Review Article

Lysine Acetylation: Elucidating the Components of an Emerging Global Signaling Pathway in Trypanosomes

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Received 17 April 2012; Revised 20 July 2012; Accepted 30 July 2012

Academic Editor: Andrea Silvana Rópolo

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In the past ten years the number of acetylated proteins reported in literature grew exponentially. Several authors have proposed that acetylation might be a key component in most eukaryotic signaling pathways, as important as phosphorylation. The enzymes involved in this process are starting to emerge; acetyltransferases and deacetylases are found inside and outside the nuclear compartment and have different regulatory functions. In trypanosomatids several of these enzymes have been described and are postulated to be novel antiparasitic targets for the rational design of drugs. In this paper we overview the most important known acetylated proteins and the advances made in the identification of new acetylated proteins using high-resolution mass spectrometry. Also, we summarize what is known so far about the acetyltransferases and deacetylases in eukaryotes, focusing on trypanosomes and their potential use as chemotherapeutic targets.

1. Introduction

Lysine (K) acetylation is a reversible and highly regulated posttranslational modification (PTM) involved in a wide range of cellular processes in eukaryotes. It has been proposed that acetylation might have the same relevance as phosphorylation, although no acetylation cascade has been reported to date [1]. During the past four decades lysine acetylation was associated with important roles in the regulation of nuclear transcription. Recently, proteome-wide analyses revealed a large number of acetylated proteins in the cytoplasm and the mitochondria, including most of the enzymes involved in the intermediate metabolism. These findings suggest a central role for an acetylation-related regulatory mechanism in cell development inside and outside the nucleus [2].

Lysine acetylation is catalyzed by acetyltransferases, which transfer the acetyl group of acetyl CoA to the ϵ -amino group of an internal lysine residue. The reverse reaction is catalyzed by deacetylases. Both types of enzymes are usually part of protein complexes and were named histone

acetyltransferases (HATs) and histone deacetylases (HDACs) because they were initially found to target histones. Later on they were shown to target nonhistone proteins as well. Allis et al. proposed in 2007 that these names should be changed to lysine acetyltransferases (KATs) and lysine deacetylases (KDACs), names that better correlate with the broader substrate specificity of these enzymes [3].

Trypanosoma cruzi, *Trypanosoma brucei*, and *Leishmania* spp. (named collectively Trityps) are trypanosomatid parasites (order Kinetoplastida) that cause millions of deaths in tropical and subtropical regions of the world [4]. Trypanosomatids branched early from the eukaryotic lineage, and this is reflected in some unique characteristics like polycistronic transcription, transsplicing, and the involvement of RNA polymerase I in the transcription of certain protein coding genes [5]. They exhibit complex life cycles, with different developmental stages alternating between vertebrate and invertebrate hosts [6–8].

Trypanosoma brucei is transmitted to humans through the bite of tse-tse flies (*Glossina* spp.) and is responsible for african sleeping sickness. In mammals, the parasite survives

free in the bloodstream and is able to evade the host immune response through antigenic variation [7]. *Trypanosoma cruzi* is transmitted through hematophagous insect vectors of the Reduviidae and Hemiptera families and is the causative agent of Chagas disease. Once in the mammalian host, it multiplies intracellularly (amastigote stage) and differentiates into the nonreplicative infective form present in the bloodstream (trypomastigote stage) [9]. *Leishmania* spp. is transmitted by the bite of sand flies (Phlebotomies) and has two main life cycle morphologies: the intracellular amastigote in the mammalian host and the promastigote in the fly. Clinical symptoms of leishmaniasis vary from cutaneous and mucocutaneous to visceral depending on the species [8].

The aim of this paper is to describe the general bases of lysine acetylation as well as the enzymes responsible for this modification, focusing on trypanosomatids. Additionally, we describe the possible use of lysine acetyltransferases and deacetylases as chemotherapeutic targets for parasitic diseases.

2. Acetylation: A General Posttranslational Modification

2.1. Acetylation of Histone and Nonhistone Proteins. Lysine acetylation was initially discovered as a posttranslational modification of histones in the 1960s [10, 11]. Histone N-terminal tails are extended from the core of the nucleosomes and are modified by acetylation and other posttranslational modifications such as phosphorylation, methylation, ubiquitination, and sumoylation. These PTMs can alter DNA-histone interactions or the binding of other proteins, such as transcription factors to chromatin. It has been suggested that distinct modifications present on histone tails may act sequentially or in combination to form a code. This “histone code” is read by other proteins or protein modules and determines a variety of nuclear events [12]. Histone acetylation occurs on the ϵ -amino groups of specific lysine residues and has emerged as a modification of major regulatory significance. This chromatin-based mechanism modulates the accessibility of genetic information during transcription, DNA replication, recombination, and repair [13]. The addition of an acetyl group neutralizes the positive charge and changes the overall size of the modified amino acid along with the local hydrophobicity of the protein. These variations have a significant impact on the conformation and function (e.g., its enzymatic activity) of the polypeptide. Acetylation on lysine residues can also generate docking sites for the binding of proteins, a topic that will be discussed later in this paper [14].

Chromatin in trypanosomatids is less condensed than in most eukaryotic cells and appears to be organized in 10 nm filaments [24–26]. These organisms have several copies of histones genes (H1, H2A, H2B, H3, and H4). However, they are extremely divergent from those found in other eukaryotes [27]. Numerous histone PTMs were reported in trypanosomatids. In general terms, *T. cruzi* histones H4 and H2A are mainly acetylated, whereas histones H3 and H2B are methylated. Histone H4 modifications have been extensively

studied in *T. cruzi*, lysine residues 4, 10, 14, and 54 are acetylated, K18 is monomethylated and arginine at position 53 is dimethylated [28]. In *T. brucei*, histones H4 K2 and K5 are also acetylated [29, 30]. The Trytryps genomes contain coding sequences for methyltransferases, acetyltransferases and deacetylases that might be responsible for the modifications of histone N-terminal domains in these organisms [31]. Apparently, in trypanosomatids different functional states of chromatin correlate with specific posttranslational histone modifications and histone variants. It has been demonstrated that there is a direct correlation between certain PTMs and transcriptional initiation/termination, cell cycle progression, and telomeric silencing [32].

Several authors went further with the characterization of histones PTMs and described in some detail the implications of acetylation on trypanosomatids histones. In *T. cruzi*, histone H4 acetylated at lysine 4 (H4K4ac) was detected in densely packed chromatin, whereas acetylation at lysines 10 and 14 (H4K10ac, H4K14ac) was present in less condensed zones. Acetylation at K10 and K14 of histone H4 increases during the rupture of the DNA double strand, which suggests an important role for this PTM in chromatin organization. In addition, H4K4ac levels decrease in the nonreplicative forms of the parasite (trypomastigotes), whereas H4K10ac and H4K14ac levels remain unchanged. This indicates that K4 might be involved in the synthesis of new chromatin during replication [33]. Although trypanosomatids histones are quite divergent from their eukaryotic counterparts, mainly in the regions that are subjected to PTMs, epigenetic regulation seems to be very important in these organisms. In fact, since Trytryps lack canonical promoter-driven transcriptional regulation, epigenetic regulation is proposed to be the major mechanisms for global transcriptional control in these organisms.

To examine the genome-wide distribution of chromatin components, chromatin immunoprecipitation and sequencing (ChIP-seq) experiments were performed in *T. brucei* and *L. major*. Siegel et al. showed that in *T. brucei* H4K10ac is enriched at polymerase II transcription start sites. The authors also reported that histone variants H2AZ and H2BV might be associated with the transcription process [37]. Thomas et al. showed that protein-coding genes in *L. major* contain acetylated histone H3 at the origins of polycistronic transcription [38]. Thus, acetylation of histone tails correlates with an increment in transcription rates. PTMs and the presence of canonical histone variants might act as chromatin signals indicating transcription start and termination points in trypanosomatids. These findings suggest that histone modifications play crucial roles in transcription initiation and termination in trypanosomes and that destabilization of nucleosomes by histone variants is an ancient and general mechanism of transcription initiation.

Acetylation of other nuclear proteins such as the High Mobility Group (HMG) proteins was reported in the past [39]. In *T. cruzi*, HMG proteins were described [40] and a High Mobility Group B (*Tc*HMGB) family member was recently characterized [41]. *Tc*HMGB has orthologues in *T. brucei* and *Leishmania*; all of them display two HMG box domains and lack the acidic C-terminal tail characteristic

TABLE 1: Acetylomes from different organisms.

	Number of acetylation sites	Number of acetylated proteins	Reference
HeLa cells and mouse liver mitochondria	388	195 (37 from the cytosolic fraction, 38 from the nuclear fraction and 133 from mitochondria)	Kim et al., 2006 [15]
<i>E. coli</i>	125	84	Yu et al., 2008 [16]
<i>E. coli</i>	138	91	Zhang et al., 2009 [17]
MV4-11 cells, A549 cells and Jurkat cells	more than 3600	1750	Choudhary et al., 2009 [18]
Human liver	1300	1047	Zhao et al., 2010 [19]
<i>S. enterica</i>	235	191	Wang et al., 2010 [20]
<i>D. melanogaster</i>	1981		Weinert et al., 2011 [21]
<i>A. thaliana</i>	91	74	Finkemeier et al., 2011 [22]
<i>T. gondii</i>	411	274	Jeffers and Sullivan Jr., 2012 [23]

of mammalian HMGBs. Instead, Tritryps HMGBs display a unique N-terminal tail of 110 amino acids. In other species, the acetylation status of HMGB proteins plays a crucial role since it can alter their function and location. Also, it seems to be a signal for extranuclear localization of the proteins as well [42–45]. TcHMGB has numerous lysine residues that are predicted to be acetylated, and seven of them are present in the predicted nuclear localization signal (NLS) on its aminoterminal domain [41].

Alpha-tubulin was the first nonnuclear protein described to be acetylated; however, the function of this PTM is not completely understood yet. In 1985, L'Hernault and Rosenbaum reported that chlamydomonas axonemal microtubules contained acetylated α -tubulin. These authors proposed that reversible tubulin acetylation might control the assembly and disassembly of axonemal microtubules [52–54]. Microtubules (MTs) are dynamic cytoskeletal filaments composed of α/β tubulin heterodimers. These structures play key roles in diverse cellular functions including structural support, organelle localization, chromosome segregation, and intracellular trafficking. Besides acetylation, microtubules may undergo other posttranslational modifications, which are proposed to generate a code similar to the histone code. It is thought that microtubule-associated proteins might read this code and regulate the MTs structure [55]. Initially, it was thought that MT stabilization was a consequence of MT acetylation [56, 57]. Later, several authors demonstrated that acetylation of MTs does not necessarily affect their stability [58, 59].

In trypanosomatids, where several tubulin PTMs have been described, acetylation of α -tubulin at lysine 40 is particularly well characterized. In Tritryps acetylated α -tubulin is found in the subpellicular microtubules that run along the inner surface of the parasites. This array of MT plays an essential role in the maintenance of the parasites shape and is remodelled during the differentiation process throughout its life cycle [60–62]. This PTM is also present in the axoneme of the flagellum and in the ephemeral microtubules of the mitotic spindle of *T. brucei* [63]. The presence of acetylated tubulin in early mitotic spindles in several organisms and the fact that protozoa like *T. brucei*

and *T. cruzi* exhibits global α -tubulin acetylation reinforces the idea that this PTM is not restricted to stable MTs.

2.2. Acetylation versus Phosphorylation. Lysine acetylation is found in all domains of life from bacteria to humans, but small progress has been made towards understanding the exact biological role of this modification. The data provided by proteome-wide analyses helps to generate new hypotheses that will increase the current understanding of lysine acetylation in a biological context. In the last ten years, several authors using high-resolution mass spectrometry identified thousands of acetylated proteins in different organisms. The proteins identified are involved in: transcription, DNA repair, chromatin remodeling, cell cycle, splicing, metabolism, cytoskeletal dynamics, apoptosis, nuclear import, protein folding, and cellular signaling among others [15–22, 64, 65] (Table 1). All this evidence suggest that the size of the “lysine acetylome” is comparable to that of the “phosphoproteome” [1, 2, 15–22, 64, 65]. Kouzarides predicted in 2000 that acetylation might rival phosphorylation as a regulator of cellular processes [1]. Even though evidence clearly shows that acetylation is widely spread in all domains of life, the philosophy of acetylation clearly differs to that of phosphorylation. In contrast to what happens with phosphorylation, an acetylation cascades do not exist. Some acetyltransferases and deacetylases were described to be acetylated themselves; however, no acetylating enzymes able to catalyze the transfer of acetyl groups from one protein to another have been described and they are not supposed to exist either. Another interesting fact is that the number of acetyltransferases and deacetylases in eukaryotes is significantly lower than that of kinases and phosphatases. These observations suggest that the acetylation-dependant regulation is much more global than phosphorylation.

Currently there is an increasing interest for acetylation in protozoa, even though information about this topic is still scattered. Until now, the most studied acetylated proteins in these organisms were histones and tubulin; however, the first proteome-wide analysis of acetylated proteins in a protozoan organism was published this year. *Toxoplasma gondii* acetylome showed that this PTM is abundant in this

parasite; 411 lysine acetylation sites were detected across 274 tachyzoite proteins. The known acetylated marks on histones and α -tubulin were detected and also a wide variety of acetylation sites in additional proteins. The majority of acetylation occurs on proteins involved in metabolism, translation, stress response, and chromatin biology [23]. An unpublished preliminary characterization performed by western blot indicates that acetylation is as widespread in *T. cruzi* as it is in *T. gondii* (unpublished results from our lab).

Finally we would like to mention bromodomains, the only known protein modules capable of recognizing acetyl lysine residues. This association regulates protein-protein interactions similar to how SH2 (Src homology 2) and PTB (phosphotyrosine-binding) modules bind to and regulate phosphotyrosine-containing proteins [66]. There are several coding sequences for bromodomain-containing factors in the *Trypanosoma* genomes [31]. Bromodomain factor 2 from *T. cruzi* (*TcBDF2*) has been characterized. It displays a nuclear localization in all life cycle stages and associates with acetylated histones H2A and H4 (having preference for H4K10ac, and probably H4K14ac residues). *TcBDF2* accumulation in epimastigotes after UV irradiation suggests a possible role for this protein in DNA repair [67]. Two more nonnuclear BDFs are currently under investigation. The presence of several putative bromodomain-containing proteins in *Trypanosoma* with different subcellular localizations supports the idea that these early-branched eukaryotes have established a network capable of recognizing lysine acetylation signals.

3. Lysine Acetyltransferases

Histone acetyltransferases (HATs) or lysine acetyltransferases (KATs) are evolutionarily conserved from yeast to humans although there is little similarity between the protein sequences of different members of this group. Many of them contain acetyl-CoA-binding domains as well as bromodomains and they form multisubunit complexes [68]. They are diverse in structure, substrate specificity, and function. Also, their classification is less clear in contrast to what happens with KDACs [65], which will be addressed later.

KATs can be classified in two groups or general classes (A type and B type), depending on their intracellular localization and substrate specificity. A-type KATs are mainly nuclear and are involved in transcription-related acetylation as well as modification of core histones. These acetyltransferases can be further grouped into three main families (GNAT, p300/CBP, and MYST), based on sequence conservation of the HAT domain and biological functions. B-type KATs have a cytoplasmatic localization, where they acetylate free histone substrates and promote their nuclear localization. KAT families have high-sequence similarity within them but poor to no sequence similarity between any two given families [69].

The first KATs reported fifteen years ago were yeast Hat1 (histone acetyltransferase 1) and Gcn5 (general control nonrepressed 5), both members of the GNAT (Gcn5-related N-acetyltransferases) family. These enzymes are important

for telomeric silencing and transcriptional initiation, respectively [70]. The second family members, CBP (CREB binding protein) and p300, are part of the same protein complex. Also, p300 contains a bromodomain and acts on histone and nonhistone substrates. The MYST (MOZ, Ybf2/Sas3, Sas2, Tip60) family members function as catalytic subunits in Tip60 (60 kDa Tat-interactive protein), HBO1 (histone acetyltransferase binding to ORC 1), and MOZ/MOF (monocytic leukemia zinc finger protein/male absent on first) complexes [65]. MYST acetyltransferases have been shown to participate in transcription, DNA replication, recombination and repair, cell cycle, and gluconeogenesis. They are generally part of multisubunit complexes *in vivo* and regulate themselves by autoacetylation [71].

Trypanosomatids encode few acetyltransferases, methyltransferases, and binding modules (e.g., bromodomains) compared to other eukaryotes. The *Trypanosoma* genomes contain six histone acetyltransferases [31]. KATs from *trypanosomatids* are very divergent when compared to those from mammals or yeast and do not contain any other recognizable binding domains. Four of them are related to the MYST family and two are of the elongator type, which belong to the GNAT family. When compared to other species, the *Trypanosoma* enzymes are only slightly conserved and not all of them have the exact same genes.

MYST family acetyltransferases are defined by a distinctly conserved domain which contains a C2HC ($CX_2CX_{12}HX_{3-5}C$) zinc finger domain and an acetyl-CoA-binding site (motif A, Q/RX₂GXG/A), homologous to that present in the GNAT family. Some members contain additional features such as chromodomains, PHD (Plant Homeo Domain) fingers and zinc fingers [71]. *Trypanosomatids* MYST acetyltransferases are quite divergent relative to members of this family in other organisms. Despite such divergence, there are common features present in *Trypanosoma* (Figure 1). For example, motif A (green boxes in Figure 1) and the critical residues for catalysis are conserved (asterisks in Figure 1). Also, a C2HC-zinc-binding domain is present (grey box in Figure 1) and is thought to be involved in catalytic activity and/or substrate recognition [72]. But, there are some unique features among *trypanosomatids* KATs. For example, in KAT2 the motif A and the zinc-binding motifs are absent. HAT4 from *T. cruzi* and *Leishmania* have insertions of variable size and number in the acetyltransferase domain, and an important residue for catalysis is changed from E to S (asterisks in Figure 1). Also, there is a chromodomain present in HAT1 and 2 in all three *Trypanosoma* suggesting a link between acetylation and methylation in these protozoan parasites.

In Table 2 we summarize the KATs present in *trypanosomatids* and their known functions, which are detailed next. *Trypanosoma brucei* contains three KATs that belong to the MYST family. Siegel et al. demonstrated that *TbKAT3* is a nuclear protein that is responsible for acetylation of H4 K4, a modification considered more structural than regulatory. Also, they observed that treatment with cycloheximide, an inhibitor of protein synthesis, leads to a complete loss of unmodified H4 K4 sites. These facts suggest that newly synthesized histone H4 with unmodified K4 is

TABLE 2: Lysine acetyltransferases (KATs) present in Tritryps.

Acetyltransferase	<i>T. brucei</i>	Accession numbers		<i>T. cruzi</i>	Family	Subcellular localization	Comments	Reference
		<i>L. major</i>						
HAT1	Tb927.7.4560	LmjF14.0140		Tc00.1047053506605.160	MYST	Nuclear (<i>T. brucei</i>)	Essential for growth, required for telomeric silencing	Kawahara et al., 2008 [34]
				Tc00.1047053511239.150				
HAT2	Tb11.01.3380	LmjF28.2270		Tc00.1047053511017.69	MYST	Nuclear (<i>T. brucei</i>)	Essential for growth, responsible for H4K10 acetylation	Kawahara et al., 2008 [34]
				Tc00.1047053509203.60				
HAT3	Tb927.10.8310	LmjF36.6990		Tc00.1047053507611.290	MYST	Nuclear (<i>T. brucei</i>)	Not essential for growth, responsible for H4K4 acetylation	Kawahara et al., 2008 [34]
				Tc00.1047053507723.110				
HAT4		LmjF13.0170		Tc00.1047053506227.160	MYST	Cytoplasmic in all life cycle stages, nuclear in postmitotic cells (<i>L. donovani</i>)	Acetylates histone H4	Kumar et al., 2012 [35]
ELP3a	Tb927.8.5770	LmjF16.0240		Tc00.1047053506743.120	GNAT	Nuclear periphery (<i>T. brucei</i>)		Alsford and Horn, 2011 [36]
				Tc00.1047053503851.10				
ELP3b	Tb927.8.3310	LmjF23.1350		Tc00.1047053509769.110	GNAT	Nucleolar (<i>T. brucei</i>)	Controls rDNA transcription	Alsford and Horn, 2011 [36]

FIGURE 1: MYST family histone acetyltransferase orthologues in trypanosomatids. Multiple sequence alignments of the core acetyltransferase domains from Trypanosoma and other organisms. Sequences were aligned with ClustalX2 followed by manual adjustment and illustrated with ESPrnt 2.2. Identical residues are white on red background, and residues shared by some organisms are black on yellow background. Asterisks indicate residues that were predicted to form part of the catalytic pocket. The gray box marks the C2HC zinc-binding domain. Motif A that binds acetyl Co-A is indicated with a green box. Tb, Trypanosoma brucei; Tc, Trypanosoma cruzi; Lm, Leishmania mayor; At, Arabidopsis thaliana; Hs, human; Sc, Saccharomyces cerevisiae; AcuC, acetoin utilization.

rapidly imported to the nucleus where it suffers a possibly irreversible acetylation. The authors also suggest that *T. brucei* may not deacetylate histones recently synthesized in the cytoplasm as other eukaryotes do and that elevated levels of H4 K4 acetylation might serve to keep chromatin in an open conformation [73]. Another report from the same year demonstrated that *TbKAT3* is dispensable, while *TbKAT1* and 2 are required for parasite growth [34]. *KAT4* coding sequence is not present in the *T. brucei* genome, but it is present in *T. cruzi* and *Leishmania* [31]. All three *TbKATs* are present in the nucleus of bloodstream and insect-stage cells and mRNA abundance experiments revealed no difference in their expression levels. *TbKAT1* knock-down cells have telomeric silencing specifically compromised, and this enzyme might be required for DNA replication. *TbKAT2* displays *in vitro* acetyltransferase activity towards H4 K10. Apparently, trypanosomes use specific MYST acetyltransferases to acetylate H4 K4 and H4 K10, which further supports the idea of a nonredundant histone code present in these parasites [34]. *Leishmania donovani* KAT4 was recently characterized. This protein has a cytoplasmic localization throughout the parasite life cycle and a nuclear localization in postmitotic cells. *LdKAT4* acetylates histone

H4 at K4 residues almost exclusively and K2 in a minor proportion. These results suggest that H4 modification might happen in the cytoplasm prior to histone transport to the nucleus or soon after mitosis inside the nuclear compartment [35]. All this evidence clearly illustrates that KATs seem to be equivalent among the three genera but play completely different roles in each organism. Moreover, these differences may reflect that they are involved in distinct cellular processes. The most obvious example of this is the fact that the nuclear *TbKAT3* is playing the same role of the cytoplasmic *LdKAT4*, they both acetylate H4 at K4. If this is true, the mechanism by which newly synthesized histone H4 is acetylated differs between these species not only due to the participation of different enzymes but due to the fact that it occurs in different cellular compartments.

The GNAT family has sequence homology limited to 4 motifs (A-D) of 15–33 amino acids each. The central core domain is structurally conserved in different organisms and interacts with coenzyme A (CoA) through motif A which plays an important role in catalysis [74]. The members of this family present in trypanosomatids are two Elp3 (Elongator protein 3) orthologues which contain a GNAT-related acetyltransferase domain. In mammals, the elongator complex

has six subunit (Elp1–6), and it was first identified due to its association with RNAP II and its role in transcriptional elongation. One of the subunits of the elongator complex, Elp3, has a characteristic GNAT acetyltransferase domain is capable of acetylating both histones and other nonhistone substrates and is conserved from archaea to humans. In fact, the Elongator complex is mainly present in the cytoplasm where it is involved in stress signaling, exocytosis, tRNA modification, and α -tubulin acetylation (reviewed in [75]). Trypanosomatids Elp3a and Elp3b sequences have an iron-sulfur cluster (radical S-adenosylmethionine or SAM, grey boxes on Figure 2) and the motif A (green boxes in Figure 2). Despite the divergence with orthologues from other organisms the amino acids required for substrate binding are conserved (asterisks and arrowheads in Figure 2). It is worth noting that only Trityps have two Elp3 orthologues, another unique feature that may reflect distinct roles for these organisms enzymes.

A recent report by Alsford and Horn describes that the Elongator proteins (Elp3) present in *T. brucei* display acetyltransferase activity. *TbElp3a* localizes at the nuclear periphery, while *TbElp3b* is concentrated in the inner part of the nucleus in both bloodstream and insect-stage cells. Using null mutants these authors showed that *TbElp3b* negatively controls transcription elongation and rDNA transcription [36]. These results indicate that all KATs from *T. brucei* are exclusively nuclear and mainly related to transcription or other nuclear processes, suggesting that at least one not yet described enzyme should be responsible for the acetylation of cytoplasmic proteins as occurs in other organisms. Preliminary results from our lab using GFP fusions in *T. cruzi* suggest that Elp3a and Elp3b are not nuclear proteins.

Finally, we would like to mention another acetyltransferase reported in 2010, named MEC-17. This KAT is a member of the GNAT family and behaves as a K40-specific acetyltransferase for α -tubulin in *Tetrahymena* and *Caenorhabditis elegans*. This enzyme contributes to touch sensation and probably acetylates other nontubulin substrates in both organisms. MEC-17 homologues are present in most eukaryotes with the exception of fungi and plants [76]. In trypanosomatids there are several hypothetical proteins similar to MEC-17, none of which have been characterized yet.

4. Lysine Deacetylases

Mammalian and yeast KDACs can be divided into two families: classical or zinc dependent and NAD⁺-dependent. Another classification divides all KDACs into four classes according to their phylogenetic conservation. Classes I, II, and IV belong to the classical family, while class III contains NAD⁺-dependent deacetylases, also termed sirtuins (SIRT) because of their homology with yeast SIR2 proteins (silent information regulator 2). Class I and II deacetylases are represented by *Saccharomyces cerevisiae* Rpd3-like (reduced potassium dependency 3) and Hda1-like (histone deacetylase 1) proteins, respectively. The members of these classes have overlapping but distinguishable roles. In humans, class I is represented by HDAC1, 2, 3, and 8; class II is represented

by HDAC4, 5, 6, 7, 9, and 10 and Class IV is represented by HDAC11. Class III (or Sirtuins) has seven members (SIRT1–7) [77], which are present from bacteria to humans. There are nuclear and cytoplasmic KDACs and only sirtuins are present in the mitochondria [78]. For example, mammalian HDAC6 is a tubulin deacetylase that regulates cell motility [79].

In Table 3 we summarize the KDACs present in trypanosomatids and their known functions. Again, the number of enzymes is small in comparison to mammals. The number of KDACs is reduced to only four enzymes and three SIR2-related proteins (SIRT2 RP). Trityps class I KDACs have 350–500 amino acid residues (~50 kDa), a deacetylase domain and a small C-terminal region that can suffer several PTMs. Figures 3 and 4 show sequence alignments of the class I and II deacetylase domain in different organisms. Class-specific motifs are indicated with black boxes and residues important for catalysis and binding zinc-cofactor are marked with asterisks and triangles, respectively. Apparently, all class I and II KDACs have insertions of variable length in different positions.

In *T. brucei* DAC1 and 2, both members of class I KDACs, are orthologues of Rpd3 from *S. cerevisiae*. DAC2 has a highly acidic C-terminal domain that might be involved in protein-protein interaction or cellular localization. This is an unusual protein because it lacks several pocket residues, which are predicted to be required for deacetylase activity (asterisks in Figure 3). Ingram and Horn, through disruption of DAC genes in bloodstream *T. brucei* cells, determined that DAC1 is essential, while DAC2 is dispensable. Class II KDACs (DAC3 and DAC4) are larger than their class I counterparts, with an Hda1-catalytic domain at the C-terminus and other domains and regulatory sites at the N-terminus. In *T. brucei* DAC3 and DAC4 are ScHda1 orthologues, and only the former is essential. *TbDAC4* mutants display a delay in the G2/M phase of the cell cycle. Based on these features the authors propose that *TbDAC1* and *TbDAC3* might be potential chemotherapeutic targets [46]. Wang et al. went further with the characterization of these deacetylases and reported that DAC1 and DAC3 are nuclear proteins that display deacetylase activity and are essential for growth in the bloodstream forms of *T. brucei*. *TbDAC1* antagonizes telomeric silencing and *TbDAC3* is required for variant surface glycoprotein expression sites (VSG ESs) silencing in bloodstream and insect stage cells [47]. The same report states that DAC2 and 4 are predominantly cytoplasmic. Detailed information about localization and function of DACs from *Leishmania* and *T. cruzi* is still lacking. DAC2 seems to be absent in *Leishmania*; however, its function might be substituted by DAC1. The fact that *TbDAC2* is not essential could support this hypothesis. There are two sequences available for DAC2 and DAC3 that correspond to alleles from Esmeraldo and non-Esmeraldo strains in *T. cruzi*.

Class III KDACs or sirtuins (SIRT) are NAD⁺-dependent deacetylases that have diverse subcellular localization. They are involved in transcriptional silencing, DNA repair, cell cycle progression, chromosome segregation, and life span [80]. Members of the SIRT family have a 250 amino acid core domain, which exhibits a 25–60% sequence identity in different organisms. SIRTs remove acetyl groups

TABLE 3: Lysine deacetylases (KDACS) present in Trityps.

Deacetylase	<i>T. brucei</i>	Accession numbers <i>L. major</i>	<i>T. cruzi</i>	Class	Subcellular localization	Comments	Reference
DAC1	Tb10.70.6220	LmjF21.0680	Tc00.1047053511911.159 Tc00.1047053508637.114	I	Predominantly nuclear proteins in bloodstream form and cytoplasmatic in the insect stage (<i>T. brucei</i>)	Antagonizes SIR2rp1-dependent telomeric silencing essential for viability	Ingram and Horn, 2002 [46]
DAC2	Tb11.01.7240		Tc00.1047053504159.80 Tc00.1047053506821.140	I	Cytoplasmatic (<i>T. brucei</i>)	Not essential for viability	Wang et al., 2010 [47] Ingram and Horn, 2002 [46]
DAC3	Tb927.2.2190	LmjF21.1870	Tc00.1047053509395.120 Tc00.1047053503653.50	II	Nuclear in all life cycle stages (<i>T. brucei</i>)	Specifically required for silencing at VSG ES promoters in both bloodstream and insect-stage cells essential for viability	Ingram and Horn, 2002 [46] Wang et al., 2010 [47]
DAC4	Tb927.5.2900	LmjF08.1090	Tc00.1047053507063.270	II	Cytoplasmatic (<i>T. brucei</i>)	Not essential for viability	Ingram and Horn, 2002 [46]
SIR2RP1	Tb927.7.1690	LmjF26.0210	Tc00.1047053507519.60 Tc00.1047053508207.150	III	Cytoplasmatic in promastigote y amastigote stages (<i>L. major</i> , <i>L. amazonensis</i> , and <i>L. infantum</i>)	Deacetylates α -tubulin and is partially associated with the microtubule network	Yahiaoui et al., 1996 [48] Tavares et al., 2008 [49]
SIR2RP2	Tb927.8.3140	LmjF23.1210		III	Nuclear in all life cycle stages (<i>T. brucei</i>)	Catalyzes ADP ribosylation and deacetylation of histones is involved in DNA repair	García-Salcedo et al., 2003 [50]
SIR2RP3	Tb927.4.2520	LmjF34.2140	Tc00.1047053447255.20 Tc00.1047053506559.80	III	Mitochondrial (<i>T. brucei</i>)		Alsford et al., 2007 [51] Alsford et al., 2007 [51]

FIGURE 2: GNAT family histone acetyltransferases orthologues in trypanosomatids. Multiple sequence alignments of the core acetyltransferase domains from Trityps and other organisms. Sequences were aligned with ClustalX2 followed by manual adjustment and illustrated with ESPrpt 2.2. Identical residues are white on red background, and residues shared by some organisms are black on yellow background. Radical SAM domain (blue box) and GNAT-type acetyltransferase domain (black box) are indicated. Motif A is marked with a green box. Arrowheads indicate the Cys residues that are part of the Fe-S cluster in the SAM domain. Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*; Lm, *Leishmania mayori*; At, *Arabidopsis thaliana*; Hs, human; Sc, *Saccharomyces cerevisiae*; Tt, *Tetrahymena thermophila*.

in nuclear, cytoplasmatic, and mitochondrial substrates. During the deacetylation reaction, acetyl-lysine and NAD^+ are converted into lysine, nicotinamide and O-acetyl-ADP-ribose [81]. Michishita et al. characterized seven human proteins homologous to Sir2 and concluded that (1) three nuclear SIRT proteins (SIRT1, SIRT6, and SIRT7) show different subnuclear localizations, being SIRT6 and SIRT7 associated with heterochromatic regions and nucleoli; (2) SIRT3, SIRT4, and SIRT5 are localized in mitochondria, an organelle linked to aging and energy metabolism; (3) cellular p53 is a major *in vivo* substrate of SIRT1 deacetylase, but not of the other six SIRT proteins; (4) SIRT1, but not the other two nuclear SIRT proteins, shows an *in vitro* deacetylase activity on histone H4 and p53 peptides; (5) overexpression of the seven SIRT proteins does not extend cellular replicative lifespan in normal human fibroblasts or prostate epithelial cells [82]. Additionally, *hSIRT2* deacetylates α -tubulin [83] and shuttles to the nucleus during G1/M phase transition regulating microtubule dynamic and cell cycle progression

[84]. Sirtuins from *T. brucei*, *cruzi*, and *Leishmania* are listed in Table 3 along with their known functions.

There are three SIRP2RPs in trypanosomatids, one on them (SIR2 RP2) is not present in *T. cruzi*. All trypanosomatid SIR2RPs lack the N-terminal portion of ScSir2, which is required for nucleolar localization, but all contain a complete catalytic domain (Figure 5). RP1s contain serine-rich motifs towards the C-terminus and RP2s have two insertions in Trityps. All trypanosomatid proteins have a zinc-binding motif (CX₂CX₂₀CX₂X type), but one of the Cys residues is absent in the three SIR2 RP3 (grey box in Figure 5). Apparently all SIR2 RP2 from Trityps are phylogenetically closer to bacterial SIRTs, while RP1 and RP3 are more related to yeast and human SIRTs, respectively [51]. In all trypanosomatid sirtuins two motifs (GAG and NID) and two specific residues (HG) essential for enzymatic activity are conserved (underlined in Figure 5).

L. mayori SIR2 RP1 was the first sirtuin homologue described in a kinetoplastid protozoa [48] and is localized

FIGURE 3: Class I deacetylases orthologues in Trypanosomatids. Alignment of core deacetylase domain from different organisms. Sequences were aligned using ClustalX and were adjusted manually and illustrated with ESript 2.2. The black boxes indicate the class specific motifs. Identical residues are white on red background and residues shared by some organisms are black on yellow background. Asterisks indicate residues predicted to form part of the catalytic pocket whereas triangles indicate zinc cofactor ligands. Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*; Lm, *Leishmania major*; At, *Arabidopsis thaliana*; Hs, human; Sc, *Saccharomyces cerevisiae*; St, *Sulfolobus tokodaii*.

in cytoplasmic granules. *Leishmania infantum* SIR2 RP1 is expressed in each developmental stage, it contains the conserved core domain and is a NAD^+ -dependent deacetylase and ADP-ribosyltransferase. Also, *LiSIR2* RP1 is cytoplasmatic, similar to human SIRT2 [83] and HDAC6 [79]. It associates with the cytoskeleton network and deacetylates α -tubulin [49]. *Leishmania amazonensis* Sir2 RP1 is expressed in cytoplasmatic vesicles of both promastigote and amastigote developmental forms. Its secretion/excretion was correlated with the glycosylation state of the protein [85]. *TbSIR2* RP1 is a nuclear and chromosome-associated protein. It is expressed throughout *T. brucei* life cycle and catalyses NAD^+ -dependent ADP ribosylation and deacetylation of histones as some human SIRTs do [50, 86]. Overexpression of *TbSIR2* RP1 is toxic to the *T. brucei* bloodstream form but not to the insect stage parasite. Moreover, it controls DNA repair and repression of RNA Pol I-mediated expression immediately adjacent to telomeres, but it is not required for antigenic variation [51]. *TbSIR2* RP2 and 3 were identified in the single mitochondrion of the parasite. These genes were disrupted, but the mutant strains did not show growth or differentiation defects [51]. The fact that *T. brucei* and *Leishmania* have mitochondrial deacetylases makes sense considering that acetylation is very important for the regulation of the energetic metabolism, as demonstrated in other organisms. It also highlights the need of an acetyltransferase activity out of the nucleus in *T. brucei*, suggesting that at least one of the nuclear KATs mentioned above might have a dual nuclear-cytoplasmatic localization as some mammalian enzymes have.

5. KDACs and KATs Inhibitors as Chemotherapeutic Agents

In the last few years, a number of KDAC and KAT inhibitors (iKADCs and iKATs) were developed and assayed against several types of cancer where these enzymes are deregulated and

generally overexpressed [87]. KATs and KDACs inhibitors are now being investigated to target a range of parasitic diseases with the advantage that some of these compounds have already been validated for other human diseases and the chemical starting points are already available. Also, these compounds are very useful tools to study the catalytic features of acetyltransferases and deacetylases, as well as their roles in different cellular pathways. KDAC inhibitors were known before the discovery of their target enzymes. For example, sodium butyrate, trichostatin A, and valproic acid were described to induce mammalian histone acetylation many years ago [88–90]. These compounds have diverse structures and mechanisms of action for inhibiting their target proteins. Inhibition of KDACs has multiple effects on the cell, such as cell cycle arrest, senescence, apoptosis, ROS production, and mitotic cell death. iKDACS are capable of reducing tumor invasiveness, angiogenesis, and metastasis *in vivo* (reviewed in [91]). Sirtuins inhibitors were also described many years ago, for example, sirtinol [92–95] and nicotinamide, which is a product of the Sir2-catalyzed reaction [96]. iKATs are less studied than iKDACS and only a small number of them are known, such as curcumin, anacardic acid, garcinol, and isothiazolones. iKATs have different target proteins and cell permeability. Recently, there have been advances in the development of cell-permeable small molecules that behave as specific modulators of HATs (reviewed in [97]).

As we stated before, current knowledge leads to think that KATs and KDACs are crucial in the life cycle of trypanosomatids, thus these enzymes could be interesting chemotherapeutic targets for treating parasitic diseases. Several inhibitors have been tested against protozoan parasites *in vitro* and *in vivo*, but there are still many critical factors that need to be considered before using these compounds clinically. Next, we describe the iKATs and iKDACS that have been tested against protozoa, focusing on trypanosomatids (Table 4).

FIGURE 4: Class II deacetylases orthologues in trypanosomatids. Alignment of the core deacetylase domain from different organisms. Sequences were aligned using ClustalX and were adjusted manually and illustrated with ESPrpt 2.2. The black boxes indicate the class-specific motif. Identical residues are white on red background, and residues shared by some organisms are black on yellow background. Asterisks indicate residues that were predicted to form part of the catalytic pocket, whereas triangles indicate zinc cofactor ligands. Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*; Lm, *Leishmania major*; At, *Arabidopsis thaliana*; Hs, human; Sc, *Saccharomyces cerevisiae*; Sy, *Synechocystis* spp.; AphaA, acetylpolymine aminohydrolase.

Apicidin (iKDAC) displays antiparasitic activity against apicomplexan parasites such as *Plasmodium falciparum* and *Toxoplasma gondii*, among others [98]. An apicidin analogue has been reported as having a potent and selective activity against *T. brucei* but is toxic to *L. donovani* and *T. cruzi*. This species-specific effect observed for *Trypanosoma* species was also seen for three other synthetic analogues [99]. Valproic acid, sodium butyrate and its derivatives all have quite poor *in vitro* activity against *P. falciparum* and *T. gondii* but are useful for studying the acetylation-dependant cellular pathways [100, 101]. Nicotinamide (nam) has antiparasitic activity against several *Leishmania* species [102]. Moreover,

nam and trichostatin A (TSA) cause growth arrest in *T. cruzi* epimastigotes at micromolar concentrations (unpublished results from our lab). TSA and SAHA (suberoylanilide hydroxamic acid) are both hydroxamate-based compounds that inhibit *T. gondii* and *P. falciparum* growth *in vitro*, but they kill mammalian cells at similar concentrations [100, 103]. Very little information is available on the activity of hydroxamate or other HDAC class I or II inhibitors against Trityps. However, as stated for other compounds, TSA is a very useful tool that helps understand how KDAC inhibition affects the parasite growth, development, and transcriptional control. Sirtinol, a Sir2 inhibitor, which is

TABLE 4: KDACs and KATs inhibitors tested in protozoan parasites.

	Structure	Target	Antiparasitic activity
KDAC inhibitors			
Apicidin	Cyclic tetrapeptide	All KDAC	Apicomplexa <i>T. gondii</i> , <i>P. falciparum</i>
Apicidin analogues	Cyclic tetrapeptide with indole modifications	All KDAC	<i>T. gondii</i> , <i>P. falciparum</i> , <i>T. brucei</i>
Valproic acid	Short-chain fatty acid	Class I KDAC	<i>P. falciparum</i> , <i>T. gondii</i>
Sodium butyrate	Short-chain fatty acid	All KDAC	<i>P. falciparum</i> , <i>T. gondii</i>
Sirtinol	2-hydroxy-1-naphthaldehyde derivative	SIR2	<i>L. infantum</i> amastigotes, <i>P. falciparum</i> , <i>T. gondii</i>
Nicotinamide	Noncompetitive inhibitor	SIR2	<i>Leishmania</i> spp., <i>T. cruzi</i> epimastigotes
Tricostain A (TSA)	Natural hydroxamate based	Class I and II KDAC	<i>T. cruzi</i> , <i>P. falciparum</i>
Suberoylanilide hydroxamic acid (SAHA)	Synthetic hydroxamate based	All KDAC	<i>P. falciparum</i>
Hydroxamic acid derivatives		All KDAC	<i>T. brucei</i>
KAT inhibitors			
Curcumin	Natural phenol	MYST family p300/CBP	<i>T. brucei</i> , <i>L. mexicana</i>
Garcinol	Polyisoprenylated benzophenone	MYST family p300 and PCAF	<i>Leishmania</i> spp.
Anarcadic acid	Salicylic acid substituted with an alkyl chain	MYST family p300 and PCAF, Tip60	<i>P. falciparum</i>

FIGURE 5: Class III deacetylases orthologues in trypanosomatids. Multiple sequence alignments of the core deacetylase domains from Trityps and other organisms. Sequences were aligned with ClustalX2 followed by manual adjustment and illustrated with ESPrnt 2.2. Identical residues are white on red background and residues shared by some organisms are black on yellow background. Asterisks indicate residues that were predicted to form part of the catalytic pocket. The grey box marks the C2HC zinc-binding domain. Circles indicate NAD⁺-binding residues and asterisks indicate catalytic residues. The GAD and NID motifs as well as the conserved HG residues important for catalysis are underlined. Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*; Lm, *Leishmania major*; At, *Arabidopsis thaliana*; Hs, human; Sc, *Saccharomyces cerevisiae*; Sy, *Synechocystis* spp.

commercially available, is capable of inducing death in *L. infantum* amastigotes [104]. Also, it was found to inhibit growth in *P. falciparum* [105]. A number of hydroxamic acid derivatives, which inhibit human deacetylases, were recently tested against *T. brucei* bloodstream form. Most of the derivatives assayed, identified by screening compound libraries,

were able to block parasite growth in the submicromolar range. The most effective compound was a member of the sulphonopiperazine series [106].

As we mentioned before, only a small number of iKATs have been reported, and even fewer have been tested against protozoan parasites. For example, curcumin has trypanosidal

activity *in vitro* [107, 108] and curcumin analogues have a potent activity against *T. brucei* bloodstream forms and *L. mexicana* amastigotes and promastigotes *in vitro* [109]. Anacardic acid blocked growth of *P. falciparum* *in vitro* by reversibly and noncompetitively inhibiting the KAT activity of recombinant PfGCN5 [110]. Other natural acetyltransferase inhibitors such as, garcinol and xhantanes were tested *in vitro* in *Leishmania*, the results were promising, but the cytotoxicity profile of these compounds needs to be assessed before evaluating them *in vivo* [111].

All these evidence supports the idea that KDACs and KATs are promising drug targets against protozoan parasites and it would be extremely valuable to fully characterize these enzymes and discover their mechanisms of action in order to assess future inhibitors. As we mentioned before, these compounds are also useful to study localization, stability and interactions of deacetylases and acetyltransferases in trypanosomatids.

6. Concluding Remarks

The flagellated protozoa *T. brucei*, *T. cruzi*, and *Leishmania* spp. represent important human and animal pathogens and are emerging as model organisms for the study of epigenetic regulation [112]. Several lines of evidence described here indicate that lysine deacetylases and acetyltransferases constitute key enzymes in trypanosomatids and some of them are essential for their viability. Even though the enzymes that exist in the three genera are considered orthologues, in some cases they do not have the same function and/or the same cellular localization. We observed that there is a tendency to extrapolate results from one species to other. In particular, from *T. brucei*, a parasite with many genetic tools including a very efficient iRNA system, to *T. cruzi*, which have limited genetic tools available. In the case of these enzymes, we highlight the need to study each organism separately to discover their mechanisms of action. Even though in many cases these extrapolations are correct, in the case of these enzymes we highlight the need to study each organism separately to discover their mechanisms of action. The search for KAT and KDAC inhibitors is a very active research field. There are several inhibitors described in the literature, which are very useful tools for unraveling the mechanisms of action and targets of these enzymes. Many of these compounds were assayed against parasites. The results presented here illustrate the fact that acetyltransferase and deacetylase inhibitors are species-specific in Trityps and that most of these compounds inhibit mammalian enzymes as well. These results further support the need to study and fully characterize each trypanosomatid KAT and KDAC in order to rationally design new drugs to battle these pathogens.

Acknowledgments

The authors would like to thank Dr. Pamela Cribb and Carla Ritagliati for their valuable inputs and corrections.

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