latent reservoirs, which include quiescent memory T cells and tissue-resident macrophages, represent a subset of cells with decreased or inactive proviral transcription. HIV proviral transcription is regulated at multiple levels including transcription initiation, polymerase recruitment, transcription elongation, and chromatin organization. How these biochemical processes are coordinated and their potential role in repressing HIV transcription along with establishing and maintaining latency are reviewed.

1. Introduction

A critical step in the HIV life cycle is transcriptional regulation of the integrated provirus. Robust transcription assures that sufficient mRNA and genomic RNA are produced for efficient virus assembly and infectivity. Repression of HIV transcription leads to the establishment of HIV latency, which creates repositories for infectious and drug-resistant viruses that reemerge upon treatment failure or interruption [1–4]. The existence of long-lived stable HIV reservoirs was demonstrated by the rebound of virus replication following highly active antiretroviral therapy (HAART) interruption [5–8]. These latent reservoirs, which include quiescent memory T cells, tissue-resident macrophages [9, 10], and potentially hematopoietic stem cells [11], although this is still controversial [12], represent long-lived subsets of cells with decreased or inactive proviral transcription. In general, studies with chronically and acutely infected cells show that mutations in Tat [13, 14], absence of cellular transcription factors [15–18], miRNA machinery [19, 20], and proviral integration into transcriptionally silent sites contribute to post-integration latency [21, 22]. Although there may not be a common mechanism that promotes HIV latency, it is critical to understand the molecular events that establish and maintain latency if strategies to reduce or purge HIV from latent reservoirs are to be devised [9, 23, 24]. HIV transcription is regulated at multiple levels including transcription initiation, polymerase recruitment, transcriptional elongation, and chromatin organization. How these events are coordinated and their role in HIV latency will be reviewed. In particular, mechanisms that contribute to repressing HIV transcription will be highlighted.

2. LTR and Transcription Factors

Although viral accessory proteins, such as Vpr, and putative elements within the HIV provirus genome may influence HIV transcription [25, 26], the dominant HIV transcriptional regulatory element is the 5′ long terminal repeat (LTR). The HIV LTR is often divided into four functional elements: the Tat activating region (TAR), which in the context of the nascent RNA forms an RNA stem loop structure that binds the virus-encoded transactivator Tat; the promoter, the enhancer, and the negative/modulatory regulatory element (Figure 1(a)). The promoter, enhancer and modulatory elements recruit a plethora of tissue specific and ubiquitously expressed host-transcription factors that function as activators, repressors, or adapter proteins (see references for detailed reviews [27–29]). AP-1, Sp1, and NF-κB

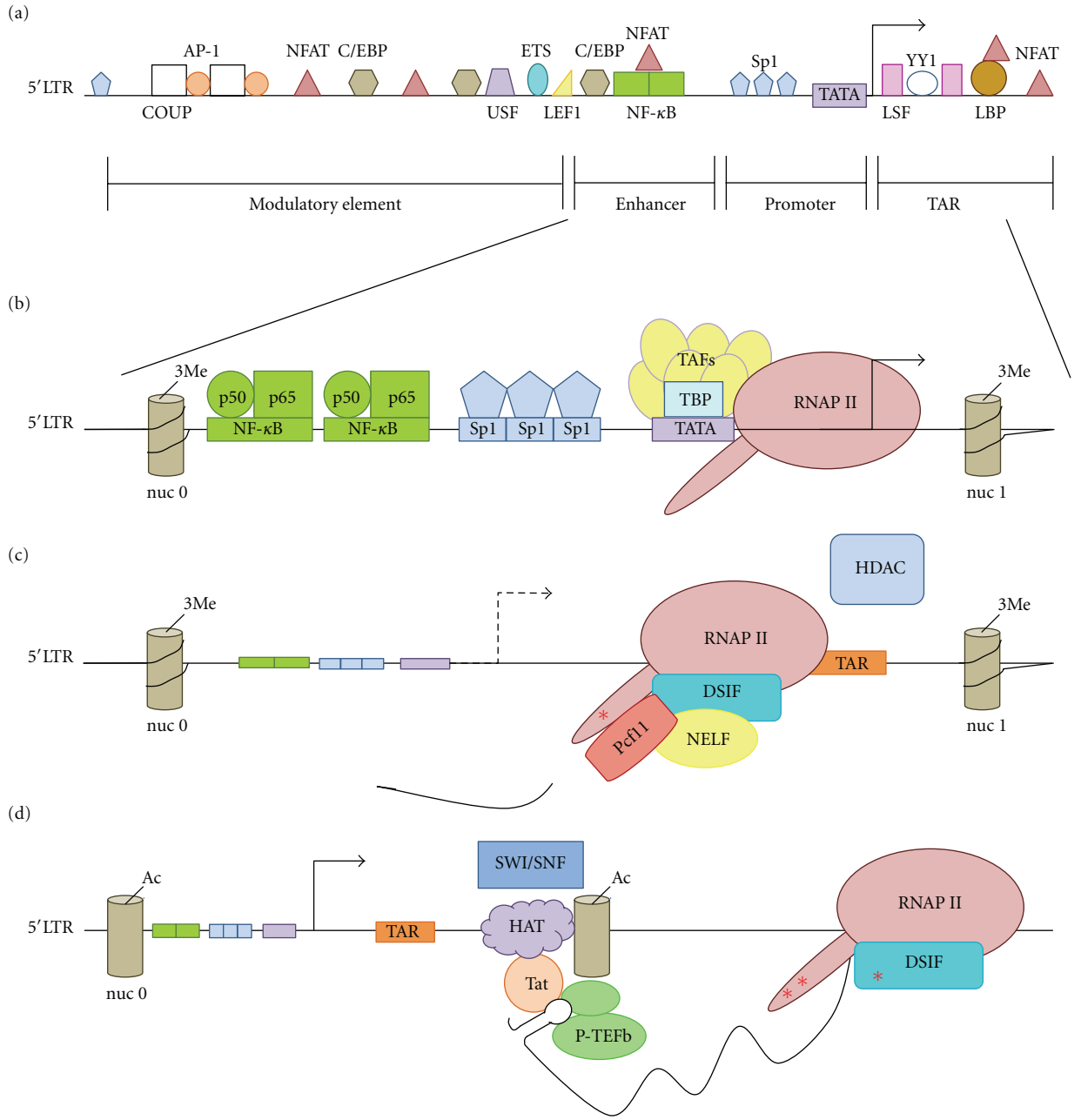

Figure 1: Regulation of HIV transcription initiation and elongation. (a) HIV LTR organization. This only represents a small subset of cis-elements and transcription factors, which bind these sites. (b) Cellular transcription factors are recruited to LTR elements and initiation complex forms at the transcriptional start site. Nucleosomes are posttranslationally modified favoring a condensed chromatin structure that impedes RNAP II transcriptional elongation. (c) RNAP II processes a short distance downstream from the transcriptional start site when DSIF and NELF induce a pause in transcription. Pcf11 reinforces this block in elongation by prematurely terminating the transcription of the short nascent RNA product. HDAC recruitment to the paused complex reinforces a transcriptionally repressed chromatin state. The red asterisk depicts phosphorylation of RNAP II CTD at serine 5 position. (d) RNAP II elongation complex is released from the transcriptional pause by the recruitment of P-TEFb, which mediates hyperphosphorylation of the CTD at serine 2 position and phosphorylation of DSIF, which induces NELF disassociation from the complex (red asterisks indicate key phosphorylation events). The recruitment of chromatin remodeling machinery such as HATs and PBAF SWI/SNF facilitates acetylation of nucleosomes, which displaces the blocking nucleosome and supports transcription elongation.

are required for efficient basal and induced HIV transcription and replication [27–32]. One major check-point in the control of HIV transcription is the availability of critical transcription factors. Several inducible transcription factors have been identified in T cell and monocytic cell lines that transactivate the HIV LTR, including AP-1 [30, 33, 34], C/EBPb [35, 36], NFAT [37–39], Ets/PU.1 [40, 41], and TCF/LEF-1 [42, 43] to cite a select few. A classic example of

transcription factor availability regulating function is the binding of NF-κB to sites within the HIV LTR [44]. Upon cell activation, the p65 subunit is released from the IκB inhibitory complex, dimerizes with the p50 NF-κB subunit, and translocates from the cytoplasm to the nucleus, where it binds the NF-κB sites in HIV LTR to mediate efficient transcription [17]. However, sequestering p65 in the cytoplasm through its interaction with IκB limits the availability of active NF-κB in the nucleus and HIV provirus transcription. Furthermore, this transcriptional repression is reinforced by p50-p50 homodimers binding the NF-κB sites and recruiting histone deacetylase complexes (HDACs), which promote a repressive chromatin state [18]. In addition, there have been reports of several cellular transcription factors that repress transcription in the context of HIV latency. They include but are not limited to, the ubiquitous factors LSF-1, YY-1 [45, 46]; Sp1 and the bHLH-zipper proto-oncogene c-Myc [47]; CTIP-2/Bcll11b, a COUP-TF interacting protein expressed in the central nervous system that interacts with Sp1 [48, 49]; CBF-1, an effector of Notch signaling that is regulated during T cell activation [50]; ligand-activated nuclear receptors [51]; FBI-1, a POZ domain, Kruppel-type zinc finger [52]. Which combination of these factors potentially establishes latency in specific cellular subsets is a critical question that needs to be addressed.

3. Chromatin and HIV Transcription

One function of transcription factors is to recruit complexes that influence chromatin organization. For example, transcriptional activators such as NF-κB, NFAT, and C/EBPβ recruit histone acetyltransferases (HATs) that modify key lysines on histone 3 and histone 4 [10, 24, 44, 53–56]. Histone acetylation, which is associated with active transcription, results in an open or accessible DNA conformation that is more amenable to the binding of additional transcriptional activators, initiation factors, and RNA polymerase II (RNAP II). SWI/SNF complexes and demethylases are recruited to promoters and enhancers by transcription factors and coactivators to remodel nucleosomes, especially around the promoter and transcriptional start sites of genes, resulting in the induction of transcription. The chromatin organization of the HIV LTR has been studied in detail (reviewed in [55–57]). The HIV LTR is flanked by two positioned nucleosomes, nuc-0 at the 5′ end of the LTR and nuc-1 that is juxtaposed to the transcriptional start site (Figure 1(b)). Induction of HIV transcription correlates with histone acetylation, recruitment of HATs [53, 58–60], PBAF containing SWI/SNF complexes [61–64], and displacement of nuc-1 [57, 61, 63–67]. These posttranscriptional modifications to the chromatin state are associated with HIV transcription.

Reversing the posttranslational modifications associated with transcriptional activation is accomplished by recruiting SWI/SNF complexes, HDACs, and/or methyltransferases, which catalyze histone trimethylation. These inhibitory modifications are proposed to contribute to a more condensed chromatin structure which impedes RNAP II processivity and transcription elongation [68, 69]. For SWI/SNF there are at least two distinctive complexes that have been described, PBAF which has been associated with transcriptional activation and BAF which has been implicated in the establishment and maintenance of HIV latency [62, 64]. Class I and II HDACs [54, 70], the methyltransferases Suv39H1, Zeste 2, and heterochromatin protein 1 (hp-1) [71, 72] have been implicated in mediating the deacetylation and trimethylation of nuc-1 and the repression of HIV transcriptional elongation. Long term repression of transcription can be reinforced by additional epigenetic changes including DNA methylation [55, 73]. In summary, posttranslational modifications of chromatin have been linked to the maintenance of latent viral reservoirs.

4. Transcriptional Interference

Although epigenetic events, such as restrictive positioned nucleosomes or DNA methylation, limit HIV transcription recent studies examining proviral integration sites have highlighted the need to consider additional models to explain repression of HIV transcription. Initial experiments by the Bushman laboratory [74–77] in which proviral integration sites in cells that were latently infected with HIV were sequenced indicated that silenced HIV preferentially integrated into transcriptionally active host genes. Similar findings were obtained in infection models with cell lines [77–80] and primary cells, as well as resting CD4 cells from patients either untreated or undergoing HAART [79, 81]. These findings indicate that active neighboring promoters are directly repressing or transcriptionally interfering with the HIV LTR [78, 80, 82]. Transcriptional interference is defined as the suppression of one transcription unit by another neighboring cis-element [83]. Suggested mechanisms that lead to interference of the HIV LTR include the adjacent promoters competing for or displacing the components of transcription initiation complexes, or collisions between transcription elongation complexes moving in opposite directions [83–88]. Although there may be a potential role for chromatin-associated factors in maintaining transcriptional interference [89], other reports from the literature would predict that there are additional critical repressive checkpoints that contribute to HIV latency [78, 82].

5. Transcriptional Elongation

Transcription factors assist with the recruitment of the general basal factors, which include the RNAP II itself, TFIID (TATA binding protein or TBP), and the TBP-associated factors (TAFs), TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH, to assemble the core promoter complex and assure proper positioning of the RNAP II at the transcriptional start site (Figure 1(b)). General transcription factors, such as TFIIH, have been implicated as playing a critical role in HIV transcription at times of low Tat expression [90]. However, recently, the concept of a "core" promoter has been challenged by the dis-covery of tissue-specific TAFs and unique preinitiation com-plexes [91] favoring models in which the factors found at core promoters and the

RNAP II are diverse and dynamic. For example, RNAP II associated protein, Gdown1, competes with TFIIF for RNAP II, therefore inhibiting transcription and promoting the assembly of a paused RNAP II complex [92, 93]. Whether the complexity associated with RNAP II recruitment and assembly reflects cell type and cell-cycle-specific requirements for HIV transcription is just starting to be investigated. However, it has been shown that Tat can influence the recruitment of TBP and associated TAFs [94] suggesting that these early transcriptional complexes are regulated by HIV infection.

Control of transcription elongation is a critical checkpoint in the regulation of a number of genes including c-myc, c-fms, hsp-70, Jun B, and HIV [95–99] and is dependent on the coordination of RNAP II activity, premature transcription termination, and chromatin structure [100]. Furthermore, several genome-wide studies with multiple organisms mapping RNAP II location have shown that 20–30% of genes have enriched RNAP II density at the 5′ end of the gene relative to the body of the gene. This was discovered for genes with both detectable or undetectable transcription [101–104] suggesting that post-RNAP II recruitment and transcriptional elongation represents a key rate-limiting transcriptional checkpoint for gene expression [105]. The interplay between the negative elongation factors, negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF), and positive elongation factors, such as P-TEFb [106], sets this checkpoint. NELF and DSIF associate with the early elongation complex and inhibit RNAP II processivity, possibly by interfering with the extrusion of the nascent transcript from the elongation complex [107]. P-TEFb, which is composed of a regulatory Cyclin T1 (CycT1) subunit and an enzymatic Cyclin-dependent kinase 9 (Cdk9) subunit, alleviates transcriptional repression by phosphorylating one or more of the components in this complex as well as the carboxy terminal domain (CTD) of RNAP II at serine 2 leading to the active engagement of RNAP II in transcription elongation [108–112]. Phosphorylation of DSIF converts DSIF from a negative to a positive elongation factor [106], whereas phosphorylation of NELF by P-TEFb reduces the ability of NELF to associate with RNA [113]. Notably, NELF dissociates from the elongation complex when the complex is transcribing the DNA *in vivo* suggesting that NELF primarily functions as an inhibitor of elongation [114] (Figure 1).

P-TEFb is a general transcription factor, which is required for efficient expression of the majority of cellular genes, and its availability and activity is carefully regulated to allow for changes in global transcriptional demand [115–117]. The regulation of P-TEFb is complex and employs multiple transcriptional and posttranslational strategies that may impact HIV transcription as well as overall cellular gene expression. One mechanism that limits P-TEFb is its association with the 7SK complex, which includes 7SK RNA, HEXIM1, HEXIM2, MePCE, and LARP7 [55, 115–117]. Release of P-TEFb from this complex during T cell activation favors enhanced HIV transcription. Furthermore, recent biochemical profiling has indicated that there are multiple P-TEFb complexes that include association with other coactivators including Brd4 [118–120], SKIP [121, 122], and components of the super elongation complex [116, 123, 124]. Although the significance of these different complexes with regard to HIV latency is still being explored, it is tempting to speculate that these additional cofactors could couple transcription elongation with other processes that influence gene expression including chromatin organization and splicing. P-TEFb activity is also regulated by phosphorylation and dephosyphorylation in the T-loop domain of Cdk9. Although the kinase responsible for Cdk9 posttranslational modification has not been reported, several phosphatases, PPM1, PP1, PP2A, PP2B have been implicated in regulating P-TEFb and HIV transcription [125–129]. Finally, P-TEFb activity is in part regulated by expression of CycT1, which is regulated at a transcriptional level in macrophages and CD4+ T cells [130].

Recruitment of P-TEFb to the HIV LTR is a critical step for transcriptional activation and this is the primary function of the viral transcriptional activator Tat. Furthermore, NELF and DSIF, which are necessary for pausing RNAP II, are both bound to the HIV LTR after initiation of viral transcription [110, 113, 131]. The NELF E subunit, which has an RNA binding domain, has been shown to bind the HIV-TAR element and inhibit Tat transactivation [113, 132]. Diminishing the Spt5 subunit of DSIF decreases HIV replication [110], whereas decreasing NELF expression releases paused polymerases on the HIV LTR and induces HIV transcription elongation in cell line models for transcriptional latency. In addition, depleting NELF induced histone acetylation and displacement of the positioned nucleosome, hinting that transcription elongation and chromatin remodeling maybe coupled processes [131].

In the context of HIV, RNAP II processivity and transcriptional elongation are highly regulated events as suggested by the accumulation of short transcripts in the cytoplasm in HIV-infected cells [96–98, 133]. Under conditions that inhibit transcription elongation, RNAP II is prone to premature termination which reenforces the block in RNAP II processivity and the accumulation of short transcripts observed in cells that have repressed HIV provirus. One possibility for this is that a termination complex is recruited to RNAP II, which destabilizes the nonprocessive RNAP II complex similar to 3′ end processing of mRNA and transcription termination. Only two proteins are known that have the capa-city to dissociate RNAP II from the DNA template: TTF2, which dissociates the elongation complex in an ATP-dependent manner during chromosome condensation of the M-phase of the cell cycle [134] and Pcf11, which is involved in 3′ end processing of mRNA and transcription termination of protein-encoding genes [135, 136]. Pcf11 has been demonstrated to dissociate transcriptionally engaged RNAP II from DNA, indicating a pivotal role in termination [137–139]. Recent reports show that Pcf11 binds to the HIV LTR and represses HIV transcription in cell line models for HIV latency [140]. Pcf11 may be recruited to the LTR by the paused RNAP II complex. In summary, HIV transcriptional elongation is limited by multiple mechanisms that include the availability of P-TEFb, processiveness of the RNAP II complex, and premature termination (Figure 1(c)).

6. Tat

The presence of a blocking nucleosome and the role of pausing and premature termination would indicate that transcriptional elongation presents a major checkpoint to HIV transcription. HIV overcomes this limitation through the function of the virally encoded transcriptional activator Tat. Tat potently activates HIV gene expression by facilitating the recruitment of P-TEFb to the HIV LTR. Tat binds the RNA stem loop structure formed by the TAR element and recruits P-TEFb through its interaction with the CycT1 subunit [141]. The Tat-P-TEFb interaction brings active Cdk9 into the proximity of the paused RNAP II complex. P-TEFb phosphorylates the CTD domain of RNAP II as well as NELF and DSIF, inducing RNAP II processivity and transcriptional elongation. In addition to directly targeting the paused RNAP II complex Tat recruits chromatin remodeling factors such as SWI/SNF complexes Brm and/or Brg-1 [63, 64, 142] as well as HATs, p300/CBP, P/CAF and GCN5 that can promote transcriptional activation through post-translational modification of histones and the remodeling of the positioned nuc-1 [59, 63]. Thus, Tat is positioned to play a cri-tical role in coordinating transcriptional elongation and chromatin remodeling to assure efficient HIV transcription. The transactivation of Tat couples HIV transcriptional elon-gation along with chromatin remodeling [21, 67] (Figure 1(d)).

Tat activity is regulated at multiple levels including transcription and posttranslational modification [143]. Tat transcription is regulated by the HIV LTR and if repressed, limited Tat will be expressed. Minimal Tat function, either due to lack of cellular factors or mutation to the Tat-TAR axis, favors repression of HIV transcription and latency [55, 143]. In addition, stochastic fluctuations in Tat transcription have been shown to overcome initial repression and induce efficient transcription elongation [144]. Post-translational modifications of Tat have been demonstrated to modulate its interactions with TAR, P-TEFb, and chromatin-remodeling complexes to assure the transactivation of Tat even under limiting conditions [145]. In particular, Tat is subject to a dynamic sequential methylation/demethylation and acetylation/deacetylation cycles. Monomethylation of lysine 51 (K51) by Set7/9/KMT7 enhances Tat binding to the TAR, whereas demethylation by LSD1/KDM1/CoREST and acetylation of neighboring lysine 50 (K50) mediated by p300/KAT3B favor the dissociation of Tat from TAR and P-TEFb [146–150]. SIRT1, the class III nicotinamide adenine dinucleotide-dependent class III protein, deacetylates Tat and represses its activity [149]. The methyltransferase, demethylase HDACs and HATS that control HIV Tat function are attractive therapeutic targets [150].

7. Conclusion and Implications

Studies using a variety of cell lines [16, 22, 151] and primary cell systems [37, 152, 153] have provided insights into the complexity of HIV transcription and the appreciation that multiple mechanisms contribute to latency [154]. Furthermore, these studies have suggested that therapeutic strategies targeting transcription may be used to purge HIV from different cellular reservoirs. Attempts to activate repressed proviral transcription present several unique challenges including the lack of a single or common event in establishment and maintenance of latency, and most factors that limit HIV transcription are general transcriptional regulators and cofactors, which are necessary for normal gene expression. Compounds that target RNAP II, P-TEFb, and chromatin remodeling factors will likely be toxic, lack specificity, and have a global impact on gene expression. The challenges that exist in translating our general understanding of HIV transcription into a viable therapeutic approach are highlighted by the recent clinical trials with HDAC inhibitors. Based on the strong evidence from cell line models of HIV latency, which showed that overcoming the repressive effects of chromatin induces HIV transcription, it was hypothesized that HDAC inhibitors could be a useful tool in purging HIV from latently infected cells [155]. Initial experiments using the HDAC inhibitor valproic acid with primary cells from HIV-positive patients were encouraging [156–158]; however, follow-up studies and a recent clinical trial have shown that valproic acid had a minimal impact on the low level of virema in the peripheral blood of ART patients [159–163]. Although these results might be viewed as discouraging, next-generation HDAC inhibitors [164, 165] in combination with other potential treatments such as methyltransferase inhibitors [166] as well as newly identified compounds discovered in recent screens [89, 153], which target HIV transcription but only partially activate T cells, may be efficacious. As we screen and develop new compounds, it will be critical to assure that they are active in multiple *in vitro* and *in vivo* models of latency to assure that the broad range of potential mechanisms that influence HIV transcription and latency are targeted [154].

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Review Article

TRIM5 and the Regulation of HIV-1 Infectivity

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The past ten years have seen an explosion of information concerning host restriction factors that inhibit the replication of HIV-1 and other retroviruses. Among these factors is TRIM5, an innate immune signaling molecule that recognizes the capsid lattice as soon as the retrovirion core is released into the cytoplasm of otherwise susceptible target cells. Recognition of the capsid lattice has several consequences that include multimerization of TRIM5 into a complementary lattice, premature uncoating of the virion core, and activation of TRIM5 E3 ubiquitin ligase activity. Unattached, K63-linked ubiquitin chains are generated that activate the TAK1 kinase complex and downstream inflammatory mediators. Polymorphisms in the capsid recognition domain of TRIM5 explain the observed species-specific differences among orthologues and the relatively weak anti-HIV-1 activity of human TRIM5. Better understanding of the complex interaction between TRIM5 and the retrovirus capsid lattice may someday lead to exploitation of this interaction for the development of potent HIV-1 inhibitors.

1. Introduction

HIV-1 was identified only two years after the first report of AIDS in 1981 [1]. The HIV-1 genome was cloned and sequenced, ORFs were identified, and functions of the gene products pinpointed. At a time when few antivirals were in clinical use, HIV-1 proteins were isolated, their activities were described, their structures were determined, and inhibitors were identified [2–5]. The first anti-HIV-1 drug, AZT, was approved for patients in 1987, and effective combinations of anti-HIV-1 drugs were in the clinic by the mid-1990s. Thanks to these anti-HIV-1 drugs, the number of AIDS cases plummeted in countries like the United States. HIV-1 infection became an outpatient disease. Yet, despite the impact of basic science on disease in individuals with HIV-1 infection, the AIDS pandemic has not gone away.

2. Ongoing Pandemic and the Need for More Basic Research

Failure to control the AIDS pandemic may be attributable to a number of factors, including the need for improvement in drugs and more ready access to those drugs that already exist. Aside from one extraordinary case of a person who underwent bone marrow transplantation with cells from a CCR5-defective donor [6], there has been no documented cure of HIV-1 infection. Aside from a small effect in one vaccination trial [7], there is no evidence for prevention of HIV-1 infection in people by a vaccine. Without prospects for curative drugs or a preventive vaccine, the cost of HIV-1 infection to individuals and to society will remain high. In New York City there are currently ~110,000 people living with HIV-1 and ~1,600 HIV-related deaths annually (NYC Dept of Health). The toll of AIDS is much greater in medically underserved regions of the world, despite improved distribution of anti-HIV-1 drugs in these places. According to the UNAIDS report concluding in 2010 (http://www.unaids.org/en/), 34 million people were living with HIV infection, and in that year alone there were 2.7 million new infections.

3. Host Factors and HIV-1 Infectivity

Much remains to be learned about the function of each of the HIV-1 gene products and the optimization of drugs that inhibit their function. In recent years the focus of much HIV-1 molecular biology research has shifted to host factors that regulate HIV-1 infection. Initially these studies

involved searches for host factors that physically interact with individual viral proteins. The cellular proteins cyclophilin A and LEDGF, for example, were found to interact with HIV-1 capsid (CA) and HIV-1 integrase (IN), respectively, [8, 9]. Both of these protein-protein interactions have been studied extensively and have offered novel approaches to HIV-1 inhibition and potential new anti-HIV-1 drug candidates [9–12].

Functional screens have also yielded information concerning host factors that regulate infection by HIV-1 and other retroviruses [13–16]. More recently, several groups have reported human genome-wide RNAi screens to identify factors that regulate HIV-1 infectivity [17–21]. Among host factors identified in these screens are host proteins such as TNPO3 that play critical roles in the poorly understood early events of HIV-1 infection that culminate in establishment of the provirus [15, 22–25]. Ultimately, information springing from the study of any one of these host factors has the potential to be exploited towards the development of drugs that disrupt HIV-1 in people.

4. Restriction Factors

Over the past 10 years, in addition to the identification of host factors that promote HIV-1 infectivity, several host factors have been discovered that block HIV-1 infection [26]. Comparative analysis of the genes encoding these proteins, which have been called restriction factors, indicates that some of them have evolved in response to challenge with pathogenic retroviruses [27, 28]. Study of these factors has offered a wealth of information concerning requirements for HIV-1 replication, novel ways that HIV-1 might be targeted therapeutically, potential paths to cure HIV-1 infection, and ways in which innate immune detection of HIV-1 might be amplified to improve vaccination protocols.

5. Fv1 and Capsid-Specific Restriction

When HIV-1 and other retroviruses undergo membrane fusion with susceptible target cells, the virion core is released into the target cell cytoplasm. The core of the virion consists of a capsid-protein lattice, within which there are two copies of the viral genome, along with the reverse transcriptase and IN proteins. An extraordinary series of experiments spanning several decades demonstrated that the retroviral CA protein lattice is the viral determinant of sensitivity to a murine-specific restriction factor called Fv1 [29, 30]. Curiously, *Fv1* encodes a retroviral Gag polyprotein [29]. The mechanism of Fv1 restriction is still unknown, but these studies established the concept of retrovirus CA-specific restriction and inspired the search for similar factors targeting HIV-1 CA.

6. Cyclophilin A and Capsid-Specific Restriction

Cyclophilin A was the first HIV-1 CA-specific host factor that was identified [9, 31]. Though cyclophilin A is not a restriction factor itself, it controls the accessibility of CA to other host factors that inhibit reverse transcription and other processes essential to the early steps of the infection cycle [32]. One apparent effect of these host factors is to influence these early steps via effects on stability of the HIV-1 virion core [15, 32–36]. The identity of these cyclophilin-regulated host factors is unknown. Additional screens have identified CPSF6 as a conditional regulator of HIV-1 infection, that acts in a capsid-specific manner [15, 37]. CPSF6 is a possible candidate for one such cyclophilin A-regulated restriction factor.

Cyclophilin A cDNAs have retrotransposed many times in evolution, in several cases creating new genes that regulate HIV-1 infectivity in a capsid-specific manner. The first of the cyclophilin A-targeted restriction factors to be identified was the TRIM5-cyclophilin A fusion protein found in South American owl monkeys [38]. A similar, though independently derived, TRIM5-cyclophilin A fusion gene that acts as a capsid-specific restriction factor was created in Asian macaques [39–42]. Nup358/RanBP2, a nuclear pore protein that possesses a cyclophilin A domain also plays a role in HIV-1 infectivity [15, 17, 19, 43].

7. The Discovery of TRIM5 as an HIV-1 CA-Specific Restriction Factor

Early studies with HIV-1 showed that infection of cells from nonhuman primates is too inefficient to establish spreading infection [44–48]. It was then shown that dominant-acting inhibitors were present in these species, and that the viral capsid was the main determinant for sensitivity [49–51]. In 2004, two groups independently identified TRIM5 orthologues as being responsible for these species-specific, capsid-specific blocks [38, 52]. The owl monkey orthologue (known as TRIM5-Cyp) targets HIV-1 capsid via its carboxy-terminal cyclophilin A domain [38, 53], and the rhesus macaque orthologue (the alpha isoform) targets HIV-1 capsid via its carboxy-terminal PRY-SPRY domain [52]. Human TRIM5alpha potently restricts EIAV and N-tropic MLV, but it only weakly inhibits HIV-1 lab strains. Differences in specificity between human and macaque TRIM5alpha map to a small block of residues in the PRY-SPRY domain [28, 32, 54, 55]. Though standard HIV-1 lab strains are only weakly inhibited by human TRIM5alpha, some primary HIV-1 isolates are much more sensitive [56, 57].

8. The Problem of CA Recognition

One of the biggest ongoing challenges for researchers studying TRIM5 is to understand the structural basis for CA recognition. TRIM5 is a multimer, and CA recognition does not occur via a simple protein-protein interaction. Rather, TRIM5 recognizes a complex surface involving the CA lattice [58, 59]. In fact, TRIM5 spontaneously forms a hexameric protein lattice, and this propensity to form a lattice is greatly stimulated in the presence of the CA lattice [60] (Figure 1). This explains why a simple binding assay has not been developed. Extensive efforts have been made by several groups to develop soluble subdomains of the CA lattice that might be used in binding studies [61, 62]. The soluble hexamer unit, for example, seems not to bind to TRIM5 [63, 64]. In contrast, promising results have been obtained

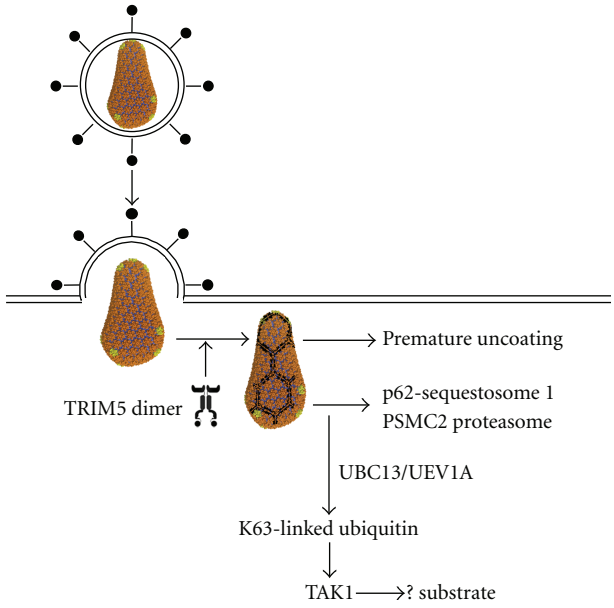

Figure 1: Schematic diagram showing current models of TRIM5-mediated restriction. Free TRIM5 probably exists as a dimer in the target cell cytoplasm. Upon interaction with the capsid of a restriction-sensitive retrovirus, the propensity of TRIM5 to form a complementary hexameric lattice is stimulated. This increases its intrinsic E3 ubiquitin ligase activity. If avidity for the retrovirus capsid is sufficient, the virion core prematurely uncoats and reverse transcription is blocked. Depending upon the proximity of particular cellular E2 enzymes, TRIM5 will either autoubiquitinate and traffic towards proteasomes, or it will activate the TAK1 kinase and downstream signaling molecules.

with a CA trimer [64]. A requirement for additional host factors such as SUMO-1 may complicate the situation with CA recognition even further [65].

9. TRIM5 and E3 Ubiquitin Ligase Activity

At latest count, the human TRIM family comprises ~100 genes [66]. Like other members of this large family, TRIM5 possesses an N-terminal RING domain, a B-box domain, and a coiled-coil domain. The B box and coiled-coil domains promote multimerization of TRIM5 required for restriction activity [67, 68]. The TRIM5 RING domain confers E3 ubiquitin ligase activity, and, in cooperation with certain E2 enzymes, TRIM5 is autocatalytic, covalently attaching ubiquitin to itself [69]. Mutations on the putative E2-interacting face which disrupt this autocatalytic activity block restriction activity [70]. Ubiquitination of TRIM5 contributes to the short half-life of this protein [71], and challenge of cells with viruses bearing restriction-sensitive capsids promotes the proteasome-dependent degradation of TRIM5 [72]. Though TRIM5-stimulated ubiquitination of viral proteins has not been detected, TRIM5 may contribute to the restriction mechanism by recruiting viral components to the proteasome for degradation (Figure 1). TRIM5 interacts biochemically with the proteasome component PSMC2 and colocalizes with proteasomes in infected cells [73]. TRIM5 also associates with the proteasomal adaptor protein p62 [74] though p62 seems to stabilize TRIM5 protein levels.

In certain experimental conditions, restriction activity has been reported in the absence of the RING domain or in the absence of ubiquitination. There are several possible explanations for these discrepancies. One possibility is that, when avidity for a particular CA is great enough, TRIM5 binding to the CA is sufficient to disassemble the virion core prior to reverse transcription [59] (Figure 1). Another possible explanation stems from the fact that TRIM5 blocks multiple steps in the restriction pathway [75]. Disruption of the RING domain rescues the TRIM5-mediated block to reverse transcription and premature uncoating but not subsequent blocks in the infection cycle that lead up to integration [76, 77].

10. TRIM5, TAK1, and Inflammation

In combination with the heterodimeric E2, UBC13/UEV1A, TRIM5 catalyzes the synthesis of unattached, K63-linked ubiquitin chains that multimerize and activate the TAK1 kinase complex [63]. These K63-linked ubiquitin chains are not generated by TRIM5 when other E2 enzymes are substituted for UBC13/UEV1A. Disruption of TAK1 or of UBC13/UEV1A prevents restriction activity. Taken together, these observations suggest that the activated TAK1 complex contributes to TRIM5-mediated restriction activity via phosphorylation of a critical cofactor (Figure 1). The identity of this putative cofactor is not known, and direct phosphorylation of CA by TAK1 has not been detected.

Coming at it from another direction, the synthesis of K63-linked ubiquitin chains that activate TAK1 is stimulated by TRIM5 interaction with a restricted capsid lattice [63]. TAK1 activation leads to NFκB and AP-1 signaling which activate inflammatory cytokine transcription. In other words, TRIM5 functions as a pattern recognition receptor specific for the retrovirus capsid lattice. The consequence of TRIM5-mediated signaling for HIV-1-associated inflammation and pathology is only now being considered.

11. Future Directions of TRIM5 Research

If a robust assay was developed for TRIM5 interaction with the retrovirus capsid lattice, it would inform attempts to influence HIV-1 CA recognition by TRIM5, and perhaps to develop HIV-1 inhibitors that increase the avidity of this specific interaction. If the avidity of human TRIM5 for the HIV-1 capsid lattice could be increased experimentally, the resulting increase in capsid-stimulated signaling might also be exploited as an adjuvant for anti-HIV-1 immunization.

Recent publicity concerning the apparent cure from HIV-1 infection of a leukemia patient in Berlin with transplantation of cells from a CCR5-mutant donor [6, 78] has generated excitement concerning prospects for curing HIV-1 infection. This case has also renewed interest in basic research concerning gene therapy against HIV-1 and the regulation of HIV-1 latency in people who are already infected with HIV-1. Concerning gene therapy, the most

promising approaches at this point involve either disruption of CCR5 [79] or transduction of hematopoietic stem cells with potent HIV-1 restriction factors such as engineered, human TRIM5-cyclophilin A fusion proteins [80].

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Review Article

Probing Retroviral and Retrotransposon Genome Structures: The "SHAPE" of Things to Come

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Understanding the nuances of RNA structure as they pertain to biological function remains a formidable challenge for retrovirus research and development of RNA-based therapeutics, an area of particular importance with respect to combating HIV infection. Although a variety of chemical and enzymatic RNA probing techniques have been successfully employed for more than 30 years, they primarily interrogate small (100–500 nt) RNAs that have been removed from their biological context, potentially eliminating long-range tertiary interactions (such as kissing loops and pseudoknots) that may play a critical regulatory role. Selective 2′ hydroxyl acylation analyzed by primer extension (SHAPE), pioneered recently by Merino and colleagues, represents a facile, user-friendly technology capable of interrogating RNA structure with a single reagent and, combined with automated capillary electrophoresis, can analyze an entire 10,000-nucleotide RNA genome in a matter of weeks. Despite these obvious advantages, SHAPE essentially provides a nucleotide "connectivity map," conversion of which into a 3-D structure requires a variety of complementary approaches. This paper summarizes contributions from SHAPE towards our understanding of the structure of retroviral genomes, modifications to which technology that have been developed to address some of its limitations, and future challenges.

1. Introduction

Cis-acting sequences within the (+) strand RNA genomes of retroviruses and long terminal repeat (LTR) containing retrotransposons control several critical events in their life cycle, including transcription [1], translation [2], dimerization [3], packaging [4], RNA export [5], and DNA synthesis [6]. Development of novel RNA-based strategies to ameliorate human immunodeficiency virus (HIV) pathogenesis would therefore benefit from an improved understanding of RNA structure and how this mediates interactions with both host and viral proteins. Historically, deciphering higher-order RNA structure has taken advantage of base- and structure-specific nucleases (e.g., RNases A, T1, T2 [7] and nuclease S1 [8]) or chemicals (e.g., dimethyl sulfate, diethyl pyrocarbonate [9, 10], and Pb^{2+} [11]). While these approaches have produced seminal advances in elucidating features of the HIV-1 and HIV-2 genomes [12–23], the necessity in most cases for multiple reaction conditions can be considered a limitation. Moreover, in almost all instances, enzymatic and chemical RNA footprinting has been performed on short RNAs prepared by *in vitro* transcription and labeled with ^{32}P, eliminating any positional context, that is, regulatory roles that might be mediated by long-range, tertiary interactions. Although this challenge has in part been addressed by Paillart et al. via *ex vivo* footprinting of virion-associated RNA with dimethyl sulfate [24], a more "user-friendly" approach capable of providing information on RNA structure both *in vitro* and *ex virio,* and with fewer base-specific reagents, would clearly be advantageous.

Selective 2′ hydroxyl acylation analyzed by primer extension (SHAPE), reported in 2005 by Merino and colleagues [25], has emerged as a facile technique that addresses many of these concerns. Since the target of the probing agent (N-methyl isatoic anhydride (NMIA) [25] or 1-methyl-7-nitroisatoic anhydride (1M7) [26]) is the ribose 2′ hydroxyl,

all four RNA bases are simultaneously probed with a single reagent. Secondly, when combined with fluorimetric detection, multiplexing and automated capillary electrophoresis, SHAPE profiles of complete, 10,000 nt retroviral genomes can be generated in a matter of weeks [27]. By comparing reactivity profiles obtained *in vitro* and *ex vivo*, these studies have also provided important information on HIV genome organization and the role played by chaperone proteins. Finally, the recent advent of the self-inactivating electrophile benzoyl cyanide (BCN) [28] opens the possibility of time-resolved SHAPE, which promises to provide important glimpses into RNA conformational dynamics.

Despite these benefits, it should be borne in mind that SHAPE effectively provides a secondary structure nucleotide "connectivity" profile; that is, it does not report directly on long-distance tertiary interactions such as kissing loops and pseudoknots and is best used in conjunction with other solution techniques, such as X-ray crystallography, NMR spectroscopy, and small angle X-ray scattering in order to generate an accurate 3-D model. Where possible, combining structural data with a genetic analysis, via construction of disruptive and complementary mutations, should be seen as an important complement. In this communication, we have reviewed the basic SHAPE methodology and its application to understanding the structure of regulatory elements of both retroviral and retrotransposon genomes. Modifications to the probing technology which have allowed us to (i) investigate tertiary interactions important for regulating nucleocytoplasmic RNA transport and (ii) combine chemical modification with tandem mass spectrometry to understand conformational dynamics of RNA/DNA hybrids containing polypurine tract (PPT) primers of (+) strand DNA synthesis, are presented. Finally, future challenges of SHAPE, including increasing sensitivity where the amount of biological material is limiting, and studying interconverting RNA structures, are also discussed.

2. SHAPE Methodology

A brief outline of SHAPE methodology is presented in Figure 1. As originally conceived, this chemoenzymatic strategy assesses local flexibility in RNA via accessibility of the ribose 2′-OH group to acylation by the electrophilic reagent NMIA. In flexible regions (such as loops, bulges, and junctions), RNA adopts conformations that will promote formation of a nucleophilic 2′-oxyanion which reacts with NMIA to form a bulky 2′-*O*-adduct [25] (Figure 1(a)). Recent modifications to the strategy have taken advantage of 1M7 [26] and BCN [28], which are more labile towards hydrolysis and self-inactivation, making them particularly advantageous for performing time-resolved footprinting. Modified RNAs are subsequently evaluated by primer extension with an RNase H-deficient reverse transcriptase, creating a cDNA library corresponding to stops at sites of adduct formation in the RNA when analyzed by high resolution gel electrophoresis (Figure 1(b)). End-labeling with ^{32}P allows primer extension products of 50–300 nt to be fractionated by conventional denaturing polyacrylamide gel electrophoresis, while multiplexing with fluorescently-labeled primers and automated capillary electrophoresis permits resolution of 500–750 nt in a single electropherogram (Figure 1(c)). Finally, autoradiograms or electropherograms are quantified and computationally deconvoluted in order to obtain the energy-minimized RNA structure (Figure 1(d)).

In contrast to the many benefits of SHAPE, analyzing sites of adduct formation by primer extension has limitations for structural studies aimed at very short RNAs. Since SHAPE information is tabulated indirectly through the length and frequency of a given cDNA, information on ~ 50 nt at the 3′ terminus of the RNA molecule is lost as a consequence of both primer binding and reduced processivity of the retroviral reverse transcriptase used for cDNA synthesis. In an attempt to address this shortcoming, Steen et al. [33] recently combined chemical acylation with sensitivity to exonucleolytic degradation, based on the observation that RNase R exonucleases processively cleave RNA in a 3′ → 5′direction. Screening several sources of RNases R identified an enzyme from *Mycoplasma genitalium* capable of processively degrading RNA, including through base-paired regions, but not beyond sites of adduct formation. The approach of RNase-directed SHAPE provides a facile and important complement to examine structural features at the termini of important regulatory RNAs. Although there is currently no commercial source for *Mycoplasma genitalium* RNase R, methods for purifying this enzyme from recombinant *E. coli* have been published [34].

3. SHA-MS Combines Chemical Acylation with Mass Spectrometry

As originally conceived, SHAPE was designed to interrogate structural features of RNA molecules ranging in size from several hundred to several thousand nucleotides. A critical feature of retrovirus and retrotransposon replication is initiation of (+) strand, DNA-dependent DNA synthesis from the polypurine tract (PPT) RNA primer. Although we have gleaned important information on PPT function using mutants of HIV RT [35–37] and targeted insertion of nucleoside analogs at, and in the vicinity of the PPT-U3 junction [38–42], the structural basis for PPT primer recognition remains elusive. Since our nucleoside analog strategy has mandated analysis of short RNA/DNA hybrids (25–30 bp), identifying structural anomalies by SHAPE becomes impractical. However, since RNA 2′-OH acylation results in a mass increment of 133 Da, we reasoned that adduct formation could be evaluated by electrospray ionization (ESI) mass spectrometry (MS). As illustrated in Figure 2(a), discrete PPT RNAs containing between one and four NMIA adducts could be detected by nanospray ESI-MS, while the DNA complement, as predicted, was insensitive to modification. Tandem mass spectrometry was subsequently used to define the positions of adduct formation indicating that, in addition to terminal ribonucleotides, which might be predicted to "fray," ribonucleotides-11 and -12 of the wild type PPT (defining position -1 as the ribonucleotide 5′ of

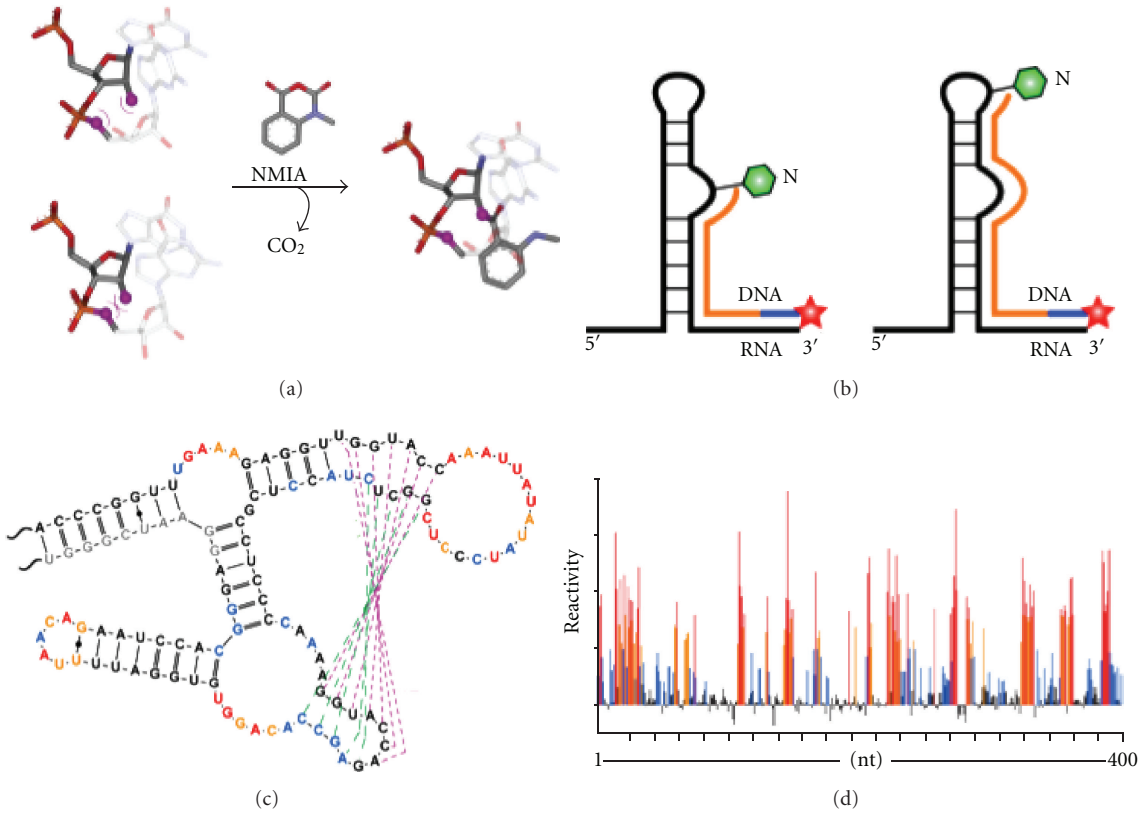

Figure 1: Overview of SHAPE technology. (a) Ribose 2′ OH of RNA at flexible, or unpaired nucleotides is selectively modified by NMIA. (b) Positions of adduct formation result in impaired primer extension during subsequent cDNA synthesis. (c) Radiolabeled or fluorescently-labeled primer extension products are resolved by high resolution polyacrylamide gel electrophoresis or automated capillary electrophoresis. (d) Electropherograms are computationally deconvoluted to obtain normalized NMIA reactivities, from which a secondary structure model is constructed.

the PPT/U3 junction) were sensitive to acylation. These positions, corresponding to bases of the mispaired or "unzipped" component of the PPT observed crystallographically [43], suggest that either mispairing alters the geometry of the ribose 2′-OH or that the unzipped region of the PPT is transiently unpaired.

The utility of our approach [29], designated selective 2′ hydroxyl acylation analyzed by mass spectrometry (SHA-MS [29]), was perhaps better demonstrated by analyzing nucleoside analog-substituted PPTs. As might be predicted, substituting template thymine −13T with the nonhydrogen bonding pyrimidine isostere 2,4-difluorotoluene (dF [41]) expanded the NMIA sensitivity profile to include ribonucleotides -11, -12, and -13. However, replacing template nucleotide-8T with dF rendered not only primer nucleotides -11 and -12 insensitive to acylation, but also the complementary primer nucleotide -8, possibly indicating a local difference in base stacking that masks the ribose 2′-OH. Surprisingly, while the PPT RNA primer of the *Saccaromyces cerevisiae* LTR-retrotransposon Ty3 was insensitive to NMIA, acylation of ribonucleotide +1G was observed. These results were in agreement with NMR data [44], suggesting that a unique geometry at the Ty3 PPT/U3 junction may contribute towards recognition specificity. When complemented with $KMnO_4$ footprinting, which differentiates between thymines in a single-stranded and duplex configuration [45], SHA-MS provides a valuable, high resolution approach to interrogate the geometry of short, purine-rich RNA/DNA hybrids where conventional probing strategies are impractical.

4. Antisense (AI)-Interfered SHAPE: Deciphering Tertiary Interactions

Originally defined as an intermolecular interactions that mediate HIV-1 RNA genome dimerization [46], kissing loops have also been identified in the genomes of hepatitis C virus [47], chrysanthemum chlorotic mottle viroid [48], and a group C enterovirus [49]. Furthermore, pseudoknots, (tertiary interactions containing at least two stem-loop structures wherein a portion of one stem is intercalated between two halves of the other) are associated with translational control via internal ribosome entry sites [50], ribosomal frameshifting [51], and tRNA mimicry [52, 53]. Analysis of the RNA transport element of the murine retrotransposon *MusD* (MTE) revealed a complex structure containing a combination of a kissing loop and a pseudoknot [30]. Such tertiary interactions are particularly challenging for SHAPE

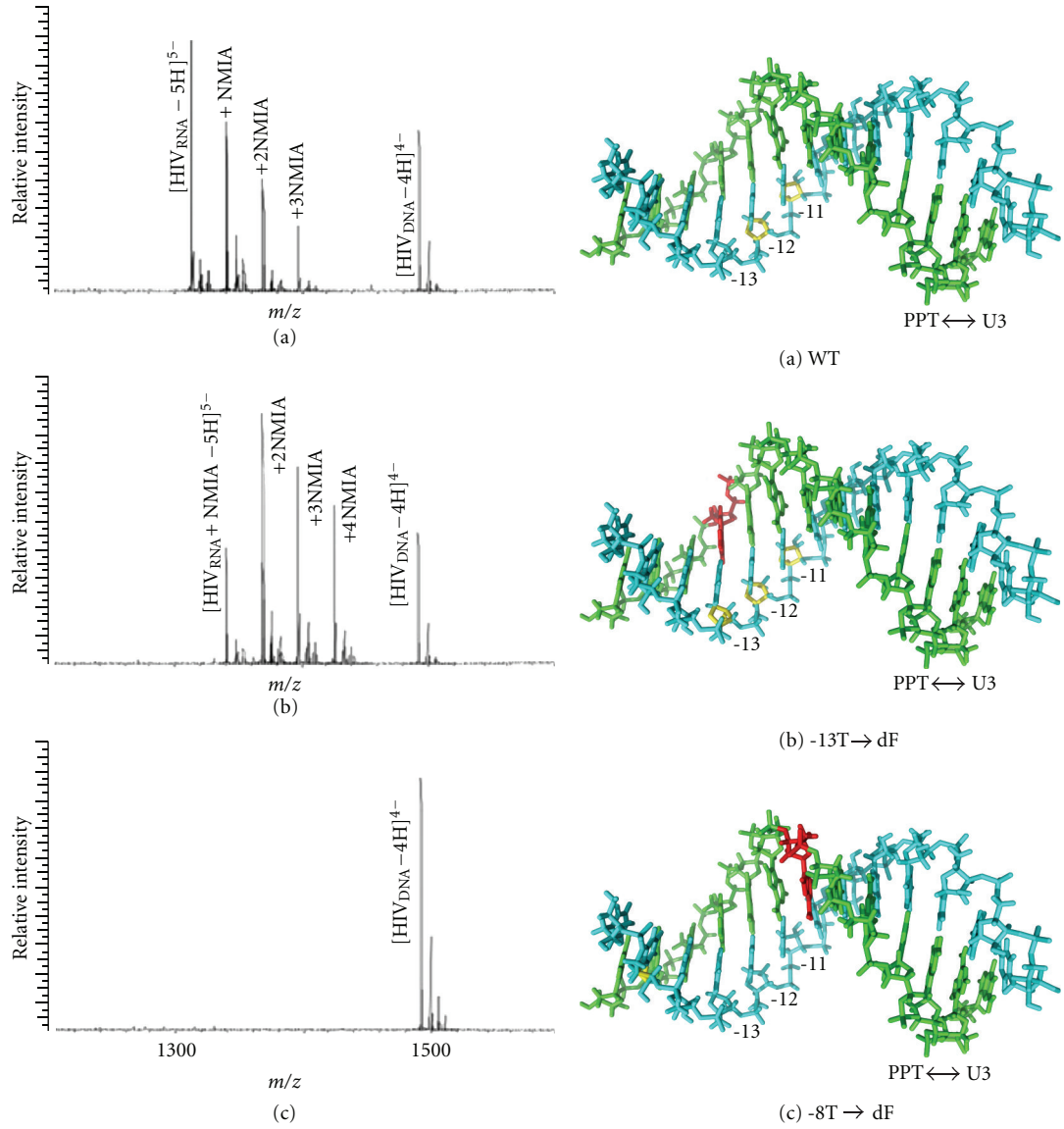

Figure 2: Examining RNA/DNA structural dynamics by combining chemical acylation with mass spectrometry. *Left*, Nano-ESI mass spectra of a model HIV-1 PPT RNA/DNA hybrid following treatment with a 10-fold (a), 50-fold (b), and 100-fold NMIA excess (c). At limiting NMIA concentrations (a) and (b), the majority of the PPT RNA is unmodified, and RNAs containing one, two, three, or four NMIA adducts can be observed, while excess acylation (c) results in overmodification of the entire RNA strand. In all cases, however, the PPT DNA complement is not modified by NMIA owing to the absence of a ribose 2′-OH group. *Right*, NMIA sensitivity of the wild type (a) and dF-modified (b) and (c) HIV-1 PPT RNA/DNA hybrids. In all cases, DNA and RNA nucleotides are represented in green and blue, respectively. NMIA-sensitive ribonucleotides are in yellow and positions of dF substitution in red. The position of the PPT/U3 junction has been indicated. Adapted from [29].

and in the first instance require manual identification. In order to verify the identity of these structures, we developed an oligonucleotides-based interfering strategy designated antisense (ai)-interfered SHAPE, the basis of which is illustrated in Figure 3(a).

This strategy involves hybridization of short (5–10 nts) oligonucleotides to the proposed RNA duplex and determining whether this induces enhanced NMIA reactivity of the displaced strand. In view of their length, antisense oligonucleotides were constructed containing 2′-O-methyl and locked nucleic acid substitutions, both of which have been shown to improve duplex stability. Such interfering oligonucleotides are invasive inasmuch that they will hybridize to their partner sequence in an RNA that has already adopted its 3D structure. When applied to the MusD MTE, an interfering octanucleotide hybridized to internal loop 8 (IL8) stimulated NMIA reactivity at several positions in its kissing partner, loop 3 (L3, Figure 3(b)). Importantly, and as suggested earlier, the L3/IL8 kissing interaction suggested by ai-SHAPE was confirmed genetically *in vivo*,

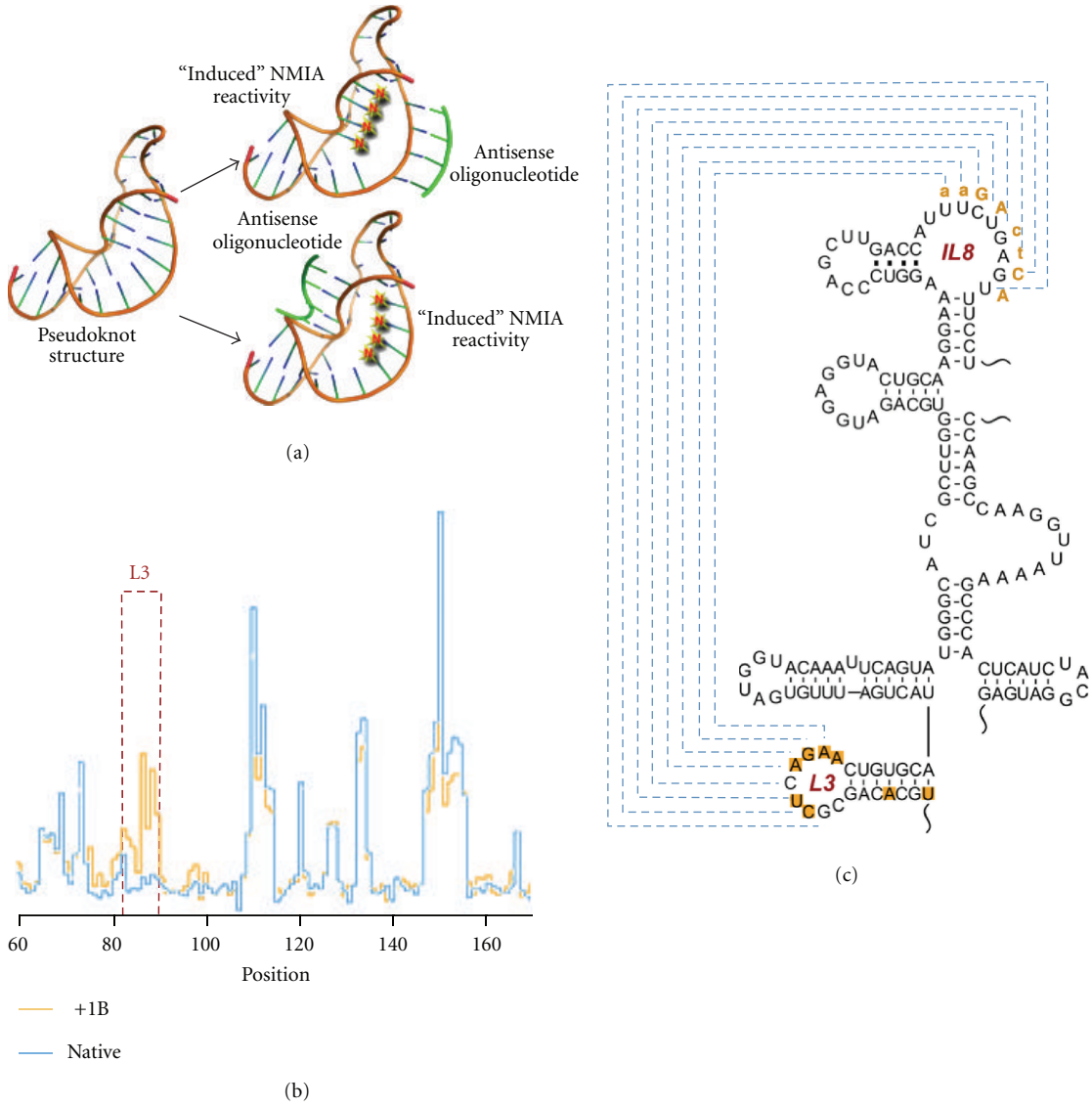

Figure 3: Examining RNA tertiary interactions by ai-SHAPE. (a) ai-SHAPE principal, that is, hybridization of an interfering oligonucleotide (green) to one partner of the proposed RNA duplex increases acylation sensitivity of its base-paired counterpart. (b) Electropherogram of NMIA reactivity of MTE nucleotides 60–170 in the absence (blue trace) and presence of the interfering oligonucleotide 1B (yellow trace). Loop L3 has been highlighted by the red box. (c) Secondary structure map for a portion of the *MusD* RNA transport element MTE, illustrating the L3/IL8 kissing interaction. The sequence of the interfering oligonucleotide hybridized to IL8 is indicated in orange, while nucleotides of loop L3 and the neighboring helix that exhibited enhanced NMIA reactivity are depicted within orange boxes adapted from [30].

where MusD-dependent nucleocytoplasmic RNA transport was abrogated and restored by disruptive and compensatory kissing loop mutations, respectively. The structure of the MusD pseudoknot was likewise confirmed by ai-SHAPE, while a genetic analysis indicated that the ability to assume a pseudoknot configuration was a more critical determinant of function than absolute nucleotide sequence.

5. Interconverting RNAs: Choosing between Dimerization and Protein Synthesis

Riboswitches, located in the noncoding region of several mRNAs, have been demonstrated to regulate RNA stability, protein synthesis, and splicing via a conformational change mediated by binding of a high-affinity ligand [54–56]. The highly-structured 5′ untranslated regions of many retroviruses can be considered formally analogous to a riboswitch, inasmuch as overlapping sequences have been proposed to mediate both genome dimerization/packaging and translation [57, 58]. An inconclusive acylation pattern in our recent SHAPE study of the 5′ UTR of the feline immunodeficiency virus (FIV) genome [31] led us to postulation that certain regions were metastable, allowing them to adopt alternative structures, a notion strengthened by the observation of two closely-migrating RNA species following fractionation by nondenaturing polyacrylamide gel electrophoresis. The

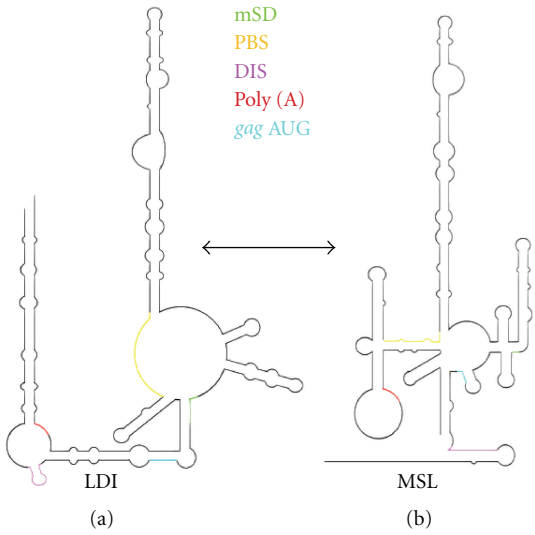

FIGURE 4: Proposed interconverting structures of the FIV 5′ leader RNA controlling genome dimerization/packaging and translation. For both the long-distance interaction (LDI) (a) and multiple stem-loops (MSL) structures (b), important regulatory sequences have been color-coded. mSD, major splice donor sequence; PBS, $tRNA_{Lys,3}$ primer binding site; DIS, dimer initiation site; Poly(A), poly (A) hairpin; *gag* AUG, *gag* initiator methionine codon. See text for fuller details adapted from [31].

hypothesis that best unified our experimental data is illustrated in Figure 4, suggesting that alternate structures for the FIV 5′ UTR mediate different events in the retrovirus life cycle.

The long range interaction (LRI) model, originally proposed by Kenyon et al. [59], exposes the putative FIV dimer initiation sequence (DIS), while the *gag* initiation codon is embedded within a short helix, and free energy calculations suggest this model would support genome dimerization and packaging. The LRI structure also exposes the tRNA primer binding site from which reverse transcription is initiated following infection. The alternative, multiple stem-loop (MSL) structure occludes the DIS, while the *gag* initiation codon is positioned within a short stem-loop, the stability of which would facilitate translation over dimerization and packaging. In the MSL, the tRNA primer binding site is also inaccessible. Support for interconverting structures of the 5′ UTR was provided by the observation that the FIV mutant AN14, demonstrated *in vivo* to have impaired packaging [60], exhibited impaired dimerization *in vitro*, while dimerization was enhanced when the RNA was stabilized in the LRI form [31]. Though a later section will address future SHAPE strategies, our study of the FIV leader RNA provides another good example of combining chemical probing with functional studies, while at the same time highlighting one of its challenges, namely, how to deal with interconverting RNAs. One potential solution might be to perform nondenaturing electrophoretic separation immediately following chemical acylation. Since SHAPE relies on single-hit kinetics, modified RNAs should still resolve as discrete species. Polymerizing gels with disruptable crosslinking agents such as N,N′-bisacryloylcystamine (BAC) or N,N-diallyltartardiamide (DATD) would allow solubilization and recovery of nucleic acid for subsequent cDNA synthesis. Should ribose acylation alter RNA conformation, in-gel probing directly following fractionation by nondenaturing electrophoresis is an alternative strategy.

6. Investigating RNA Tertiary Structure with "Threading Intercalators"

Understanding RNA structure-function relationship requires accurate three-dimensional structure modeling methods. At present, there is a substantial gap in obtaining high-throughput 3D information for RNA molecules larger than 150 nts. The techniques frequently used to obtain atomic resolution of RNAs, such as NMR spectroscopy and X-ray crystallography, have restrictions that preclude structural analysis. In NMR spectroscopy, the excited signal from individual atomic nuclei becomes congested and difficult to analyze with the increasing size of RNA molecule. Even though X-ray crystallography does not suffer from size limitations, obtaining crystals for flexible and diverse RNA structures represents a great challenge. These difficulties however, are now being addressed by combining SHAPE with methidiumpropyl-EDTA- (MPE-) directed through-space hydroxyl radical cleavage, as outlined schematically in Figure 5. In the past, MPE has been successfully applied as a tool for footprinting binding sites of small molecules on heterogeneous DNA [61], RNA folding analysis [61, 62] and examining RNA-binding properties of phospho- and dephospho-RNA-dependent protein kinase [63]. Recently, Gherghe et al. successfully combined SHAPE with MPE-directed hydroxyl radical cleavage to study $tRNA^{Asp}$ tertiary structure [64].

MPE is a methidium intercalator moiety tethered to EDTA that preferentially intercalates at G-C rich helices in RNA at sites adjacent to a single nucleotide bulge. The intercalated MPE occupies roughly the same space as a single base pair and is oriented in the motif such that the EDTA moiety points toward the bulge. Upon addition of Fe(II) and a reducing agent, ferrous ion binds the EDTA and generates short-lived hydroxyl radicals that cleave proximal regions of the RNA backbone [65]. The MPE binding site can be placed at RNA helical motifs by replacing four consecutive base pairs with CGAG/C(C/U)G motif [64]. Provided that this replacement is compatible with the native structure of RNA, cleavage at positions proximal in space to the unique location of the bound MPE affords information about the nucleotides neighboring the intercalating ligand. Cleavage intensity at each position can be calculated as a ratio relative to the mean value for all intensities, after subtracting background cleavage observed for the native RNA sequence that does not contain an MPE binding site. Subsequently, MPE-directed through-space cleavage experiments yield high quality, long range constrains that refine nucleotide positions in RNA to atomic resolution of 4 Å rmsd [64]. As a result, the combined experimental and computational approach has the potential to yield native-like models for functionally crucial RNA

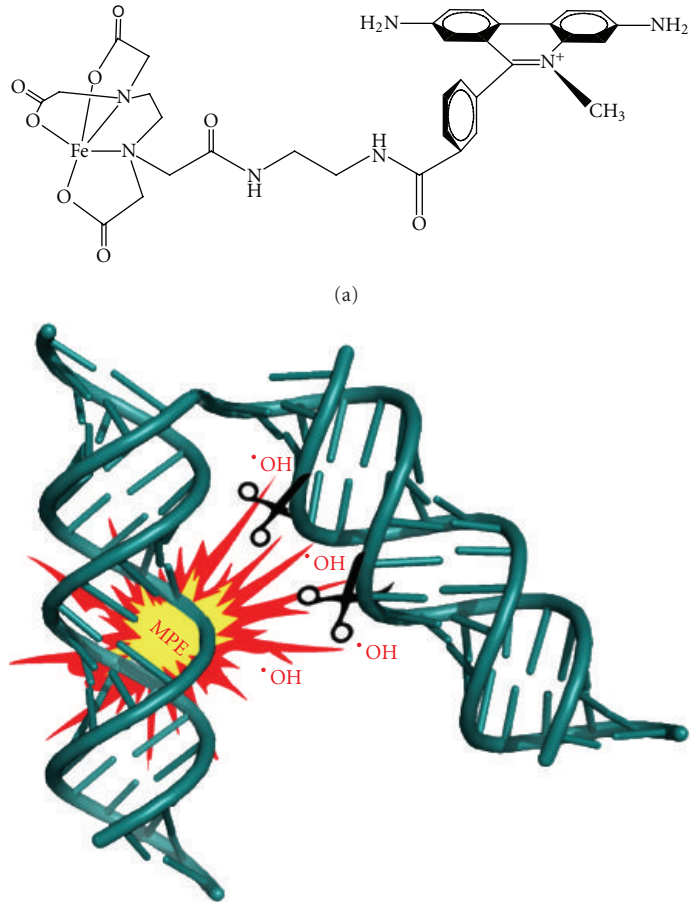

Figure 5: (a) Structure of the threading intercalator, MPE. (b) Examining RNA tertiary interactions by through-space hydroxyl radical cleavage (–OH) with the threading intercalator methidiumpropyl EDTA (MPE). Once a SHAPE profile for the RNA under investigation is determined, an MPE intercalation site is introduced by replacing four consecutive nucleotides with the CGAG/C(C/U)G recognition motif. SHAPE is then repeated to determine that sequence changes are nonperturbing, after which site-directed hydroxyl radical cleavage is performed to identify neighboring sites in the RNA. Repeating this process with independent RNAs containing unique MPE intercalation sites cumulatively provides information on tertiary interactions.

molecules. Currently, MPE is not commercially available, and its application to through-space cleavage has only been demonstrated with a well-characterized RNA (yeast $tRNA^{Asp}$). However, synthesis of MPE has been reported, and this strategy opens the intriguing possibility of developing "molecular rulers" by introducing linkers of different length between the intercalating and hydroxyl radical generating moieties.

7. Bringing It All Together-Determining Full Genome Structures by SHAPE

Most structural analyses have historically targeted small RNA motifs (<500 nt) in artificial contexts and, in the absence of complementary genetic and phylogenetic data, may not accurately relate their structures to the biology of the larger RNAs from which they were derived. In contrast, SHAPE provides an unprecedented opportunity to view an entire RNA molecule, giving the researcher the opportunity to connect simple elements to the components of larger RNA motifs. This concept has recently been exemplified through the application of SHAPE to decode the structure of the entire HIV-1 genome (~9750 nucleotides) at single-nucleotide resolution [27]. This seminal study determined that, although the HIV-1 genome is less structured than ribosomal RNA, it nonetheless contains independent RNA folding domains. Some functionally significant RNA motifs were shown to belong to the larger elements, an example of which is the *gag-pol* ribosomal frameshift signal, which constituted one component of a three-helix structure (P1-P2-P3). The slippery sequence forms one of the three helices (P2), while two others (P1 and P3) are stabilized by an anchoring stem with two bulges. Additional RNA elements were identified in protein-coding regions of the genome, from which it has been tentatively postulated that RNA structure constitutes an additional organizational level of the genetic code. Since many proteins appear to fold co-translationally, highly structured RNA might induce pausing of the translational machinery, promoting protein folding

in a more native-like conformation. In contrast, highly unstructured regions were observed in hypervariable regions of the HIV-1 genome, which have important roles in viral host evasion. These unstructured regions were shown as separated from the rest of the genome by stable helices that have been proposed to function as structural "insulators."

The versatility of SHAPE extends to studying viral RNA not only in the context of the intact genome, but also at different biological states, providing information with respect to RNA conformational changes underlying different stages of viral life cycle. As an example, Wilkinson et al. [66] have provided structural information on the HIV-1 leader RNA in four biological states, namely (i) *in vivo*, (ii) *ex vivo*, where genomic RNA had been gently deproteinized, (iii) *in vivo*, but where important interactions between the nucleocapsid protein (NC) and genomic RNA had been compromised by covalent modification with aldrithiol-2 (AT-2 [67]), and (iv) genomic RNA prepared by *in vitro* transcription. This study concluded that the first 1000 nt of the HIV-1 genome exists in a single, predominant conformation in all four states. RNA of noncoding regions that regulate different steps of viral life cycle was distinguished by significantly lower sensitivity to acylation (predictive of secondary structure) than coding regions. A comparison of acylation profiles for the *in vivo* state with those following covalent modification by AT-2 defined several high affinity NC recognition sites, consistent with the role of this critical RNA chaperone in governing packaging of viral RNA. All NC binding sites were characterized by a G-rich single-stranded sequence flanked by stable helices. Additionally, RNA motifs where NC increases local flexibility were also identified, comprising single-stranded A/U-rich motifs adjacent to a duplex in which the first base pair includes a guanosine nucleotide. Collectively, this genome-probing approach suggests that local protein interactions can be organized by the long-range architecture of RNA. Although a limited region of the genome of the formerly known gammaretrovirus xenotropic murine leukemia virus related virus (XMRV) was examined using this strategy, it yielded similar conclusions on high affinity NC binding sites [68]. Future studies directed towards whole-genome structural analysis would, however, benefit from development of methods that enhanced SHAPE sensitivity, thereby reducing the culture volumes of potentially biohazardous material required. Efforts in this direction are discussed in the following section.

8. Increasing SHAPE Sensitivity for *In Vivo* Structure Analysis

In most instances, RNA structural analysis is performed on material either made synthetically or via *in vitro* transcription, where the amount of starting material is not a major consideration. Although *in vivo* and *ex vivo* analysis of the entire HIV-1 genome has been reported [27], this has required virus isolation from substantial culture volumes and is not readily adaptable to routine laboratory procedures. Thus, in circumstances where the amounts of biological material may be both biohazardous and limiting, methods of increasing SHAPE sensitivity that have broader applicability would be a major advantage. Efforts in this direction are summarized below.

8.1. (i) SHAPE-Seq. Approximately 1–3 pmol of RNA is usually needed to accurately map a reactivity spectrum for any given RNA molecule [69]. This limits the application of SHAPE to biological samples for which significant amounts of RNA are available. The recently-described SHAPE-Seq technology provides a means of signal intensification to address this limitation [32]. This innovative methodology, which merges SHAPE with a multiplexed hierarchical bar coding and deep sequencing strategy, is outlined schematically in Figure 6.

Initially, input RNA templates are bar-coded with a unique sequence. Such barcodes comprise tetranucleotide sequences that are placed in the 3′ structural cassette and introduced prior to *in vitro* transcription. Subsequently, these RNA templates are mixed and refolded under desired conditions. After folding, the mixture is divided into two pools, one of which is treated with modifying agent, while the second treated with a control solvent. Primer extension is subsequently performed with an end-labeled DNA primer tagged at the 5′ end with tetranucleotide "handle" sequence. This handle allows the user to distinguish between cDNA fragments derived from the positive or control reactions. Additionally, the 5′ tail of the reverse transcription primer contains an Illumina adapter necessary for paired-end sequencing. As a result, reverse transcription generates a bar-coded library of uniquely-sized cDNAs corresponding to stops at sites of adduct formation in the target RNA. The process is followed by hydrolysis of RNA and single-stranded (ss) cDNA ligation to incorporate the second Illumina adapter. Single-stranded cDNA ligation is achieved using a thermostable ligase (circLigase, Epicentre Biotechnologies, Madison, WI) and a blocking group on the 3′ end of the adapter to prevent concatemerization [70]. Finally, 9 to 12 cycles of PCR, employing primers that bind to the Illumina adapter sequences, amplify the cDNA library before multiplex paired-end deep sequencing of primer extension products. Since the RNA modification position and the identity barcode are on opposite ends of the cDNA fragments, only 50 nucleotides need to be read on each terminus. After sequencing, the reads are separated first by handle sequence, then barcode, and subsequently aligned to probed RNAs.

When compared to conventional SHAPE, SHAPE-Seq permits rapid, fully-automated analysis and eliminates the necessity for manual, time-consuming data manipulations associated with quantification of fluorescently-labeled cDNAs by capillary electrophoresis. By ligating single-stranded cDNA products with 5′ adapters followed by PCR-amplification, with minute amounts of RNA needed to generate the reactivity spectrum of a given RNA, SHAPE-Seq represents a more generally-applicable and sensitive technique studying RNA samples that are limiting, from a biohazardous source, or both. For example, it was shown for the RNase P specificity domain that with as little as 0.1 pmol

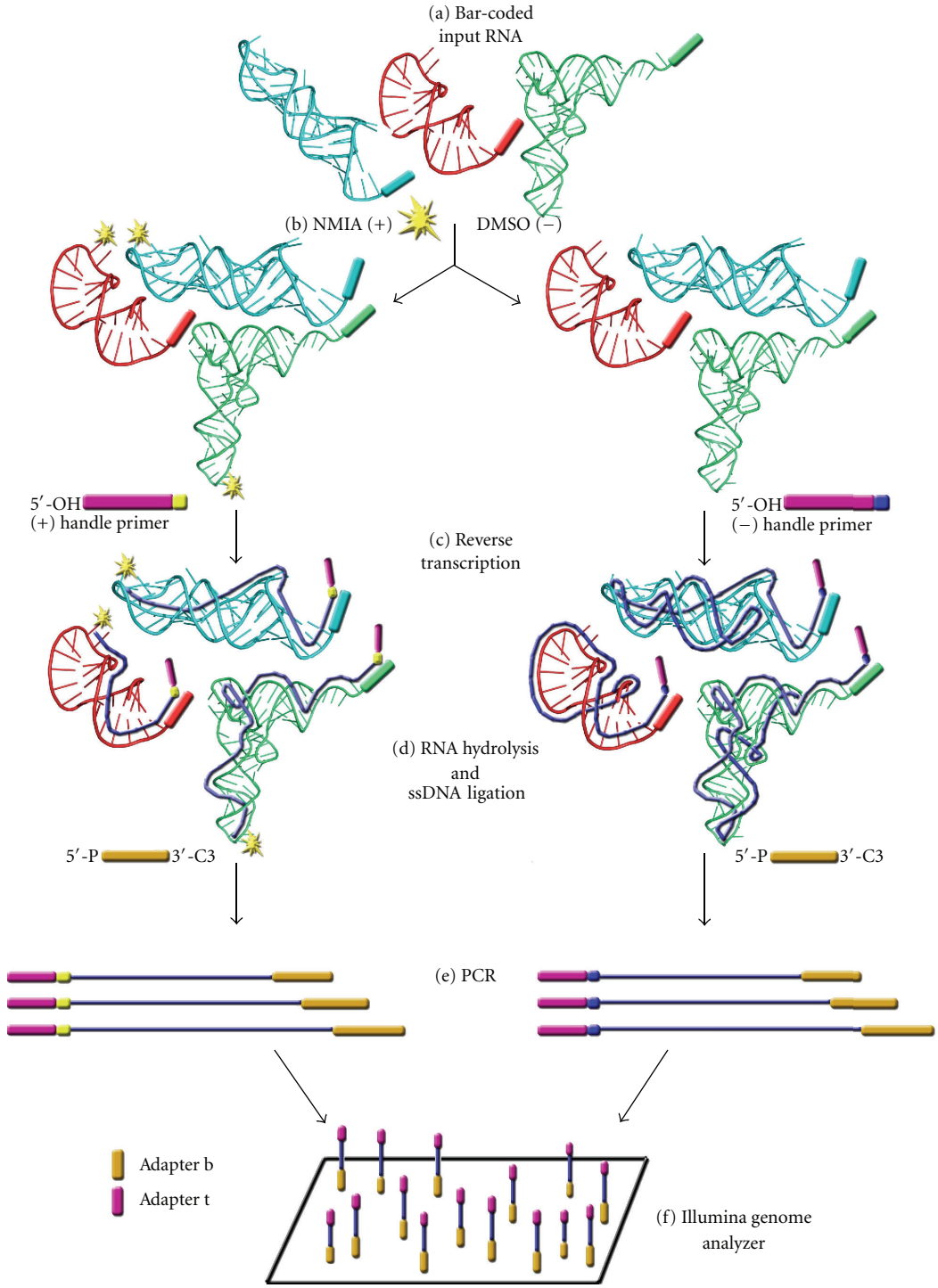

Figure 6: Summary of SHAPE-Seq methodology. (a) Input RNAs are bar-coded during *in vitro* transcription, followed by refolding under desired conditions and modification with SHAPE reagent (NMIA, 1M7). (b) The mixture is split into NMIA-treated and control pools. (c) Reverse transcription is performed with end-labeled primer containing a "handle" at the 5′ end and an Illumina adapter t. (d) The process is followed by hydrolysis of RNA and single-stranded (ss) cDNA ligation to incorporate the second Illumina adapter b. (e) After 9 to 12 cycles of PCR amplification, the cDNA library is analyzed by multiplex paired-end deep sequencing (f) adapted from [32].

of input RNA, SHAPE-Seq reactivities of over 800 bar-coded RNA species could be inferred [32]. SHAPE-Seq has the additional advantage of being able to simultaneously determine structural information from many RNAs through direct sequencing of the 3′ RNA bar codes. Although the additional steps of SHAPE-Seq, (adapter ligation, PCR amplification, sequencing) might result in decreased sensitivity to some structural effects, as has been observed for the UUCG tetraloop of RNase P, this is offset with the ability of this technique to study structural changes involving

interaction of various species within a population of RNA molecules.

8.2. (ii) Femtomole SHAPE. Using a two-color automated capillary electrophoresis with subfemtomole sensitivity, Grohman et al. [68] have recently reported *in vivo* analysis of a short portion of the formerly known XMRV genome. In contrast to earlier *in vivo* studies that required 1–3 pmole of input RNA, acylation profiles could be obtained with as little as 50 fmole aliquots of genomic RNA. As might be predicted, structural features of the XMRV leader RNA were similar to the extensively-studied counterpart Moloney murine leukemia virus, although binding sites unique to the XMRV nucleocapsid protein were proposed. More importantly, this study, which required in-house construction of a dedicated two-color capillary electrophoresis instrument, opens the exciting prospect of future functional studies on low abundance RNAs of clinical significance.

9. Future Perspectives

Rather than giving an exhaustive review of projects that have made use of SHAPE, which have included structures of wild type and mutant variants of the HIV-1 Rev response element [71], NC binding sites of the HIV-2 leader RNA [72], and RNA control of foamy virus protease activity [73], we have attempted here to highlight variations in this novel technology which facilitate interrogation of retroviral RNAs varying in size from 25–30 nt to intact, 9.5 kb retroviral genomes. The unequivocal benefit of this strategy is its ability to interrogate all four RNA bases with a single reagent, requiring thereafter simply fractionation of cDNA products. However, we should stress that SHAPE, while predictive of RNA structure, is best used with complementary genetic, phylogenetic, chemical modification (Pb^{2+} cleavage, ai-SHAPE and threading intercalators) and biophysical approaches (X-ray crystallography, NMR spectroscopy and small angle X-ray scattering). The benefits of capillary electrophoresis-based high throughput SHAPE must also be balanced by the demand this makers on the number of fluorescent oligonucleotide primers required for multiplexing, and the necessity for expensive instrumentation, features that also hold for femtomole SHAPE and SHAPE-Seq. Moreover, Kladwang and coworkers [74] compared SHAPE and crystallographic data for six RNAs and demonstrated significantly high (~20%) false negative and discovery rates, as well as several helix prediction errors, concluding that helix-by-helix confidence estimates may be critical for interpreting results from this powerful methodology. These issues notwithstanding, SHAPE should be seen as the beginning, and not the end, of an exciting path towards understanding the architecture of retroviral RNA genomes and the contribution this makes to biological function.

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Review Article

TRIM22: A Diverse and Dynamic Antiviral Protein

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The tripartite motif (TRIM) family of proteins is an evolutionarily ancient group of proteins with homologues identified in both invertebrate and vertebrate species. Human TRIM22 is one such protein that has a dynamic evolutionary history that includes gene expansion, gene loss, and strong signatures of positive selection. To date, TRIM22 has been shown to restrict the replication of a number of viruses, including encephalomyocarditis virus (EMCV), hepatitis B virus (HBV), and human immunodeficiency virus type 1 (HIV-1). In addition, TRIM22 has also been implicated in cellular differentiation and proliferation and may play a role in certain cancers and autoimmune diseases. This comprehensive paper summarizes our current understanding of TRIM22 structure and function.

1. Introduction

The TRIM gene family encodes a diverse group of proteins that are involved in many biological and antiviral processes. There are currently 100 known TRIM genes in the human genome and many of these genes are upregulated by multiple, distinct stimuli [1–3]. Historically, TRIM genes have been researched mainly for their antiviral properties; however this paradigm is changing. Two recent reports discussing the role of TRIM genes in autoimmunity and cancer highlight the importance of the TRIM family in the development of nonviral diseases [4, 5]. Many TRIM genes also have a dynamic evolutionary history and the TRIM family has been shown to undergo extensive gene duplication in both primates and teleost fish [1, 6]. In addition, several TRIM genes have experienced strong positive selection in primates [7]. Although the forces behind TRIM evolution remain unclear, it is possible that the TRIM family has evolved and continues to evolve, in response to new viral pathogens or endogenous danger signals. This paper provides an overview of the TRIM22 gene and summarizes its structure, evolution, expression, and antiviral activities.

2. Structure

TRIM proteins typically contain a conserved RBCC motif, which consists of an amino-terminal RING domain, one or two B-box domains, and a predicted coiled-coil region. Approximately 60% of TRIM proteins, including TRIM22, also contain a carboxyl-terminal domain B30.2 domain (Figure 1) [8, 9]. The RING domain of TRIM22 has homology with E3 ligases and has been shown to possess E3 ubiquitin ligase activity [9, 10]. The catalytic cysteine residues Cys15 and Cys18 are essential for this activity and mediate the transfer of ubiquitin to target proteins (Figure 1) [11, 12]. TRIM22 can also modify itself with ubiquitin which leads to proteasomal degradation [10, 11]. Interestingly, the *TRIM* family represents one of the largest groups of E3 ubiquitin ligases and E3 ligase activity seems to be crucial for *TRIM*-mediated carcinogenesis [4]. In addition, E3 ligase activity is important for many *TRIM*-mediated antiviral activities and for TRIM22, it is required for the inhibition of EMCV, HBV, and HIV-1 [11, 13, 14].

TRIM proteins typically contain one or two B-box domains, although B-box 1 is never present without B-box 2, and the two domains have different consensus sequences

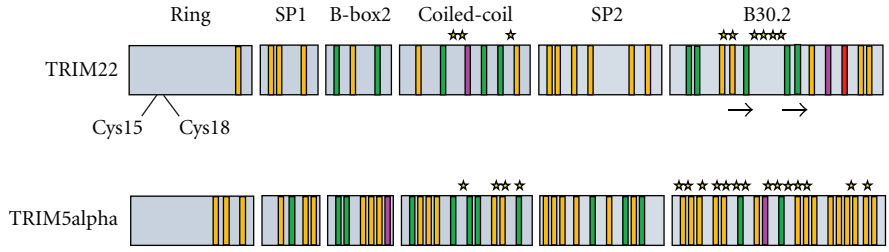

Figure 1: Structure and variability of TRIM22 and TRIM5α protein domains. TRIM22 contains an amino-terminal RING domain, one B-box domain (B-box 2), a coiled-coil region, and a carboxyl-terminal B30.2 domain (SP1 = Spacer 1 and SP2 = Spacer 2). Two cysteine residues (Cys15 and Cys18) in the RING domain are required for the E3 ligase activity of TRIM22, and a number of positively selected amino acids are found in the coiled-coil and B30.2 domains. The location and spacing of positively selected amino acids in TRIM22 are similar to those found in TRIM5α, which may reflect species-specific pathogenic pressures. The approximate location of positively selected amino acids in TRIM22 and TRIM5α is denoted with a star, and the location of the β2-β3 surface loop of TRIM22 is also indicated (arrows). Single nucleotide polymorphisms (SNPs) in the coding regions of TRIM22 and TRIM5α are shown as vertical bars, along with the type of mutation that each SNP can generate (green: nonsynonymous mutations; yellow: missense mutations; pink: frameshift mutations; red: nonsense mutations).

[15, 16]. TRIM22 contains one B-box domain (B-box 2), of which no clear function has been assigned (Figure 1). Certain B-box 2 mutations have been shown to affect viral recognition by other TRIM proteins, such as TRIM5α. Similar to TRIM22, TRIM5α has been shown to inhibit HIV-1 replication albeit at an earlier stage in the viral lifecycle. Interestingly, the human orthologue of TRIM5α only modestly inhibits HIV-1 replication whereas the rhesus orthologue of TRIM5α (rhTRIM5α) has potent anti-HIV-1 activity [17]. Several mechanisms of rhTRIM5α-mediated HIV-1 inhibition have been proposed; however, the favoured mechanism involves rhTRIM5α binding to the HIV-1 core and disruption of the normal uncoating process (reviewed in [18, 19]). For rhTRIM5α, the RING and B-box 2 domains promote its dimerization and higher-order self-association on the HIV-1 capsid [17]. It is unknown whether the B-box 2 domain of TRIM22 is required for higher-order self-association; however, it has been shown to play a role in the nuclear localization of TRIM22 [20].

The coiled-coil domain contains multiple predicted hypersecondary structures and intertwined α-helices [21]. In TRIM proteins, the coiled-coil domain is thought to promote homo-oligomerization, as its deletion prevents TRIM protein self-association [22]. Homo-oligomerization can be important for the formation of higher-molecular-weight complexes that define specific subcellular structures, such as nuclear bodies [21, 22]. Although the role of the coiled-coil region of TRIM22 remains unclear, self-association is a function of the coiled-coil region in other TRIM proteins. For example, the coiled-coil region of rhTRIM5α is required for rhTRIM5α trimerization and may be involved in the formation of cytoplasmic bodies. Importantly, rhTRIM5α trimerization is thought to drive its interaction with the HIV-1 capsid and the coiled-coil region is required for rhTRIM5α-mediated HIV-1 restriction [17, 23]. TRIM22 has also been shown to form trimers and to restrict HIV-1 replication but it is unknown whether the coiled-coil domain is required for these processes [14, 22, 24–26].

The B30.2 domain of TRIM proteins consists of two separate domains called the PRY and SPRY domains that form a putative protein-protein interaction site [27, 28]. This interaction site is likely important for the antiviral activities of TRIM22 and other TRIM proteins. Indeed, the B30.2 domain of rhTRIM5α is required for trimerization and HIV-1 restriction [23]. Three hyper-variable regions in the B30.2 domain of rhTRIM5α are thought to form the binding surface for the HIV-1 capsid protein [29]. In addition, these hypervariable regions confer the virus specificity of rhTRIM5α. The B30.2 domain of TRIM22 also contains these three hypervariable regions but their role in HIV-1 restriction has not yet been established. Similar to rhTRIM5α, the hypervariable regions in TRIM22 are highly polymorphic and contain a large number of positively selected amino acids (Figure 1) [7]. It will be interesting to learn if the B30.2 domain of TRIM22 confers specificity for targets such as viral pathogens. Notably, the B30.2 domain is required for the formation of nuclear bodies [13, 20, 30].

3. Evolution of TRIM22

The human *TRIM22* gene is located on chromosome 11, immediately adjacent to the *TRIM5*, *TRIM6,* and *TRIM34* genes [7, 31]. The origins of *TRIM22,* and the entire *TRIM5/6/22/34* gene cluster, can be traced back to the Cretaceous period, sometime after the divergence of Metatherian (marsupial) and Eutherian (placental) mammals (Figure 2). Previous studies have shown that the *TRIM5/6/22/34* locus is absent in Metatherian mammals such as opossum and chicken but presents in the major Eutherian groups containing cow, dog, and human [7]. Thus, this gene cluster must have emerged after the Metatherian-Eutherian division but before the separation of the major Eutherian groups. Taken together, this dates the birth of *TRIM22* (along with *TRIM5, TRIM6 and TRIM34*) to approximately 90–180 million years ago (Figure 2) [7].

The *TRIM5/6/22/34* gene cluster likely arose through tandem gene duplication, as these four *TRIM* genes are close human paralogs and because major gene rearrangements have been documented in this chromosomal region

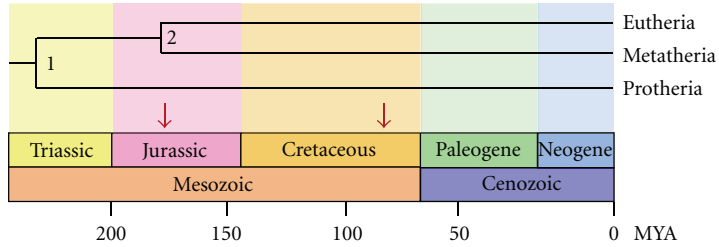

Figure 2: Timeline of Metatherian and Eutherian mammalian evolution showing the emergence of *TRIM22*. The divergence of Metatherian (marsupial) and Eutherian (placental) mammals occurred approximately 180 million years ago in the Jurassic period of the Mesozoic era. The *TRIM22* gene emerged sometime after this division, as it is absent in Metatherian mammals but present in all major Eutherian groups. In addition, since *TRIM22* is present in all Eutherian mammals, it must have emerged before further Eutherian division occurred (approximately 90 million years ago). Taken together, this dates the birth of *TRIM22* to approximately 90–180 million years ago. The predicted window of time for *TRIM22* emergence in Eutherian mammals is demarcated with two red arrows. MYA: millions of years.

[1, 7, 32]. Gene duplication plays a major role in evolution and *TRIM* genes have been shown to undergo extensive gene duplication in both primates and teleost fish [1, 6]. One of the most important outcomes of gene duplication is neofunctionalization, whereby one copy of the duplicate gene acquires a novel, beneficial function, and the other copy of the gene retains its original function [33–35]. This type of gene manipulation is a potent driver of evolution because it allows an organism to create new, potentially advantageous genes without disrupting the integrity of the original gene.

Recently, a genomic analysis of a different branch of the *TRIM* gene family identified several *TRIM* genes on chromosome 11 that have given rise to multiple *TRIM* paralogs in humans and African apes [1]. A group of 7 *TRIM* genes that are present in all Eutherian mammals (*TRIM43*, *TRIM48*, *TRIM49*, *TRIM51*, *TRIM53*, *TRIM64*, and *TRIM77*) were shown to spawn 11 new *TRIM* genes in certain primates and 6 new *TRIM* genes in humans, primarily through segmental duplications [1]. These new *TRIM* genes have presumably evolved and adapted to react against more recently emerged pathogenic threats. In addition, a Han Chinese woman with 12 new *TRIM* genes was identified, documenting for the first time *TRIM* gene copy number variation in humans [1]. Given its role in antiviral immunity, *TRIM22* probably emerged in a similar manner as a means of counteracting new viral pathogens; however the exact selective pressures giving rise to the *TRIM22* gene remain unclear.

According to a recent study, *TRIM* genes can be divided into two main groups based on their structural similarities and evolutionary properties [36]. Group 1 members have two B-box domains, have variable C-terminal domains, and are represented in both vertebrate and invertebrate species. In contrast, Group 2 members have only one B-box domain (B-box 2), are characterized by a C-terminal SPRY domain, and are found only in vertebrates. In addition, Group 2 genes are younger and smaller and evolve more rapidly than Group 1 genes [36]. Compared to some other *TRIM* genes, *TRIM22* is young and has evolved under strong positive selection, thus *TRIM22* (along with the *TRIM5/6/22/34* gene cluster) is classified as a Group 2 gene. Interestingly, the authors suggest that Group 2 genes may act as *TRIM* gene reservoirs, spawning new genes to respond to species-specific changes at the host-pathogen interface. Consistent with this interpretation, there are a number of positively selected amino acids in TRIM22 which all cluster at predicted virus interaction sites in the coiled-coil and B30.2 domains (Figure 1) [7, 36].

Within the *TRIM5/6/22/34* gene cluster, *TRIM22* and *TRIM5* have a unique evolutionary relationship. In some Eutherian groups, such as cow, there are multiple copies of the *TRIM5* gene and no *TRIM22* gene. However in others such as dog, the *TRIM22* gene is present and the *TRIM5* gene is absent [7]. In addition, the strong positive selection that each of these two genes has experienced over millions of years has occurred in a mutually exclusive manner. This type of anticorrelative pattern is probably due to genetic linkage between the two genes, whereby positive selection of an advantageous mutation in one gene indirectly leads to the selection of a linked mutation in the other [7]. The location and spacing of positively selected amino acids in TRIM22 is very similar to those found in TRIM5α (Figure 1). In both proteins, the positively selected amino acids are located in the coiled-coil and B30.2 domains, which is interesting because their amino acid sequences are actually the least similar in these regions. The majority of positively selected amino acids in TRIM22 are found within the β2-β3 surface loop of the B30.2 domain, an area that is important for HIV-1 recognition in TRIM5α (Figure 1) [7, 37, 38]. It is possible that TRIM22 and TRIM5α once possessed a similar antiretroviral mechanism, and that they evolved over time to respond to species-specific pathogenic pressures. Indeed, many studies have shown that rhesus TRIM5α, but not human TRIM5α, can potently inhibit HIV-1 replication [18, 39]. In contrast, human TRIM22 can inhibit HIV-1 replication and thus may have evolved to compensate for the loss of TRIM5α's anti-HIV function.

The *TRIM22* gene has a dynamic evolutionary history that includes gene expansion, gene loss, and strong signatures of positive selection in primates [1, 6, 7, 36]. The high number of nonsynonymous mutations found in *TRIM22*, along with its classification as a Group 2 *TRIM* gene, suggests that this gene continues to evolve at a rapid pace. Given the volatile state of other *TRIM* genes in chromosome 11, it is possible that the *TRIM5/6/22/34* gene cluster takes part in

Table 1: Summary of the localization patterns observed for TRIM22.

Localization	Pattern	Cell Type	Epitope Tag	Reference
Cytoplasm	Diffuse	293T	GFP or V5/His	[40]
	Diffuse	COS7	GFP or V5/His	[40]
	Diffuse	HeLa	Endogenous	[40]
	Diffuse with speckles/bodies	HeLa	GFP	[22]
	Diffuse	HeLa	GFP or V5/His	[40]
	Diffuse	PBMCs	Endogenous	[40]
	Diffuse with speckles/bodies	U2OS	GFP	[22]
Cytoplasm & Nucleus	Nucleoplasmic, with nuclear bodies[1]	ABC28	Endogenous	[30]
	Diffuse throughout, or nuclear bodies[2]	HeLa	EGFP	[30]
	Nucleoplasmic, with nuclear bodies	HeLa	Endogenous	[30]
	Diffuse, with cytoplasmic bodies[3]	HeLa	FLAG	[43]
	Nucleoplasmic with NB[4]	MCF7	EGFP, EYFP, or FLAG	[30]
	Nucleoplasmic, with nuclear bodies	MCF7	Endogenous	[30]
	Nucleoplasmic and cytoplasmic	T47D	Endogenous	[30]
	Diffuse with speckles[5–7]	U2OS	Endogenous	[42]
Nucleus	Aggregates/bodies	293	Myc	[41]
	Aggregates/bodies	COS7	Myc	[10]
	Diffuse with speckles/bodies	HepG2	Endogenous	[13]
	Diffuse with speckles/bodies	HepG2	Myc	[13]
	Diffuse with bodies	MCF7	FLAG	[20]

[1] Some colocalization with fibrillarin (Nucleoli).
[2] Pattern changes with cell cycle phase: (G0/G1: nuclear bodies; S-phase: nuclear speckles and cytoplasmic; mitosis: diffuse throughout cell).
[3] TRIM22 plasmid was coexpressed with Rhesus TRIM5α.
[4] Partial colocalization with Cajal bodies.
[5] Potential colocalization with calnexin (Endoplasmic reticulum).
[6] Localization was primarily cytoplasmic when cells were fixed with paraformaldehyde, or both cytoplasmic and nuclear when fixed with ice-cold methanol.
[7] Partial colocalization with the centrosome.

gene and/or segmental duplication in humans. Presumably, individuals with an increased number of these *TRIM* genes may have an augmented antiviral response and could be particularly adept at controlling retroviral infections. Similar to copy number variations, a number of single nucleotide polymorphisms (SNPs) exist in *TRIM22* that may influence its antiviral capacity or biological function for that matter. For instance, there are two documented frameshift mutations and one documented nonsense mutation in the National Center for Biotechnology Information SNP database for the *TRIM22* gene (Figure 1). If present, these SNPs would generate different truncated versions of the TRIM22 protein, which may alter its structure, E3 ubiquitin ligase activity and/or antiviral function. There are also twenty documented missense mutations in the *TRIM22* gene, the majority of which are found in its B30.2 domain (Figure 1). Many of these SNPs have the potential to impact TRIM22 function and their presence or absence may contribute to individual differences in TRIM22-mediated activities.

4. Biological Functions of TRIM22

4.1. TRIM22 Localization. There are several contradictory reports detailing the subcellular localization of TRIM22. Some reports have observed that TRIM22 localizes predominantly to the cytoplasm [22, 40] or to the nucleus [10, 13, 20, 41], whereas other reports have observed that TRIM22 can localize to both the cytoplasm and the nucleus (Table 1) [30, 42, 43]. The pattern of localization also varied between diffuse, speckled, and aggregated. A number of explanations have been given in the literature for the differences in localization, including whether the expression was endogenous (e.g., IFN-treatment) or exogenous (e.g., overexpression). In addition, the method of fixation and the type of epitope tag used for detection have also been reported to affect the localization pattern. Given the diverse range of cell lines used in these studies, it is also possible that cell type-specific factors influence the localization of TRIM22.

A number of determinants affecting TRIM22 localization have been identified. A bipartite nuclear localization signal (NLS) located in the Spacer 2 domain of TRIM22 was shown to be necessary, but not sufficient, for nuclear localization [20]. Although there are no known NLSs present in the B30.2/SPRY domain, several groups have shown that this domain is required for nuclear localization [13, 20, 40, 41]. More specifically, Val 493 and Cys 494 of the B30.2 domain were shown to be critical for nuclear localization and the formation of nuclear bodies [20]. In an independent study, amino acids Ser 395, Lys 396, and Ser 400 located in variable loops 1 and 3 of the B30.2 domain were shown to be important for certain localization patterns of TRIM22 [40].

In some cell types, TRIM22 localizes in the nucleus as punctate bodies, which have been shown to partially colocalize with Cajal bodies [20]. Cajal bodies play important roles in RNA processing and modification as well as in cell cycle progression [44]. TRIM22 also interacts with p80-coilin, which is a major component of Cajal bodies. Similar to Cajal bodies, TRIM22 localization has been shown to change during the cell cycle. In G0/G1 TRIM22 localizes

in nuclear bodies, in S-phase it localizes in a more diffuse and speckled pattern throughout the nucleus, and during mitosis it assumes a diffuse pattern in both the nucleus and cytoplasm [30]. In an independent study, TRIM22 was shown to colocalize with the centrosome independently of the cell cycle and also with vimentin-containing aggresome-like structures next to the endoplasmic reticulum [42]. From these data, it appears that multiple factors influence the localization of TRIM22, possibly indicating that TRIM22 has several biological roles.

4.2. Antiviral Function of TRIM22. Several reports including published transcriptional profiling datasets (e.g., GDS1096, GDS3113, and GDS596) deposited in the Gene Expression Omnibus database repository (http://www.ncbi.nlm.nih.gov/gds) show that TRIM22 is ubiquitously expressed in several human tissues and is highly upregulated in response to Type I and II interferons (Table 2) [7, 13, 14, 24, 25, 45–50]. Interestingly, the 5′ flanking region of the *TRIM22* gene contains two regions matching the consensus sequence for an IFN-stimulating response element (ISRE) and a third region matching that for an IFN-γ activation site (GAS); however ISRE1 or GAS is not required for IFN-γ induction of TRIM22. In contrast, the ISRE2 plus six upstream nucleotides (extended ISRE) is capable of binding IFN regulatory factor 1 (IRF1) in a manner dependent on the chromatin remodelling enzyme Brahma-related gene 1 (BRG1) [48, 49]. Furthermore, this extended ISRE appears to be important for both stimulation by IFN-α and IFN-γ as well as for basal TRIM22 expression [48]. The significant upregulation of TRIM22 in response to IFNs, together with the finding that TRIM22 has evolved under strong positive selection for millions of years, suggests that TRIM22 plays an important fundamental role in cell biology. To date, several lines of evidence suggest that this role is as an antiviral factor.

Human TRIM22 was first discovered by Tissot and Mechti in 1995 during a search for IFN-induced genes in Daudi cells, where exogenous expression of TRIM22 was shown to downregulate transcription from the HIV-1 LTR [45]. Although this was performed using a luciferase reporter gene under the transcriptional control of the HIV-1 LTR and not in the context of the entire HIV-1 proviral genome, it provided the first evidence suggesting that TRIM22 blocks HIV-1 transcription and replication. In 2006, Bouazzaoui et al. showed that TRIM22 was highly upregulated in primary monocyte-derived macrophages (MDMs) in response to HIV-1 infection, IFNα treatment, or stimulation with lipopolysaccharide (LPS). They provided the first evidence that TRIM22 can restrict HIV-1 replication *in vitro* by showing that exogenous expression of TRIM22 inhibited HIV-1 infection by 50–90% in 293T cells modified to express the CD4 and CCR5 receptors and in primary MDM. Furthermore, cotransfection of TRIM22 with a three-plasmid system for replication-defective HIV-1 resulted in reduced infectious titres of pseudotyped virus, suggesting that TRIM22 inhibited a late stage of HIV-1 pseudoparticle production and/or subsequent infection with the pseudotyped virus [24].

In 2008, Barr et al. showed that TRIM22 was an integral part of the Type I interferon-induced inhibition of HIV-1 replication and provided the first mechanistic data for the inhibition of HIV-1 replication by TRIM22. TRIM22 expression in several human cell lines potently inhibited HIV-1 replication, and interestingly, analysis of Gag production in those cells revealed that TRIM22 may inhibit HIV-1 replication by two different mechanisms. In the HOS and HeLa cell lines, TRIM22 inhibited HIV-1 particle production by interfering with the trafficking of the Gag polyprotein to the plasma membrane. Since TRIM22 and Gag proteins interact, and that the E3 ligase activity of TRIM22 is required for this restriction [14], it is possible that TRIM22 posttranslationally modifies Gag, resulting in altered Gag trafficking to the plasma membrane. In the U2OS and 143B cell lines, TRIM22 inhibited HIV-1 particle production by inhibiting the accumulation of intracellular Gag protein [14]. Although no mechanism of restriction was identified in U2OS or 143B cells, several possibilities could explain the decrease in intracellular Gag protein levels, including inhibition of transcription from the LTR as previously suggested [25, 45], or degradation of the Gag RNA and/or polyprotein. Given that TRIM22 exhibits cell type-specific differences in localization (as discussed earlier), it is likely that the mechanism of TRIM22-induced restriction of HIV-1 particle production is cell type-specific and/or dependent on the subcellular localization of TRIM22. Future experiments are required to further elucidate the mechanism of TRIM22-induced inhibition of HIV-1 particle production (Figure 3).

TRIM22 was also independently identified and shown to inhibit HIV-1 replication by several laboratories [25]. Following observations made by Franzoso et al. in 1994 that clones of the U937 promonocytic cell line were either permissive or nonpermissive to HIV-1 replication, Kajaste-Rudnitski et al. (2011) identified *TRIM22* as the only known restriction factor that was expressed in the nonpermissive and absent from the permissive U937 cells. Using a luciferase reporter plasmid under the control of the HIV-1 LTR, they showed that LTR-mediated transcription was decreased 7–10-fold in nonpermissive clones. They also showed that by knocking down *TRIM22* expression in nonpermissive cells, the levels of transcription from the LTR approached those observed in permissive cells. Exogenous expression of TRIM22 in permissive clones also decreased LTR transcription to levels comparable to those observed in nonpermissive clones. Further investigation revealed that TRIM22 inhibited basal and phorbol myristate acetate-ionomycin-induced HIV-1 transcription. These effects were independent of NFκB, HIV-1 Tat and the E3 ubiquitin ligase activity of TRIM22 [25]. It is important to note that all direct evidence showing that TRIM22 inhibits HIV-1 transcription has been through the use of LTR-driven reporter constructs. It will be important to test the effects of TRIM22 on HIV-1 LTR transcription in the context of full-length replication-competent HIV-1.

In 2011, Singh et al. provided the first clinically relevant evidence supporting a role for TRIM22 as an anti-HIV-1 effector *in vivo*. They showed that expression of

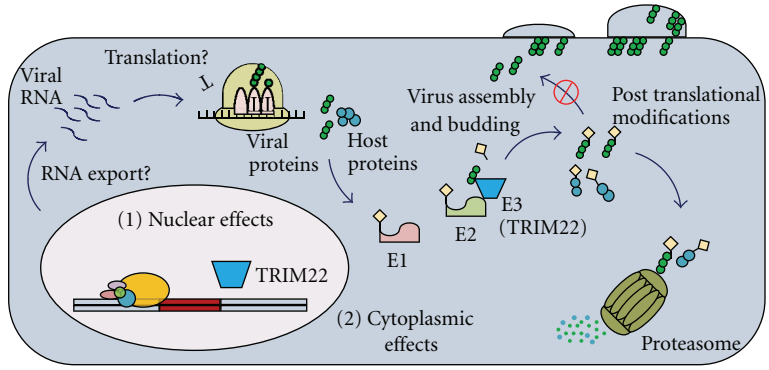

Figure 3: Possible mechanisms of TRIM22 antiviral functions. Based on current reports, TRIM22 can inhibit viral replication through nuclear-associated effects such as inhibiting viral transcription. Although not investigated to date, RNA export and translation are also potential targets of TRIM22. Given its E3 ligase activity, TRIM22 may posttranslationally modify host or viral proteins that are required for viral assembly and/or budding. Posttranslational modifications occur when an E1 activating enzyme (E1), E2 conjugating enzyme (E2), and E3 ligase protein (E3) work together to transfer ubiquitin or ubiquitin-like molecules to a target protein. These modifications could target the protein for proteasomal degradation or alter its subcellular localization or ability to interact with other proteins or DNA.

TRIM22 in peripheral blood mononuclear cells (PBMCs) of HIV-1-infected individuals was significantly increased in patients after HIV-1 infection. Importantly, infected patients expressing higher *TRIM22* levels exhibited significantly lower viral loads and significantly higher CD4+ T-cell counts [26]. These findings are quite significant, as this suggests that TRIM22 has a potential effect on the severity and/or progression of HIV-1 infection. Additional research on the role of TRIM22 during primary infection will be important to provide a greater understanding of the effects TRIM22 may have on HIV-1 replication *in vivo*.

The antiviral activities of TRIM22 are not limited to HIV-1. In 2009, Eldin et al. identified TRIM22 as a potent inhibitor of encephalomyocarditis virus (EMCV) replication. TRIM22 was shown to interact with the EMCV 3C protease via the C-terminal domain of TRIM22, and expression of TRIM22 corresponded with increased ubiquitination of the 3C protease (Figure 3). 3C protease is essential for successful viral replication and has several roles, including processing of the viral polyprotein and inhibition of the host immune defences [11]. There are also reports that TRIM22 may play an important role in protecting the liver from viral pathogens. In 2009, Gao et al. reported that TRIM22 is highly upregulated in response to type I or II IFN in the hepatocellular carcinoma cell line HepG2. Cotransfection of plasmids encoding TRIM22 and replication-competent hepatitis B virus (HBV) inhibited the accumulation of HBV antigens in the supernatants of cells and significantly reduced levels of intracellular HBV RNA and DNA replication intermediates. Similar results were observed in the sera of mice during codelivery of plasmids to mouse livers, showing that TRIM22 can restrict HBV infection in an *in vivo* system. Using a luciferase reporter plasmid, they showed that TRIM22 downregulates expression from the HBV core promoter (Figure 3). This mechanism of action was dependent on the nuclear localization of TRIM22 and its E3 ubiquitin ligase activity [13]. Although there is no direct evidence for a protective role of TRIM22 against HBV in primates, *TRIM22* expression is significantly upregulated during clearance of HBV in chimpanzees [51]. Moreover, TRIM22 expression is significantly upregulated during clearance of hepatitis C virus (HCV) in chimpanzees [52]. These findings are paralleled in human infections, as *TRIM22* is significantly upregulated in cirrhotic liver from HCV patients and patients with mild chronic HCV infection and no fibrosis [53]. Further research is needed to assess the role of TRIM22 in inhibiting HBV and HCV *in vivo*.

In further support of the notion that TRIM22 is involved in the host antiviral response, TRIM22 expression is modulated in response to several other viruses and viral antigens (Table 2). TRIM22 expression is upregulated in response to infection with rubella virus [54] and Epstein-Barr virus (EBV) [55] and downregulated during infection with human papillomavirus type 31 [56]. A couple intriguing reports elude to the possibility that TRIM22 may also contribute to viral latency. Exogenous expression of TRIM22 significantly upregulates expression of the EBV latent membrane protein 1 (LMP-1) [55]. LMP-1 is required for latency during EBV infection and appears to induce an antiviral state by upregulating expression of several ISGs via an IFN- and STAT1-independent mechanism. The Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen (LANA) also activates several ISGs including TRIM22, which was shown to be upregulated by LANA both in culture and in tissues from KSHV lesions. LANA also repressed transcription from the HIV-1 LTR, an NFκB consensus sequence, and the SV40 promoter [57]. Furthermore, TRIM22 is expressed in resting T cells, which are known reservoirs of latent HIV-1, and is strongly repressed during T-cell activation [47]. Although much more research is needed to directly implicate TRIM22 in viral latency, it is tempting to hypothesize that TRIM22 contributes to viral latency.

4.3. Other Functions of TRIM22. Several reports in the literature suggest that TRIM22 may have a role in other biological processes, such as cell differentiation and proliferation. One

Table 2: Summary of factors that alter TRIM22 expression.

Stimulation	Change	Tissue	Reference
Cytokines			
IFN-α	increase	CEM, Jurkat, and THP-1 cells	[26]
IFN-α	increase	H9 cells	[47]
IFN-α	increase	HepG2 cells	[13]
IFN-α	increase	Primary MDM	[24]
IFN-α	increase	U937	[25]
IFN-α	increase	U-937-4 cells	[46]
IFN-α/β	increase	Daudi, and HeLa cells	[45]
IFN-β	increase	HOS cells	[14]
IFN-γ	increase	HeLa cells	[30, 45]
IFN-γ	increase	HepG2 cells	[13, 48, 49]
IFN-γ	increase	MCF7 cells	[30]
IL-1-β	increase	Coronary artery endothelium	[58]
IL-2	increase	CD4+, CD8+, NK cells	[50]
IL-15	increase	CD4+, CD8+, NK cells	[50]
Progesterone	increase	ABC28, and T47D cells	[30]
TNF-α	increase	Coronary artery endothelium	[58]
Antigens/Infections			
EBV infection[1]	increase	BL41-EBV cells[1]	[55]
EBV LMP-1	increase	DG75 cells	[55]
Hepatitis B virus infection[2]	increase	Liver tissue[2]	[51]
Hepatitis C virus infection[2]	increase	Liver tissue[2]	[52]
Hepatitis C virus infection	increase	Liver tissue	[53]
HIV-1 infection	increase	Immature DC	[55]
HIV-1 infection	increase	Primary MDM	[24]
HIV-1 infection	increase	Primary PBMCs	[26]
HIV-1 Tat	increase	Immature DC	[55]
HPV infection	decrease	Human keratinocytes	[56]
KSHV infection	increase	KSHV lesion	[57]
KSHV LANA	increase	BJAB cells	[57]
LPS	increase	Primary MDM	[24]
Rubella virus infection	increase	ECV304 cells	[54]
Activation/Differentiation/Cell Cycle			
1α,25-dihydroxyvitamine D3[3]	increase	Primary MDM	[24]
Anti-CD2	increase	Primary T cells	[47]
Anti-CD2/CD28	decrease	Primary T cells	[47]
Anti-CD2/CD28/CD3	decrease	CD4+, CD8+, NK cells[4]	[50]
All-trans retinoic acid	increase	HL60 and NB4 cells	[46]
All-trans retinoic acid	increase	Primary MDM	[24]
p53	increase	K562 and U-937-4 cells[5]	[46]
p73	increase	U-937-4 cells	[46]
Pioglitazone	increase	Primary MDM	[24]
UV-irradiation[6]	increase	MCF-7 cells	[46]
Disease			
SLE	decrease	CD4+ T cells from SLE patient	[59]

Table 2: Continued.

Stimulation	Change	Tissue	Reference
Wilms tumor	decrease	Tumor tissue	[60, 61]

[1] BL41 cells that are latently infected with EBV.
[2] From infected chimpanzees.
[3] Hormonally active form of Vitamin D.
[4] Only reached significance in CD8+ cells.
[5] Cells lack endogenous p53 but stably express a plasmid encoding p53 under control of a temperature-sensitive promoter. Cells were grown at the permissive temperature (32°C) to induce p53 expression.
[6] UV-irradiation induces p53 expression.

group showed that the expression of TRIM22 is directly activated by p53 in myeloid cells via a functional p53-response element in intron 1 of the *TRIM22* gene [46]. They also showed that the p53-family member p73 can bind to this response element and activate *TRIM22* expression [46]. Since p73 has been linked to the differentiation of leukemic cells [62], the authors speculated that TRIM22 may be involved in cell differentiation. Another group reported that TRIM22 expression is significantly upregulated during differentiation of the promyelocytic cell line NB4 [63]. They also showed that TRIM22 expression is high in monocytes and early granulocytes but decreases in the lymphocyte and late granulocyte populations and is undetectable in erythroid cells [63]. Obad et al. (2004) provided the first direct evidence supporting an antiproliferative role for TRIM22 by showing that overexpression of TRIM22 in the promonocytic cell line U937 resulted in decreased clonogenic growth [46]. An inverse correlation between TRIM22 expression and cell differentiation has also been reported, showing that TRIM22 is highly expressed in human immature $CD34^+$ bone marrow progenitor cells, but declines in mature populations [63]. Despite the correlations of TRIM22 expression levels with cell differentiation and proliferation, the evidence lacks key experiments such as loss-of-function studies (i.e., TRIM22 knockdown) to conclusively implicate TRIM22 as a key player in any of these processes.

A couple of reports have associated TRIM22 with human disease. Downregulation of TRIM22 expression is associated with progression, relapse and increased mortality in cases of Wilms tumor [60, 61]. Although TRIM22 is a p53-responsive gene and may promote cell-cycle arrest [46], its role in tumour development and progression, including Wilms tumor, is yet to be determined. The involvement of TRIM proteins in cancer is not unprecedented. TRIM13, 24, and 29, which are also involved in p53 regulation, have also been implicated as important regulators for carcinogenesis. Moreover, TRIM19/PML may act as a tumour suppressor protein (reviewed in [4]). TRIM22 expression is also down-regulated in CD4+ T cells from patients with active systemic lupus erythematosus (SLE) [59]. Although it is also unclear what role TRIM22 plays in this disease, it is notable that several other TRIM proteins, including TRIM 21, 25, 56, and 68, have been linked to SLE and other autoimmune diseases [5]. It will be interesting to learn more about the role (if any) TRIM22 plays in these and other human diseases.

Although it is clear that TRIM22 is an exciting and dynamic protein, it appears that we have only begun to understand its role in cellular biology and antiviral immunity. A rich evolutionary history, together with its potential involvement in numerous biological processes, suggests that TRIM22 is an important and multifarious protein. Despite its importance, the function of TRIM22 remains poorly understood, and a number of issues will need to be addressed in future research. One discrepancy that needs clarification is the disparate observations and contradictory reports surrounding TRIM22 subcellular localization. In particular, we need to understand why TRIM22 localization is so heterogeneous, as this may provide useful insight into its biological function. Another priority will be to consolidate previous reports on the antiviral mechanism of TRIM22. In the case of HIV-1, it will be important to determine the stage(s) of the virus lifecycle that TRIM22 targets. In this regard, future studies that identify the host and/or virus targets of TRIM22 will be extremely useful. In addition, it will be interesting to discover if TRIM22 has antiviral activity against additional viruses, and to determine the role it plays in other nonviral diseases. Overall, its breadth of involvement in antiviral immunity, combined with the range of possible mechanisms by which TRIM22 acts, presents a number of exciting research opportunities. Future work on TRIM22 will help us understand this important player in the host antiviral response and contribute to our knowledge of host-pathogen interactions.

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