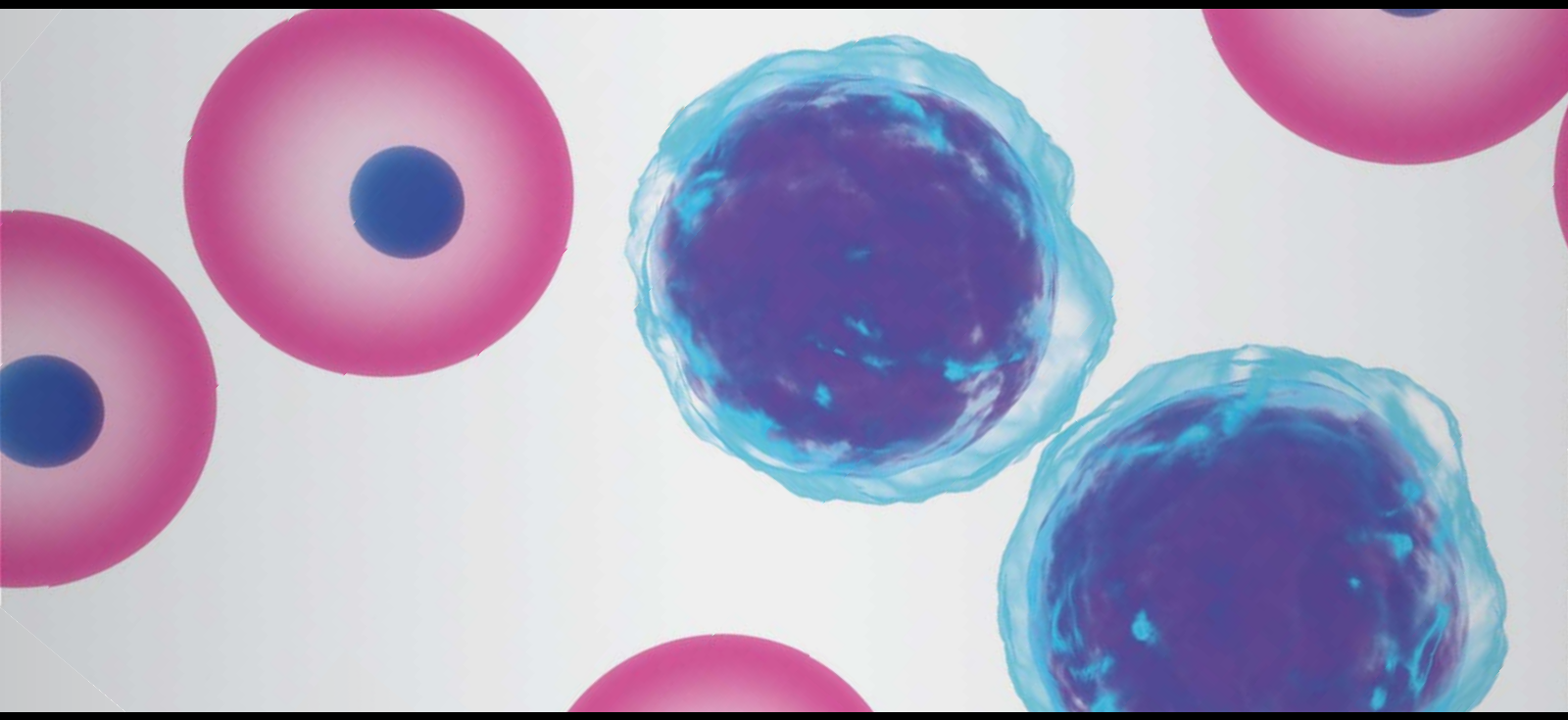
Leukemia Research and Treatment

Differentiation and Cell Survival of Myeloid Leukemia Cells

Guest Editors: George P. Studzinski, Geoffrey Brown, Michael Danilenko, Philip Hughes, and Ewa Marcinkowska



Differentiation and Cell Survival of Myeloid Leukemia Cells

Leukemia Research and Treatment

Differentiation and Cell Survival of Myeloid Leukemia Cells

Guest Editors: Krasimir Vasilev, Haifeng Chen, and Patricia Murray

Editorial Board

Contents

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 370375, 2 pages
doi:10.1155/2012/370375

Editorial

Differentiation and Cell Survival of Myeloid Leukemia Cells

George P. Studzinski,[1] Geoffrey Brown,[2] Michael Danilenko,[3] Philip Hughes,[2] and Ewa Marcinkowska[4]

[1] *Department of Pathology and Laboratory Medicine, UMDNJ-New Jersey Medical School, Newark, NJ 07103, USA*
[2] *School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT, UK*
[3] *Department of Clinical Biochemistry, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel*
[4] *Faculty of Biotechnology, University of Wroclaw, Tamka 2, Wroclaw, Poland*

Correspondence should be addressed to George P. Studzinski, studzins@umdnj.edu

Received 3 June 2012; Accepted 3 June 2012

The landscape of treatment for acute myeloid leukemia (AML) is currently a grim one. Apart from the AML subtype characterized by the 15:17 chromosome translocation known as acute promyelocytic leukemia (APL), which has shown lasting remissions when treated with all-trans retinoic acid (ATRA), especially when supplemented with the mildly toxic compound arsenic trioxide (ATO), mortality from the disease remains high. Thus, research into novel regimens of therapy is needed to supplement the current reliance on toxic compounds such as AraC and daunorubicin in the treatment of these diseases. Despite some spectacular clinical successes, ATRA-based differentiation therapy is not without its problems due to the induction of potentially life-threatening toxicities and the acquisition of therapeutic resistance in some patients. G. Brown and P. Hughes summarize the current state of knowledge in a comprehensive review entitled "*Retinoid differentiation therapy for common types of acute myeloid leukemia*" and suggest ways in which retinoid-based therapies can be improved by the inclusion of additional agents to increase the sensitivity of APL cells towards ATRA. This is followed in this issue by an example of cutting-edge research into the molecular basis for the efficacy of ATRA/ATO therapy for APL. B. Ozpolat et al. describe that at least part of the anti-APL effect of ATRA/ATO treatment can be explained by the inhibition of protein translation by these compounds. The report of these studies, entitled "*PKCδ regulates translation initiation through PKR and eIF2α in response to retinoic acid in acute myeloid leukemia cells*" also indicates that ATRA/ATO inhibit the PI3K/AKT/mTOR pathway, leading to an upregulation of the PKC delta/PKR axis. Both these articles add to our knowledge of the mechanism of ATRA in the treatment of myeloid leukemias and add to the debate on how this treatment may be improved. These new approaches may have importance outside the realms of leukemia treatments and may also lead to improved use of retinoids as therapies for other solid tumors.

Chronic myeloid leukemia (CML) has considerably better prognosis than AML, and as with APL, treatment can be targeted to a hybrid gene, here Bcr-Abl, which results from a reciprocal translocation between chromosomes 9 and 22. Specific kinase inhibitors, such as Imatinib, have been identified and offer front-line treatment for CML. Unfortunately, resistance to kinase inhibitors frequently develops, and G. N. de Moraes et al. in a review entitled "*The interface between BCR-ABL-dependent and -independent resistance signaling pathways in chronic myeloid leukemia*" analyze the known causes for this resistance and offer several feasible molecular targets, which may overcome the development of resistance to kinase inhibitors in CML.

The lessons from these two therapeutic successes are not easily transferred to other subtypes of myeloid leukemia, as the molecular lesions that can be attacked to eradicate the malignant cells appear to be "moving targets." The mutations vary from AML case to case; secondary mutations appear and are often multiple, so current therapy largely depends on "brute-force" cell killing by administration of highly toxic agents, which have varying differential sensitivity for leukemic and normal cells. It seems, therefore, that alternate

strategies are needed. The "by-pass the genetic lesion" suggested approach (G. P. Studzinski et al., PMID: 16046262) is based on the induction of cell differentiation by compounds such as vitamin D derivatives (VDDs). Here, instead of targeting a genetic lesion, the therapeutic agent induces the expression of transcription factors, which activate alternative but dormant routes to cell differentiation. Unfortunately, as pointed out by Harrison and J. A. Bershadskyi in a clinical perspective "*Clinical experience using vitamin D and analogs in the treatment of myelodysplasia and acute myeloid leukemia: A review of the literature*," attempts to improve the therapy of AML with VDDs alone have not been successful so far. Further research on the mode of action of differentiation-inducing agents is, therefore, needed and the mechanisms involved should be explored in depth, to gain insights how to improve the differentiation regimens. One example of such exploration is provided in this issue by E. Gocek et al., who show multiple levels of regulation of the differentiation process in the report "*Regulation of leukemic cell differentiation through the vitamin D receptor at the levels of intracellular signal transduction, gene transcription, protein trafficking and stability*." Another report on VDD action "*Cell-type-specific effects of silibinin on vitamin D-induced differentiation of acute myeloid leukemia cells are associated with differential modulation of RXRα levels*" by R. Wassermann et al. is more directly translational. This article addresses the potential pitfall of cell type specificity of biological responses. When attempts are made to enhance the effects of VDDs by combining them with an antioxidant silibinin, enhanced differentiation is seen in one AML subtype, but inhibition of differentiation is seen in another AML subtype. Advance knowledge of such antagonism is essential for future clinical trials.

An exciting recent development in our understanding of the molecular basis of myeloid disorders is the realization that small noncoding RNAs play a role in these diseases. Y. Yuan et al. summarize a variety of such recent data in an article "*MicroRNAs in acute myeloid leukemia and other blood disorders*." It seems that the information these and other studies described in this issue will provide a beginning of a long road that will eventually lead to much improved therapy, targeted or not, for myeloid leukemia.

George P. Studzinski
Geoffrey Brown
Michael Danilenko
Philip Hughes
Ewa Marcinkowska

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 125814, 8 pages
doi:10.1155/2012/125814

Review Article

Clinical Experience Using Vitamin D and Analogs in the Treatment of Myelodysplasia and Acute Myeloid Leukemia: A Review of the Literature

Jonathan S. Harrison[1,2] and Alexander Bershadskiy[1,2]

[1] *Division of Hematology, Department of Medicine, Robert Wood Johnson Medical School, New Brunswick, NJ 08901, USA*
[2] *The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, New Brunswick, NJ 08901, USA*

Correspondence should be addressed to Jonathan S. Harrison, harrisjs@umdnj.edu

Received 1 March 2012; Revised 5 April 2012; Accepted 17 April 2012

Academic Editor: George P. Studzinski

Despite progress in understanding the biology of acute myeloid leukemia (AML), and despite advances in treatment, the majority of patients with AML die from the disease. The observation that Vitamin D can induce AML blast cells *in vitro* to differentiate along the monocytic lineage was made 30 years ago; however, it remains to translate this into a clinically meaningful strategy. This is a review of published clinical experience regarding the use of Vitamin D and its analogs, either alone or in combination with other agents, to treat AML. In many of these reports, investigators included patients with myelodysplasia (MDS) as well as AML patients in their treatment cohorts; therefore reports of Vitamin D and its analogs in treating MDS are included. This review documents heterogeneity in selection criteria for patients treated in these studies, the spectrum of Vitamin D analogs used in various studies, and the differing dosing strategies employed by investigators. Despite examples of occasional clinical efficacy, barriers remain to the successful application of Vitamin D in the treatment of MDS and AML. These include the lack of definition of a particularly sensitive target population, and the as yet unknown optimal choice of Vitamin D analog and dosing schedule.

1. Introduction

Despite significant progress over the past several decades in understanding the biology of acute myeloid leukemia (AML) and despite important advances in treatment approaches, the majority of patients who develop acute myeloid leukemia will still die from their disease. A recent analysis by Southwest Oncology Group investigators of data from 1344 patients with newly diagnosed AML enrolled into SWOG studies between 1986 and 2009, excluding Acute Promyelocytic Leukemia, illustrated this point dramatically. In that study, overall survival at 4 years was only 49% for patients with favorable cytogenetics; overall survival was approximately 25% for those with intermediate-prognosis cytogenetics, and only 9% for those with unfavorable cytogenetics [1]. Among the most important advances made in recent decades in the treatment of AML was the recognition of the exquisite sensitivity of Acute Promyelocytic Leukemia (APL) to differentiation therapy using all-trans-retinoic acid (ATRA). This observation was, shortly thereafter, accompanied by the identification of the Retinoic Acid Receptor Alpha (RARA) gene as a partner in the balanced translocation that drives APL. The dual recognition of a novel mechanism of disease—in the case of APL, overexpression of RARA, a protein that participates in proliferation and differentiation of hematopoietic cells—along with the identification of a compound, ATRA, that is a derivative of a vitamin, has provided a powerful paradigm for cancer therapy. This parallels the dramatic progress that has been made in the past fifteen years in the use of rationally designed, small molecules that target specific intracellular signal transduction pathways in cancer, best exemplified by the development of Imatinib, and followed by many new agents effective in a variety of solid tumors. The remarkable improvement in the rates of remission and survival for patients with APL has stimulated our laboratory, and many others to explore a similar approach for the treatment of non-APL subsets of AML, using vitamin D and analogs (collectively referred

to henceforth as vitamin D and Deltanoids—"VDD"). Investigations have focused on either VDD alone, or in combination with other compounds, in an effort to induce differentiation of leukemia cells, and allow for less toxic chemotherapy, as is the case now in the treatment of patients with APL. The observation that vitamin D can induce acute myeloid leukemia blast cells *in vitro* to differentiate along the monocytic lineage was made approximately 30 years ago [2]; however, it remains to translate this observation into a clinically meaningful strategy. The barriers to translating this observation from the bench to the bedside include the induction of hypercalcemia VDD, as well as the need to identify which, if any, subset of patients with AML will be sensitive to VDD. The current understanding of the biologic basis for VDD effects in AML *in vitro* and *ex vivo* has recently been reviewed in detail [3]. The following is a review of published clinical experience that has accumulated to date regarding the use of VDD either alone or in combination with cytotoxic agents, other differentiating agents, or both. In many of these reports, the investigators included patients with a diagnosis of myelodysplasia (MDS), as well as AML, in their treatment cohorts, and for this reason, the effects of VDD in MDS patients are included in the discussion below.

2. Clinical Reports of Single-Agent VDD Treatment

Given the *a priori* expectation that there would be a low probability of a favorable clinical response to the use of single agent vitamin D or its derivatives to treat AML, it is readily understandable that there are few published reports of their use as single agents in such patients. However, in the late 1980's investigators at Kagawa Medical School in Japan reported a series of 3 patients treated using oral administration of from 4.5 up to 15 micrograms/day of 1 alpha (OH) vitamin D3 [4]. Two of these patients carried a diagnosis of AML, and the third patient had a diagnosis of the myelodysplastic syndrome refractory anemia with excess blasts. The authors reported a decrease in bone marrow blast percentage for each of the three patients, with transient hypercalcemia developing in one patient and resolving within three days of cessation of vitamin D3. Shortly thereafter, Takahashi and colleagues [5] reported their experience using alfacalcidol in a cohort of 13 patients, of whom two patients had AML—one patient with APL, and one with FAB-M4 histology by report. Patients received single-agent alfacalcidol at doses that ranged from 0.25 microgram/day up to 10 microgram/day orally, for at least one month of therapy; drug was administered only intermittently in order to avoid hypercalcemia. The authors reported that the patient with AML FAB-M4 had a minor response, utilizing response criteria developed by Koeffler [6]; the patient with APL did not respond. Of note, among the patients with a diagnosis of MDS, there were 3 partial responses and 2 minor responses. Nakayama reported a single case of AML responding favorably to treatment using 1 alpha(OH)D3 in a brief communication [7]. In this report, the patient was an 81-year-old man with pancytopenia on presentation, whose initial marrow exam reportedly showed 20% myeloblasts. He was treated using 1 alpha (OH) vitamin D3 at a dose of 6 micrograms daily by mouth as single-agent therapy. He achieved a normalization of blood counts by 4 weeks of therapy, with normalization of marrow morphology. The response was lost after the dose of 1 alpha (OH) vitamin D3 was tapered, and reescalation of the dose failed to achieve a second response. Hypercalcemia did not occur. These appear to be the only reports published to date of single agent VDD used as therapy for AML.

There are at least six reports in the literature of single agent VDD treatment of patients with a diagnosis of myelodysplastic syndrome. In 1985, Koeffler, a leading investigator in this field, and colleagues administered 2 microgram/day of 1,25(OH)2D3 to 18 patients with myelodysplastic syndrome in an attempt to improve their hematopoiesis [6]. They reported that during therapy, peak peripheral blood granulocyte, platelet, and macrophage concentrations were slightly elevated as compared to baseline levels. Eight patients had a partial or minor peripheral blood response during the administration of 1,25(OH)2D3. However, no patient showed significant improvement of peripheral blood cell or marrow blast cell counts by the end of the study (greater than or equal to 12 weeks) as compared to their starting levels. Nine of the 18 patients developed hypercalcemia. In this paper, *ex vivo* treatment of dysplastic myeloid cells did demonstrate differentiation in response to 1,25(OH)2D3. In the 1989 report of Takahashi and colleagues from Japan discussed earlier, in addition to treating two patients with AML using alfacalcidol at doses ranging from 0.25 microgram/day up to 10 microgram/day orally for at least one month, there were 11 patients with various subtypes of myelodysplastic syndromes in the FAB classification schema. Of these, three patients showed partial responses, 2 patients showed minor responses and remainder of the patients did not respond. The hematological improvement of 6 responders was transient, reported by the authors to range from 1 to 2 months; however, one patient with low-risk MDS demonstrated an improvement in blood counts that persisted for more than 1 year ([5], op cit). In 1991, Motomura and colleagues published a clinical trial in a series of 30 patients with myelodysplasia. In 15 patients, 4–6 micrograms/day of 1-hydroxyvitamin D3 was administered, for a median duration of 17 months; a control group had no therapy. Leukemia-free survival of the D3-treated group was reported to be statistically superior to the control group; 7 patients in the control group developed acute leukemia, in contrast to only one in the D3 treated group [8]. In 1998, Mellibovsky and colleagues [9] described their experience in treating a series of 19 patients with low-to-intermediate grade MDS, as determined by the Bournemouth criteria [10], using single agent VDD. The first five patients in this study received 266 microgram calcifediol three times per week; the remaining twelve patients received Calcitriol, in doses ranging from 0.25 microgram/day increasing up to a maximum of 0.75 microgram/day by mouth, provided that plasma calcium concentrations remained in the normal range. Of the total of nineteen patients, 12 were men and seven women; mean patients age was 74 years. Patients were categorized by

the FAB classification scheme; seven had refractory anaemia with ringed sideroblasts, five had refractory anaemia, one had refractory anaemia with excess of blasts, and six had chronic myelomonocytic leukemia. All the patients were in a low to intermediate risk group. Mean follow-up period was 26 months, range 9–75. Responders were defined as having experienced either a granulocyte or platelet count increase by 50%, hemoglobin increase of 1.5 g/dL, or transfusion needs decreased by 50%. Response was observed in 11 of the 19 patients. In the calcifediol treated group, one case responded, three were nonresponders, and one showed progression of disease. One responder was maintained on calcifediol for two years, stopped at his own request, and experienced a fall in hemoglobin, which then improved after resuming calcifediol. In the calcitriol group, 10 were responders (two with major response), and four were nonresponders. No correlation was observed between baseline levels of vitamin D metabolites and the presence of response. No hypercalcaemia was observed. These authors concluded that treatment with VDD could induce long-standing responses in blood counts in some low-intermediate risk MDS patients without inducing hypercalcemia.

In 1993, Yoshida and colleagues published a multicenter randomized clinical trial of alfacalcidol (1 alpha hydroxyvitamin D3) meant to evaluate the therapeutic effect of alfacalcidol in patients with MDS. Twenty-three evaluable patients were randomized to receive either a single daily oral dose of 6 micrograms of alfacalcidol, or supportive care as a control. Treatment was continued, whenever possible, for a period of 6 months. Response was assessed by weekly blood counts, clinical course, and repeated marrow examinations. No significant difference was noted between the alfacalcidol and control groups [11]. Three of the 13 patients in the alfacalcidol group and two of the 10 patients in the control group experienced progression of disease. One patient with refractory anemia showed a favorable response to alfacalcidol in all three hematopoietic cell lineages; the response, however, was not maintained, and discontinuation of the drug resulted in a worsening of pancytopenia which was refractory to a second course of alfacalcidol therapy. Hypercalcemia was the major toxic side effect of alfacalcidol therapy. These authors concluded that single-agent alfacalcidol therapy does not yield a significant clinical beneficial in patients with MDS. Most recently, Juckett and colleagues at the University of Wisconsin published a phase II clinical trial of single agent doxercalciferol, a vitamin D2 analogue, in 15 patients with MDS [12]. Patients were treated using doxercalciferol 12.5 microgram by mouth daily for 12 weeks. Nine of 15 patients completed the prescribed course, and of these six had stable disease. No patient had a favorable clinical response based upon International Working Group criteria, and eight patients experienced progressive disease while on therapy. Of interest, however, two patients with chronic myelomonocytic leukemia (CMML) had a marked rise in monocytes documented while on study, and one patient experienced hypercalcemia. These authors concluded that a twelve-week course of therapy using single-agent doxercalciferol has very limited activity in patients with MDS.

3. Clinical Reports of VDD Combined with Other Agents

Several groups have reported the clinical use of VDD in combination with other agents intended to force differentiation of pathologic myeloid progenitor cells, in a few series without the inclusion of classical cytotoxic antineoplastic agents. Most commonly, a VDD has been combined with a retinoid, with or without, a third agent or even a fourth agent. In 1991, Blazsek and colleagues in France reported their experience in treating two patients; one patient had APL in relapse and responded to single-agent all-trans-retinoic acid (a relatively new observation as of that writing). However, the second patient that they described in that report carried a diagnosis of MDS, and experienced a sustained hematologic response to treatment using the combination of prednisone with 1 alpha,25-dihydroxyvitamin D3 (1 alpha,25D3) as well as 13-*cis*-retinoic acid [13]. In this paper, 1 alpha,25D3 was administered at a dose of 0.25 micrograms by mouth three times per day for 30 days, in combination with prednisone 40 mg per day for the first 15 days and *cis*-Retinoic Acid 20 mg by mouth daily for 30 days. This patient continued the vitamin D for an 8-month period and the retinoic acid for a total of two and one-half years, apparently with a sustained response.

In 2007, the Finnish Leukemia Group reported the results of a phase II clinical trial of the combination of valproic acid together with 13-*cis*-retinoic acid and 1,25-dihydroxyvitamin D3 in the treatment of MDS, including several cases chronic myelomonocytic leukemia and several cases of refractory anemia with excess blasts-2 (RAEB-2). Valproic acid, an antiseizure medication, has pleotropic effects, including inhibition of the P-glycoprotein; however, it was chosen in this instance because of the observation that Valproic Acid can inhibit histone deacetylase activity. Oral Valproic acid was titrated to achieve serum concentrations between 500 and 700 micromolar, and 13-*cis*-retinoic acid was administered at a dose of 10 mg by mouth twice daily. The VDD used in this study was Etalpha at a dose of 13 microgram by mouth daily. There were no episodes of hypercalcemia observed, although the majority of patients experienced the hypertriglyceridemia associated with the use of 13-*cis*-retinoic acid. Three of the nineteen patients experienced some hematologic improvement—a rise in hemoglobin and neutrophil count in one patient with chronic myelomonocytic leukemia, an improvement in platelet count in one patient with RAEB-2, and an improvement in hemoglobin level in one patient with refractory cytopenias with multilineage dysplasia [14].

A number of groups have attempted to combine vitamin D or its analogs with low-dose conventional antineoplastic chemotherapy in an effort to improve blood counts in patients with either AML, MDS, or both. Most commonly, Cytarabine has been used in combination with a VDD, sometimes with additional agents added to the regimen. In 1988, Hellström and colleagues in Sweden reported a series of 62 evaluable patients with either MDS or AML who were treated with various combinations of lowdose Cytarabine, alpha-interferon (IFN), 1 alpha-hydroxyvitamin D3 (vit D3),

and retinoic acid. The Cytarabine was dosed initially at 15 mg/meter squared body surface area subcutaneously daily, and the interferon was administered initially at 3 million units subcutaneously daily. The 1 alpha-hydroxyvitamin D3 was dosed at 1 microgram per day by mouth in two divided doses, escalated until mild hypercalcemia was noted, with the dose then adjusted to maintain a serum calcium below 2.90 millimoles/liter. The overall response rate was 44%. Of these, 50% responded favorably to the combination of IFN, vitamin D3, and retinoic acid, a combination that they termed IDR. This was felt by the authors to be comparable to the response rate of 43% for low-dose Cytarabine alone [15].

The same group then performed a prospective, randomized Phase III clinical trial, in which 63 evaluable patients with myelodysplastic syndromes and 15 with acute myeloid leukemia were randomized between low-dose Cytarabine (arm A) as a single agent, and low-dose Cytarabine in combination with 13-*cis*-retinoic acid (13-CRA) and 1 alpha-hydroxy-vitamin D3 (1 alpha D3) (arm B). The doses of these agents were the same as used in their prior report described above ([15], op cit). The authors stated that 69 patients were evaluable and of these 18 (26.1%) responded to therapy [16]. The addition of 13-CRA and 1 alpha D3 had no significant favorable influence on neither survival of the patients, remission rates, nor duration of remissions. 12/27 patients (44%) in arm A and 6/29 patients (20%) in arm B progressed from MDS to AML during the course of the study ($P = 0.0527$). Arm B gave significantly more sideeffects than arm A ($P = 0.005$). The authors concluded that a clear cut therapeutic effect of the addition of 13-CRA and 1 alpha D3 to Cytarabine on MDS was not supported by this study. However, they also stated their interpretation that an inhibitory effect on AML development—that is, progression from MDS to AML—in some MDS subgroups could not be excluded by their data [16].

De Rosa and colleagues in Italy reported, in 1992, their experience treating forty-four patients with high-risk primary myelodysplastic syndromes. This cohort was treated with a combination of low-dose Cytarabine, retinoic acid, and vitamin D3 [17]. Morphological subtypes, using the FAB classification schema, were refractory anemia with excess of blasts (RAEB) in 16, RAEB in transformation (RAEB-T) in 20, and chronic myelomonocytic leukemia (CMML) in eight patients. In this series, Cytarabine was administered at a dose of 10 mg per meter squared body surface area twice daily for 15 consecutive days. The 13-*cis*-retinoic acid was given at a dose of 20 mg per meter squared body surface area for 21 days by mouth, and vitamin D3 was given at 0.75 microgram per day in three divided doses of 0.25 microgram per oral dose, also for 21 days. Cycles were repeated every four to six weeks. The therapy was continued in responders until relapse or death. The results were compared to those of a matched control group of 44 patients given supportive care only. In the treated group the overall response rate was 50% (75% in RAEB, 50% in RAEB-T, and 0% in CMML) and the survival was significantly better in the control group ($P < 0.025$). Comparing separately each FAB subgroup suggested that the treatment prolonged the survival in the RAEB-T subgroup ($P < 0.002$), but not the other two groups. The median duration of response was 15 months and the survival in responders was statistically better than in nonresponders ($P < 0.0001$). Myelosuppression was the most important side effect; however, no deaths related to treatment were noted. These authors concluded that this approach was useful for treatment of patients with high-risk myelodysplasia.

Several years later, Ferrero and colleagues from Italy described their results in treating 53 MDS patients with a combination of *cis*-retinoic acid (cRA, 20 to 40 mg/day) and 1,25 alpha (OH)2 cholecalciferol [(OH)2D3, 1–1.5 micrograms/day] with or without intermittent 6-thioguanine (30 mg/m^2/day). The 6-thioguanine was administered only to patients with bone marrow blast counts greater than or equal to 5%. The authors reported that treatment was well tolerated, without major toxicity. Among 25 patients with bone marrow blasts less than 5%, they observed one complete response, eight partial responses, and four minor responses (overall response rate 52%) with a median response duration of 8 months (2 ± 24). Median survival, which did not correlate with response, was projected by the authors, as of the time of publication, to be 76 months overall [18]. Thirty-one patients with BM blast excess (> or = 5%), including three of the previous group who progressed to refractory anemia with excess of blasts (RAEB), were treated with the three-drug protocol. One complete, 12-partial, and six minor responses were obtained (response rate 61%) with a median response duration of 6 months (2–29+). A significant difference in survival ($P < 0.005$) was observed between the 19 responders (median 25 months) and the 12 nonresponders (median 9 months). A reduction in the transfusion need was observed in 41% of the transfusion-dependent patients with blast excess and in 53% of those without blast excess. Therefore, these authors did conclude that combined differentiating therapy seems more effective than single-agent treatments.

The same group subsequently reported their experience treating 26 patients with a diagnosis of AML, as well as 4 patients with MDS ineligible for standard chemotherapy. The regimen used was a combination of 13-*cis*-retinoic acid at 20 to 40 mg by mouth daily, together with dihydroxy-vitamin D3 (Rocaltrol) 1 microgram by mouth daily, along with 6-thioguanine 40 mg by mouth daily, and Cytarabine 8 mg per meter squared body surface area twice weekly by subcutaneous injection. The Cytarabine was administered during the initial two to three weeks of treatment. The median age of this group of patients was 72.5 years, and they had been deemed ineligible for standard chemotherapy. The response rate was 50%, with 27% complete remission. The median survival of the whole group and responders was 7.5 (1−47+) and 16.5 months (3.5−47+), respectively [19].

In a somewhat similar approach, Slapek and colleagues at Tufts University Medical Center published their experience in treating a series of twenty-nine patients, ranging from 62 to 82 years of age, all with a diagnosis of AML, treated using a 21-day course of continuous infusion Cytarabine at 20 mg per meter squared body surface area per day, together with 1,25-dihydroxyvitamin D3 (calcitriol) at a dose of 0.25 microgram by mouth twice daily. The calcitriol was continued until progression of disease or until the patient

went off study. Hydrea was also given daily by mouth at a dose of 500 mg, started one day prior to the initiation of the Cytarabine infusion, and continued for 21 days as well. Ten patients had an antecedent myelodysplastic syndrome. Calcitriol was continued as the only postremission therapy. Thirteen patients (45%) obtained a complete remission, and 10 patients (34%) had a partial response for an overall 79% response rate [20]. There were three early deaths. The median remission duration was 9.8 months. Overall median survival was 12 months for all patients and 14 months for responding patients. All responding patients had marked bone marrow hypoplasia. Twenty patients received part or all of their chemotherapy as outpatients. These authors concluded that this regimen had acceptable toxicity and can result in prolonged remissions in elderly, high-risk patients with AML.

Building upon work described above, Ferrero and colleagues in Europe recently reported on the use of recombinant human erythropoietin in combination with the previously investigated regimen of 13-*cis*-retinoic acid and dihydroxy-vitamin D3 in the management of anemia in MDS patients. They treated 63 MDS patients (excluding refractory anaemia with excess blasts, type 2 (RAEB2)) with the combination of 13-*cis*-retinoic acid and dihydroxy-vitamin D3 with, or without, the further addition of 6-thioguanine. Most patients were categorized as refractory cytopenia with multilineage dysplasia and RAEB1, in the WHO classification scheme, with intermediate 1 International Prognostic Scoring System (IPSS) score [21]. All patients had, at baseline, a hemoglobin of <9.5 gm/dL, and 70% required regular erythrocyte transfusions prior to beginning this therapy. The treatment dosing schedule included 13-*cis*-retinoic acid at 20 mg/day by mouth, and 1,25 di(OH) vitamin D3 at 1 microgram/day by mouth. Eleven of the 16 RAEB1 patients also received intermittent, low-dose 6-thioguanine (40 mg/d for 21 days every 5 weeks). rHuEPO (alpha epoetin in the majority, beta epoetin in a minority of patients) was added at different dosages and schedules according to practices of the different participating institutions and different time periods of treatment. Until 2002, 10,000 unit formulations only were available, and patients received weekly doses ranging from 10,000 units subcutaneously three times weekly up to 10,000 units subcutaneously daily. From 2002 onwards, 40,000 unit formulations of alpha erythropoietin became available, and dosages ranged from 40,000 units/week to 40,000 U every 3 to 4 days. Median weekly dose overall was 60,000 units per week (range: 30,000–80,000 units/week). All patients were treated for at least 6 months, and in the case of response, until disease progression or death. The treatment started within 12 months from diagnosis in 52 patients and between 15 and 48 months (median: 21) from diagnosis in 11 patients was well tolerated, and erythroid response rate according to new International Working Group criteria [23] was 60%:50% in RAEB1 and 64% in non-RAEB patients. The weekly recombinant human erythropoietin dose administered ranged from 30,000 units per week to as much as 80,000 units per week. Median response duration was 16 months, and median survival reached 14 months for RAEB1 and 55 months for non-RAEB patients, with a significant difference in the latter between responders and nonresponders (median 82 months versus 44 months; $P = 0.036$). No patient experienced clinically significant hypercalcemia.

In a recent novel approach, Akiyama and colleagues in Japan recently reported the results of a Phase II trial of the combination of either vitamin K monotherapy, or vitamin K in combination with 1-alpha hydroxy-vitamin D3 for the treatment of patients with either low or intermediate-1 risks MDS. A total of 24 patients were enrolled into this study in total [22]. The overall response rate to vitamin K monotherapy (45 mg/day) after 16 weeks was 13% (5/38) including 4 cases with improvement of both anemia and thrombocytopenia and improvement in 1 case with thrombocytopenia. They then enrolled and evaluated 20 out of 33 vitamin K-monotherapy nonresponders for vitamin K plus vitamin D3 (0.75 microg/day) combination therapy. The overall response rate at 16 weeks after initiation of vitamin K plus vitamin D3 was 30% (6/20). Hematologic improvement for hemoglobin (Hb) was observed in 6 out of 11 patients (55%) and Hematologic improvement for thrombocytopenia in 3 out of 11 patients (27%), respectively. No Hematologic Improvement was observed for neutropenia in vitamin K monotherapy, nor in response to vitamin K plus vitamin D3 combination therapy. There was a suggestion in the data that International Prognostic Scoring System for MDS scores and absolute neutrophil counts positively correlated with favorable response, and Hemoglobin levels inversely correlated with the response to vitamin K plus vitamin D3 combination therapy. These authors concluded that vitamin K plus vitamin D3 combination therapy appears to be a promising treatment approach for improving anemia and thrombocytopenia in MDS patients with low/intermediate-1 MDS.

4. Discussion

A summation of the numbers of patients treated in the reports cited above documents that more than 85 patients with a diagnosis of AML, and a total of more than 350 patients with a diagnosis of MDS, have been treated using a vitamin D derivative either alone, or in combination with additional agents—as is evident from Table 1. Among this population, response rates have been highly variable, and this may be attributed to several factors. AML is a heterogeneous disease, with dramatic differences in the biologic subtypes, both with respect to the mutational events that drive the disease, as well as the prognosis of the disease based upon cytogenetics, molecular features, and the treatment paradigm employed. At one end of the spectrum, AML with core binding factor mutations generally have a very favorable prognosis when treated using high-dose Cytarabine as a part of therapy; at the other end of the spectrum, AML with MLL-1 gene mutations generally have an abysmal prognosis in the absence of allogeneic hematopoietic transplantation. Many of the reports reviewed herein were published prior to the recognition in the late 1990's of the core-binding factor mutations and their prognostic and treatment implications;

Table 1: Clinical reports of vitamin D in the treatment of patients with AML and MDS.

Vitamin D derivative	Dose schedule	Duration of therapy	Concurrent agents	Disorder treated	Number treated	Response	First author	Reference
1 alpha(OH) vitamin D3	4.5 to 15 microgram/day	4 weeks	Single agent	AML MDS	2 1	Transient decline in marrow blasts Transient decline in marrow blasts	Irino	[4]
Alfacalcidol	0.25 to 10 microgram/day	≥4 weeks	Single agent	AML MDS	2 11	1 minor response 3 partial and 1 minor response	Takahashi	[5]
1 alpha(OH) vitamin D3	1 microgram/day	>4 weeks	Single agent	AML	1	1 major response	Nakayama	[7]
$1,25(OH)_2$ vitamin D3	2 microgram/day	12 weeks	Single agent	MDS	18	8 transient, partial responses	Koeffler	[6]
1 hydroxy vitamin D3	4 to 6 microgram/day	17 months	Single agent	MDS	15	Improved PFS compared to control	Motomura	[8]
Calcifediol	266 microgram 3 days/week	Up to 2 years	Single agent	MDS	5	1 major response	Mellibovsky	[9]
Calcitriol	0.25 to 0.75 /day	Up to 2 years	Single agent	MDS	14	10 responders, 2 major	Mellibovsky	[9]
Alfacalcidiol	6 microgram/day	6 months	Single agent	MDS	13	1 transient response	Yoshida	[11]
Doxercalciferol	12.5 microgram/day	12 weeks	Single agent	MDS	15	No formal response	Petrich	[12]
$1,25(OH)_2$ vitamin D3	0.75 microgram/day	12 weeks	Prednisone + 13 cis-Retinoic Acid	MDS	1	1 major response	Blazsek	[13]
$1,25(OH)_2$ vitamin D3	13 microgram/day	16 weeks	Valproic acid	MDS	19	3 major response	Siitonen	[14]
1 alpha(OH) vitamin D3	1 microgram/day	Variable	Cytarabine ± IFN ± Retinoids	AML	15	5 responses (including stable disease)	Hellström	[15]
1 alpha(OH) vitamin D3	1 microgram/day	Variable	Cytarabine ± IFN ± Retinoids	MDS	47	22 responses (including stable disease)	Hellström	[15]
1 alpha(OH) vitamin D3	1 microgram/day	Variable	Cytarabine ± 13 cis-Retinoic Acid	MDS	69	13 responses	Hellström	[16]
1 alpha(OH) vitamin D3	1 microgram/day	Variable	Cytarabine ± 13 cis-Retinoic Acid	AML	15	5 responses	Hellström	[16]
$1,25(OH)_2$ vitamin D3	0.75 microgram/day	Variable	Cytarabine + 13 cis-Retinoic Acid	MDS	44	22 responses	De Rosa	[17]
$1,25(OH)_2$ vitamin D3	1 to 1.5 microgram/day	Until progression	13 cis-Retinoic Acid + 6TG	MDS	31	19 responses (patients with >5% blasts)	Ferrero	[18]
Dihydroxy-vitamin D3	1 microgram/day	Until progression	Cytarabine + 13 cis-Retinoic Acid	AML	26	15 responses among all treated	Ferrero	[19]
Dihydroxy-vitamin D3	1 microgram/day	Until progression	Cytarabine + 13 cis-Retinoic Acid	MDS	4	15 responses among all treated	Ferrero	[19]
$1,25(OH)_2$ vitamin D3	0.5 microgram/day	Until progression	Cytarabine	AML	29	23	Slapak	[20]
1,25 di(OH) vitamin D3	1 microgram/day	>6 months	13-cis retinoic acid, 6TG, Epo	MDS	63	38	Ferrero	[21]
1-alpha hydroxy-vitamin D3	0.75 microgram/day	16 weeks	Vitamin K	MDS	20	6	Akiyama	[22]

this is similarly so for the impact of the MLL-1 gene, to identify just two of many important observations regarding AML. The heterogeneity of MDS is even greater with respect to biologic features, disease natural history, and prognosis, as compared to AML. How the biologic heterogeneity of these diseases impacts upon the clinical responsiveness of an individual patient has not been investigated to date, but it is very likely that adverse molecular events play a role in the relative unresponsiveness of some patients disease to vitamin D therapy. It is clear that some patients described in the clinical treatment reports above have benefited from vitamin D, or vitamin D analog therapy; however, dissecting the specific features that could predict responsiveness is difficult based on the relatively limited biologic data provided in the reports above. *A priori*, one might expect that patients with lower-grade (low IPSS score) MDS would be more likely to benefit from vitamin D therapy than patients with higher risk MDS; however, in the report of De Rosa and colleagues ([17], op cit), that was not the case, and patients with RAEB-T appeared more likely to benefit from vitamin D therapy, when compared to historical controls. However, this has not been formally tested to date in a prospective, randomized clinical trial with fully matched controls. The only prospective, randomized clinical trial summarized above was a small study, not powered to detect a modest impact of vitamin D, and randomized patients between low-dose Cytarabine (arm A) as a single agent, and low-dose Cytarabine in combination with 13-cis-retinoic acid (13-CRA) and 1 alpha-hydroxy-vitamin D3 ([16], op cit). Consequently, the effects, if any, of the vitamin D agent would have been obscured by the use of 13-CRA even if the numbers of patients had been larger.

A further issue in determining the efficacy of a VDD in the treatment of AML and MDS is the heterogeneity in the vitamin D agent chosen, which has varied from one report to another, as well as the dose and dosing schedule variations. The heterogeneity in the choice of vitamin D agent and the dosing schedule makes aggregating the data from the disparate trials extremely difficult. In order to glean more meaningful insight into the potential role for VDD in the treatment of AML and MDS, an optimal VDD, as well as an optimal dosing regimen, will ideally need to be defined in formal Phase I and Phase II studies, and then subjected to testing in a large, randomized Phase III clinical trial. *In vitro*, concentrations of vitamin D that induce differentiation are typically in the range of 1 nanomolar 1,25(OH)2 vitamin D3 [24]; however, conventional VDD administered in a dose that would achieve such a blood level carries a significant risk of inducing clinically significant hypercalcemia. Noncalcemic VDD agents have been developed [25], but to date have not been tested in patients with MDS or AML.

5. Summary

There is extensive preclinical data establishing the ability of vitamin D and its analogs to induce immature myeloid hematopoietic cells to terminally differentiate into mature monocytic cells, at which point these cells are no longer able to proliferate. However, in the 19 reports summarized in this paper, only a minority of patients experienced either a transient or a persistent improvement in blood counts in response to VDD-based therapy. The observation that a meaningful number of patients did experience some degree of objective, favorable response demonstrates the potential for clinical use of VDD as differentiation therapy in the management of MDS and AML. However, substantial progress remains to be made before this approach can be included in the formal armamentarium of treatments for MDS and AML.

References

[1] B. C. Medeiros, M. Othus, M. Fang, D. Roulston, and F. R. Appelbaum, "Prognostic impact of monosomal karyotype in young adult and elderly acute myeloid leukemia: the Southwest Oncology Group (SWOG) experience," *Blood*, vol. 116, no. 13, pp. 2224–2228, 2010.

[2] C. Miyaura, E. Abe, and T. Kuribayashi, "1α,25-dihydroxyvitamin D3 induces differentiation of human myeloid leukemia cells," *Biochemical and Biophysical Research Communications*, vol. 102, no. 3, pp. 937–943, 1981.

[3] M. Kim, L. Mirandola, A. Pandey et al., "Application of vitamin D and derivatives in hematological malignancies," *Cancer Letters*, vol. 319, no. 1, pp. 8–22, 2012.

[4] S. Irino and T. Taoka, "Treatment of myelodysplastic syndrome and acute myelogenous leukemia with Vit. D3 [1α(OH)D3]," *Japanese Journal of Cancer and Chemotherapy*, vol. 15, no. 4, pp. 1183–1190, 1988.

[5] T. Takahashi, S. Ichiba, Y. Okuno et al., "Therapeutic effectiveness of vitamin D3 in patients with myelodysplastic syndromes, leukemias and myeloproliferative disorders," *The Japanese Journal of Clinical Hematology*, vol. 30, no. 1, pp. 1–10, 1989.

[6] H. P. Koeffler, K. Hirji, and L. Itri, "1,25-Dihydroxyvitamin D3: in vivo and in vitro effects on human preleukemic and leukemic cells," *Cancer Treatment Reports*, vol. 69, no. 12, pp. 1399–1407, 1985.

[7] S. Nakayama, T. Ishikawa, H. Yabe, K. Nagai, S. Kasakura, and H. Uchino, "Successful treatment of a patient with acute myeloid leukemia with 1α(OH)D3," *Acta Haematologica Japonica*, vol. 51, no. 6, pp. 1026–1030, 1988.

[8] S. Motomura, H. Kanamori, A. Maruta, F. Kodama, and T. Ohkubo, "The effect of 1-hydroxyvitamin D3 for prolongation of leukemic transformation-free survival in myelodysplastic syndromes," *American Journal of Hematology*, vol. 38, no. 1, pp. 67–68, 1991.

[9] L. Mellibovsky, A. Díez, E. Pérez-Vila et al., "Vitamin D treatment in myelodysplastic syndromes," *British Journal of Haematology*, vol. 100, no. 3, pp. 516–520, 1998.

[10] G. J. Mufti, J. R. Stevens, and D. G. Oscier, "Myelodysplastic syndromes: a scoring system with prognostic significance," *British Journal of Haematology*, vol. 59, no. 3, pp. 425–433, 1985.

[11] Y. Yoshida, S. Oguma, H. Uchino, T. Maekawa, and T. Nomura, "A randomized study of alfacalcidol in the refractory myelodysplastic anaemias. A Japanese cooperative study," *International Journal of Clinical Pharmacology Research*, vol. 13, no. 1, pp. 21–27, 1993.

[12] A. Petrich, B. Kahl, H. Bailey, K. Kim, N. Turman, and M. Juckett, "Phase II study of doxercalciferol for the treatment of myelodysplastic syndrome," *Leukemia and Lymphoma*, vol. 49, no. 1, pp. 57–61, 2008.

[13] I. Blazsek, C. Farabos, M. Musset et al., "Retinoic acid in mono- or combined differentiation therapy of myelodysplasia and acute promyelocytic leukemia," *Biomedicine and Pharmacotherapy*, vol. 45, no. 4-5, pp. 169–177, 1991.

[14] T. Siitonen, T. Timonen, E. Juvonen et al., "Valproic acid combined with 13-cis retinoic acid and 1,25-dihydroxyvitamin D3 in the treatment of patients with myelodysplastic syndromes," *Haematologica*, vol. 92, no. 8, pp. 1119–1122, 2007.

[15] E. Hellström, K. H. Robèrt, G. Gahrton et al., "Therapeutic effects of low-dose cytosine arabinoside, alpha-interferon, 1α-hydroxyvitamin D3 and retinoic acid in acute leukemia and myelodysplastic syndromes," *European Journal of Haematology*, vol. 40, no. 5, pp. 449–459, 1988.

[16] E. Hellström, K. H. Robèrt, J. Samuelsson et al., "Treatment of myelodysplastic syndromes with retinoic acid and 1α-hydroxy-vitamin D3 in combination with low-dose ara-C is not superior to ara-C alone. Results from a randomized study," *European Journal of Haematology*, vol. 45, no. 5, pp. 255–261, 1990.

[17] L. De Rosa, A. Montuoro, and A. De Laurenzi, "Therapy of "high risk" myelodysplastic syndromes with an association of low-dose Ara-C, retinoic acid and 1,25-dihydroxyvitamin D3," *Biomedicine and Pharmacotherapy*, vol. 46, no. 5-7, pp. 211–217, 1992.

[18] D. Ferrero, B. Bruno, P. Pregno et al., "Combined differentiating therapy for myelodysplastic syndromes: a phase II study," *Leukemia Research*, vol. 20, no. 10, pp. 867–876, 1996.

[19] D. Ferrero, E. Campa, C. Dellacasa, S. Campana, C. Foli, and M. Boccadoro, "Differentiating agents + low-dose chemotherapy in the management of old/poor prognosis patients with acute myeloid leukemia or myelodysplastic syndrome," *Haematologica*, vol. 89, no. 5, pp. 619–620, 2004.

[20] C. A. Slapak, J. F. Desforges, T. Fogaren, and K. B. Miller, "Treatment of acute myeloid leukemia in the elderly with low-dose cytarabine, hydroxyurea, and calcitriol," *American Journal of Hematology*, vol. 41, no. 3, pp. 178–183, 1992.

[21] D. Ferrero, A. Darbesio, V. Giai et al., "Efficacy of a combination of human recombinant erythropoietin + 13-cis-retinoic acid and dihydroxylated vitamin D3 to improve moderate to severe anaemia in low/intermediate risk myelodysplastic syndromes," *British Journal of Haematology*, vol. 144, no. 3, pp. 342–349, 2009.

[22] N. Akiyama, K. Miyazawa, Y. Kanda et al., "Multicenter phase II trial of vitamin K_2 monotherapy and vitamin K_2 plus 1α-hydroxyvitamin D_3 combination therapy for low-risk myelodysplastic syndromes," *Leukemia Research*, vol. 34, no. 9, pp. 1151–1157, 2010.

[23] B. D. Cheson, P. L. Greenberg, J. M. Bennett et al., "Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia," *Blood*, vol. 108, no. 2, pp. 419–425, 2006.

[24] G. P. Studzinski, X. Wang, Y. Ji et al., "The rationale for deltanoids in therapy for myeloid leukemia: role of KSR-MAPK-C/EBP pathway," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 97, no. 1-2, pp. 47–55, 2005.

[25] R. Wieder, S. C. Novick, B. W. Hollis et al., "Pharmacokinetics and safety of ILX23-7553, a non-calcemic-vitamin D 3 analogue, in a phase I study of patients with advanced malignancies," *Investigational New Drugs*, vol. 21, no. 4, pp. 445–452, 2003.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 482905, 17 pages
doi:10.1155/2012/482905

Research Article

PKCδ Regulates Translation Initiation through PKR and eIF2α in Response to Retinoic Acid in Acute Myeloid Leukemia Cells

Bulent Ozpolat,[1] Ugur Akar,[1] Ibrahim Tekedereli,[1] S. Neslihan Alpay,[1] Magaly Barria,[1] Baki Gezgen,[1] Nianxiang Zhang,[2] Kevin Coombes,[3] Steve Kornblau,[2] and Gabriel Lopez-Berestein[1]

[1] *Department of Experimental Therapeutics, Unit 422, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[2] *Department of Leukemia, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*
[3] *Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA*

Correspondence should be addressed to Bulent Ozpolat, bozpolat@mdanderson.org and Gabriel Lopez-Berestein, glopez@mdanderson.org

Received 14 February 2012; Revised 8 May 2012; Accepted 10 May 2012

Academic Editor: George P. Studzinski

Translation initiation and activity of eukaryotic initiation factor-alpha (eIF2α), the rate-limiting step of translation initiation, is often overactivated in malignant cells. Here, we investigated the regulation and role of eIF2α in acute promyelocytic (APL) and acute myeloid leukemia (AML) cells in response to all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO), the front-line therapies in APL. ATRA and ATO induce Ser-51 phosphorylation (inactivation) of eIF2α, through the induction of protein kinase C delta (PKCδ) and PKR, but not other eIF2α kinases, such as GCN2 and PERK in APL (NB4) and AML cells (HL60, U937, and THP-1). Inhibition of eIF2α reduced the expression of cellular proteins that are involved in apoptosis (DAP5/p97), cell cycle (p21Waf1/Cip1), differentiation (TG2) and induced those regulating proliferation (c-myc) and survival (p70S6K). PI3K/Akt/mTOR pathway is involved in regulation of eIF2α through PKCδ/PKR axis. PKCδ and p-eIF2α protein expression levels revealed a significant association between the reduced levels of PKCδ ($P = 0.0378$) and peIF2 ($P = 0.0041$) and relapses in AML patients ($n = 47$). In conclusion, our study provides the first evidence that PKCδ regulates/inhibits eIF2α through induction of PKR in AML cells and reveals a novel signaling mechanism regulating translation initiation.

1. Introduction

Differentiation block or arrest is one of the major characteristics of acute myeloid leukemia (AML) [1]. All-*trans* retinoic acid (ATRA), an active metabolite of vitamin A, is a potent inducer of cellular differentiation and growth arrest in various tumor cell lines and has been successfully used in the treatment of acute promyelocytic leukemia (APL) [1–5]. The success of ATRA in the treatment of APL introduced the concept of differentiation therapy in treating malignant diseases [1]. Arsenic trioxide (ATO), an FDA approved drug, induces both differentiation and apoptosis in APL and AML cells [5]. The molecular events that are involved in underlying mechanism of these drugs are not completely elucidated. Understanding the pathways regulating cell proliferation and differentiation may help designing new molecularly targeted therapies in AML.

Translation initiation is a highly regulated process of translation in response to cellular stress and mitogenic stimulation [6–11]. Increased translation and protein synthesis are associated with cell proliferation and malignant disease [6, 7]. Translational regulation plays a vital role in the expression of oncogenic, and growth-regulatory, differentiation, and apoptosis related proteins and is considered one of the important but understudied feature of malignant phenotype [6–10, 12, 13].

Increased activity of eukaryotic translation initiation factor-2α (eIF2α) is the rate-limiting step of translation initiation and phosphorylation of eIF2α at serine 51 converts eIF2 to a competitive inhibitor of eIF2B, resulting in the inhibition of translation [6, 13–16]. Transfection of cells with eIF2α has been shown to cause malignant transformation of normal cells, suggesting that eIF2α plays a critical role in cellular pathways controlling cell proliferation [10, 11, 17–27]. Phosphorylation of eIF2α on serine 51 (Ser51) by eIF2α kinases, such as PKR, GCN2, and PERK, leads to the increased affinity of eIF2α for eIF2B and converts the phosphorylated eIF2α into an inhibitor of the GDP-GTP exchange factor, thereby inhibiting eIF2α activity and translation initiation [14]. While reducing global translation, phosphorylation of eIF2α also induces preferential translation of specific mRNAs that assist in the regulation of genes involved in metabolism and apoptosis [25].

We and others reported that ATRA and ATO inhibit translation initiation through multiple posttranscriptional mechanisms, including downregulation of translation factors and upregulation of repressors of translation initiation, such as PDCD4 and DAP5/p97 in APL cells [28, 29]. However, the posttranscriptional mechanisms regulating in APL and AML cells remain largely unknown.

Protein kinase C (PKC) is a family of serine/threonine protein kinases that are key regulatory enzymes in signal transduction [30]. The PKC family is divided in three groups, based on the differences in their sequence homology and cofactors required for their activation. The conventional PKCs (α, βI, βII, and γ) are activated by calcium and 1,2-diacyl-*sn*-glycerol (DAG), whereas the novel class of PKCs (δ, ε, θ, η) are calcium independent but DAG dependent. The atypical PKCs (λ, ζ, and υ) do not require calcium or DAG for their activation. Depending on the cell type, PKCδ can function as a tumor suppressor, a proapoptotic factor, and can regulate cell proliferation and cell survival functions [30]. The role of PKCδ in regulation of translational machinery is not well understood.

In the present study, we investigated the regulation of eIF2α in APL and AML cells. We found that PKCδ regulates eIF2α activity by phosphorylating it at Ser-51 through PKR, an eIF2α kinase. We also found PI3K/Akt/mTOR pathway is involved in regulation of eIF2α through PKCδ/PKR. Overall, our data provided the first evidence that PKCδ regulates phosphorylation/activity of eIF2α through PKR in APL and AML cells, revealing a novel role of PKCδ signaling and regulation of translation initiation.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions. The human promyelocytic cell line NB4, (AML-M3 type by the FAB classification) harboring t(15;17), was purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). AML cell lines including myeloblastotic HL60 (M2-AML), myelomonocytic U937 (M4/M5-AML), and THP-1 (M4-AML) cells were purchased from American Type Culture Collection (Manassas, VA). Primary human hematopoietic progenitor cells (CD34+ bone marrow progenitor cells) were purchased from Cambrex Bioscience Inc. (Walkersville, CA). The cells were grown in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum at 37°C under 5% CO_2 in a humidified incubator. ATRA, arsenic trioxide (ATO), and rottlerin were purchased from Calbiochem, La Jolla, CA.

2.2. Evaluation of Cell Differentiation. Cell differentiation was identified by examining expression of granulocytic (CD11b and CD11c) and monocytic (CD14) differentiation markers, morphologic changes, electron microscopy images, and reformation of PML nuclear bodies. Cells were collected from 2 to 5 days after treatment and washed with phosphate-buffered saline solution (PBS). Cells (5×10^5) in 100 μL of PBS were incubated for 30 min with fluorescein isothiocyanate (FITC)—labeled or phycoerythrin (PE)—labeled anti-CD11b, anti-CD11c, or anti-CD14 antibodies (1 : 200; BD Biosciences, San Jose) and for 20 min with PE-labeled isotope control IgG2a (Becton Dickinson) on ice in the dark, as described by the manufacturer. The percentages of $CD11b^+$, $CD11c^+$, and CD14+ cells were determined by fluorescence-activated cell sorting (FACS) analysis at the Flow Cytometry and Cellular Imaging Facility, The University of Texas MD Anderson Cancer Center. Background staining was determined from the cells stained with the isotype control antibodies.

2.3. Cell Growth Assays. Cells were seeded at 1×10^5 cells/mL in RPMI medium in six-well tissue culture plates (Costar, Cambridge, MA). After dilution with saline from a 10 mM stock in DMSO, cells were treated with ATRA at a final concentration of 1 μM and incubated for 3 days. The maximum concentration of DMSO was kept at less than 0.001% (v/v). Cell viability was determined by two methods: trypan blue (Sigma) exclusion and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) dye reduction assay [31]. Briefly, after cells were incubated with ATRA or ATO, 10 μL of MTT solution (10 mg/mL in PBS) was added to each well (in triplicate). The plates were then incubated for 4 h at 37°C, and the formazan crystals formed were dissolved by adding 100 μL of 0.04 N HCl in 2-propanol. Plates were read at 490 nm by a microplate reader (Molecular Devices, Sunnyvale, CA). In the trypan blue exclusion test, cells with intact membranes exclude the dye, whereas cells without intact membranes take up the coloring agent. For this assay, a cell suspension was prepared and mixed with an equal amount of a 0.4% trypan blue solution. Cell viability was assessed within 1-2 min by calculating the percentage of unstained (i.e., viable) cells. Untreated controls were used to determine the relative viability in both assays.

2.4. Western Blot Analysis. NB4, HL60, THP-1, and U937 cells were harvested from exponentially growing cell cultures. After treatment, the cells were collected, centrifuged, and lysed with a lysis buffer. Total protein concentration of the resulting whole-cell lysates was determined using a DC protein assay kit (Bio-Rad, Hercules, CA). In the experiments

in which the PKCδ inhibitor rottlerin (4 μM) were used, the cells were incubated with the inhibitor for 4 h and then with ATRA for the indicated time periods. Aliquots containing 30 μg of total protein from each sample were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to the membranes as described previously [32]. The membranes were blocked with 5% dry milk in TBST (100 mM Tris-HCL [pH 8.0], 150 mM NaCl, and 0.05% Tween-20), probed with primary antibodies diluted in TBST containing 5% dry milk, and incubated at 4°C overnight. We used primary antibodies against eIF2, phosphorylated (p) eIF2e (Ser51), p-eIF4E (Ser209), p-4E-BP1 (Thr37/40), Akt (Ser473), P-PERP (The981), p-P70S6K (Thr421/Ser424) (all, Cell Signaling Technology, Danvers, MA), and p-PKR (Thr446) (Santa Cruz Biotechnology, Santa Cruz, CA); $p21^{Waf1/Cip1}$, tissue transglutaminase 2 (TG2), and GCN2 also were purchased from Santa Cruz Biotechnology. eIF2α antibodies were diluted 1 : 1,000 in TBST. After being washed, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Life Science, Cleveland, OH). Mouse anti-β-actin and donkey anti-mouse secondary antibodies were purchased from Sigma to examine β-actin expression for equal loading. The bands were visualized by the enhanced chemiluminescence method (KPL, Gaithersburg, MD). Images were scanned and quantitated by a densitometer using the Alpha Imager application program (both from Alpha Innotech, San Leandro, CA). All experiments including treatments were repeated at least three times.

2.5. RNA Isolation and RT-PCR Analysis. Cells were seeded in six-well plates (1×10^6 cells/mL) and treated with ATRA at a final concentration of 1 μM. The cells were collected at various time points, and total cellular RNA was isolated with Trizol reagent (Life Technologies). cDNA was obtained from 5 μg of total RNA using a Superscript II RT kit (Life Technologies) as previously described [32]. Briefly, 5 μL of the total 20 μL of reverse-transcribed product was used for polymerase chain reaction (PCR) in 1× PCR buffer containing 1.5 mM $MgCl_2$, 250 μM dNTPs, 0.5 units of Taq polymerase (Life Technologies), and 100 ng of primers for PKCδ (Santa Cruz Biotechnology) or β-actin (Sigma-Genosys, Houston, TX). The following programs were used for PCR amplification of PDCD4: 1 cycle at 94°C for 2 min; 35 cycles of denaturation at 94°C for 1 min; annealing at 55–65°C for 1 min; extension at 72°C for 1 min. A cycle of 72°C for 7 min was added to complete the reaction. The reaction products were analyzed on 2% agarose gels containing ethidium bromide, and cDNA synthesis was verified by detection of the β-actin transcript.

2.6. Knockdown of PKCδ and eIF2α by siRNA. Targeted downregulation of eIF2α and PKCδ was achieved by using double-stranded small-interfering RNA (siRNA), which were purchased from Santa Cruz Biotechnology and Invitrogen Inc. The control and FITC-labeled siRNA were purchased from Qiagen (Valencia, CA). Transfection of siRNA was performed by using an optimized nucleofection protocol according to the manufacturer's instructions (Amaxa Inc., Gaithersburg, MD). Exponentially growing NB4 cells were harvested and 2×10^6 cells were used for siRNA transfection experiments. Cells were also transfected with control (nonsilencing) siRNA [35]. Under these conditions, we consistently reached a transfection efficiency of 70% without significant reduction of viability in the cell lines. Untransfected cells and cells treated with transfection reagent or control siRNA were used as controls. Target protein expression was determined 24, 48, 72, and 96 h after transfection by Western blot analysis. Fresh medium containing 1 μM ATRA was added for the analysis at 72 h. After the treatment, the cells were harvested to assess differentiation markers by FACS analysis.

2.7. Reverse Phase Protein Array (RPPA). The assay was performed at MD Anderson Cancer Center in collaboration with Dr. Steven Kornblau using AML samples under an approval protocol and consent. Paired samples from the same patients isolated at the time of diagnosis and relapse from the same patients were analyzed by RPPA assay as previously described [36, 37].

2.7.1. Statistical Analysis. The results are expressed as means ± standard deviations of three or more experiments. Statistical analysis was performed using the two-tailed Student's t-test for paired data. P values less than 0.05 were considered significant. Comparison of the protein levels detected by reverse phase protein array (RPPA) in pairs (each pair are from the same patient) of "newly diagnosed" and "relapsed" AML Samples was analyzed by paired t-test to assess the difference.

3. Results

3.1. Evaluation of ATRA- and ATO-Induced Effects. We first determined the effects of ATTA and ATO on cell differentiation and apoptosis of NB4 cells. NB4 cells (M3-AML) are true APL cells that express $t(15:17)$ PML-RARα. NB4 cells that were treated with ATRA (1 μM) at the indicated time points underwent granulocytic differentiation, as indicated by induction of CD11b and CD11c expression detected by FACS analysis (Figures 1(a), 1(b), and 1(c)). Induction of differentiation was also evidenced by PML nuclear body reformation (data not shown), May-Grünwald-Giemsa staining and transmission electron microscopy that ATRA-treated cells acquired granulocytic morphology: decreased nuclear/cytoplasm ratio, the appearance of cytoplasmic granules, and nuclear lobulation (Supplementary Figure 1). ATO induced growth inhibition by cell toxicity assay (Figure 1(d)) and apoptosis by activation of caspases 9 and 3 as well as cleavage of PARP was detected (Supplementary Figure 2) [5, 38].

3.2. ATRA and ATO Induce Ser-51 Phosphorylation of eIF2α in APL Cells. Previous studies suggest that growth inhibition and terminal differentiation are associated with inhibition of total protein synthesis and translational suppression by

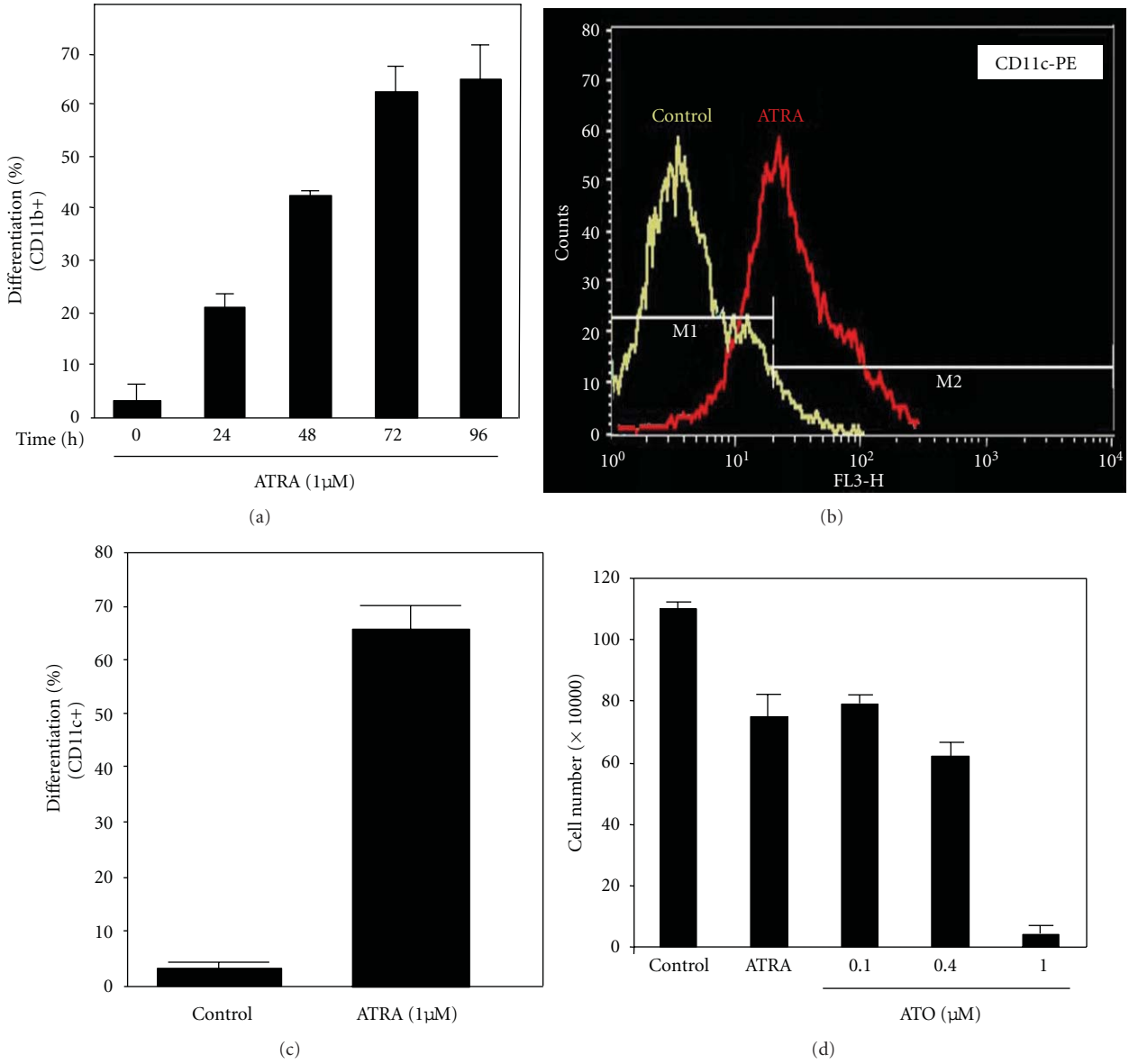


Figure 1: ATRA- and ATO-mediated effects in APL cells. APL (NB4) cells were kept in growth medium with ATRA (1 μM) or without ATRA for the indicated time periods. The cells were stained with monoclonal anti-CD11b (a) or anti-CD11c ((b) and (c)) antibodies to detect induction of granulocytic differentiation and analyzed by flow cytometry. (d) ATO inhibits growth of NB4 cells. NB4 cells were treated with ATO at the indicated concentrations for 48 h and viable cells were counted as described in Section 2. ATO-induced apoptosis was determined by the activation of caspases 9 and 3 as well as cleavage of PARP in NB4 cells (see Supplementary Figure 2 in Supplementary Material available online at doi: 10.1155/2012/482905).

differentiation-inducing agents in APL, AML, and other cells [28, 33, 34, 39, 40]. We have previously reported the evidence that ATRA modulates the expression of proteins involved in suppression of translation initiation during granulocytic differentiation of APL cells [32–34]. Phosphorylation of serine 51 on the alpha subunit of eukaryotic initiation factor eIF2 is a well-documented mechanism of inhibition of translation and global inhibition of protein synthesis under a variety of conditions in variety of cells including APL and AML cells [14–16, 40, 41]. Therefore, we first investigated whether ATRA induces phosphorylation of eIF2α for inhibition of translation and protein expression in APL cells. ATRA treatment induced marked phosphorylation (Ser 51) of eIF2α in the cells at the differentiation-inducing concentrations of ATRA (0.1 or 1 μM) in NB4 cells (Figure 2(a)). ATRA-induced phosphorylation of eIF2α started at 24 h, reached its maximum at 48 h to 72 h of treatment, and correlated well with the level of differentiation observed in NB4 cells (Figure 1(a)). Total and unphosphorylated eIF2α expression were also elevated during differentiation. Densitometric analysis showed that p-eIF2α/eIF2α and p-eIF2α/β-actin ratios were increased about 7- to 10-fold (Figure 2(a), lower

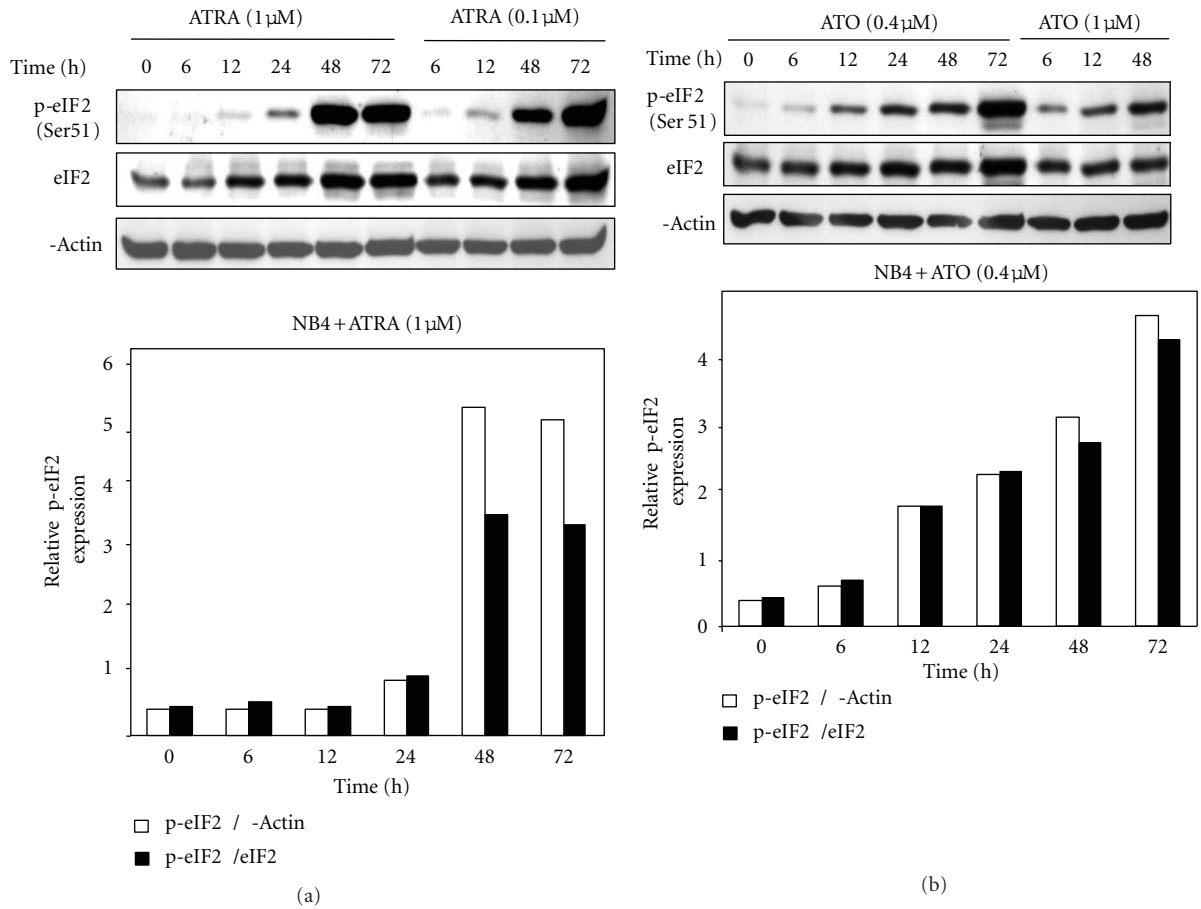


Figure 2: ATRA and ATO induce phosphorylation of eIF2α. (a) NB4 cells were treated with ATRA (0.1 μM or 1 μM) for the indicated time periods or left untreated. Equal amounts of total cell lysate were analyzed by SDS-PAGE and immunoblotted with antiphospho (Ser51) eIF2α or eIF2α antibodies. β-Actin was used as loading control for the Western blots. The panel (below) represents densitometric analysis shows relative expression of p-eIF2α after normalization to total eIF2α and actin levels. (b) ATO (0.4 μM or 1 μM) induces phosphorylation (Ser51) of eIF2α and total eIF2α in a time-dependent manner in NB4 cells. Densitometry analysis (below) represents relative p(Ser51)-eIF2α expression seen in the blots.

panel). Exposure of NB4 cells to ATO (0.4 and 1 μM) for 6–72 h also resulted in Ser-51 phosphorylation of eIF2α in NB4 cells as indicated by Western blot analysis (Figures 2(b) and 2(c)), suggesting that ATRA and ATO have similar effects in inducing phosphorylation of eIF2α.

3.3. ATRA- and ATO-Induced Phosphorylation of eIF2α Is Mediated PKR but Not PERK and GCN2. PKR is an eIF2α kinase that is known to phosphorylates eIF2α on the Ser51 residue [25, 42–44]. To determine whether ATRA- and ATO-induced phosphorylation of eIF2α is mediated by PKR activity in APL cells we examined PKR expression. We found that ATRA and ATO induce marked PKR expression in NB4 cells (Figure 3(a)). We also observed activity of PKR as indicated by p-PKR (Thr448) was also induced (Figure 3(b)). We also observed similar effects in HL60 cells that undergo granulocytic differentiation by ATRA and ATO (Figure 3(c)).

GCN2 and PERK induce eIF2α phosphorylation in response to stress and unfolded protein response (UPR) [25]. We next examined whether other eIF2α kinases, including GCN2 and PERK, are involved eIF2α regulation during ATRA. Neither ATRA nor ATO treatment resulted in induction of GCN2 and PERK in NB4 cells (Figures 3(d) and 3(e)). We did not detect any change in PERK levels, suggesting no UPR type of response is involved in the process (Figure 3(e)).

To show a link between PKR and phosphorylation of eIF2α, we knocked down PKR by a specific siRNA and found that inhibition of PKR completely blocked ATRA-induced phosphorylation (Ser51) of eIF2α in NB4 cells (Figure 3(f)). To further eliminate possibility that PERK is not involved in ATRA-induced regulation of eIF2α, we knocked down of PERK by a specific siRNA and did not detect any change in phosphorylation status of eIF2α (Supplementary Figure 3). Overall, these findings suggested that PKR plays a major role in the phosphorylation of eIF2α.

3.4. ATRA and ATO Induce p-Ser51 eIF2α and PKR in AML Cells. We next investigated whether ATRA induces phosphorylation of eIF2α in AML cells during terminal differentiation. It is well established that ATRA induces monocytic differentiation of U937 (M4/M5-AML) [45] and

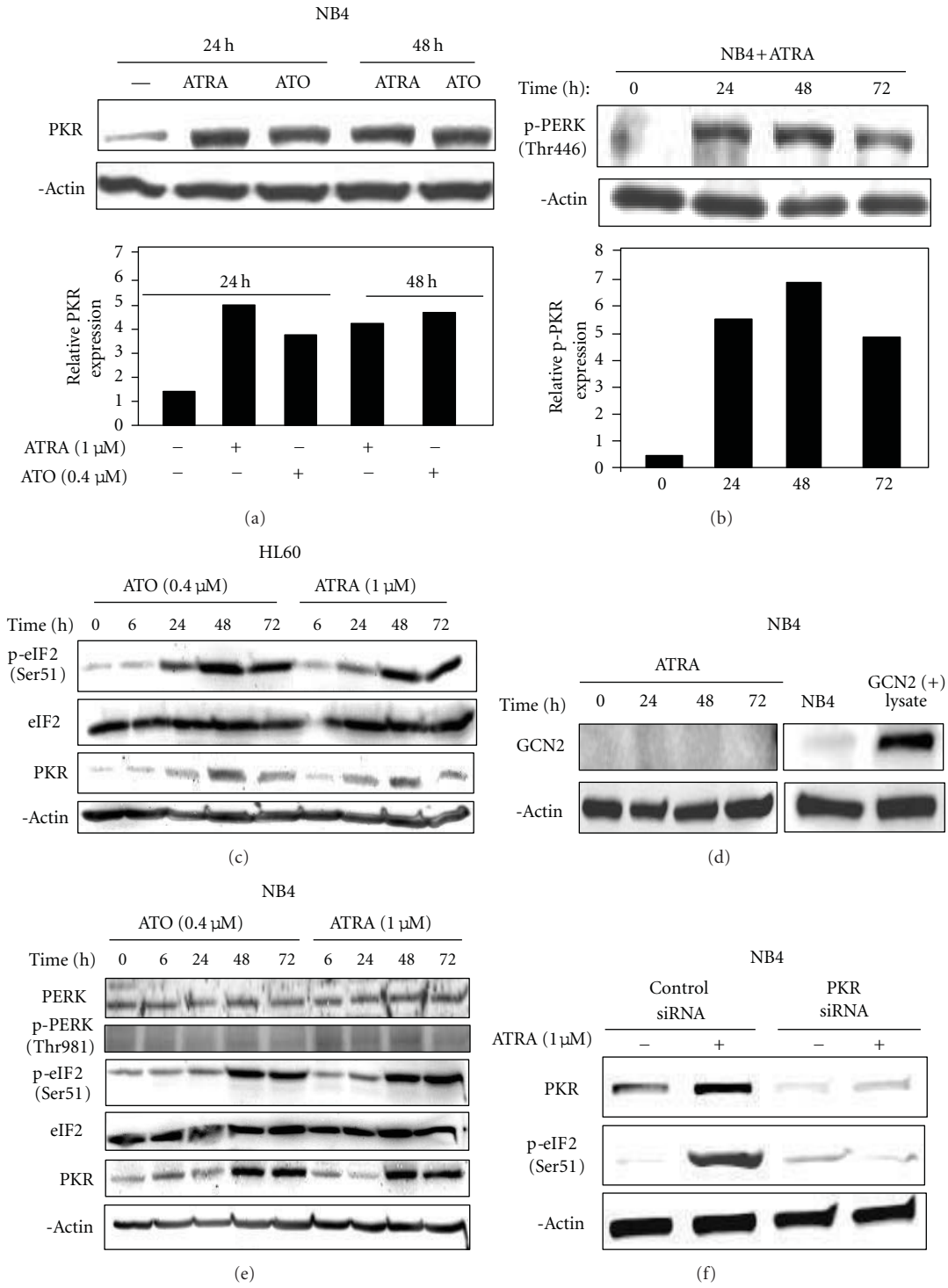


Figure 3: PKR regulates phosphorylation (Ser51) of eIF2α. (a) NB4 cells were treated with either ATRA (1 μM) or ATO (0.4 μM) at the indicated time points and PKR levels were detected by Western blot using PKR specific antibody. Densitometry analysis (lower panel) represents relative PKR expression after normalizing to actin expression. (b) NB4 cells were treated with either ATRA (1 μM) at the indicated time points and p-(Thr446) PKR levels were detected by Western blot. (c) HL60 cells were treated with either ATO (0.4 μM) or ATRA (1 μM) at the indicated time points and PERK, p-(Thr981)PERK, p-eIF2α, and PKR levels were detected by Western blot. (d) Expression of GCN2, an eIF2α kinases, is not induced by ATRA in NB4 cells. GCN2 positive cell lysate were used as positive controls for anti-GCN2 antibody and Western blot analysis. (e) ATO and ATRA do not induce PERK eIF2α kinase. NB4 cells were treated with either ATO (0.4 μM) or ATRA (1 μM) at the indicated time points and PERK, p-(Thr981)PERK, p-eIF2α, and PKR levels were detected by Western blot. (f) Knockdown of PKR by a specific siRNA blocks the ATRA-induced phosphorylation of eIF2α in NB4 cells by Western blot. Bar graph represents inhibition and the relative expression of PKR (PKR/β-actin) and p-eIF2α (p-eIF2α/β-actin) after siRNA treatments by densitometry analysis of Western blot bands.

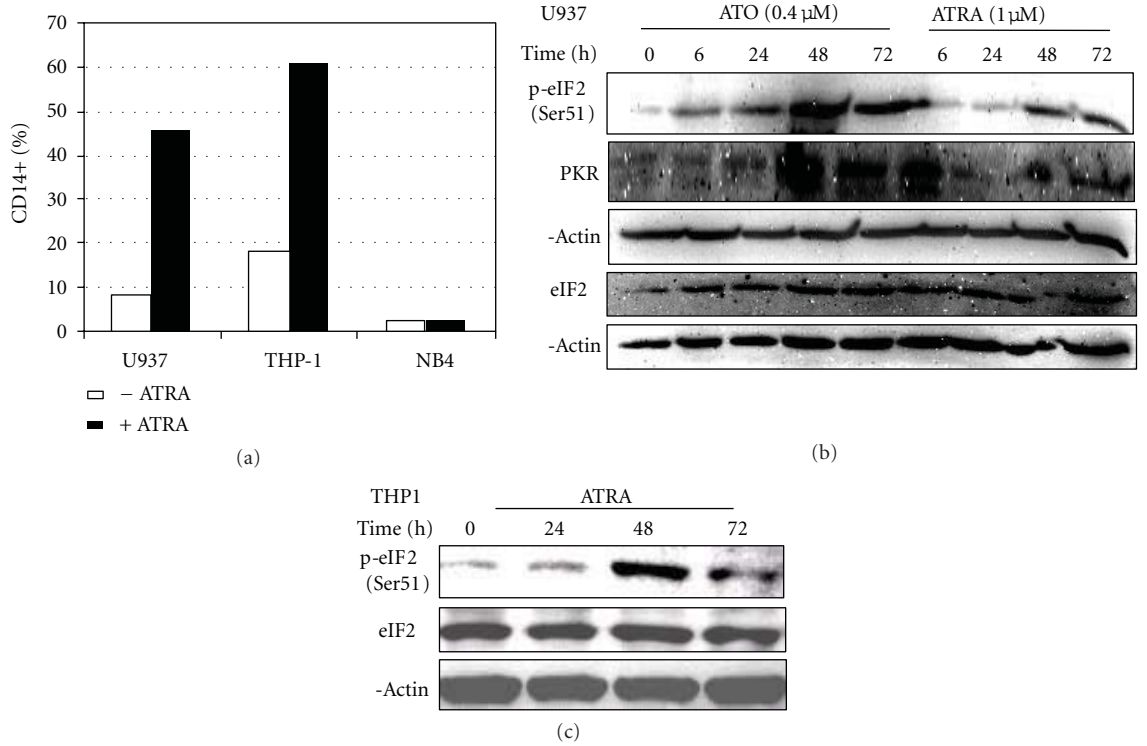


Figure 4: ATRA and ATO induce p-eIF2α during monocytic differentiation of AML cells. (a) ATRA (1 μM) induces monocytic differentiation in U937 and THP-1 cells but not in NB4 cells as indicated by induction of CD14 expression on the cells detected by FACS analysis. (b) ATRA and ATO induce phosphorylation (Ser51) of eIF2α expression in U937 AML cells. The U937 cells were treated with either ATRA (1 μM) or ATO (0.4 μM) for the indicated time periods and analyzed by Western blot. (c) ATRA (1 μM) also induces phosphorylation (Ser51) of eIF2α expression in THP-1 monocytic AML.

THP-1 (M4-AML) [46, 47] cells. Monocytic differentiation of ATRA (1 μM) treatment of U937 and THP-1 cells was shown by CD14 expression by FACS analysis (Figure 4(a)). ATRA (1 μM) treatment of THP-1 and U937 cells led to an induction of phosphorylation (Ser51) of eIF2α, which is associated with marked PKR expression (Figures 4(b) and 4(c)). ATO (0.4 uM) also induced p-Ser51 eIF2α and PKR expression. These findings provided further evidence that phosphorylation of eIF2α is regulated by PKR by ATRA and ATO.

3.5. PKCδ Is Involved in Ser51-Phosphorylation of eIF2α in APL Cells. To identify the signaling mechanism that regulates/activates PKR in response to ATRA or ATO we examined PKCδ, a serine/threonine kinase that is induced during ATRA-induced differentiation in APL cells [30, 48]. We found that ATRA and ATO induce PKCδ expression, which is closely correlated with the increased phosphorylation (Ser 51) of eIF2α in NB4 cells by Western blot analysis (Figure 5(a)). Upregulation of PKCδ expression was also associated with phosphorylation of PKCδ on threonine 505 (Thr-505) in the activation loop (Figure 5(b)). RT-PCR analysis suggested that ATRA (1 μM) induces PKCδ expression at the transcriptional level (Figure 5(c)). ATRA also induced PKCδ expression also correlated with increased phosphorylation of eIF2α in U937 AML cells (Figure 5(d)), indicating that PKC activation is not APL cell line specific event.

To determine whether PKCδ plays a role in the regulation of eIF2α phosphorylation (Ser-51), we inhibited PKCδ using a PKCδ inhibitor rottlerin (at 4 μM specifically inhibits PKCδ) [49, 50]. Rottlerin treatment blocked basal levels and ATRA-induced phosphorylation of eIF2α in NB4 cells (Figure 5(e)). Densitometric analysis revealed that inhibition of PKCδ led to 5-fold reduction in p-eIF2α levels (lower panel). Overall, these findings suggest that PKCδ is involved in regulation of phosphorylation of eIF2α.

3.6. PKCδ Regulates Phosphorylation of eIF2α in HSC and AML Cells. We also investigated whether PKCδ regulates eIF2α activity in normal human hematopoietic stem cells (HSC) isolated from bone marrow of a healthy donor. Western blot analysis showed that inhibition of PKCδ by rottlerin (4 μM) markedly blocked ATRA-induced phosphorylation of eIF2α in HSCs, HL60 myeloblastic (AML-M2), and THP1 monocytic AML cells (AML-M5) (Figures 6(a)–6(c)). Rottlerin treatment also caused a slight inhibition of total eIF2α. These findings indicated that PKCδ plays a role in regulation of eIF2α not only in APL and AML as well as normal HSCs.

3.7. PKCδ Regulates Phosphorylation of eIF2α through PKR. PKR, a component of the antiviral defense, is of the kinases

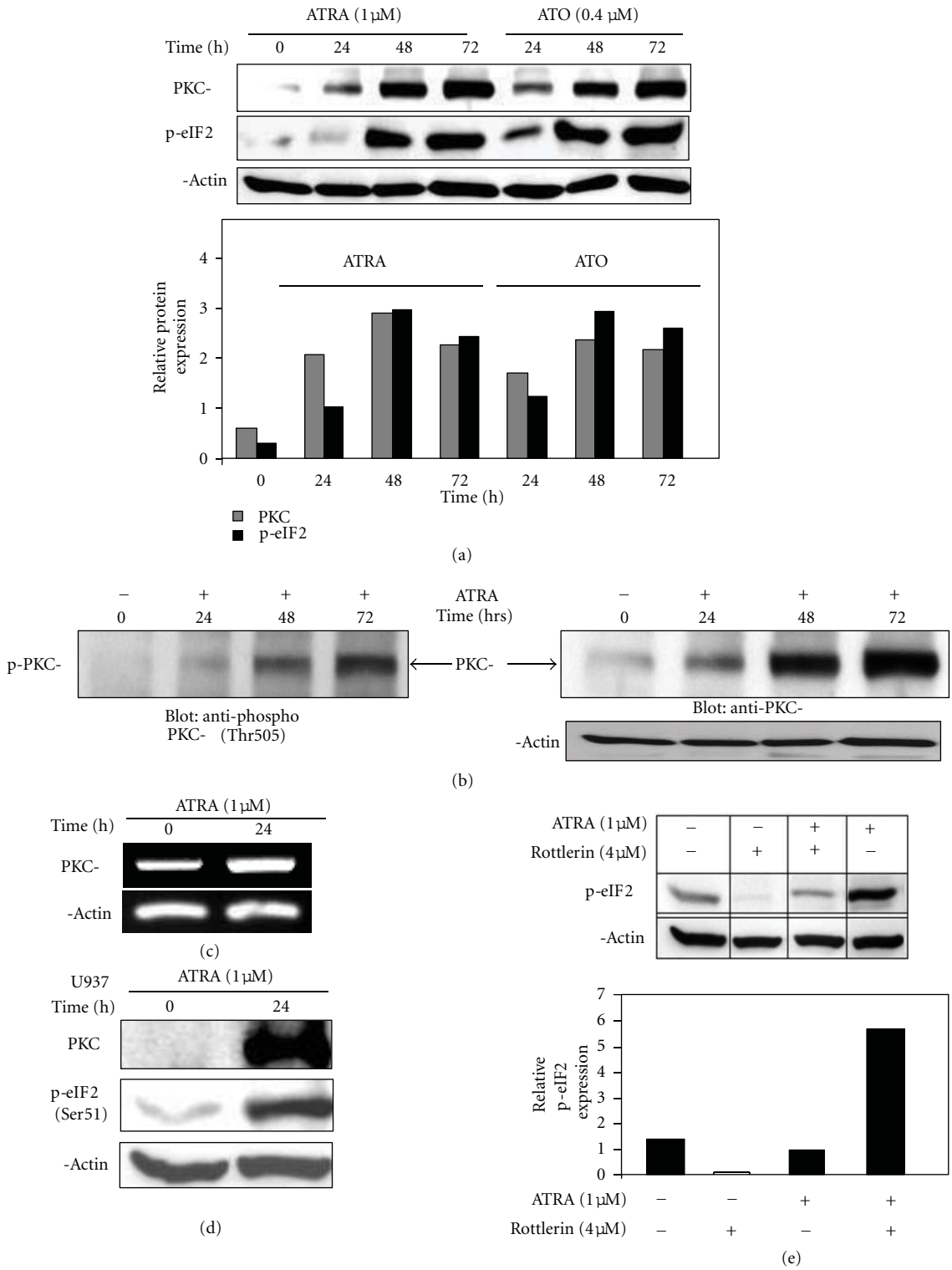


Figure 5: PKCδ regulates the phosphorylation (Ser51) of eIF2α by ATRA and ATO in APL cells. (a) NB4 cells were left untreated or incubated with ATRA (1 μM) or ATO (0.4 μM), for the indicated time periods. Equal amounts of total cell lysate were analyzed by SDS-PAGE and immunoblotted with specific antibodies against PKCδ or p-eIF2α. β-Actin was used as a loading control. (b) ATRA (1 μM) induces phosphorylation (Thr505) of PKCδ at the activation domain detected by Western blot in NB4 cells. (c) ATRA (1 μM) induces PKCδ mRNA expression. After 24 h of ATRA treatment, NB4 cells were collected and total cellular RNA was extracted to detect PKC by RT-PCR using specific primers. The reaction products were analyzed on 2% agarose gels. cDNA synthesis and equal loading were verified by detection of the β-actin transcript. (d) ATRA (1 μM) induces phosphorylation of eIF2α in U937 (M4/M5-AML) cells. (e) Inhibition of PKCδ by rottlerin inhibits basal and ATRA-induced phosphorylation (Ser51) of eIF2α. Exponentially growing NB4 cells were collected and pretreated with specific PKCδ inhibitor rottlerin (4 μM) for 4 h before adding ATRA (1 μM) into the culture medium. Equal amount of total cell lysates were analyzed by SDS-PAGE and immunoblotted with specific antibodies against p-eIF2α, as described in Section 2.

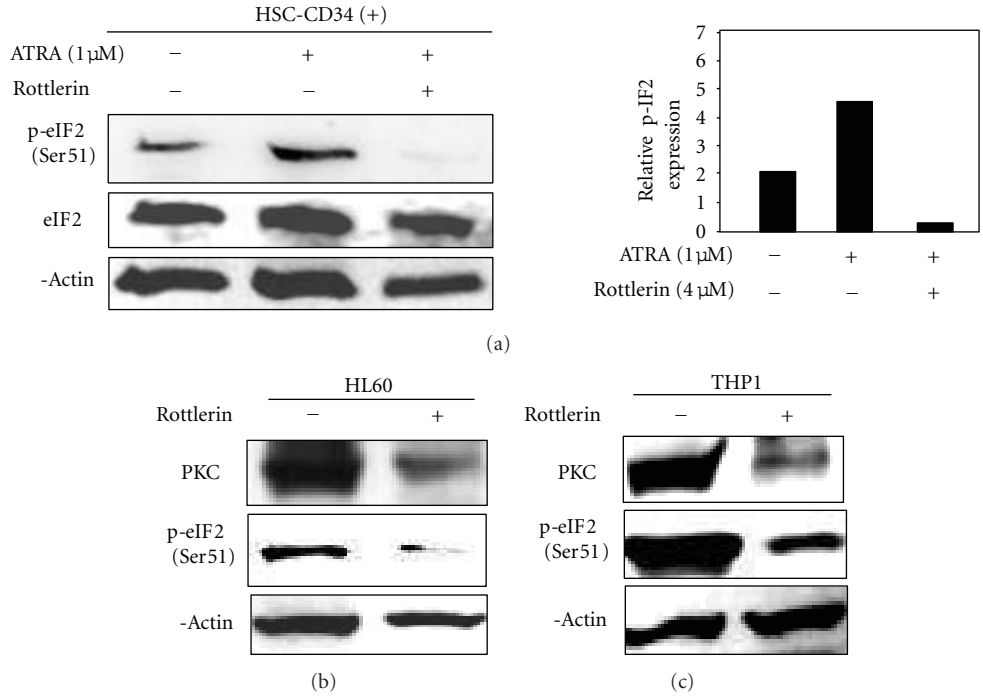


Figure 6: PKCδ mediates ATRA-induced phosphorylation (Ser51) of eIFα in normal CD34+HCC and AML cells. (a) All cells were treated with ATRA (1 μM) in the presence or absence of rottlerin for 24 h and p-(Ser51)-eIF2α was examined by Western blot analysis. ATRA induced-eIF2α phosphorylation in human normal CD34+ bone marrow HCC cells. Densitometric analysis shows (right panel) relative expression of p-eIF2α protein levels after normalization to β-actin expression level. Inhibition of PKCδ by rottlerin blocked the Ser51-phosphorylation eIF2α in HCC cells (a), HL60 (M2-AML) (b), and THP1 (M4-AML) cells (c).

known to phosphorylate eIF2α at Ser51 to inhibit its activity and translation initiation and protein synthesis [25]. Because our data previously indicated that inhibition of PKR (Figure 3(f)) or PKCδ (Figure 5(d)) prevented basal and ATRA-induced phosphorylation of eIF2α, we hypothesized that PKCδ, a Serine/Threonine kinase, may be responsible for activation of PKR through phosphorylation on Thr446. Therefore, we next examined whether PKCδ regulates PKR phosphorylation. Pretreatment of NB4 cells with PKCδ inhibitor rottlerin significantly inhibited ATRA-induced Thr446-phosphorylation of PKR (Figure 7(a)). The bar graph next to Figure 7(a) shows densitometric analysis of Figure 7(a). Figure 6(b) confirms that rottlerin treatment inhibited PKCδ in response to ATRA in NB4 cells. To provide further link between PKCδ and PKR we knockdown PKCδ by a specific siRNA and found that inhibition of PKCδ expression and reduced activity (phosphorylation) of PKR (Thr446) (Figure 7(c) and densitometry, right panel), indicating that PKCδ regulates PKR activity/phosphorylation in APL cells.

To evaluate the role of PKCδ signaling in cell differentiation of APL cells we specifically inhibited PKCδ and assessed ATRA-induced (48 h) differentiation in NB4 cells. Inhibition of PKCδ by rottlerin significantly inhibited ATRA-induced granulocytic differentiation in cells (Figure 8). While ATRA (1 μM for 48 h) treatment led to about 43.4% cell differentiation in NB4 cells, preincubation with rottlerin reduced ATRA-induced differentiation to 5.2% ($P < 0.05$). This finding suggested that PKCδ plays a key role in ATRA-induced granulocytic differentiation.

3.8. Inhibition of Mammalian Target of Rapamycin (mTOR) Induces p-(Ser51) eIF2α through PKCδ/PKR Axis. Inhibition of mammalian target of rapamycin (mTOR) signaling has been shown to potentiate the effects of ATRA and ATO to induce growth arrest and differentiation of APL (NB4) and AML cells in vitro and in vivo models [51, 52]. mTOR signaling is one of the major regulators of translation in cancer cells by altering 4EBP-1 and eIF4E activity [53]. We have previously shown that ATRA and ATO inhibit the activity PI3K/Akt/mTOR and p70S6 kinase [33, 34]. To investigate whether mTOR regulates eIF2α through PKR we inhibited the PI3K/Akt/mTOR pathway by a specific mTOR inhibitor rapamycin (20 nM). Treatment of NB4 cells with rapamycin for 24 h markedly induced p-PKR and total PKR expression that is closely associated with phosphorylation (Ser51) of eIF2α (Figure 9(a)). Inhibition of PI3K pathway by a specific inhibitor LY294002 (20 uM) [33] also resulted in increased p-(Ser51) eIF2α and PKCδ in NB4 cells (Figure 9(b)), indicating that the PI3K/Akt/mTOR pathway is involved in phosphorylation of eIF2α by PKCδ/PKR in APL cells.

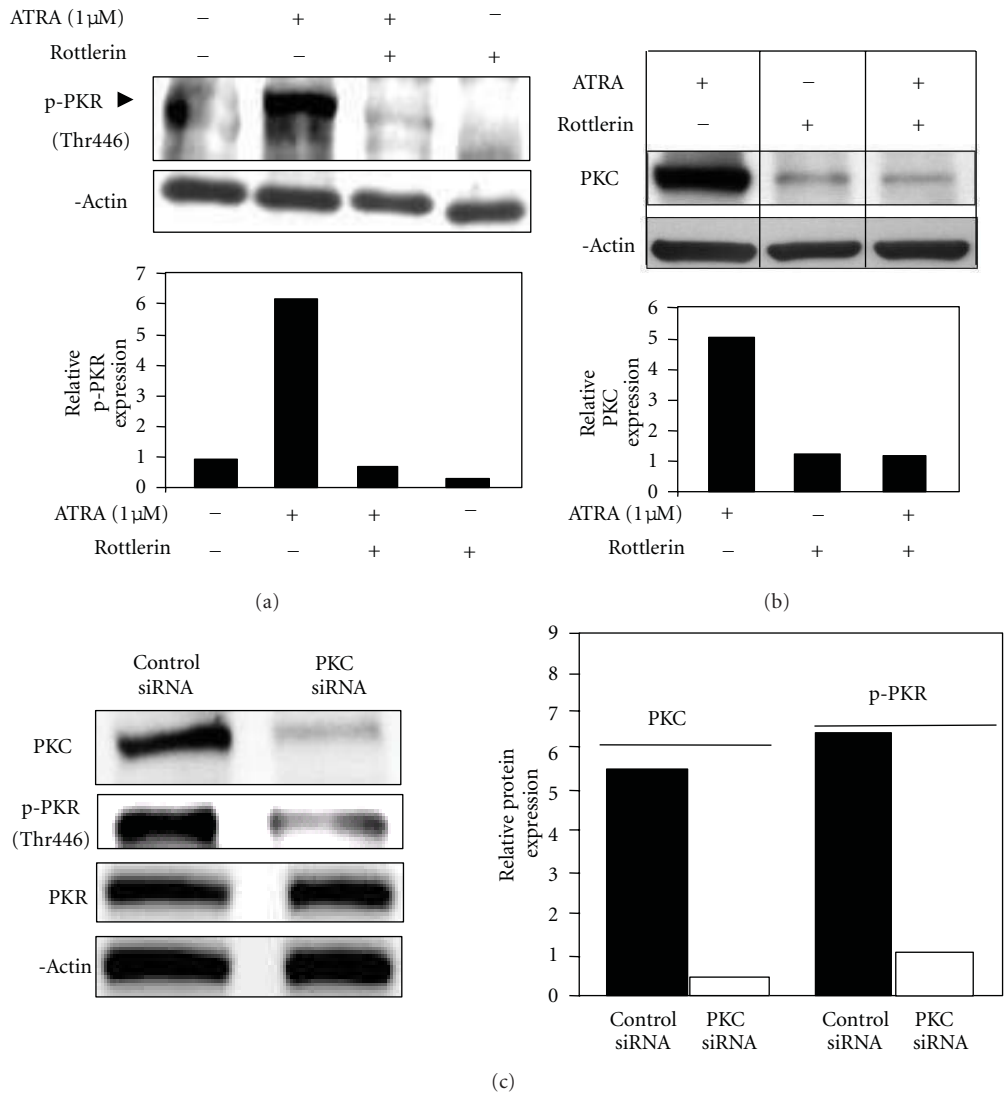


Figure 7: Inhibition of PKCδ blocks ATRA-induced PKR and Ser51-phosphorylation of eIF2α. (a) Inhibition of PKCδ blocked ATRA-induced phosphorylation (Thr446)/activation of PKR. NB4 cells were pretreated with PKCδ inhibitor rottlerin (4 μM) for 4 h before adding ATRA (1 μM). (b) Rottlerin (4 M) inhibits ATRA-induced expression of PKCδ in NB4 cells detected by Western blot analysis. (c) Knockdown of PKCδ by a specific siRNA results in reduction of phosphorylation (Thr446) of PKR in NB4 cells. Bar graph represents inhibition of relative expression of PKCδ (PKCδ/β-actin) and p-PKR (p-PKR/β-actin) based on densitometric analysis of Western blots.

3.9. eIF2α Phosphorylation Leads to Differential Expression of Important Cellular Proteins. Studies have shown that ATRA regulates important regulatory proteins such as cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ [54], death-associated protein 5 (DAP5/p97) [55, 56], c-myc [23, 57], p-P70S6K [58], and TG2 [46, 47, 59]. To gain some insight about the ATRA-mediated downstream changes in response to eIF2α regulation we knocked down eIF2α by siRNA and examined if expression of these proteins is altered. Knockdown of eIF2α inhibited ATRA-induced upregulation of $p21^{Waf1/Cip1}$, DAP5/p97, and TG2 (Figure 10(a)). We also observed inhibition of ATRA-induced downregulation of c-myc and p-P70S6K, a downstream mediator of PI3K/Akt/mTOR pathway (Figure 10(a)). These findings suggest that eIF2α in fact is involved in expression of some of the proteins involved in cell cycle arrest, proliferation, survival, differentiation, and apoptosis.

3.9.1. PKCδ and p-eIF2α Protein Expressions Are Associated with Relapses in AML Patient. PKCδ and p-eIF2α protein expression was assessed by RPPA assay in 47 paired samples from newly diagnosed and relapsed AML patients to determine if the protein level changes when the disease status changes. The comparison of PKCδ (Figures 11(a) and 11(c)) and p-eIF2α protein (Figures 11(b) and 11(d)) expression and distributions of the protein levels between pairs (newly diagnosed and relapsed) were analyzed. Data suggest that there is a significant relationship between the reduced levels

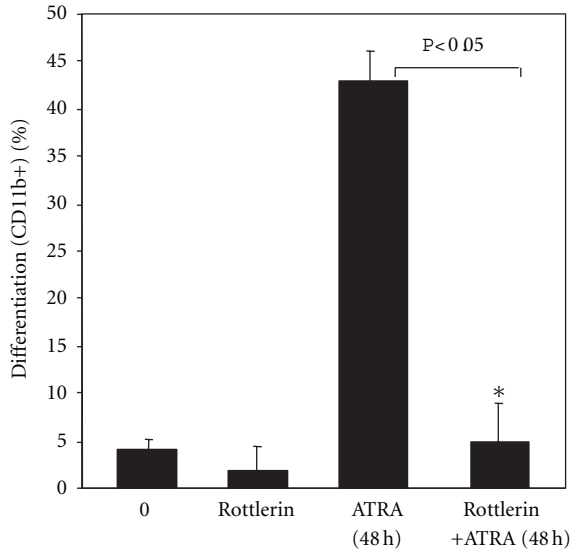


Figure 8: PKCδ plays a critical role in ATRA-induced terminal differentiation of APL cells. Inhibition of PKCδ blocked ATRA-induced granulocytic differentiation of NB4 cells. NB4 cells were pretreated with rottlerin (4 μM) for 4 h before adding ATRA (1 μM). After 48 h of ATRA treatment, cells were collected and analyzed for CD11b positivity as a measure of granulocytic differentiation.

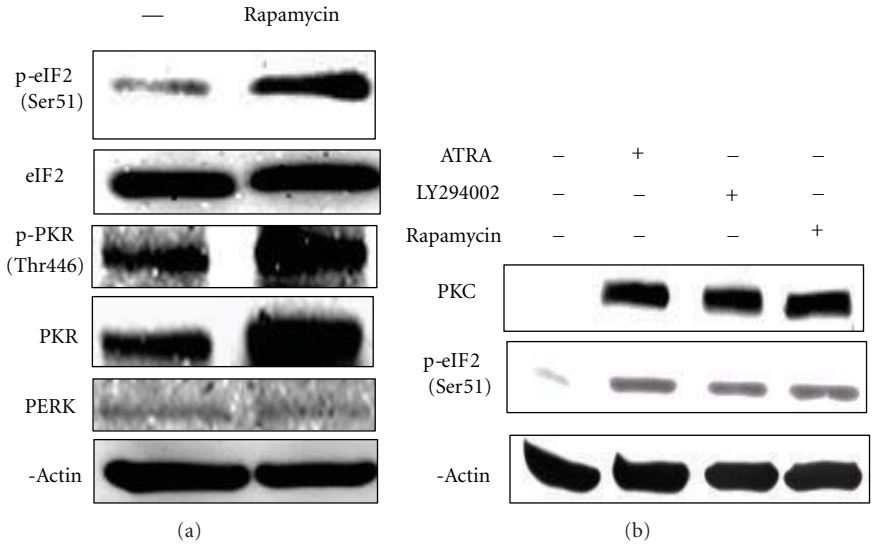


Figure 9: mTOR signaling is involved in regulation of (Ser51) of eIF2α through PKC and PKR. (a) Inhibition of PI3K/Akt/mTOR signaling pathway by rapamycin (20 nM), a specific mTOR inhibitor, led to induction of phosphorylation eIF2α in NB4 cells. Rapamycin treatment results in induction of p-(Ser51) eIF2α and PKR and p(Thr446)-PKR in NB4 cells. (b) NB4 cells were pretreated with a specific PI3K inhibitor (LY294002, 20 μM) induced PKCδ and p-eIF2α levels. ATRA Equal amounts of total cell lysate were subjected to Western blot analysis to detect p-eIF2α, p-Akt, and PKCδ expression.

of PKCδ ($P = 0.0378$) and p-eIF2α ($P = 0.0041$) and relapses in AMP patients, suggesting that higher levels of PKCδ and p-eIF2α is associated with better response.

4. Discussion

Overactivity of translation initiation factors, such as eIF2α, eIF4E, and eIF4G, results in malignant transformation, indicating that regulation of the activity of these factors is critical in controlling survival pathways and cell proliferation [6–13, 22]. Previous reports including ours suggest that growth inhibition and terminal cell differentiation are associated with suppression of translation initiation [28, 32–34, 39, 60]. However, molecular mechanisms responsible for regulation of eIF2α during these events have not been elucidated.

The present study provides the first evidence that PKCδ regulates activity of eIF2α through induction of PKR, an eIF2α kinase, but not PERK and GCN2, leading to inhibition

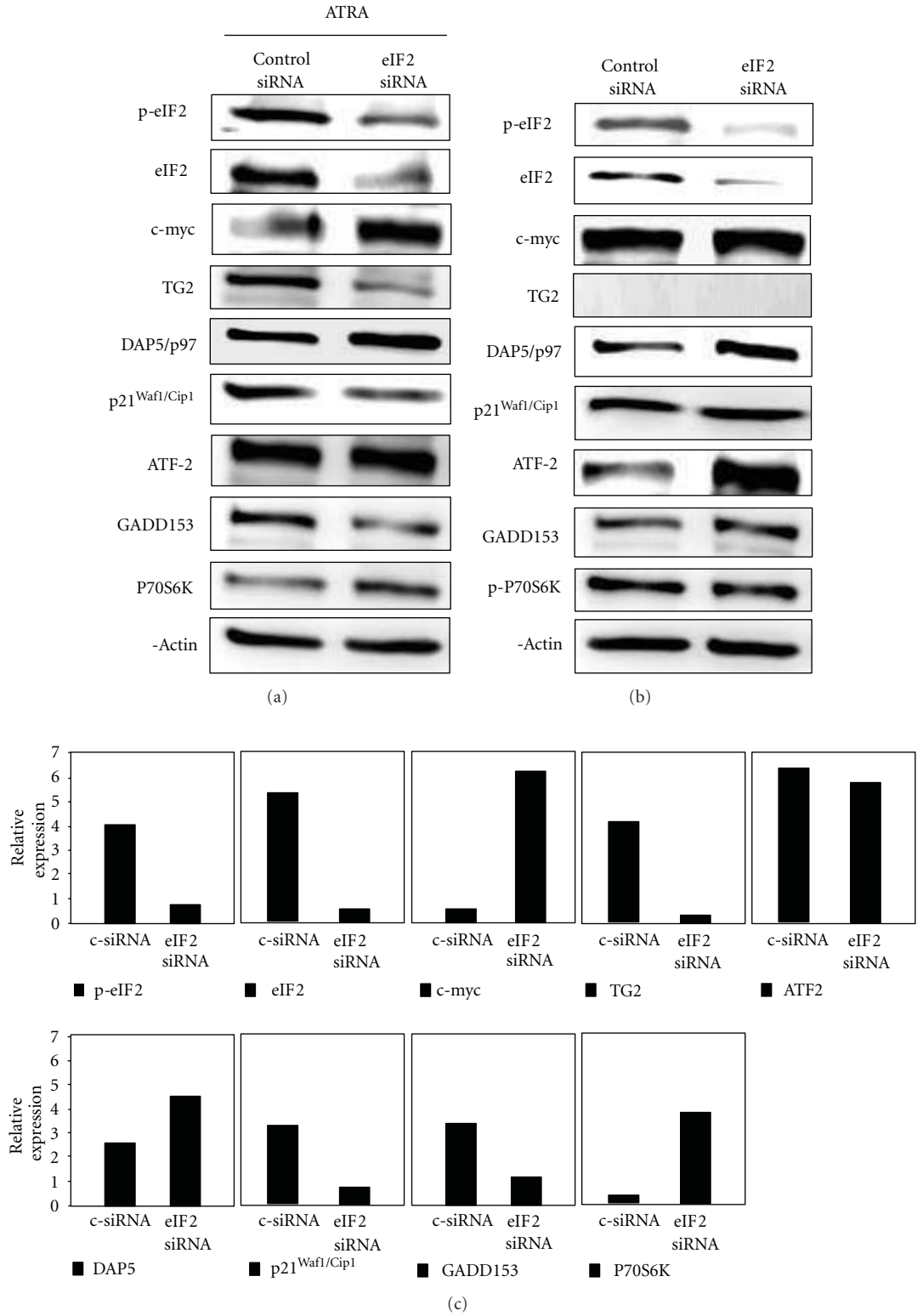


Figure 10: eIF2α is involved in ATRA-mediated expression of c-myc, DAP5, TG2, and P70S6 in NB4 cells. NB4 cells were transfected with control or eIF2α siRNA for 48 h in the absence (a) or in the presence of ATRA (1 μM, 48 h) (b). (c) Densitometry analysis (lower panel) represents relative expression of Western blot bands after treatments with control or eIF2α siRNA in the presence of ATRA. Protein bands were normalized expression based actin levels.

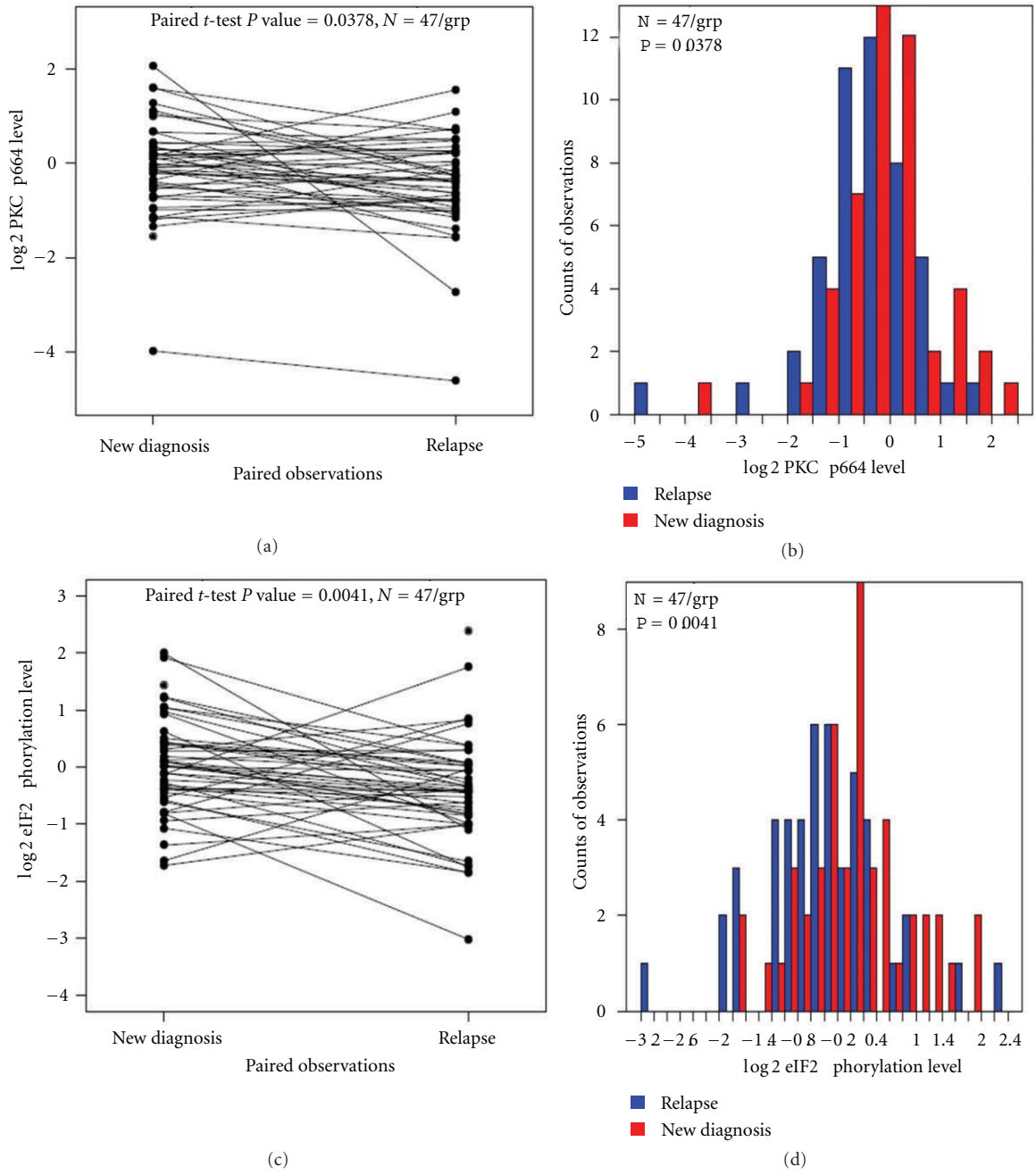


Figure 11: Reduced PKCδ and p-eIF2α protein expression is associated with relapses in AM patients. PKCδ and p-eIF2α protein expression was assessed by RPPA in 47 paired samples from newly diagnosed and relapsed AML patients to determine if the protein level changes when the disease status changes. The comparison of PKCδ and p-eIF2α protein expression ((a) and (c)) and distributions ((b) and (d)) of the protein levels between pairs (newly diagnosed and relapsed) were plotted. Data suggest that there is a significant association between the reduced levels of PKCδ ($P = 0.0378$) and p-eIF2α ($P = 0.0041$) and relapses in AMP patients.

of translation initiation during terminal differentiation of APL and AML cells (Figure 12). Moreover, our study suggested that PI3K/Akt pathway and its downstream mediator mTOR, a major hub of translational control, is involved in regulation of eIF2α through PKCδ/PKR axis. Overall our findings revealed a novel mechanistic insight on actions of ATRA and ATO in regulation of eIF2α, the rate limiting step of translation initiation in APL and AML cells.

PKCδ, a serine/threonine kinase, can function as a tumor suppressor and a proapoptotic factor and can regulate cell proliferation and cell survival functions [30]. Induction of PKCδ has been previously shown in APL cells [48]. PKCδ

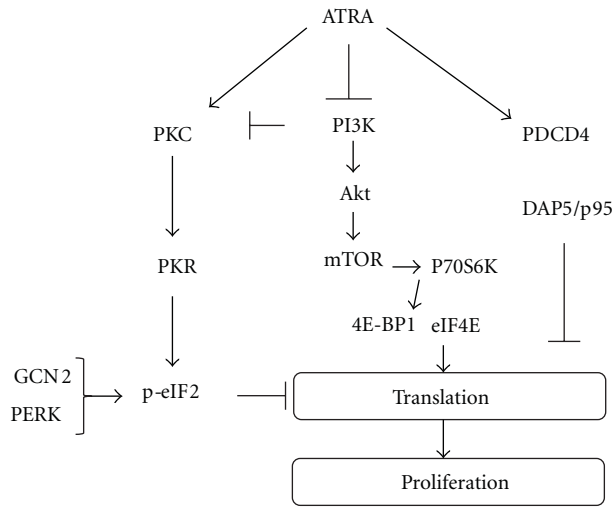


Figure 12: PKCδ is involved in regulation of translational initiation by eIF2α through PKR axis in AML cells. ATRA and ATO inhibit translation initiation by inducing inactivation (phosphorylation at Ser-51) of eIF2α. Suppression of translation initiation is also regulated by other mediators, including induction of translational suppressors (PDCD4 and DAP5) and inhibition of 4E-BP1 [32–34]. The eIF2α kinases other than PKR, such as GCN2 and PERK, are not involved in regulation of eIF2α during ATRA-induced differentiation. eIF2α is involved in regulation of ATRA-regulated cellular proteins in cell cycle arrest ($p21^{Waf1/Cip1}$), proliferation (c-myc), survival (p70S6K), and apoptosis (DAP5/p97) and differentiation (TG2).

is critical to ATRA-induced terminal differentiation because inhibition of PKCδ by rottlerin resulted in almost complete blockage of ATRA-induced differentiation (Figure 8). Our data also indicated that induction of PKCδ signaling is critical for regulation of eIF2α, linking for the first time PKCδ with the regulation of translation initiation, which is often over activated in AML cells. Most importantly, our data suggested that PKCδ regulates PKR and eIF2α and thereby revealing a novel function of PKCδ.

PKR, GCN2, and PERK are eIF2α kinases that are known to phosphorylate and inhibit the activity of eIF2α [9, 25]. However, we found that ATO and ATRA treatments induce PKR but not GCN2 and PERK. The activation of unfolded protein response (UPR) is manifested by phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) and eIF2α. In our study we did not detect any change in the activity (or phosphorylation) of PERK on phosphorylation of eIF2α, suggesting that ATRA and ATO do not induce UPR response. PKR has been shown to be induced by interferon in myeloid leukemia cells [44]. Interferon is known to inhibit translation and protein synthesis during viral infections, limiting production of viral particles. It is possible that ATRA-induced interferon secretion from the cells may lead to upregulation and activation of PKR in APL by autocrine mechanisms. This hypothesis remains to be tested by future studies.

PI3K/Akt/mTOR signaling pathway is overactivated in APL and AML cells and plays an important role in proliferation, drug resistance, inhibition of apoptosis in cancer cells [50, 61]. mTOR signaling is a critical inducer of translational activity by phosphorylating 4EBP-1 and releasing eIF4E from 4EBP1 and increasing activity translation initiation complex [53]. We found that ATRA and ATO inhibits phosphorylation of 4E-BP1 in NB4 APL cells (unpublished observation). Dephosphorylated 4E-BP1 inhibits translation initiation by binding to eIF4E, which normally binds to the cap-structure of mRNA to form an initiation complex [62, 63]. Reduced phosphorylation of 4E-BP1 by ATRA facilitates its binding to eIF4E, leading to inhibition of eIF4E. We have also shown that ATRA and ATO inhibit the activity PI3K/Akt/mTOR and p70S6 kinase in APL cells [33, 34]. The current study is in agreement with our previous findings that ATRA inhibits translation initiation by multiple mechanisms, including inhibition of initiation factors and induction of PDCD4 and DAP5 (inhibitors of translation initiation), inhibition of p-4E-BP1 and EF4E (Figure 11) [32–34]. DAP5 and PDCD4, a novel tumor suppressor protein, were recently identified as inhibitors of translation initiation. PDCD4 binds to eIF4A and specifically inhibits its helicase activity [64]. DAP5 functions as a repressor of translation initiation by forming translationally inactive complexes with eIF4A and eIF3 [56]. Overall, data suggest that translation initiation and protein synthesis are suppressed by several mechanisms by ATRA and ATO in APL cells.

Several specific mRNAs have been found to be selectively regulated in response to inhibition of eIF2α. These selectively upregulated proteins include ATF-2, ATF-3, ATF-4, GADD34, and CHOP/GADD153 [25, 28, 65], suggesting that even during inhibition of translation some of the mRNAs are being translated. Our study suggest that eIF2α is involved in expression of $p21^{Waf1/Cip1}$, DAP5, and TG2 in response to ATRA during differentiation, supporting the hypothesis that subset of mRNAs encoding critical proteins are differentially regulated by eIF2α. On the other hand, increased expression of eIF2α in response to growth induction by c-myc [23] and transformed cells [24] suggest that eIF2α plays a critical role in regulation of cell proliferation and is strictly regulated because of its oncogenic potential.

In conclusion, a better understanding of translation initiation and posttranscriptional mechanisms may help identify novel targets for targeted therapies. Antitumor agents such as rapamycin, a specific mTOR inhibitor, or related compounds that inhibit translation by inhibiting phosphorylation of 4E-BP1 and P70S6K have been shown to induce remissions even in AML patients with relapsed disease, suggesting that the targeted inhibition of mRNA translational pathways might offer therapeutic benefits for patients with certain malignancies.

Authors' Contribution

B. Ozpolat and U. Akar contributed equally to this work.

Acknowledgments

The authors would like to thank Pierette Lo for editing the paper. This study was supported in part by grants from the Ladies Leukemia League (B. Ozpolat) and the National Cancer Institute (U54 RFA CA096300) (G. Lopez-Berestein).

References

[1] L. Altucci and H. Gronemeyer, "The promise of retinoids to fight against cancer," *Nature Reviews Cancer*, vol. 1, no. 3, pp. 181–193, 2001.

[2] M. Lanotte, V. Martin-Thouvenin, S. Najman, P. Balerini, F. Valensi, and R. Berger, "NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3)," *Blood*, vol. 77, no. 5, pp. 1080–1086, 1991.

[3] T. R. Breitman, S. J. Collins, and B. R. Keene, "Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid," *Blood*, vol. 57, no. 6, pp. 1000–1004, 1981.

[4] C. Chomienne, P. Fenaux, and L. Degos, "Retinoid differentiation therapy in promyelocytic leukemia," *FASEB Journal*, vol. 10, no. 9, pp. 1025–1030, 1996.

[5] Z. X. Shen, G. Q. Chen, J. H. Ni et al., "Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (AFL): II. Clinical efficacy and pharmacokinetics in relapsed patients," *Blood*, vol. 89, no. 9, pp. 3354–3360, 1997.

[6] M. J. Clemens and U. A. Bommer, "Translational control: the cancer connection," *International Journal of Biochemistry and Cell Biology*, vol. 31, no. 1, pp. 1–23, 1999.

[7] A. E. Willis, "Translational control of growth factor and proto-oncogene expression," *International Journal of Biochemistry and Cell Biology*, vol. 31, no. 1, pp. 73–86, 1999.

[8] C. O. Brostrom and M. A. Brostrom, "Regulation of translational initiation during cellular responses to stress," *Progress in Nucleic Acid Research and Molecular Biology*, vol. 58, pp. 79–125, 1998.

[9] M. J. Clemens, "Initiation factor eIF2α phosphorylation in stress responses and apoptosis," *Progress in Molecular and Subcellular Biology*, vol. 27, pp. 57–89, 2001.

[10] M. J. Clemens, "Targets and mechanisms for the regulation of translation in malignant transformation," *Oncogene*, vol. 23, no. 18, pp. 3180–3188, 2004.

[11] O. Donze, R. Jagus, A. E. Koromilas, J. W. B. Hershey, and N. Sonenberg, "Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells," *The EMBO Journal*, vol. 14, no. 15, pp. 3828–3834, 1995.

[12] V. M. Pain, "Initiation of protein synthesis in eukaryotic cells," *European Journal of Biochemistry*, vol. 236, no. 3, pp. 747–771, 1996.

[13] T. E. Dever, "Gene-specific regulation by general translation factors," *Cell*, vol. 108, no. 4, pp. 545–556, 2002.

[14] A. Sudhakar, A. Ramachandran, S. Ghosh, S. E. Hasnain, R. J. Kaufman, and K. V. A. Ramaiah, "Phosphorylation of serine 51 in initiation factor 2α (eIF2α) promotes complex formation between eIF2α(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B," *Biochemistry*, vol. 39, no. 42, pp. 12929–12938, 2000.

[15] B. Datta, R. Datta, S. Mukherjee, and Z. Zhang, "Increased phosphorylation of eukaryotic initiation factor 2α at the G2/M boundary in human osteosarcoma cells correlates with deglycosylation of p67 and a decreased rate of protein synthesis," *Experimental Cell Research*, vol. 250, no. 1, pp. 223–230, 1999.

[16] A. Leroux and I. M. London, "Regulation of protein synthesis by phosphorylation of eukaryotic initiation factor 2α in intact reticulocytes and reticulocyte lysates," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 79, no. 7 I, pp. 2147–2151, 1982.

[17] A. Lazaris-Karatzas, K. S. Montine, and N. Sonenberg, "Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap," *Nature*, vol. 345, no. 6275, pp. 544–547, 1990.

[18] A. DeBenedetti and R. E. Rhoads, "Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 21, pp. 8212–8216, 1990.

[19] A. DeBenedetti and A. L. Harris, "EIF4E expression in tumors: its possible role in progression of malignancies," *International Journal of Biochemistry and Cell Biology*, vol. 31, no. 1, pp. 59–72, 1999.

[20] T. Fukuchi-Shimogori, I. Ishii, K. Kashiwagi, H. Mashiba, H. Ekimoto, and K. Igarashi, "Malignant transformation by overproduction of translation initiation factor eIF4G," *Cancer Research*, vol. 57, no. 22, pp. 5041–5044, 1997.

[21] C. Bauer, I. Diesinger, N. Brass, H. Steinhart, H. Iro, and E. U. Meese, "Translation initiation factor eIF-4G is immunogenic, overexpressed, and amplified in patients with squamous cell lung carcinoma," *Cancer*, vol. 92, no. 4, pp. 822–829, 2001.

[22] M. Böhm, K. Sawicka, J. P. Siebrasse, A. Brehmer-Fastnacht, R. Peters, and K. H. Klempnauer, "The transformation suppressor protein Pdcd4 shuttles between nucleus and cytoplasm and binds RNA," *Oncogene*, vol. 22, no. 31, pp. 4905–4910, 2003.

[23] I. B. Rosenwald, D. B. Rhoads, L. D. Callanan, K. J. Isselbacher, and E. V. Schmidt, "Increased expression of eukaryotic translation initiation factors eIF-4E and eIF-2α in response to growth induction by c-*myc*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 13, pp. 6175–6178, 1993.

[24] I. B. Rosenwald, "Upregulated expression of the genes encoding translation initiation factors eIF-4E and eIF-2α in transformed cells," *Cancer Letters*, vol. 102, no. 1-2, pp. 113–123, 1996.

[25] R. C. Wek, H. Y. Jiang, and T. G. Anthony, "Coping with stress: EIF2 kinases and translational control," *Biochemical Society Transactions*, vol. 34, no. 1, pp. 7–11, 2006.

[26] I. Topisirovic, M. L. Guzman, M. J. McConnell et al., "Aberrant eukaryotic translation initiation factor 4E-dependent mRNA transport impedes hematopoietic differentiation and contributes to leukemogenesis," *Molecular and Cellular Biology*, vol. 23, no. 24, pp. 8992–9002, 2003.

[27] J. Eberle, K. Krasagakis, and C. E. Orfanos, "Translation initiation factor eIF-4A1 mRNA is consistently overexpressed in human melanoma cells in vitro," *International Journal of Cancer*, vol. 71, no. 3, pp. 396–401, 1997.

[28] A. M. Krichevsky, E. Metzer, and H. Rosen, "Translational control of specific genes during differentiation of HL-60 cells," *The Journal of Biological Chemistry*, vol. 274, no. 20, pp. 14295–14305, 1999.

[29] N. Meani, S. Minardi, S. Licciulli et al., "Molecular signature of retinoic acid treatment in acute promyelocytic leukemia," *Oncogene*, vol. 24, no. 20, pp. 3358–3368, 2005.

[30] D. N. Jackson and D. A. Foster, "The enigmatic protein kinase Cδ: complex roles in cell proliferation and survival," *FASEB Journal*, vol. 18, no. 6, pp. 627–636, 2004.

[31] J. E. Coligan, A. M. Kruisbeck, D. H. Margulies, E. M. Shevach, and W. Strober, *Current Protocols in Immunology*, vol. 1, Wiley-Interscience, New York, NY, USA, 1995.

[32] M. N. Harris, B. Ozpolat, F. Abdi et al., "Comparative proteomic analysis of all-*trans*-retinoic acid treatment reveals systematic posttranscriptional control mechanisms in acute promyelocytic leukemia," *Blood*, vol. 104, no. 5, pp. 1314–1323, 2004.

[33] B. Ozpolat, U. Akar, M. Steiner et al., "Programmed cell death-4 tumor suppressor protein contributes to retinoic acid-induced terminal granulocytic differentiation of human myeloid leukemia cells," *Molecular Cancer Research*, vol. 5, no. 1, pp. 95–108, 2007.

[34] B. Ozpolat, U. Akar, M. Harris et al., "All-*trans*-retinoic acid and arsenic trioxide (ATO)-induced supression of translational initiation involves death associated protein 5 (DAP5/p97/NAT1) in leukemia cell differentiation and apoptosis," *Apoptosis*, vol. 4, pp. 1–11, 2008.

[35] C. N. Landen Jr., A. Chavez-Reyes, C. Bucana et al., "Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery," *Cancer Research*, vol. 65, no. 15, pp. 6910–6918, 2005.

[36] S. M. Kornblau, Y. Qiu, W. Chen et al., "Proteomic profiling of 150 proteins in 511 acute myelogenous leukemia (AML) patient samples using reverse phase proteins arrays (RPPA) reveals recurrent proteins expression signatures with prognostic implications," *Blood*, vol. 112, no. 11, pp. 281–282, 2008.

[37] S. M. Kornblau, N. Singh, Y. Qiu, W. Chen, N. Zhang, and K. R. Coombes, "Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia," *Clinical Cancer Research*, vol. 16, no. 6, pp. 1865–1874, 2010.

[38] M. Giannì, M. H. M. Koken, M. K. Chelbi-Alix et al., "Combined arsenic and retinoic acid treatment enhances differentiation and apoptosis in arsenic-resistant NB4 cells," *Blood*, vol. 91, no. 11, pp. 4300–4310, 1998.

[39] A. Grolleau, N. Sonenberg, J. Wietzerbin, and L. Beretta, "Differential regulation of 4E-BP1 and 4E-BP2, two repressors of translation initiation, during human myeloid cell differentiation," *Journal of Immunology*, vol. 162, no. 6, pp. 3491–3497, 1999.

[40] A. Kentsis, E. C. Dwyer, J. M. Perez et al., "The RING domains of the promyelocytic leukemia protein PML and the arenaviral protein Z repress translation by directly inhibiting translation initiation factor eIF4E," *Journal of Molecular Biology*, vol. 312, no. 4, pp. 609–623, 2001.

[41] M. S. Sheikh and A. J. Fornace, "Regulation of translation initiation following stress," *Oncogene*, vol. 18, no. 45, pp. 6121–6128, 1999.

[42] R. J. Kaufman, "Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls," *Genes and Development*, vol. 13, pp. 1211–1233, 1999.

[43] R. C. Wek, "EIF-2 kinases: regulators of general and gene-specific translation initiation," *Trends in Biochemical Sciences*, vol. 19, no. 11, pp. 491–496, 1994.

[44] E. A. Kohlhepp, M. E. Condon, and A. W. Hamburger, "Recombinant human interferon α enhancement of retinoic-acid-induced differentiation of HL-60 cells," *Experimental Hematology*, vol. 15, no. 4, pp. 414–418, 1987.

[45] Y. Atsumi, R. C. Dodd, F. W. Maddux, S. J. Citron, and T. K. Gray, "Retinoids induce U937 cells to express macrophage phenotype," *American Journal of the Medical Sciences*, vol. 292, no. 3, pp. 152–156, 1986.

[46] J. Drach, G. Lopez-Berestein, T. McQueen, M. Andreeff, and K. Mehta, "Induction of differentiation in myeloid leukemia cell lines and acute promyelocytic leukemia cells by liposomal all-trans-retinoic acid," *Cancer Research*, vol. 53, no. 9, pp. 2100–2104, 1993.

[47] Z. Balajthy, K. Csomós, G. Vámosi, A. Szántó, M. Lanotte, and L. Fésüs, "Tissue-transglutaminase contributes to neutrophil granulocyte differentiation and functions," *Blood*, vol. 108, no. 6, pp. 2045–2054, 2006.

[48] S. Kambhampati, Y. Li, A. Verma et al., "Activation of protein kinase Cδ by all-*trans*-retinoic acid," *The Journal of Biological Chemistry*, vol. 278, no. 35, pp. 32544–32551, 2003.

[49] M. Gschwendt, H. J. Muller, K. Kielbassa et al., "Rottlerin, a novel protein kinase inhibitor," *Biochemical and Biophysical Research Communications*, vol. 199, no. 1, pp. 93–98, 1994.

[50] A. M. Martelli, M. Nyåkern, G. Tabellini et al., "Phosphoinositide 3-kinase/Akt signaling pathway and its therapeutical implications for human acute myeloid leukemia," *Leukemia*, vol. 20, no. 6, pp. 911–928, 2006.

[51] C. Nishioka, T. Ikezoe, J. Yang, S. Gery, H. P. Koeffler, and A. Yokoyama, "Inhibition of mammalian target of rapamycin signaling potentiates the effects of all-*trans* retinoic acid to induce growth arrest and differentiation of human acute myelogenous leukemia cells," *International Journal of Cancer*, vol. 125, no. 7, pp. 1710–1720, 2009.

[52] G. Tabellini, P. L. Tazzari, R. Bortul et al., "Phosphoinositide 3-kinase/Akt inhibition increases arsenic trioxide-induced apoptosis of acute promyelocytic and T-cell leukaemias," *British Journal of Haematology*, vol. 130, no. 5, pp. 716–725, 2005.

[53] X. M. Ma and J. Blenis, "Molecular mechanisms of mTOR-mediated translational control," *Nature Reviews Molecular Cell Biology*, vol. 10, no. 5, pp. 307–318, 2009.

[54] T. Casini and P. G. Pelicci, "A function of p21 during promyelocytic leukemia cell differentiation independent of CDK inhibition and cell cycle arrest," *Oncogene*, vol. 18, no. 21, pp. 3235–3243, 1999.

[55] S. Yamanaka, X. Y. Zhang, M. Maeda et al., "Essential role of NAT1/p97/DAP5 in embryonic differentiation and the retinoic acid pathway," *The EMBO Journal*, vol. 19, no. 20, pp. 5533–5541, 2000.

[56] H. Imataka, H. S. Olsen, and N. Sonenberg, "A new translational regulator with homology to eukaryotic translation initiation factor 4G," *The EMBO Journal*, vol. 16, no. 4, pp. 817–825, 1997.

[57] A. Melnick and J. D. Licht, "Deconstructing a disease: RARα, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia," *Blood*, vol. 93, no. 10, pp. 3167–3215, 1999.

[58] J. Chung, C. J. Kuo, G. R. Crabtree, and J. Blenis, "Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases," *Cell*, vol. 69, no. 7, pp. 1227–1236, 1992.

[59] E. A. Chiocca, P. J. A. Davies, and J. P. Stein, "Regulation of tissue transglutaminase gene expression as a molecular model for retinoid effects on proliferation and differentiation," *Journal of Cellular Biochemistry*, vol. 39, no. 3, pp. 293–304, 1989.

[60] T. Suzuki, Y. Koyama, H. Ichikawa et al., "1,25-Dihydroxyvitamin D3 suppresses gene expression of eukaryotic translation initiation factor 2 in human promyelocytic leukemia HL-60 cells," *Cell Structure and Function*, vol. 30, no. 1, pp. 1–6, 2005.

[61] C. Billottet, L. Banerjee, B. Vanhaesebroeck, and A. Khwaja, "Inhibition of class I phosphoinositide 3-kinase activity impairs proliferation and triggers apoptosis in acute promyelocytic leukemia without affecting ATRA-induced differentiation," *Cancer Research*, vol. 69, no. 3, pp. 1027–1034, 2009.

[62] T. A. Lin, X. Kong, T. A. J. Haystead et al., "PHAS-I as a link between mitogen-activated protein kinase and translation initiation," *Science*, vol. 266, no. 5185, pp. 653–656, 1994.

[63] A. Pause, G. J. Belsham, A. C. Gingras et al., "Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function," *Nature*, vol. 371, no. 6500, pp. 762–767, 1994.

[64] H. S. Yang, A. P. Jansen, A. A. Komar et al., "The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation," *Molecular and Cellular Biology*, vol. 23, no. 1, pp. 26–37, 2003.

[65] X. Wang and D. Ron, "Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase," *Science*, vol. 272, no. 5266, pp. 1347–1349, 1996.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 603830, 11 pages
doi:10.1155/2012/603830

Review Article

MicroRNAs in Acute Myeloid Leukemia and Other Blood Disorders

Yao Yuan,[1] Siddha Kasar,[1] Chingiz Underbayev,[1] Sindhuri Prakash,[1] and Elizabeth Raveche[1,2]

[1] *New Jersey Medical School, Newark, NJ 08731, USA*
[2] *Pathology and Laboratory Medicine, New Jersey Medical School, MSB C512, Newark, NJ 07103, USA*

Correspondence should be addressed to Elizabeth Raveche, raveches@umdnj.edu

Received 6 March 2012; Accepted 17 April 2012

Academic Editor: Michael Danilenko

Common blood disorders include hematopoietic cell malignancies or leukemias and plasma cell dyscrasia, all of which have associated microRNA abnormalities. In this paper, we discuss several leukemias including acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL) and identify altered microRNAs and their targets. Immune disorders with altered blood levels of antibodies include autoimmune disorders, such as systemic lupus erythematosus (SLE) with associated anti-self-autoantibodies and immunoglobulin A nephropathy (IgAN) also have related microRNA abnormalities. The alterations in microRNAs may serve as therapeutic targets in these blood disorders.

1. Introduction

MicroRNAs are small (20–22 nt), evolutionarily conserved, noncoding single-stranded RNAs discovered in the 1990s [1, 2], functioning to target 3′ untranslated region (UTR) of mRNAs in antisense sequence specific way and regulate genes posttranscriptionally for degradation or translation suppression. MicroRNAs target 1–3% of all eukaryotic genes yet regulating ∼30% of protein-coding genes [3]. The miRNAs are first transcribed by RNA polymerase II in the nucleus as large primary transcript (pri-miRNA) [4], either from independent genes or from clustered genes encoding several miRNAs [5] and further processed into ∼70 nt pre-miRNA with hairpin structure by Drosha, a RNase III type endonuclease (RN3) in the nucleus. Alternatively, in the nucleus, a small class of "mintron" without the stem-loop and the flanking single-strand structure as in pri-miRNA required for Drosha processing, could be generated by passing Drosha-dependent pathway [6]. In the cytoplasm, ∼20 bp miRNA/miRNA* duplex are generated by Dicer, another RN3 endonuclease. One of the miRNA duplex strands is further incorporated into protein-RNA complex called RNA-induced silencing complex (RISC), although in some cases, both arms of the pre-miRNA hairpin could generate mature miRNAs [7–9]. miRNAs interact with target mRNA by sequence complementarity, and in perfect base pairing usually triggers endonucleolytic mRNA cleavage [10]; however, in most situations, such base pairing is imperfect, resulting in translational suppression. The key component of this RISC machinery is Ago protein family (Ago 1–4), but only Ago 2 is known to have the catalytic enzyme function [11, 12]. Besides Ago proteins, GW182 protein is also recruited to the RISC complex and together localize in cytoplasmic foci called processing bodies (P bodies or GW bodies), where mRNA is sequestered from being translated [13–16]. There are different experimental and bioinformatics approaches to predict miRNA targets. At a minimum, the precise matching to 3′UTR of mRNA in multiple copies should be within the first 2–8 bases from the 5′ end of the mature miRNA, called the "seed region" [17–20]. To date, over 2000 human miRNAs have been annotated in the Sanger miRBASE (Release 18, http://www.mirbase.org/cgi-bin/browse.pl?org=hsa). The miRNA network is highly re dundant, since a single miRNA may have multiple target mRNAs, and in turn, a single mRNA could be targeted by many miRNAs [21]. Various miRNAs have been shown to be involved in a myriad of cellular processes including

differentiation, metabolism, apoptosis, and development [22]. Physiologically, and pathologically, miRNAs have been reported to play roles in cancers, inflammatory responses, diabetes, and autoimmunity [23, 24].

2. MicroRNAs in Hematopoietic Stem Cells

Multiple evidence suggest that microRNAs play a significant role in the posttranscriptional genetic regulation in stem and progenitor cells. They are involved in a number of hematological malignancies such as acute lymphoblastic leukemia, acute myeloid leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, diffuse large-B-cell lymphoma, and others [25]. Therefore, miRNA profiling is critical in order to distinguish stem cells of the different origins, developmental stages, and genetic conditions [26]. Furthermore, it can help classify cancer cell samples and develop appropriate therapeutic strategies [27]. Recent studies have demonstrated a causative role for miRNAs in malignant diseases development in the hematopoietic system. For instance, overexpression of miR-155 or miR-29a in the mouse hematopoietic system leads to a myeloproliferative disorder [28] or leukemia [29], respectively. On the other hand, tumor suppressor miRNAs such as miR-15a/16-1 are found to be deleted in a subset of lymphomas [30] and have been shown to cause chronic lymphocytic leukemia in mice [31, 32]. MicroRNA-125b has been demonstrated to cause pathological myeloid cells expansion in a dose-dependant manner [33], and miR-155 is known to induce polyclonal expansion followed by B-cell malignancy development [34]. In another study on human umbilical cord blood, two particular miRNAs-hsa-miR-520h and hsa-miR-526b*- levels appeared to be elevated. Interestingly, ABCG2, an important factor of stem cells maintenance, is a known target of hsa-miR-520h [35].

3. MicroRNAs in the Immune System

Proper regulation of immune response is critical in preventing immunopathology and autoimmune disorders. Studies have implicated important functions of miRNA on hematopoietic development as well as innate and adaptive immune responses. Toll-like receptor (TLR) signaling leads to transcriptional activation of a large class of proinflammatory cytokines as well as multiple miRNAs. For example, miR-146a and miR-155 have been shown to be upregulated upon exposure to LPS in the monocytic leukemia cell line THP-1. More importantly, two key components of TLR4 signaling pathway, TRAF6 and IRAK1 have been verified to be targets for miR-146a [36]. This study for the first time profiled the miRNAs alterations in TLR signaling and proposed the miRNAs as negative regulators of TLR activation. MiR-155 is another well-studied microRNA reported to be activated by several TLR pathways [36, 37], and its negative regulatory role during TLR-mediated activation has also been addressed [38, 39]. More interestingly, IL-10 is shown to inhibit TLR-induced miR-155 [40]. To understand global miRNAs' importance in B and T development, studies were performed in which knocking out Dicer at different stages of B and T development resulted in blockage of further differentiation [41–43]. In addition, miR-155 has been found to be one of the most important miRNAs in both B and T cells as well as antigen presentation by dendritic cells (DCs) and is required for normal germinal center (GC) response [44, 45], B-cell class switching [46], Th1/Th2 polarization, and Treg development both in the thymus and peripheral [47].

4. MicroRNA in Autoimmune Diseases

Considering the importance of miRNAs in the immune system raises the question whether or not there is direct link between miRNAs abnormalities and immune disorders or autoimmune diseases. Interestingly, the discovery in 2002 of GW bodies (GWBs), where miRNA-mRNA reside for degradation was from serum from an autoimmune patient with motor and sensory neuropathy [48]. Subsequently, anti-GWB autoantibodies in the serum have been identified from patients with various autoimmune disorders [49], indicating an involvement of general miRNA pathway and autoantibody production. Dysregulated miRNA expression has been associated with autoimmunity, for example, miR-146a was underexpressed in PBMC from SLE patients when compared with healthy control. The study further showed that miR-146a is a negative regulator of type I interferon (IFN) pathway by targeting interferon regulatory factor (IRF) 5 and signal transducers and activators of transcription (STAT) 1, thus the decrease in miR-146a may contribute to the increased type I IFN signaling pathway observed in SLE [50]. A recent study in murine models (MRL-lpr, C57BL/6-lpr, and NZB/NZW F1) of SLE using a combination of microarray and real-time RT-PCR approaches, Dai et al. identified that miR-182-96-183 cluster, miR-31, and miR-155 are among those consistently upregulated miRNAs across different genetic background strains of mice [51]. In addition to important contribution of miR-155 to physiological immune response, its activity in autoimmune circumstances was also investigated. A murine experimental autoimmune encephalomyelitis (EAE) model with *mir-155* $^{-/-}$ was shown to be resistant to EAE pathology. Thus, unregulated miR-155 may be a link between inflammation and cancer via inducing a high proliferation rate resulting in increased mutations [52]. The miR-17-92 cluster locating in human chromosome 13q31 is known as an onco-miR, and this genomic region is often amplified in lymphomas and other cancer, and the mature miR-17-92 expression is highly elevated in malignant cells [27, 53–55]. Results showed that miR-17-92 targets phosphatase and tensin homolog (PTEN, tumor suppressor) and Bim (proapoptotic molecule) mRNA directly resulting in lymphoproliferative and autoimmune diseases [56]. In a current study from our lab on microRNA abnormalities in NZB/NZW F1 lupus model by using type I and type III interferons (IFN-α and IFN-λ) as exogenous disease accelerators, we identified upregulation of several microRNAs correlated to disease severity, yet not with the IFN treatment. MiR-15a was one of the most significant elevated microRNAs as autoimmunity developed in these mice and the level of splenic miR-15a was correlated to the level of anti-dsDNA IgG, in addition, the cellular level of miR-15a

was also reflected in the plasma (manuscript accepted for publication).

5. MicroRNAs in Hyperimmunoglobulinemias

Multiple myeloma (MM) is characterized by a clonal expansion of plasma B cells in the bone marrow or in extramedullary sites which results in high levels of monoclonal immunoglobulins in the serum [57]. Cytogenetic abnormalities are present in many MM cases, characterized by either hyperdiploidy with the presence of trisomies of odd chromosomes or nonhyperdiploidy with chromosomal aberrations and translocations involving the IgH locus on chromosome 14. In addition to these advancements in understanding MM pathogenesis, studies on the role of microRNAs in recent years have shown them to be key players in MM development not only in the sustenance of malignant cells but also in the initiation of malignancy due to methylation of microRNAs that function as tumor suppressors. Studies investigating microRNAs in MM began in 2007 with the discovery that interleukin 6 (IL-6) indirectly induces the transcription of miR-21 through signal transducer and activator of transcription 3 (STAT3) transcription in the human myeloma cells line. The same upstream enhancer controls miR-21 and STAT3 transcription, and STAT3 controls the transcription of survivin, Bcl2, and Mcl-1. Thus, Stat3 exerts its antiapoptotic affect through the induction of miR-21 [58]. A microRNA microarray analysis in 20 myeloma samples revealed that miR-335 and miR-342-3p were upregulated and may be involved in plasma cell homing and other interactions in the bone marrow [59]. Subsequent studies uncovered various microRNAs that are key players in MM. For example, miR-106b-25 cluster, miR-181a/b, and miR-32 target a histone acetyltransferase, P300/CBP-associated factor (PCAF) that reversibly acetylates transcriptional regulators including p53, thus accounting for the low levels of PCAF observed in MM cells. Also miR-17–92 downregulates Bim, a proapoptotic molecule, and miR-19a/b target SOCS-1, a silencer of the STAT3, thus enhancing the oncogenicity of MM cells [60, 61]. As seen in CLL, miR-15a/16-1 is seen to be downregulated in multiple myeloma [62]. Normally encoded within the DLEU2, a gene frequently deleted in lymphocytic leukemia, miR-15a/16-1 activity is central to the antiproliferative activity of DLEU2 [63], as miR-15a/16-1 inhibits cyclinD1, cyclinD2, and CDC25a [62]. Also, several microRNAs have been seen to target the p53 gene. For example, miR-25 and miR-30d are increased in MM and target the 3′UTR of the p53 gene [64]. Also, MM cells have low levels of miR-192, miR-194, and miR-215 which targets MDM2, a p53 antagonist, thereby lowering p53 levels and increasing oncogenic potential [65]. Moreover, the promoter of miR-34b/c, a transcriptional target of p53 was found to be hypermethylated and thus inactivated in multiple myeloma cell lines. Such epigenetic modifications are observed to be causal in other microRNAs as well. Hypermethylation of the promoters of various tumor suppressors such as miR-124-1 (a target of CDK6) [66], miR-203 (a target of cyclic-responsive element-binding protein which increases proliferation) [67], and miR-29b (a target of Mcl-1 which antagonizes IL-6) increase the tumorigenicity of myeloma cells [68]. Extranodal marginal zone lymphomas are most associated with mucosal-associated lymphoid tissue (MALT) and are characterized by clonal proliferation of plasma cells that produce the immunoglobulin A isotype. Investigations involving microRNAs have found the miR-203 promoter to be hypermethylated in samples of gastric lymphoma, and this microRNA targets the c-abl1 oncogene, thus enabling tumor growth and proliferation [69]. In addition, miR-150 and miR-155 were upregulated, while miR-184, miR-205 and miR-200a/b/c (which targets cyclin E2) were downregulated [70]. In another hyperimmunoglobulin disorder, Immunoglobulin A nephropathy (IgAN) is characterized by the deposition of immune complexes in the kidney mesenchyme causing renal injury and usually coincides with mucosal infections [57]. These immune complexes are composed of IgA1 molecules that are galactose-deficient, causing a conformational change in the molecule, and autoantibodies (IgA or IgG) form to its exposed epitopes [71]. Since miR-155 and miR-146 are involved in B lymphocyte development, their levels were examined in 43 IgAN biopsy specimens and urine samples. The results showed that miR-146 and miR-155 were high in IgAN biopsy and urine sediment, suggesting their role in IgAN pathogenesis [72].

6. MicroRNAs in Acute Myeloid Leukemia (AML)

MicroRNAs (miRNAs) have been well studied in various cancers including leukemias [73, 74]. Acute myeloid leukemia (AML) is a hematopoietic progenitor cell-originated malignant disorder affecting the myeloid lineage, which could be classified into subtypes based on the differentiation stages of the malignant cells found in peripheral blood and in bone marrow [75]. Among various symptoms and manifestations identified in association with AML, one of the most common characteristics involved in ∼50% of AML patients is a group of cytogenetic abnormalities, which is considered to be contributing to the disease heterogeneity and with prognostic significance [76]. Other AML patients without detectable chromosomal abnormalities may display mutations or dysregulations in specific genes, a signature ubiquitously found in cancers [77–79]. MicroRNA signatures in AML have been sought, and many groups of researchers performed large-scale profiling of miRNA expression in different populations of AML patients. In the first study where AML patient samples were compared to acute lymphoblastic leukemia (ALL), both groups with similar chromosomal alterations, 27 miRNAs were reported to be different between the two groups [80]. Importantly, miR-146a was inversely correlated to overall survival in both AML and ALL [81]. However, these studies focused on miRNA profile distinguishing AML from ALL, which was not sufficient for understanding the abnormalities of miRNAs expression exclusive to AML.

Another study compared 122 AML samples to CD34+ cells from 10 normal controls. Among the 122 AML samples, 60 cases were untreated and 54 relapsed or refractory [82]. By microarray profiling, 26 microRNAs were downregulated

in AML samples. Several of these downregulated miRNAs in AML were also underexpressed in mature myeloid cells suggesting that miRNAs related to the differentiation patterns in AML (miR-126, miR-130a, miR-93, miR-125a, and miR-146). In correlating cytogenetic abnormalities with miRNA expression, 14 downregulated and 8 upregulated miRNAs were associated with 11q23 translocation versus all other AML, including the downregulation of miR-196 and miR-15a, and overexpression of miR-21 in $t(6;11)$ with worse prognosis [82]. In AML patients ($n = 36$) achieving complete remission the levels of miR-15a/16 were upregulated. Subsequently, in 2 patients in which relapse occurred, miR-15a decreased. All-trans retinoic acid (ATRA) *in vitro* treatment in AML cell lines and primary leukemic cells induced miR-15a/16 upregulation, in addition, miR-15a/16 enhanced the effects of ATRA inducing leukemic cell differentiation [83]. Despite the poor overall survival of these AML patients, the study showed several associations between miRNA expression and the outcome of patients, especially the overexpression of miR-199a and miR-191, identified in AML with trisomy 8 and associated with poor outcome. This study was the first to identify the distinct miRNAs profile between AML patients and normal control, and the subsets of miRNAs related to cytogenetic groups and disease outcome [82].

Almost at the same time, a study with 215 heterogeneous AML samples was performed to demonstrate the signatures of miRNAs expression in cytogenetic and molecular subtypes [84]. A group of upregulated miRNAs were prominent in $t(15;17)$ cases. In contrast, $t(8;21)$ was characterized by downregulated miRNA alterations, for example, tumor suppressor let-7. In molecular subgroups of AML, nucleophosmin *(NPM1)* mutations, which represent the most common molecular abnormality in AML, are associated with overexpression of homeobox genes (*HOX*) [85]. Upregulation of miR-10a, miR-10b, miR-196a and miR-196b, was identified in AML carrying *NPM1* mutations, and these miRNAs were located within the *HOX* genes. Although miR-196a directly targets *HOXB8* mRNA [86], the upregulated miR-196a in this AML subgroups may represent a breakage in the regulation loop between miRNAs and *HOX* genes [84]. Consistent with other studies, miR-155 was significantly upregulated in AMLs with internal tandem duplications of Flt3*(FLT3*-ITD), corroborating the oncogenic effect of miR-155 in myeloid cells in addition to such effects in lymphoid lineages [84, 87, 88]. In comparing AML to normal CD34+ cells, upregulation of miR-21 in AMLs was found, consistent with other studies and further strengthening the importance of miR-21 in AML [82, 84].

Interestingly, in an analysis of AML subgroups the $t(8;21)$ and inv(16) were grouped together by miRNA profile, supporting the notion that both subgroups belong to core-binding factor (CBF) AMLs, suggesting some common pathways shared by CBF-AMLs [89]. Overexpression of miR-224, miR-368, and miR-382 was restricted to the $t(15;17)$ samples, while miR-17-92 cluster was overexpressed exclusively in mixed-lineage leukemia (MLL) rearrangements [89]. In addition, in a study of 100 AMLs, comparing leukemic samples to normal bone marrow, miR-155 and miR-181a were upregulated [90]. MiR-181a has been reported to target $p27^{Kip1}$ in AML cell lines, resulting in an abrogation of 1, 25-dihydroxyvitamin D3 (1,25D) induced differentiation in AML cell lines [91]. A recent study classified AML cases into favorable, moderate, and poor as the predicted outcome according to the karyotype. MiR-181a high expression was suggested to be associated with better-risk groups suggesting a potential therapeutic approach involving manipulation of miR-181a level in AML patients. In contrast to elevated miR-181a as favorable prognostic factor, miR-155 upregulation predicts poor prognosis in AML [92].

7. MicroRNAs in Chronic Lymphocytic Leukemia (CLL)

CLL is characterized by the accumulation of malignant B-1 cells ($CD5^+CD19^+CD20^{dull}CD23^+IgM^{dull}$) in peripheral lymphoid organs, bone marrow, and peripheral blood [93]. It accounts for 30% of all leukemias in the Western world, making it the most common lymphoid malignancy with mainly elderly with disease. CLL is broadly classified into aggressive ($Zap70^{hi}$-unmutated IgH) and indolent ($Zap70^{low}$-mutated IgH) [94]. CLL cells have genomic instability, chromosomal alterations and have several characteristic genetic abnormalities. Prominent among them are 11q23 deletions (ATM; miR-34b/c cluster), trisomy 12 (increased MDM2), 17p deletion (TP53), and 13q14 deletions (miR-15a/16-1) [95]. Dysregulation of several microRNAs like miR-15a/16-1, miR-34 cluster, miR-155, miR-29, and miR-181b has been implicated in the pathogenesis of CLL. The most common genetic abnormality in CLL patients is the deletion of 13q14 region (50–60% of CLL cases) that encodes a crucial microRNA locus, *miR-15a/16*-1 [30, 96]. Decreased miR-15a/16-1 confers a growth advantage as these microRNAs target key cell cycle regulatory and antiapoptotic proteins such as cyclin D1 and Bcl2 [97, 98]. It is interesting to note that NZB mice (spontaneously occurring mouse model of CLL) also exhibit a 50% reduction in the level of miR-15a/16-1, that is associated with a point mutation and deletion in the 3′ flanking region of miR-16-1 [99]. Moreover targeted deletion of the miR-15a/16-1 locus or a larger surrounding minimal deleted region (MDR) led to the development of CLL in mice, further confirming the tumor suppressor function of this locus [32]. Other microRNAs are abnormal in CLL including miR-29 and miR-181, which target Tcl1, a gene that is highly elevated in aggressive CLL [100]. MiR-29 expression is decreased in aggressive CLL, while it is increased in indolent CLL as compared to normal volunteers [100, 101]. Thus, the same microRNA can function as both an oncogene and as a tumor suppressor in CLL. MiR-34a/b/c is decreased in patients with 11q deletions. Normally, upon transactivation by TP53, miR-34 expression would result in decreased Zap70 [102]. MiR-34a has also been shown to target E2F1 and B-Myb oncogenes in CLL as well as AML [103]. MiR-155, miR-150, and miR-21 expression is increased in B-CLL cells as compared to normal B cells [23, 104]. Increased miR-155 levels are associated with increased Zap70 expression and faster progression. v-Myb is

Table 1: MiR-15/107 group involvement in common blood disorders[§].

Blood disorders	MiR-15/107 group alterations	Abnormalities associated	Effects of miRNAs
AML	MiR-15a/16 decreased in *t*(11q23) AML patients [82], elevated in patients with complete remission, and decreased with relapse [83]		MiR-15a/16 enhanced ATRA effects inducing AML cell differentiation [83]
APL	MiR-15a/16 upregulation and miR-107 downregulation in a cohort of APL patients [135]		Patients showed increased miR-15/107 during remission, and miR-15/107 upregulation was induced by ATRA *in vitro* in APL cells [135]
CLL	MiR-15a/16 underexpressed in CLL patients with 13q14 deletion and NZB mice (CLL model) [96, 99]	Uncontrolled B-1 cell proliferation [97]	Overexpression of miR-15a/16 in CLL murine model resulted in exclusive elimination of malignant B-1 cells [136]
	MiR-195 upregulation reported from a study of 9 CLL patients compared to normal controls [137]		Not determined
	MiR-107 downregulated in CLL patients [138]		Underexpression of miR-107 resulted in overexpression of oncogenic PLAG-1 protein [138]
MM	MiR-15a/16 decreased in MM patients [62]	Uncontrolled clonal plasma cell proliferation and proangiogenesis in bone marrow [62]	MiR-15a/16 targeted cell cycle regulators, inhibited NF-κB pathway, and downregulated proangiogenic genes [62]
SLE	miR-15a upregulated in spleen cells from NZB/NZW F1 mice (SLE model), when disease fully developed (manuscript accepted for publication)	Elevated autoreactive antibody producing cells terminally differentiated plasma cells	MiR-15a enhanced plasma cell differentiation

[§]AML: acute myeloid leukemia; APL: acute promyelocytic leukemia; CLL: chronic lymphocytic leukemia; MM: multiple myeloma; SLE: systemic lupus erythematosus; PLAG-1: pleomorphic adenoma gene.

found to be elevated in CLL patients, and it stimulates the miR-155 host gene [105]. The oncogenic potential of miR-155 is further supported by the development of B-cell malignancies in Eμ-mmu-miR-155 transgenic mice [34]. Using a poorly understood mechanism, microRNAs are secreted into body fluids such as serum and urine, and their levels can be used as noninvasive biomarkers for diagnosis and monitoring of cancer and various other diseases [106, 107]. In a recent, study it was shown that elevated miRNA levels in serum may offer early CLL detection and differentiation between Zap70 status [108]. The authors further concluded that increased expression of miR-150, miR-29a, miR-222, and miR-195 can be used as a highly sensitive diagnostic test for CLL.

8. Conclusion

In this paper, a variety of blood disorders were discussed in terms of microRNA abnormalities observed. One microRNA family of interest stood out as a potential regulator of cell fate (Table 1 and Figure 1). Recently, miR-15 family members (miR-15a/b, miR-16, miR-103, miR-107, miR-195, and miR-497) have been grouped together due to their identical "AGCAGC" sequence at 5′ end "seed region (nucleotides 2–7)" [109, 110], which offers this miRNA group various overlapping functions in gene-regulatory pathways and disease scenarios, especially in cancers. MiR-15/107 gene group could be upregulated by tumor suppressor p53 [102], altered by various cell stress [111–115], or inhibited by Myc [116, 117]. A broad spectrum of mRNAs is targeted by miR-15/107, importantly, miR-15/16 paralogs regulate cell cycle via targeting of Cyclin D1 [97] and induce apoptosis via targeting of Bcl-2 [118], and miR-107 also induces cell cycle arrest [119]. The tight involvement of miR-15/107 in cell growth and cell fate control, and their upstream regulators, such as p53 and Myc, which by themselves are important players in tumorigenesis [120, 121], revealed critical mechanisms for abnormalities in cancer development, including leukemias. Indeed, all members from miR-15/107 group have been identified to be altered in various tumor cells [122–125]. Specifically, underexpression of miR-15a/16 as a result of deletion or mutation of *mir-15a/16* loci has been linked to the pathogenesis of CLL [96, 99, 118], similarly in AML and MM, where the downregulation of miR-15a/16 was associated with the loss of control for malignant cells differentiation and proliferation [62, 83]. In contrast, in SLE which is characterized by elevated plasma cell differentiation contributing to increased autoantibody production [126, 127], splenic miR-15a was increased, and this was significantly correlated with autoantibody levels in lupus-like autoimmune mouse model (manuscript accepted for publication), suggesting a role of miR-15a upregulation in cell cycle arrest in order for plasma cell differentiation.

Future directions may be directed toward stem cell transplantation for many of these blood disorders. Cellular transplantation therapy holds a huge potential for a variety of

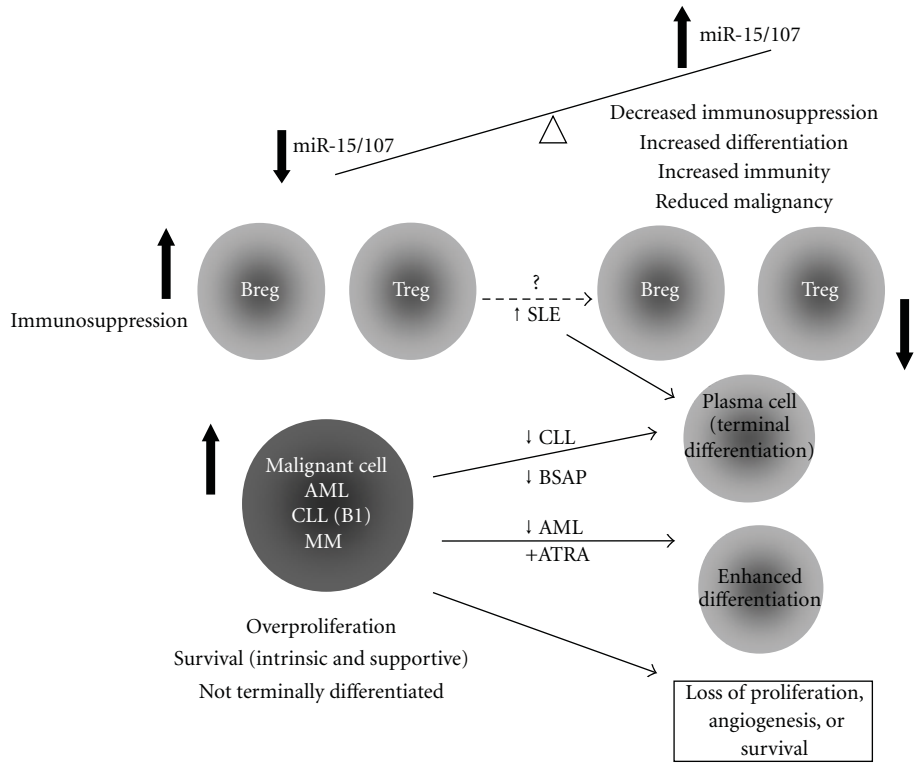


Figure 1: Schematic mechanism of miR-15/107 family alterations in hematopoietic disorders. Decreased expression of miR-15/107 family members is found in malignant cells from AML (acute myeloid leukemia), CLL (chronic lymphocytic leukemia), and MM (multiple myeloma) patients. The underexpression of miR-15/107 may also contribute to increased immunosuppressive regulatory B (Breg) and T cells (Treg), which further promote the expansion and survival of malignant cells. In contrast, with increased miR-15/107, there may be a loss of immunosuppression that leads to SLE (systemic lupus erythematosus) development and antitumor responses. In the therapeutically induced differentiated AML cells, and terminally differentiated B cells, plasma cells (with decreased B-cell-specific activator protein (BSAP), the negative regulator of miR-15a/16-1), miR-15/107 family members would be upregulated, leading to the loss of malignant potential and an increase in differentiation function (SLE).

degenerative, genetic, and malignant conditions treatment. Hematopoietic stem cell transplantation is the most widely used form of such a therapy, but many patients do not benefit from that because of the lack of a suitable HLA-matched donor [128]. In this sense, patient-specific autologous pluripotent stem cells generation would provide a great opportunity to combine gene therapy with autologous cell transplantation to treat different human conditions including hematological disorders such as AML. For this reason, robust protocols for the generation of safe autologous induced pluripotent stem (iPS) cells are strongly needed. To this end, microRNAs represent an attractive tool for both iPS generation efficiency enhancement and gene targeting approaches. It is known that expression of embryonic stem (ES) cell-specific microRNAs such as miR-294 promotes iPS cells induction from somatic cells [129]. Recently, it has even been demonstrated that the expression of miR-302/367 cluster can directly reprogram mouse and human somatic cells to a pluripotent stem cell state in the absence of the commonly used reprogramming factors [130]. Alternatively, inhibition of tissue-specific miRNAs would also enhance iPS generation, which has been confirmed by antisense silencing of a prodifferentiation let-7 miRNA [131]. Another application of microRNAs lies in promoting patient-specific iPS differentiation towards the required cell lineage, for example HSC expansion. MiR-145 has been shown to induce ES cell differentiation by inhibiting the expression of Sox2, Oct4, Klf4, and c-Myc, key reprogramming factors, and led to an increase of HSC number *in vivo* by more than 8 fold [132–134]. Nevertheless, HSC expansion from iPS cells by means of microRNAs needs to be further developed. Hopefully, the recently achieved success in the production of iPS cells with the use of miRNAs will pave the way for successful *in vitro* expansion of HSCs with miRNAs.

Abbreviations

miRNA/miR:	MicroRNA
AML:	Acute myeloid leukemia
ALL:	Acute lymphoblastic leukemia
CLL:	Chronic lymphocytic leukemia
SLE:	Systemic lupus erythematosus
IgAN:	Immunoglobulin A nephropathy
MM:	Multiple myeloma
TLR:	Toll-like receptor
IFN:	Interferon

3′UTR: 3′untranslated region
NZB: New Zealand black
ATRA: All-trans retinoic acid
HOX: Homeobox genes
NPM: Nucleophosmin
FLT3-ITD: Internal tandem duplications of Flt3
CBF: Core-binding factor
iPS cell: Induced pluripotent stem cell
HSC: Hematopoietic stem cells
ES cell: Embryonic stem cells.

Acknowledgment

This work was supported by NCI R01CA129826 (E. Raveche).

References

[1] R. C. Lee, R. L. Feinbaum, and V. Ambros, "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14," *Cell*, vol. 75, no. 5, pp. 843–854, 1993.

[2] B. Wightman, I. Ha, and G. Ruvkun, "Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans," *Cell*, vol. 75, no. 5, pp. 855–862, 1993.

[3] W. Filipowicz, S. N. Bhattacharyya, and N. Sonenberg, "Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?" *Nature Reviews Genetics*, vol. 9, no. 2, pp. 102–114, 2008.

[4] V. N. Kim, "MicroRNA biogenesis: coordinated cropping and dicing," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 5, pp. 376–385, 2005.

[5] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, and T. Tuschl, "Identification of novel genes coding for small expressed RNAs," *Science*, vol. 294, no. 5543, pp. 853–858, 2001.

[6] J. G. Ruby, C. H. Jan, and D. P. Bartel, "Intronic microRNA precursors that bypass drosha processing," *Nature*, vol. 448, no. 7149, pp. 83–86, 2007.

[7] N. Bushati and S. M. Cohen, "MicroRNA functions," *Annual Review of Cell and Developmental Biology*, vol. 23, pp. 175–205, 2007.

[8] T. M. Rana, "Illuminating the silence: understanding the structure and function of small RNAs," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 1, pp. 23–36, 2007.

[9] T. Du and P. D. Zamore, "MicroPrimer: the biogenesis and function of microRNA," *Development*, vol. 132, no. 21, pp. 4645–4652, 2005.

[10] M. W. Jones-Rhoades, D. P. Bartel, and B. Bartel, "MicroRNAs and their regulatory roles in plants," *Annual Review of Plant Biology*, vol. 57, pp. 19–53, 2006.

[11] J. Liu, M. A. Carmell, F. V. Rivas et al., "Argonaute2 is the catalytic engine of mammalian RNAi," *Science*, vol. 305, no. 5689, pp. 1437–1441, 2004.

[12] G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, and T. Tuschl, "Human argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs," *Molecular Cell*, vol. 15, no. 2, pp. 185–197, 2004.

[13] T. Eystathioy, A. Jakymiw, E. K. Chan, B. Séraphin, N. Cougot, and M. J. Fritzler, "The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies," *RNA*, vol. 9, no. 10, pp. 1171–1173, 2003.

[14] L. Ding and M. Han, "GW182 family proteins are crucial for microRNA-mediated gene silencing," *Trends in Cell Biology*, vol. 17, no. 8, pp. 411–416, 2007.

[15] A. Eulalio, I. Behm-Ansmant, and E. Izaurralde, "P bodies: at the crossroads of post-transcriptional pathways," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 1, pp. 9–22, 2007.

[16] R. Parker and U. Sheth, "P bodies and the control of mRNA translation and degradation," *Molecular Cell*, vol. 25, no. 5, pp. 635–646, 2007.

[17] J. G. Doench and P. A. Sharp, "Specificity of microRNA target selection in translational repression," *Genes & Development*, vol. 18, no. 5, pp. 504–511, 2004.

[18] J. Brennecke, A. Stark, R. B. Russell, and S. M. Cohen, "Principles of microRNA-target recognition," *PLoS Biology*, vol. 3, no. 3, article e85, 2005.

[19] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[20] A. Grimson, K. K. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim, and D. P. Bartel, "MicroRNA targeting specificity in mammals: determinants beyond seed pairing," *Molecular Cell*, vol. 27, no. 1, pp. 91–105, 2007.

[21] A. Esquela-Kerscher and F. J. Slack, "Oncomirs—microRNAs with a role in cancer," *Nature Reviews Cancer*, vol. 6, no. 4, pp. 259–269, 2006.

[22] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[23] G. A. Calin, C. G. Liu, C. Sevignani et al., "MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 32, pp. 11755–11760, 2004.

[24] P. S. Eis, W. Tam, L. Sun et al., "Accumulation of miR-155 and BIC RNA in human B cell lymphomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 10, pp. 3627–3632, 2005.

[25] E. Barbarotto and G. A. Calin, "Potential therapeutic applications of miRNA-based technology in hematological malignancies," *Current Pharmaceutical Design*, vol. 14, no. 21, pp. 2040–2050, 2008.

[26] C. P. Arnold, R. Tan, B. Zhou et al., "MicroRNA programs in normal and aberrant stem and progenitor cells," *Genome Research*, vol. 21, no. 5, pp. 798–810, 2011.

[27] J. Lu, G. Getz, E. A. Miska et al., "MicroRNA expression profiles classify human cancers," *Nature*, vol. 435, no. 7043, pp. 834–838, 2005.

[28] R. M. O'Connell, D. S. Rao, A. A. Chaudhuri et al., "Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder," *Journal of Experimental Medicine*, vol. 205, no. 3, pp. 585–594, 2008.

[29] Y. C. Han, C. Y. Park, G. Bhagat et al., "MicroRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia," *Journal of Experimental Medicine*, vol. 207, no. 3, pp. 475–489, 2010.

[30] G. A. Calin, C. D. Dumitru, M. Shimizu et al., "Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15524–15529, 2002.

[31] E. Salerno, B. J. Scaglione, F. D. Coffman et al., "Correcting miR-15a/16 genetic defect in New Zealand black mouse model of CLL enhances drug sensitivity," *Molecular Cancer Therapeutics*, vol. 8, no. 9, pp. 2684–2692, 2009.

[32] U. Klein, M. Lia, M. Crespo et al., "The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia," *Cancer Cell*, vol. 17, no. 1, pp. 28–40, 2010.

[33] R. M. O'Connell, A. A. Chaudhuri, D. S. Rao, W. S. Gibson, A. B. Balazs, and D. Baltimore, "MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 32, pp. 14235–14240, 2010.

[34] S. Costinean, N. Zanesi, Y. Pekarsky et al., "Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in Eμ-miR155 transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 18, pp. 7024–7029, 2006.

[35] R. Liao, J. Sun, L. Zhang et al., "MicroRNAs play a role in the development of human hematopoietic stem cells," *Journal of Cellular Biochemistry*, vol. 104, no. 3, pp. 805–817, 2008.

[36] K. D. Taganov, M. P. Boldin, K. J. Chang, and D. Baltimore, "NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 33, pp. 12481–12486, 2006.

[37] T. Ruggiero, M. Trabucchi, F. De Santa et al., "LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages," *FASEB Journal*, vol. 23, no. 9, pp. 2898–2908, 2009.

[38] M. Ceppi, P. M. Pereira, I. Dunand-Sauthier et al., "MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 8, pp. 2735–2740, 2009.

[39] B. Tang, B. Xiao, Z. Liu et al., "Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation," *FEBS Letters*, vol. 584, no. 8, pp. 1481–1486, 2010.

[40] C. E. McCoy, F. J. Sheedy, J. E. Qualls et al., "IL-10 inhibits miR-155 induction by toll-like receptors," *The Journal of Biological Chemistry*, vol. 285, no. 27, pp. 20492–20498, 2010.

[41] B. S. Cobb, T. B. Nesterova, E. Thompson et al., "T cell lineage choice and differentiation in the absence of the RNase III enzyme dicer," *Journal of Experimental Medicine*, vol. 201, no. 9, pp. 1367–1373, 2005.

[42] S. A. Muljo, K. M. Ansel, C. Kanellopoulou, D. M. Livingston, A. Rao, and K. Rajewsky, "Aberrant T cell differentiation in the absence of dicer," *Journal of Experimental Medicine*, vol. 202, no. 2, pp. 261–269, 2005.

[43] S. B. Koralov, S. A. Muljo, G. R. Galler et al., "Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage," *Cell*, vol. 132, no. 5, pp. 860–874, 2008.

[44] A. Rodriguez, E. Vigorito, S. Clare et al., "Requirement of bic/microRNA-155 for normal immune function," *Science*, vol. 316, no. 5824, pp. 608–611, 2007.

[45] T. H. Thai, D. P. Calado, S. Casola et al., "Regulation of the germinal center response by microRNA-155," *Science*, vol. 316, no. 5824, pp. 604–608, 2007.

[46] E. Vigorito, K. L. Perks, C. Abreu-Goodger et al., "MicroRNA-155 regulates the generation of immunoglobulin class-switched plasma cells," *Immunity*, vol. 27, no. 6, pp. 847–859, 2007.

[47] S. Kohlhaas, O. A. Garden, C. Scudamore, M. Turner, K. Okkenhaug, and E. Vigorito, "Cutting edge: the foxp3 target miR-155 contributes to the development of regulatory T cells1," *Journal of Immunology*, vol. 182, no. 5, pp. 2578–2582, 2009.

[48] T. Eystathioy, E. K. Chan, S. A. Tenenbaum, J. D. Keene, K. Griffith, and M. J. Fritzler, "A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles," *Molecular Biology of the Cell*, vol. 13, no. 4, pp. 1338–1351, 2002.

[49] R. A. Bhanji, T. Eystathioy, E. K. Chan, D. B. Bloch, and M. J. Fritzler, "Clinical and serological features of patients with autoantibodies to GW/P bodies," *Clinical Immunology*, vol. 125, no. 3, pp. 247–256, 2007.

[50] Y. Tang, X. Luo, H. Cui et al., "MicroRNA-146a contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins," *Arthritis & Rheumatism*, vol. 60, no. 4, pp. 1065–1075, 2009.

[51] R. Dai, Y. Zhang, D. Khan et al., "Identification of a common lupus disease-associated microRNA expression pattern in three different murine models of lupus," *PloS ONE*, vol. 5, no. 12, article e14302, 2010.

[52] E. Tili, J. J. Michaille, D. Wernicke et al., "Mutator activity induced by microRNA-155 (miR-155) links inflammation and cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 12, pp. 4908–4913, 2011.

[53] A. Ota, H. Tagawa, S. Karnan et al., "Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma," *Cancer Research*, vol. 64, no. 9, pp. 3087–3095, 2004.

[54] B. Zhang, X. Pan, G. P. Cobb, and T. A. Anderson, "microRNAs as oncogenes and tumor suppressors," *Developmental Biology*, vol. 302, no. 1, pp. 1–12, 2007.

[55] H. Tagawa and M. Seto, "A microRNA cluster as a target of genomic amplification in malignant lymphoma," *Leukemia*, vol. 19, no. 11, pp. 2013–2016, 2005.

[56] C. Xiao, L. Srinivasan, D. P. Calado et al., "Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes," *Nature Immunology*, vol. 9, no. 4, pp. 405–414, 2008.

[57] V. Kumar, A. K. Abbas, J. C. Aster, and S. L. Robbins, *Robbins Basic Pathology*, Elsevier Saunders, Philadelphia, Pa, USA, 9th edition, 2013.

[58] D. Löffler, K. Brocke-Heidrich, G. Pfeifer et al., "Interleukin-6-dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer," *Blood*, vol. 110, no. 4, pp. 1330–1333, 2007.

[59] D. Ronchetti, M. Lionetti, L. Mosca et al., "An integrative genomic approach reveals coordinated expression of intronic miR-335, miR-342, and miR-561 with deregulated host genes in multiple myeloma," *BMC Medical Genomics*, vol. 1, article 37, 2008.

[60] F. Pichiorri, S. S. Suh, M. Ladetto et al., "MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 35, pp. 12885–12890, 2008.

[61] L. Chen, C. Li, R. Zhang et al., "MiR-17-92 cluster microRNAs confers tumorigenicity in multiple myeloma," *Cancer Letters*, vol. 309, no. 1, pp. 62–70, 2011.

[62] A. M. Roccaro, A. Sacco, B. Thompson et al., "MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma," *Blood*, vol. 113, no. 26, pp. 6669–6680, 2009.

[63] M. Lerner, M. Harada, J. Lovén et al., "DLEU2, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1," *Experimental Cell Research*, vol. 315, no. 17, pp. 2941–2952, 2009.

[64] M. Kumar, Z. Lu, A. A. Takwi et al., "Negative regulation of the tumor suppressor p53 gene by microRNAs," *Oncogene*, vol. 30, no. 7, pp. 843–853, 2011.

[65] F. Pichiorri, S. S. Suh, A. Rocci et al., "Downregulation of p53-inducible microRNAs 192, 194, and 215 impairs the p53/MDM2 autoregulatory loop in multiple myeloma development," *Cancer Cell*, vol. 18, no. 4, pp. 367–381, 2010.

[66] K. Y. Wong, C. C. So, F. Loong et al., "Epigenetic inactivation of the miR-124-1 in haematological malignancies," *PLoS ONE*, vol. 6, no. 4, article e19027, 2011.

[67] K. Y. Wong, R. Liang, C. C. So, D. Y. Jin, J. F. Costello, and C. S. Chim, "Epigenetic silencing of MIR203 in multiple myeloma," *British Journal of Haematology*, vol. 154, no. 5, pp. 569–578, 2011.

[68] Y. K. Zhang, H. Wang, Y. Leng et al., "Overexpression of microRNA-29b induces apoptosis of multiple myeloma cells through down regulating Mcl-1," *Biochemical and Biophysical Research Communications*, vol. 414, no. 1, pp. 233–239, 2011.

[69] V. J. Craig, S. B. Cogliatti, H. Rehrauer, T. Wündisch, and A. Müller, "Epigenetic silencing of microRNA-203 dysregulates ABL1 expression and drives helicobacter-associated gastric lymphomagenesis," *Cancer Research*, vol. 71, no. 10, pp. 3616–3624, 2011.

[70] J. Cai, X. Liu, J. Cheng et al., "MicroRNA-200 is commonly repressed in conjunctival MALT lymphoma, and targets cyclin E2," *Graefe's Archive for Clinical and Experimental Ophthalmology*, vol. 250, no. 4, pp. 523–531, 2012.

[71] M. Tomana, J. Novak, B. A. Julian, K. Matousovic, K. Konecny, and J. Mestecky, "Circulating immune complexes in IgA nephropathy consist of IgA1 with galactose-deficient hinge region and antiglycan antibodies," *The Journal of Clinical Investigation*, vol. 104, no. 1, pp. 73–81, 1999.

[72] G. Wang, B. C. Kwan, F. M. Lai, K. M. Chow, P. K. Li, and C. C. Szeto, "Elevated levels of miR-146a and miR-155 in kidney biopsy and urine from patients with IgA nephropathy," *Disease Markers*, vol. 30, no. 4, pp. 171–179, 2011.

[73] I. Bhatti, A. Lee, J. Lund, and M. Larvin, "Small RNA: a large contributor to carcinogenesis?" *Journal of Gastrointestinal Surgery*, vol. 13, no. 7, pp. 1379–1388, 2009.

[74] S. P. Nana-Sinkam and C. M. Croce, "MicroRNA in chronic lymphocytic leukemia: transitioning from laboratory-based investigation to clinical application," *Cancer Genetics and Cytogenetics*, vol. 203, no. 2, pp. 127–133, 2010.

[75] B. L. Betz and J. L. Hess, "Acute myeloid leukemia diagnosis in the 21st century," *Archives of Pathology and Laboratory Medicine*, vol. 134, no. 10, pp. 1427–1433, 2010.

[76] K. Mrózek, N. A. Heerema, and C. D. Bloomfield, "Cytogenetics in acute leukemia," *Blood Reviews*, vol. 18, no. 2, pp. 115–136, 2004.

[77] K. Mrózek, G. Marcucci, P. Paschka, S. P. Whitman, and C. D. Bloomfield, "Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification?" *Blood*, vol. 109, no. 2, pp. 431–448, 2007.

[78] G. Marcucci, K. Mrózek, and C. D. Bloomfield, "Molecular heterogeneity and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics," *Current Opinion in Hematology*, vol. 12, no. 1, pp. 68–75, 2005.

[79] A. Renneville, C. Roumier, V. Biggio et al., "Cooperating gene mutations in acute myeloid leukemia: a review of the literature," *Leukemia*, vol. 22, no. 5, pp. 915–931, 2008.

[80] S. Mi, J. Lu, M. Sun et al., "MicroRNA expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 50, pp. 19971–19976, 2007.

[81] Y. Wang, Z. Li, C. He et al., "MicroRNAs expression signatures are associated with lineage and survival in acute leukemias," *Blood Cells, Molecules, and Diseases*, vol. 44, no. 3, pp. 191–197, 2010.

[82] R. Garzon, S. Volinia, C. G. Liu et al., "MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia," *Blood*, vol. 111, no. 6, pp. 3183–3189, 2008.

[83] S. M. Gao, J. Yang, C. Chen et al., "miR-15a/16-1 enhances retinoic acid-mediated differentiation of leukemic cells and is up-regulated by retinoic acid," *Leukemia & Lymphoma*, vol. 52, no. 12, pp. 2365–2371, 2011.

[84] M. Jongen-Lavrencic, S. M. Sun, M. K. Dijkstra, P. J. Valk, and B. Löwenberg, "MicroRNA expression profiling in relation to the genetic heterogeneity of acute myeloid leukemia," *Blood*, vol. 111, no. 10, pp. 5078–5085, 2008.

[85] M. Alcalay, E. Tiacci, R. Bergomas et al., "Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance," *Blood*, vol. 106, no. 3, pp. 899–902, 2005.

[86] S. Yekta, I. H. Shih, and D. P. Bartel, "MicroRNA-directed cleavage of HOXB8 mRNA," *Science*, vol. 304, no. 5670, pp. 594–596, 2004.

[87] R. Garzon, M. Garofalo, M. P. Martelli et al., "Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 10, pp. 3945–3950, 2008.

[88] S. P. Whitman, K. Maharry, M. D. Radmacher et al., "FLT3 internal tandem duplication associates with adverse outcome and gene- and microRNA-expression signatures in patients 60 years of age or older with primary cytogenetically normal acute myeloid leukemia: a cancer and leukemia group B study," *Blood*, vol. 116, no. 18, pp. 3622–3626, 2010.

[89] Z. Li, J. Lu, M. Sun et al., "Distinct microRNA expression profiles in acute myeloid leukemia with common translocations," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 40, pp. 15535–15540, 2008.

[90] A. Dixon-McIver, P. East, C. A. Mein et al., "Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia," *PLoS ONE*, vol. 3, no. 5, article e2141, 2008.

[91] X. Wang, E. Gocek, C. G. Liu, and G. P. Studzinski, "MicroRNAs181 regulate the expression of p27Kip1 in human myeloid leukemia cells induced to differentiate by 1,25-dihydroxyvitamin D3," *Cell Cycle*, vol. 8, no. 5, pp. 736–741, 2009.

[92] Y. D. Zhu, L. Wang, C. Sun et al., "Distinctive microRNA signature is associated with the diagnosis and prognosis of acute leukemia," *Medical Oncology*. In press.

[93] M. Hallek, B. D. Cheson, D. Catovsky et al., "Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the international workshop on chronic lymphocytic leukemia updating the National Cancer Institute-working group 1996 guidelines," *Blood*, vol. 111, no. 12, pp. 5446–5456, 2008.

[94] J. G. Gribben and S. O'Brien, "Update on therapy of chronic lymphocytic leukemia," *Journal of Clinical Oncology*, vol. 29, no. 5, pp. 544–550, 2011.

[95] H. Döhner, S. Stilgenbauer, A. Benner et al., "Genomic aberrations and survival in chronic lymphocytic leukemia," *The New England Journal of Medicine*, vol. 343, no. 26, pp. 1910–1916, 2000.

[96] G. A. Calin and C. M. Croce, "Genomics of chronic lymphocytic leukemia microRNAs as new players with clinical significance," *Seminars in Oncology*, vol. 33, no. 2, pp. 167–173, 2006.

[97] E. Salerno, B. J. Scaglione, F. D. Coffman et al., "Correcting miR-15a/16 genetic defect in New Zealand black mouse model of CLL enhances drug sensitivity," *Molecular Cancer Therapeutics*, vol. 8, no. 9, pp. 2684–2692, 2009.

[98] A. Cimmino, G. A. Calin, M. Fabbri et al., "miR-15 and miR-16 induce apoptosis by targeting BCL2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 39, pp. 13944–13949, 2005.

[99] E. S. Raveche, E. Salerno, B. J. Scaglione et al., "Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice," *Blood*, vol. 109, no. 12, pp. 5079–5086, 2007.

[100] Y. Pekarsky, U. Santanam, A. Cimmino et al., "Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181," *Cancer Research*, vol. 66, no. 24, pp. 11590–11593, 2006.

[101] U. Santanam, N. Zanesi, A. Efanov et al., "Chronic lymphocytic leukemia modeled in mouse by targeted miR-29 expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 27, pp. 12210–12215, 2010.

[102] M. Fabbri, A. Bottoni, M. Shimizu et al., "Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of b-cell chronic lymphocytic leukemia," *Journal of the American Medical Association*, vol. 305, no. 1, pp. 59–67, 2011.

[103] G. Zauli, R. Voltan, M. G. di Iasio et al., "miR-34a induces the downregulation of both E2F1 and B-Myb oncogenes in leukemic cells," *Clinical Cancer Research*, vol. 17, no. 9, pp. 2712–2724, 2011.

[104] V. Fulci, S. Chiaretti, M. Goldoni et al., "Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia," *Blood*, vol. 109, no. 11, pp. 4944–4951, 2007.

[105] K. Vargova, N. Curik, P. Burda et al., "MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia," *Blood*, vol. 117, no. 14, pp. 3816–3825, 2011.

[106] C. H. Lawrie, S. Gal, H. M. Dunlop et al., "Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma," *British Journal of Haematology*, vol. 141, no. 5, pp. 672–675, 2008.

[107] X. Chen, Y. Ba, L. Ma et al., "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," *Cell Research*, vol. 18, no. 10, pp. 997–1006, 2008.

[108] E. Moussay, K. Wang, J. H. Cho et al., "MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 16, pp. 6573–6578, 2011.

[109] J. R. Finnerty, W. X. Wang, S. S. Hébert, B. R. Wilfred, G. Mao, and P. T. Nelson, "The miR-15/107 group of microRNA genes: evolutionary biology, cellular functions, and roles in human diseases," *Journal of Molecular Biology*, vol. 402, no. 3, pp. 491–509, 2010.

[110] P. T. Nelson, W. X. Wang, G. Mao et al., "Specific sequence determinants of miR-15/107 microRNA gene group targets," *Nucleic Acids Research*, vol. 39, no. 18, pp. 8163–8172, 2011.

[111] J. Pothof, N. S. Verkaik, W. van Ijcken et al., "MicroRNA-mediated gene silencing modulates the UV-induced DNA-damage response," *EMBO Journal*, vol. 28, no. 14, pp. 2090–2099, 2009.

[112] B. Zhang and X. Pan, "RDX induces aberrant expression of microRNAs in mouse brain and liver," *Environmental Health Perspectives*, vol. 117, no. 2, pp. 231–240, 2009.

[113] J. B. Redell, Y. Liu, and P. K. Dash, "Traumatic brain injury alters expression of hippocampal microRNAs: potential regulators of multiple pathophysiological processes," *Journal of Neuroscience Research*, vol. 87, no. 6, pp. 1435–1448, 2009.

[114] R. Kulshreshtha, M. Ferracin, S. E. Wojcik et al., "A microRNA signature of hypoxia," *Molecular and Cellular Biology*, vol. 27, no. 5, pp. 1859–1867, 2007.

[115] K. J. Yin, Z. Deng, M. Hamblin et al., "Peroxisome proliferator-activated receptor δ regulation of miR-15a in ischemia-induced cerebral vascular endothelial injury," *Journal of Neuroscience*, vol. 30, no. 18, pp. 6398–6408, 2010.

[116] T. C. Chang, D. Yu, Y. S. Lee et al., "Widespread microRNA repression by Myc contributes to tumorigenesis," *Nature Genetics*, vol. 40, no. 1, pp. 43–50, 2008.

[117] X. Zhang, X. Chen, J. Lin et al., "Myc represses miR-15a/miR-16-1 expression through recruitment of HDAC3 in mantle cell and other non-Hodgkin B-cell lymphomas," *Oncogene*, vol. 31, no. 24, pp. 3002–3008, 2012.

[118] G. A. Calin, A. Cimmino, M. Fabbri et al., "MiR-15a and miR-16-1 cluster functions in human leukemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 13, pp. 5166–5171, 2008.

[119] Y. Takahashi, A. R. Forrest, E. Maeno, T. Hashimoto, C. O. Daub, and J. Yasuda, "MiR-107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines," *PLoS ONE*, vol. 4, no. 8, article e6677, 2009.

[120] R. G. Wickremasinghe, A. G. Prentice, and A. J. Steele, "P53 and notch signaling in chronic lymphocytic leukemia: clues to identifying novel therapeutic strategies," *Leukemia*, vol. 25, pp. 1400–1407, 2011.

[121] A. Albihn, J. I. Johnsen, and M. A. Henriksson, "MYC in oncogenesis and as a target for cancer therapies," *Advances in Cancer Research*, vol. 107, pp. 163–224, 2010.

[122] G. A. Calin, M. Ferracin, A. Cimmino et al., "A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia," *The New England Journal of Medicine*, vol. 353, no. 17, pp. 1793–1801, 2005.

[123] Y. Guo, Z. Chen, L. Zhang et al., "Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma," *Cancer Research*, vol. 68, no. 1, pp. 26–33, 2008.

[124] H. M. Heneghan, N. Miller, R. Kelly, J. Newell, and M. J. Kerin, "Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for

detecting noninvasive and early stage disease," *Oncologist*, vol. 15, no. 7, pp. 673–682, 2010.

[125] D. Li, Y. Zhao, C. Liu et al., "Analysis of MiR-195 and MiR-497 expression, regulation and role in breast cancer," *Clinical Cancer Research*, vol. 17, no. 7, pp. 1722–1730, 2011.

[126] A. Hutloff, K. Büchner, K. Reiter et al., "Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 50, no. 10, pp. 3211–3220, 2004.

[127] S. Lacotte, H. Dumortier, M. Décossas, J. P. Briand, and S. Muller, "Identification of new pathogenic players in lupus: autoantibody-secreting cells are present in nephritic kidneys of (NZBxNZW)F1 mice," *Journal of Immunology*, vol. 184, no. 7, pp. 3937–3945, 2010.

[128] G. Q. Daley, "Towards the generation of patient-specific pluripotent stem cells for combined gene and cell therapy of hematologic disorders," *Hematology*, vol. 2007, no. 1, pp. 17–22, 2007.

[129] S. K. Mallanna and A. Rizzino, "Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells," *Developmental Biology*, vol. 344, no. 1, pp. 16–25, 2010.

[130] F. Anokye-Danso, C. M. Trivedi, D. Juhr et al., "Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency," *Cell Stem Cell*, vol. 8, no. 4, pp. 376–388, 2011.

[131] A. Marson, S. S. Levine, M. F. Cole et al., "Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells," *Cell*, vol. 134, no. 3, pp. 521–533, 2008.

[132] M. Sachdeva, S. Zhu, F. Wu et al., "P53 represses c-Myc through induction of the tumor suppressor miR-145," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 9, pp. 3207–3212, 2009.

[133] S. Guo, J. Lu, R. Schlanger et al., "MicroRNA miR-125a controls hematopoietic stem cell number," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 32, pp. 14229–14234, 2010.

[134] U. Bissels, A. Bosio, and W. Wagner, "MicroRNAs are shaping the hematopoietic landscape," *Haematologica*, vol. 97, no. 2, pp. 160–167, 2012.

[135] S. Careccia, S. Mainardi, A. Pelosi et al., "A restricted signature of miRNAs distinguishes APL blasts from normal promyelocytes," *Oncogene*, vol. 28, no. 45, pp. 4034–4040, 2009.

[136] S. Kasar, E. Salerno, Y. Yuan et al., "Systemic in vivo lentiviral delivery of miR-15a/16 reduces malignancy in the NZB de novo mouse model of chronic lymphocytic leukemia," *Genes & Immunity*, vol. 13, no. 2, pp. 109–119, 2011.

[137] D. L. Zanette, F. Rivadavia, G. A. Molfetta et al., "miRNA expression profiles in chronic lymphocytic and acute lymphocytic leukemia," *Brazilian Journal of Medical and Biological Research*, vol. 40, no. 11, pp. 1435–1440, 2007.

[138] C. P. Pallasch, M. Patz, J. P. Yoon et al., "miRNA deregulation by epigenetic silencing disrupts suppression of the oncogene PLAG1 in chronic lymphocytic leukemia," *Blood*, vol. 114, no. 15, pp. 3255–3264, 2009.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 939021, 11 pages
doi:10.1155/2012/939021

Review Article

Retinoid Differentiation Therapy for Common Types of Acute Myeloid Leukemia

Geoffrey Brown and Philip Hughes

School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Correspondence should be addressed to Geoffrey Brown, g.brown@bham.ac.uk

Received 3 February 2012; Accepted 5 March 2012

Academic Editor: George P. Studzinski

Many cancers arise in a tissue stem cell, and cell differentiation is impaired resulting in an accumulation of immature cells. The introduction of all-*trans* retinoic acid (ATRA) in 1987 to treat acute promyelocytic leukemia (APL), a rare subtype of acute myeloid leukemia (AML), pioneered a new approach to obtain remission in malignancies by restoring the terminal maturation of leukemia cells resulting in these cells having a limited lifespan. Differentiation therapy also offers the prospect of a less aggressive treatment by virtue of attenuated growth of leukemia cells coupled to limited damage to normal cells. The success of ATRA in differentiation therapy of APL is well known. However, ATRA does not work in non-APL AML. Here we examine some of the molecular pathways towards new retinoid-based differentiation therapy of non-APL AML. Prospects include modulation of the epigenetic status of ATRA-insensitive AML cells, agents that influence intracellular signalling events that are provoked by ATRA, and the use of novel synthetic retinoids.

1. Introduction

There is an increasing need to devise milder treatments for older patients with cancer. The proportion of older people in the population is steadily increasing, and 23% of the UK population is projected to be aged ≥65 by 2034, with 5% aged ≥85 [1]. More than half of cancer patients are aged ≥65 years, and around 40% of older persons will be diagnosed with some form of cancer. Treatment of these patients poses a real challenge to the health care service, more so as the population as whole ages. For older persons, an immediate resort to eradicating cancer *via* aggressive chemotherapy is neither well tolerated nor necessary. Differentiation therapy to reduce tumour load followed by milder chemotherapy provides an alternative approach. It is also important to bear in mind that many patients aged ≥65 years are excluded from aggressive trials, by coexisting age-related conditions, for example, declining bone marrow [2, 3] and hepatic function [4]. Trials of new combinations of drugs in leukemia include only younger patients able to tolerate multidrug chemotherapy. For patients in their 80s with malignancies, differentiation therapy that might merely aim to control disease for the patient's natural lifespan is perhaps a more realistic target.

AML, which accounts for ~80% of adult acute leukemias [5], involves the proliferation, abnormal survival, and arrest of cells at a very early stage of myeloid cell differentiation. Alongside this expansion of leukemia cells, the production of normal red cells, platelets, and neutrophils is reduced: these deficits are also life threatening as in the case of infections relating to neutropenia. Current cytotoxic chemotherapy for AML results in a remission rate of 60 to 80% for patients <60 years of age. However, most patients relapse with treatment-resistant disease, and 5-year survival rates are low around 30% [6, 7]. Survival is worse for patients >60 years of age, largely because these patients cannot tolerate intensive treatment and the ensuing further ablation of normal

haematopoiesis [8–10]. Thirty-five percent of AML patients are aged ≥75 years, and the median age at diagnosis is 72 [11]. These patients are very hard to treat with current regimens, and whilst younger patients have benefited from more intensive approaches to treatment, there have not been substantial improvements to outcomes for the elderly [12]. Only around 5% of elderly patients survive long-term when treated by conventional means [13], and stem cell transplantation is not an option for many patients [14].

There is a pressing need to improve survival rates between 5 to 30% and particularly the outcome for elderly patients which has not changed much during the last 20 years [8]. One of the reasons why current chemotherapies for AML are failing is that in endeavoring to eradicate the leukemia cells normal haematopoiesis is compromised substantially, with patients requiring blood and platelet transfusion. Hence, there is a compelling case for persevering with the development of new therapies that target the failure of AML cells to differentiate, are more efficacious in this regard, and have low haematological toxicity. Though AML is somewhat a rare malignancy, the various subtypes of AML provide an excellent test bed for new differentiation therapies, and proven new regimens might have a beneficial effect in treating more prevalent cancers that are presently incurable.

2. The Success of ATRA in Acute Promyelocytic Leukaemia

Retinoids are a class of naturally occurring compounds that are structurally related to vitamin A (or retinol). Retinoids regulate a wide range of biological processes, including development, differentiation, proliferation, and apoptosis [15]. ATRA is the active metabolite of vitamin A and mediates its biological effects by activating one or more of the closely related retinoic acid receptors (RARα, RARβ, and RARγ) that function as ligand-dependent transcriptional regulators. These receptors form heterodimers with rexinoid receptors (RXRα, β, and γ) and bind to retinoid responsive response elements (RAREs) located in the promoter region of retinoid target genes to stimulate gene transcription [15]. Primitive human haematopoietic cells, such as $CD133^{+ve}$/$CD34^{+ve}$/$lineage^{-ve}$ cells that are used to restore haematopoiesis after ablation of leukemia cells, express RAR α1, α2, and γ1. It is well known that activation of RARα drives the differentiation of normal myeloid progenitor cells and myeloid cell lines towards neutrophils, and examination of *in vitro* models of ATRA-driven myeloid differentiation has identified genes that play important roles in this process, including transcription factors and regulators of survival versus apoptosis [16].

APL accounts for around 5–10% of cases of AML. This subtype of AML is characterised by cells having a promyelocytic morphology [17] and the chromosome translocation t(15; 17), resulting in fusion of the retinoic acid receptor α (RARα, on chromosome 17) and promyelocytic leukemia (PML, on chromosome 15) genes [18]. The RARα gene fuses with other genes in variants of APL, for example, the promyelocytic leukemia zinc finger gene (PLZF, on chromosome 11) as a result of t(11; 17) to generate PLZF-RARα [19]. The RARα fusion proteins block differentiation at the promyelocytic stage, by disrupting wild-type RARα cellular signalling and promoting survival of myeloid precursor cells [20]. Cells from APL patients are exquisitely responsive to induction of cell differentiation by ATRA by virtue of this treatment leading to degradation of the RARα/PML. ATRA combined with chemotherapy is a longstanding and highly successful way of treating APL. This has been well documented [21–25] and will only be described briefly here.

Treatment of APL patients with ATRA- and anthracycline-based chemotherapy results in cure rates up to 80%. Patients who relapse (up to 15%) receive intensive chemotherapy, with or without ATR, and around 90% of patients achieve a second remission. However, in most cases this is not durable and allogeneic or autologous stem cell transplantation is then recommended [26–28]. Arsenic trioxide drives rapid degradation of RARα/PML within APL cells and induces differentiation and apoptosis and inhibits the proliferation of a variety of neoplastic cells [29–31]. In 2002, arsenic trioxide was introduced to treat APL patients who had relapsed and refractory APL, and more recent studies have revealed the effectiveness of arsenic trioxide as a primary and single curative agent [32–34].

ATRA has been highly successful in the treatment of APL, but the promise of extending the efficacy of ATRA-based differentiation therapy to other types of AML, and other leukemias and cancers, has still to be fulfilled. In the following sections we examine some of the recent research strategies that are seeking to extend and improve differentiation therapy for AML.

3. Aberrant Epigenetic Gene Regulation and Unresponsiveness of AML Cells to ATRA

One of the reasons why non-APL AML cells respond poorly to ATRA is that target genes that are important to the ATRA-driven differentiation pathway are not properly activated as to their transcription. In the case of non-APL AML cells, ATRA fails to activate the *RARA2* gene which is important for differentiation [35]. Also, RARα2 expression is reduced in AML cells, relative to normal $CD33^{+ve}$ cells, and this is related to a diminution in histone H3K4 dimethylation ($H3K4^{me2}$) on the *RARA2* gene promoter [36], whereby dimethylation is associated with activation of transcription. The H3K4me1/me2 lysine-specific demethylase 1 (LSD1/KDMI) is highly expressed in AML cells [37] and various other tumour cells [38, 39]. ASXL1, a cofactor for RAR, recruits LSD1 to repress RAR target gene promoters.

Recently, Zelent and coworkers have examined the importance of LSD1 in the lack of responsiveness of non-APL AML cells to ATRA [40]. Inhibitors of LSD1 include tranylcypromine (*trans*-2-phenylcycloprolamine) [41] and the biguanide polyamine analogue 2d (1,15-bis{N5-[3,3-(diphenyl)propyl]-N1-biguanido}-4,12-diazapentadecane) [42]. Tranylcypromine is an antidepressant (sold under the brand names Parnate and Jatrosom) and is well tolerated. Inhibitors of LSD1 and ATRA synergised to drive differentiation of primary human AML cells and enhance H3

lysine-4 dimethylation and the expression of myeloid differentiation-associated genes. ATRA and inhibitors of LSD1 when used alone had a limited effect on primary AML cells. Knockdown of LSD1 (shRNA) in HL-60 and TEX (human cord blood immortalised by expression of the *TLS-ERG* oncogene) cells confirmed that this enzyme attenuates the responsiveness of AML cells to ATRA. Importantly, ATRA plus tranylcypromide target leukemia-initiating cells as revealed by diminished engraftment when primary AML samples were treated with the two agents before and after transplantation into NOD.*SCID* gamma mice. The ability of inhibitors of LSD1 to restore responsiveness of non-APL AML cells to ATRA and the anti-leukemic effect of ATRA in combination with an inhibitor of LSD1 indicate a promising new way forward to differentiation therapy of non-APL AML.

DNA methylation at certain gene promoter regions, by DNA methyltransferase enzymes adding methyl groups to CpG sites, may contribute to leukemogenesis by silencing tumour suppressor genes [43, 44]. Such aberrant gene silencing may also be more common in older persons [45]. The use of inhibitors of DNA methyltransferases in the treatment of myelodysplastic syndromes and AML has focused on the nucleoside analog of cytidine azacitidine. The US Food and Drug Administration has approved the use of this agent to treat myelodysplastic syndromes and treatment of patients with higher-risk myelodysplastic syndrome with azacitidine results in a significant survival advantage as compared with conventional care regimens [46]. Azacitidine also prolongs overall survival in elderly AML patients with a low blast count in their bone marrow [47], and treatment of elderly AML patients with outpatient azacitidine resulted in a response rate of around 20% [48].

Lenalidomide, an immunomodulatory agent approved for use in myelodysplastic syndromes and myeloma [49], has epigenetic modifying properties [50] and appears to upregulate tumour suppressor genes that are activated by azacitidine [51]. Treatment of high-risk myelodysplastic syndrome patients with a combination of azacitidine and lenalidomide resulted in a complete response in 44% of patients [52]. Recently, Pollyea and co-workers have examined the prospect of using azacitidine and lenalidomide sequentially to induce remission in elderly and previously untreated patients with AML [53]. Eighteen patients received treatment to determine safety, efficacy, and biological predictors of response. Marked genome-wide DNA demethylation occurred, and ten of the sixteen evaluable patients responded with seven patients achieving a complete remission or remission with incomplete recovery of blood counts. These results as to biological and clinical activity are very promising, and the extent to which sequential azacitidine and lenalidomide will be beneficial in elderly and untreated AML patients, and such patients with a low disease burden, awaits the outcome from an ongoing phase 2 study.

Epigenetic therapies are an important consideration as to rationales for the induction and maintenance of responses in elderly AML patients and for treatments that are tolerable. There is still more to unravel in the use of histone demethylase inhibitors, to drive expression of myeloid differentiation-associated genes, and/or inhibitors of DNA methyltransferases, to drive growth arrest. Interestingly, lenalidomide-provoked epigenetic modifications appear to involve a LSD1-mediated process [49]. Lenalidomide provokes cell cycle arrest in cell lines that typify Burkitt's lymphoma and multiple myeloma by increasing the level of expression of p21(WAF-1), and transcription factors that bind to CpG-rich promoter regions are involved in this process. Lenalidomide-induced up regulation of p21(WAF-1) was reduced by silencing of LSD1, suggesting the involvement of this lysine-specific histone demethylase in a priming switch from methylated to acetylated H3 on the p21(WAF-1) promoter.

4. Improving ATRA Sensitivity by Inhibiting the Activity of an Aldoketoreductase

ATRA can be used to drive differentiation of non-APL myeloid leukemia cell lines, but this often requires a much higher concentration to obtain the same degree of differentiation as APL cell lines. One way in which the concentration of ATRA required for differentiation of both APL and non-APL cells can be reduced is by inhibiting the activity of the aldoketoreductase AKR1C3. Our laboratory first became interested in this enzyme following observations that inhibition of AKR1C3 with the nonsteroidal anti-inflammatory drug indomethacin or the progestogen medroxyprogesterone acetate (MPA) increased the responsiveness of the human promyeloid cell line HL-60 to both ATRA and 1α,25-dihydroxyvitamin D_3 [54, 55] and that AML cell lines express AKR1C3 at a high level [56]. Overexpression of AKR1C3 in HL-60 cells led to resistance to ATRA- and 1α,25-dihydroxyvitamin D_3-mediated differentiation, confirming the enzyme as a novel regulator of nuclear receptor-regulated cell differentiation [57].

AKR1C3 is a multifunctional NADPH-dependent oxidoreductase that plays a role in the metabolism of androgens, oestrogens, prostaglandins, retinoids, and xenobiotics [58]. Hence, AKR1C3 can potentially control the supply of ligands to several classes of nuclear hormone receptors that modulate the survival, proliferation, and differentiation of hematopoietic cells. There is also evidence to implicate AKR1C3 in leukemogenesis. Activating polymorphisms of the AKR1C3 gene has been associated with an increased chance of developing childhood myeloid leukaemia [59], and increased expression of AKR1C3 has been observed in a patient with myelodysplastic syndrome who went on to develop AML-M2 [60]. Workers in the petrochemical industry have a higher-than-normal risk of developing myeloid leukemia, and smoking is a risk factor for myelodysplatic syndromes and AML [61]. A main factor appears to be an increased production of carcinogenic activated polycyclic aromatic hydrocarbon metabolites which cause oxidative DNA damage and DNA strand breakage. Birtwistle and co-workers [62] have recently shown for primary AML cells and in a model system that elevated AKR1C3 expression leads to conversion of model polycyclic aromatic hydrocarbons into compounds that induce DNA damage.

AKR1C3-mediated metabolism of prostaglandins (PGDs) provides a rationale to the influence of this enzyme on cell differentiation (Figure 1). PGD_2 is a substrate for AKR1C3, due to its 11-ketoprostaglandin reductase activity, and would be preferentially metabolised to its 9α,11β-epimer PGF_2 [58], which enhances proliferation of several myeloid leukemic cell lines. However, endogenous PGD_2 is also relatively unstable and will be rapidly and efficiently non-enzymatically converted first to PGJ_2 and then, in a stepwise manner, to 15-Deoxy-Δ12,14-PGJ_2. 15-deoxy-Δ12,14-PGJ_2 is a ligand for the peroxisome proliferator-activated receptor-γ (PPARγ) and can suppress cell proliferation and enhance differentiation of myeloid leukemic cells [57]. Desmond and co-workers [57] have shown for myeloid progenitor cells that express a high level of AKR1C3 that PGD_2 catabolism can be switched from the generation J-series prostanoids that would enhance differentiation and suppress proliferation towards the production of the pro-proliferative PGF_2.

The PPARγ ligand troglitazone can sensitize HL60 cells to the differentiating and anti-proliferative effects of ATRA and 1α,25-dihydroxyvitamin D_3 [63]. However, there are concerns about toxicity associated with high-dose PPARγ agonist therapy. Fibrates such as clofibrate and bezafibrate are agonists of PPARα [64, 65] and modest potentiating agents. Importantly, fibrates have a good toxicity profile and are well tolerated by patients. In this regard, Murray and co-workers have used a combination of bezafibrate (to agonise PPARα) and medroxyprogesterone acetate (to inhibit AKR1C3) to treat a small number of elderly patients with myelodysplastic syndrome and AML [66]. Improvements in the hematological profile were observed, and there were limited signs of toxicity. Whether the addition of ATRA would further improve the therapeutic outcome has still to be examined.

AKR1C3 may have a direct affect on ATRA-provoked cell differentiation by lowering the intracellular concentration of ATRA. Low cellular levels of ATRA have recently been shown to be a feature of a variety of malignant cells [67]. In HL-60 cells AKR1C3 can act as a retinaldehyde reductase, to promote conversion of retinaldehyde into retinol and, as such, decrease the level of cellular ATRA [68]. Ruiz and co-workers have suggested that activity of AKR1C3 plays a role in driving proliferation of HL-60 cells: this can be blocked by a combination of an AKR1C3 inhibitor and a retinoid acid receptor antagonist [68]. The pro-proliferative retinoid signal might well be an extremely low concentration of endogenous ATRA, provoked by activity of AKR1C3, and for the following reasons. When HL-60 and NB4 cells are grown serum-free (that is free of exogenous retinoid), sub-nanomolar concentrations of ATRA enhance colony formation by single cells and the proliferation of cells in bulk cultures [69–71]. At very low ATRA concentrations it is likely that any RAR signalling is mediated *via* preferential activation of RARγ, favouring cell proliferation/survival as opposed to differentiation [72, and see later novel synthetic retinoids]. In this case and as observed by Ruiz and co-workers, an antagonist of RAR, particularly of RARγ, would be expected to interfere with cell growth [73]. As to this possible mode of action of AKR1C3, again inhibiting enzyme activity is important for increasing the sensitivity of myeloid leukemia cells to ATRA.

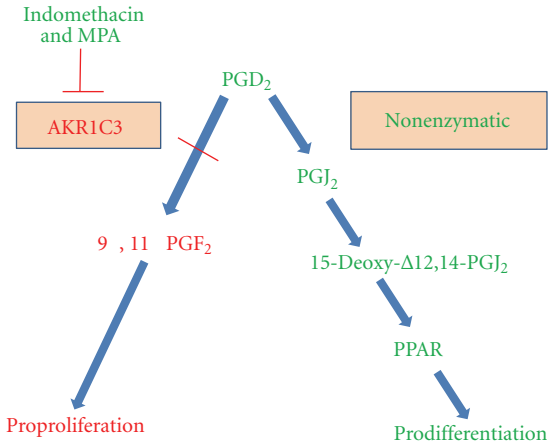


Figure 1: Influence of inhibiting the activity of AKR1C3 on PGD_2 metabolism. Inhibiting the activity of AKR1C3 by the use of indomethacin or medroxyprogesterone acetate (MPA) interferes with prostaglandin D_2 (PGD_2) metabolism towards 9α,11β PGF_2 and favours nonenzymatic metabolism towards J-series prostanoids and the PPARγ ligand 15-deoxy-Δ12,14-PGJ_2.

5. Potentiating ATRA-Stimulated Differentiation by Inhibiting Glycogen Synthase Kinase

As the name suggests, the constitutively active serine/threonine kinase glycogen synthase kinase 3 (GSK-3) plays a role in glycogen biosynthesis and insulin action by phosphorylating and inactivating glycogen synthase. However, GSK-3 is now known to phosphorylate a wide range of proteins and play a role in intracellular signalling that is initiated by various stimuli, as GSK-3 is functionally inactivated when phosphorylated *via* the growth factor receptor-activated RAS-MAP kinase, ERK5/RSK-2 and PI3K-PKB/AKT signalling pathways [74]. Therefore, compounds that inhibit the activity of GSK-3 are very likely to affect many biological processes. Of importance to differentiation therapy is that inhibitors of GSK-3 appear to have opposing effects on the proliferation and be survival and commitment to differentiation of normal and leukemic hematopoietic stem cells (HSCs). This may provide a treatment that targets transformed cells and spares their normal counterpart.

Pharmacological inhibition or genetic depletion of GSK-3 has been associated with increased self-renewal and reduced commitment to differentiation of HSCs in normal mice, and Huang and co-workers have shown that GSK-3 activity inhibits signalling through the WNT pathway to enhance lineage commitment of HSC [75]. In transformed hematopoietic cells, the situation appears to be different. Overexpression and overactivation of GSK-3 are associated with an unfavorable prognosis in AML [76]. Sustained proliferation of MLL-transformed leukemia cells is dependent on activity of GSK-3, leading Wang and co-workers [77] to

propose that GSK-3 acts as a tumor promoter in this model system. In keeping, treatment of MLL-transformed cells *ex vivo* with inhibitors of GSK-3 led to arrest of growth in G1 and an increase in the rate of spontaneous differentiation towards myeloid cells. GSK-3 inhibitors also enhanced the survival of mice with these leukemias. In a follow-up study, Wang and co-workers showed that GSK-3 controls the formation of a HOX/MEIS1/CREB complex which recruits the coactivators CBP and TORC to form a molecular complex that appears to promote self-renewal and survival of the transformed cells [78]. The transformed cells were driven into apoptosis following treatment with the GSK-3 inhibitor lithium chloride. Other workers have confirmed that leukemic HSCs are more sensitive to induction of apoptosis by GSK-3 inhibitors than normal HSCs [79, 80].

The possible use of GSK-3 inhibitors in differentiation therapy for acute myeloid leukemia dates back to around 1993. Sartorelli's group showed that high concentrations of lithium chloride induced growth arrest and myeloid differentiation of the murine myelomonocytic cell line WEHI-3B D+ and HL-60 cells [81]. In the case of HL-60 cells, lithium chloride induction of neutrophil differentiation was markedly enhanced by the addition of a low amount of ATRA, and no such interaction was seen with agents that drive HL-60 cells to differentiate towards monocytes [81]. These findings have been revisited in recent years, and several structurally unrelated GSK-3 inhibitors have now been shown to potently inhibit the growth and drive differentiation of a variety of primary leukemic cells and leukemic cell lines [82–87]. Importantly, genes that are upregulated (Id1, CEBPε, Stat1, p21, and p27) or downregulated (CDK8, c-myc) during ATRA-stimulated myeloid differentiation of HL60 and NB4 cells and whose products are important for cell differentiation, are similarly regulated by inhibitors of GSK-3 [86, 87].

Levels of RARα are linked to the activity of GSK-3β. Sartorelli's group has shown that inhibitors of GSK-3 prevent ATRA-mediated degradation of RARα, thereby potentiating ATRA-stimulated differentiation of AML cell lines [88, 89]. A recent study provides a molecular basis for this observation. Si and co-workers have shown that GSK-3β phosphorylates RARα on multiple serine residues, and phosphorylation of RARα reduces its transcriptional activity. GSK3 phosphorylation of certain proteins enhances their proteasomal degradation, and it is possible that this is the case for RARα as treatment of myeloid leukemia cells with GSK-3 inhibitors led to enhanced expression of RARα [86]. Accordingly, GSK-3 inhibitors potentiated RARα-mediated transcriptional activity which could be explained by increased expression of RARα and by GSK-3β inhibitor-mediated upregulation of the expression of the RAR transcriptional coactivators p300, SRC-1, and CBP [86]. Transcriptional activity of RARα and kinase activity of GSK-3β are also linked as follows (Figure 2). In the retinoid-sensitive AML lines HL-60 and NB4, but not the retinoid insensitive K562 cell line, ATRA treatment was associated with a time- and concentration-related phosphorylation of an N-terminal serine of GSK-3β [86]. This inhibitory phosphorylation of GSK-3β is an early step in the ATRA-induced differentiation of myeloid leukemia cells, in keeping with the importance of inhibition of activity of GSK-3β to cell differentiation.

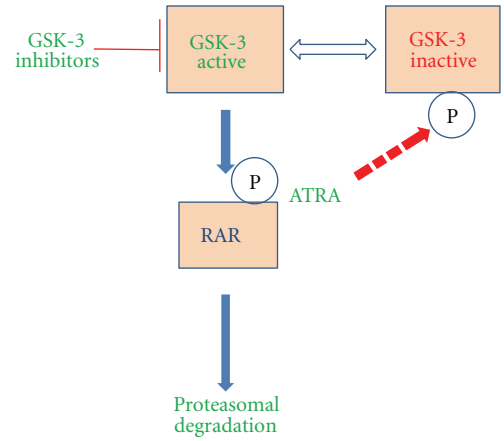


Figure 2: Inhibition of the activity of GSK-3β is important for ATRA sensitivity, The figure shows one way in which activity of GSK-3β affects ATRA responsiveness of myeloid cells and how transcriptional activity of RARα and kinase activity of GSK-3β are linked *via* ATRA-related inhibitory phosphorylation (P) of GSK-3β.

The GSK-3 inhibitors lithium chloride and valproate are already in clinical use, and the above studies indicate that a combination of these agents and a RARα agonist may enhance the effectiveness of differentiation therapy for both ATRA-sensitive and insensitive AML. In addition, beneficial effects from inhibiting the activity of GSK-3β might also relate to the involvement of this enzyme in sustaining proliferation of leukemia cells (see above).

6. Potentiation of ATRA-Stimulated Differentiation of Myeloid Leukemic Cells by Inhibitors of Inosine 5′-Monophosphate Dehydrogenase

Inosine 5′-monophosphate dehydrogenase (IMPDH) is the rate limiting step in the *de novo* synthesis of guanine nucleotides. This enzyme catalyses NAD^+-dependent oxidation of inosine 5′-monophosphate to xanthosine 5′-monophosphate, which is subsequently aminated to guanosine-5′-monophosphate which is converted to guanosine triphosphate (GTP) and deoxyguanosine triphosphate (dGTP). As such, IMPDH plays an important role in the maintenance of the intracellular levels of guanosine nucleotides; especially GTP, and GTP, and dGTP are essential for the synthesis of DNA and RNA. It has long been known that depletion of guanosine nucleotides accompanies differentiation of a variety of cell types. For example, myeloid differentiation of HL-60 and other myeloid leukemic cells is accompanied by a reduction in the cellular GTP content and differentiation can be blocked by the addition of exogenous guanosine [90]. IMPDH activity and GTP levels appear to be much higher in leukemic blast cells than in their normal counterparts, which might contribute to the failure of blast cells to complete their differentiation programme.

Table 1: Preferential binding of synthetic retinoid analogs to subtypes of RAR. The equilibrium binding affinities of each retinoid analog were determined against baculovirus expressed RAR or RXR isoforms by displacement of [^{3}H]-ATRA.

	Binding affinities (ED_{50} in nM) against RARs and RXRs			
Retinoid analog	RARα	RARβ	RARγ	RXRs
RAR agonists				
Pan-RAR (AGN191183)	15.7	7.2	6.7	9,113 (α)–2,556 (γ)
RARα (AGN195183)	20.1	>5,000	>5,000	>10,000
RAR$\beta\gamma$ (AGN190168)	>1,000	14.2	135	>10,000
RARγ (AGN205327)	3,766	734	32	>10,000
RAR antagonists				
Pan-RAR (AGN194310)	4.3	5	2	>10,000
RARα (AGN196996)	3.9	4,036	>10,000	>10,000
RAR$\beta\gamma$ (AGN194431)	300	6	20	>10,000
RARγ (AGN205728)	2,400	4,248	3	>10,000

Twenty-five years ago Knight and co-workers [91] showed that ATRA-mediated myeloid differentiation of HL-60 and RDFD2-25 myeloid leukemic cells was associated with a rapid decrease in IMPDH activity. A fall in IMPDH activity was not observed in a retinoid-insensitive variant of the RDFD2-25 cell line, which suggests that the fall in IMPDH activity is an important aspect of the differentiation process. Commensurate with this finding is that depletion of intracellular GTP levels *via* the use of a variety of IMPDH inhibitors, such as mycophenolate mofetil, tiazofurin and 3-hydrogenkwadaphnin, leads to differentiation and/or apoptosis of myeloid leukemic cell lines [92–94]. Also, treatment of HL-60 and NB4 cells with a combination of ATRA and an IMPDH inhibitor resulted in a greater degree of neutrophil differentiation, followed by apoptosis, than when either agent was used alone [95, 96]. Overall, these studies suggest that a combination of IMPDH inhibitors (e.g., mycophenolate mofetil), which are already in clinical use, with ATRA may provide a more effective treatment for both APL and non-APL AML.

7. Enhancing ATRA Responsiveness by Inhibiting Telomerase Activity

Recent studies suggest that sensitivity of cell lines that typify APL to the combination of ATRA and arsenic trioxide is due to a synergy at the level of inhibition of telomerase activity. Myeloid leukemic stem and progenitor cells exhibit a higher degree of telomerase activity than their normal counterparts which may contribute to their increased capacity for self-renewal and reduced sensitivity to differentiating agents. Shortened telomere length and elevated telomerase activity in cells from APL patients are indicative of extensive proliferative capacity and correlate with disease progression and relapse. Thus, elevated telomerase activity may serve as prognostic factors for a subset of APL patients with more aggressive disease and poor outcome and those who may not respond favourably to arsenic therapy [97].

Overexpression of telomerase in normal HCSs changes these cells into ones resembling a leukemic stem cell, suggesting a role for telomerase in the leukemogenic process. The catalytic subunit of telomerase is telomerase reverse transcriptase (TERT). In the case of HL-60 cells, a decrease in the expression of the hTERT gene and a concomitant reduction in telomerase activity is a relatively early event following exposure to differentiating concentrations of ATRA. Genetic knockdown of hTERT is sufficient to induce growth arrest and eventually drives HL-60 cells into apoptosis [98–102]. Downregulation of hTERT expression following retinoid treatment has also been observed in maturation-resistant APL cell lines and non-APL AML cell lines, but in these cell lines cotreatment with a specific RARα agonist and a retinoid X receptor- (RXR-) specific agonist (a rexinoid) was required. In non-APL AML cells there was no indication of an increased level of granulocytic differentiation following retinoid/rexinoid treatment, suggesting that retinoid/rexinoid-mediated down-regulation of telomerase was targeting a pathway that is important for survival [103, 104]. That down regulation of hTERT gene expression appears to be a key early event in ATRA-mediated growth arrest, and apoptosis induction of both retinoid-sensitive and insensitive APL cell lines and non-APL myeloid leukemic cells, suggests that a combination of ATRA (± a rexinoid) with telomerase inhibitors may have enhanced antileukemic properties in APL patients and also be beneficial in non-APL AML.

8. The Use of Novel Synthetic Retinoids

A complication during ATRA treatment of APL is retinoic acid syndrome (RAS). The full-blown syndrome is life-threatening as patients may develop renal failure or respiratory distress and require admission to intensive care. At the earliest sign of RAS, treatment with intravenous dexamethasone is the recommended course of action, and ATRA is temporarily discontinued in the case of severe RAS [21, 105]. Respiratory distress and fever can be very common; in a retrospective analysis of 102 APL patients who received ATRA as an induction regimen with or without conventional chemotherapy, 87.5% of patients developed respiratory distress and fever [106].

ATRA is promiscuous as regards binding to retinoid receptors. ATRA binds with high affinity to the α, β, and γ sub-types of RARs, and to RXRs by virtue of isomerising within cells to 9-*cis*-retinoic acid. As to the importance of ligand-activation of RARα for myeloid cell differentiation [107], synthetic retinoids have been developed that only bind to this RAR sub-type. Even though there are toxicities associated with ATRA, the success in treating APL somewhat precludes using a RARα-specific agonist instead of ATRA. However, a RARα specific agonist and also synthetic antagonists of RARs are worthy of consideration for future new therapies.

We and others have examined the biological activity of isoform-selective synthetic agonists and antagonists of RARs with a view to widening the scope as to the use of retinoids to drive growth arrest, differentiation, and/or apoptosis of malignant cells [108, 109]. The extents to which the retinoids we have studied over a number of years are selective for a particular receptor subtype(s) are shown in Table 1.

As shown in Table 1, the compound AGN195183 is a highly selective agonist of RARα; it binds to RARα with a low nanomolar affinity (ED_{50} 20.1 nM), affinities to *RAR*β and γ are much higher (ED_{50} > 5,000), and it does not bind to RXRs (ED_{50} > 10,000). Treatment of HL-60 cells with the RARα agonist, rather than agonising all RARs, is sufficient to drive differentiation towards neutrophils [108]. Hence, AGN195183 is suitable for use in differentiation therapy of AML and might circumvent some of the toxicities that are associated with the use of ATRA.

Antagonising, rather than agonising, RARs may be important for the development of milder treatments for elderly patients with leukemia and other cancers. Some time ago we showed that antagonising all RARs (AGN194310) inhibited the growth of patients' prostate cancer cells more effectively than normal prostate epithelium. Treatment of prostate cancer cells in liquid culture led to growth arrest in the G1 phase of the cell cycle followed by apoptosis. Moreover, the pan antagonist appears to be effective against tumor-initiating cells as nM concentrations of compound inhibited colony formation on plates by the small fraction of clonogenic cells [73, 110, 111]. Recently, we have shown that antagonising RARγ is sufficient to inhibit the growth of prostate cancer cells [*manuscript submitted*]. In keeping with this perceived importance of RARγ for survivability and/or proliferation of primitive cells, Purdon and coworker have shown that activation of RARγ plays a role in self-renewal of HSC as these cells are reduced in RARγ-null mice [72]. The use of a demethylase inhibitor (that is in clinical use and which restores ATRA responsiveness), plus an RARα agonist (for differentiation) and an RARγ antagonist (to inhibit growth) or the inhibitor plus either single retinoid, is interesting considerations to enhance the efficacy of differentiation therapy for ATRA-unresponsive AML.

9. Concluding Remarks

Healthcare authorities are already considering how to provide an appropriate standard of care for a population that in the future will overall be much older. A key aspect to this issue is the treatment of leukemia and other malignancies, particularly the extent to which elderly patients are able to tolerate current intensive therapeutic regimens. Differentiation therapy, and combined with less-aggressive chemotherapy, may provide milder treatments, and as outlined above there are promising ways forward for rendering differentiation therapy more efficacious.

References

[1] http://www.statistics.gov.uk/hub/population/aging/older-people.

[2] T. Ogawa, M. Kitagawa, and K. Hirokawa, "Age-related changes of human bone marrow: a histometric estimation of proliferative cells, apoptotic cells, T cells, B cells and macrophages," *Mechanisms of Ageing and Development*, vol. 117, no. 1-3, pp. 57–68, 2000.

[3] D. Herndler-Brandstetter, B. Weinberger, G. Pfister, D. Weiskopf, and B. Grubeck-Loebenstein, "The aging of the adaptive immune system," *Current Immunology Reviews*, vol. 7, no. 1, pp. 94–103, 2011.

[4] D. L. Schmucker, "Age-related changes in liver structure and function: implications for disease?" *Experimental Gerontology*, vol. 40, no. 8-9, pp. 650–659, 2005.

[5] D. A. Pollyea, H. E. Kohrt, and B. C. Medeiros, "Acute myeloid leukaemia in the elderly: a review," *British Journal of Haematology*, vol. 152, no. 5, pp. 524–542, 2011.

[6] M. S. Tallman, D. G. Gilliland, and J. M. Rowe, "Drug therapy for acute myeloid leukemia," *Blood*, vol. 106, no. 4, pp. 1154–1163, 2005.

[7] H. E. Kohrt and S. E. Coutre, "Optimizing therapy for acute myeloid leukemia," *Journal of the National Comprehensive Cancer Network*, vol. 6, no. 10, pp. 1003–1016, 2008.

[8] A. K. Burnett, D. Milligan, A. Goldstone et al., "The impact of dose escalation and resistance modulation in older patients with acute myeloid leukaemia and high risk myelodysplastic syndrome: the results of the LRF AML14 trial," *British Journal of Haematology*, vol. 145, no. 3, pp. 318–332, 2009.

[9] E. Estey, "Acute myeloid leukemia and myelodysplastic syndromes in older patients," *Journal of Clinical Oncology*, vol. 25, no. 14, pp. 1908–1915, 2007.

[10] A. Kuendgen and U. Germing, "Emerging treatment strategies for acute myeloid leukemia (AML) in the elderly," *Cancer Treatment Reviews*, vol. 35, no. 2, pp. 97–120, 2009.

[11] G. Juliusson, P. Antunovic, A. Derolf et al., "Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry," *Blood*, vol. 113, no. 18, pp. 4179–4187, 2009.

[12] H. Dombret, E. Raffoux, and C. Gardin, "Acute myeloid leukemia in the elderly," *Seminars in Oncology*, vol. 35, no. 4, pp. 430–438, 2008.

[13] S. S. Farag, K. J. Archer, K. Mrózek et al., "Pretreatment cytogenetics add to other prognostic factors predicting complete remission and long-term outcome in patients 60 years of age or older with acute myeloid leukemia: results from Cancer and Leukemia Group B 8461," *Blood*, vol. 108, no. 1, pp. 63–73, 2006.

[14] H. J. Deeg and B. M. Sandmaier, "Who is fit for allogeneic transplantation?" *Blood*, vol. 116, no. 23, pp. 4762–4770, 2010.

[15] E. Samarut and C. Rochette-Egly, "Nuclear retinoic acid receptors: conductors of the retinoic acid symphony during development," *Molecular and Cellular Endocrinology*, vol. 348, no. 2, pp. 348–360, 2012.

[16] S. J. Collins, "Retinoic acid receptors, hematopoiesis and leukemogenesis," *Current Opinion in Hematology*, vol. 15, no. 4, pp. 346–351, 2008.

[17] J. M. Bennet, D. Catovsky, M. T. Daniel et al., "Proposals for the classification of acute leukaemias," *British Journal of Haematology*, vol. 33, pp. 451–458, 1976.

[18] A. Kakizuka, W. H. Miller, K. Umesono et al., "Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor, PML," *Cell*, vol. 66, no. 4, pp. 663–674, 1991.

[19] S. J. Chen, A. Zelent, J. H. Tong et al., "Rearrangements of the retinoic acid receptor alpha and promyelocytic leukemia zinc finger genes resulting from t(11;17)(q23;q21) in a patient with acute promyelocytic leukemia," *Journal of Clinical Investigation*, vol. 91, no. 5, pp. 2260–2267, 1993.

[20] F. Grigiani, P. F. Ferrucci, U. Testa et al., "The acute promelocytic leukaemia-specific PML-RARA fusion protein inhibits differentiation and promotes survival of myeloid precursor cells," *Cell*, vol. 74, pp. 423–431, 1993.

[21] M. A. Sanz, D. Grimwade, M. S. Tallman et al., "Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet," *Blood*, vol. 113, no. 9, pp. 1875–1891, 2009.

[22] E. Lengfelder, S. Saussele, A. Weisser, T. Büchner, and R. Hehlmann, "Treatment concepts of acute promyelocytic leukemia," *Critical Reviews in Oncology/Hematology*, vol. 56, no. 2, pp. 261–274, 2005.

[23] K. Petrie, A. Zelent, and S. Waxman, "Differentiation therapy of acute myeloid leukemia: past, present and future," *Current Opinion in Hematology*, vol. 16, no. 2, pp. 84–91, 2009.

[24] L. Altucci and H. Gronemeyer, "The promise of retinoids to fight against cancer," *Nature Reviews Cancer*, vol. 1, no. 3, pp. 181–193, 2001.

[25] D. Grimwade, A. R. Mistry, E. Solomon, and F. Guidez, "Acute promyelocytic leukemia: a paradigm for differentiation therapy," *Cancer Treatment and Research*, vol. 145, pp. 219–235, 2010.

[26] C. Castagnola, M. Lunghi, A. Corso et al., "Management of acute promyelocytic leukemia relapse in the ATRA era," *Haematologica*, vol. 83, no. 8, pp. 714–717, 1998.

[27] X. Thomas, H. Dombret, C. Cordonnier et al., "Treatment of relapsing acute promyelocytic leukemia by all-trans retinoic acid therapy followed by timed sequential chemotherapy and stem cell transplantation," *Leukemia*, vol. 14, no. 6, pp. 1006–1013, 2000.

[28] M. A. Sanz, M. Labopin, N. C. Gorin et al., "Hematopoietic stem cell transplantation for adults with acute promyelocytic leukemia in the ATRA era: a survey of the European Cooperative Group for Blood and Marrow Transplantation," *Bone Marrow Transplantation*, vol. 39, no. 8, pp. 461–469, 2007.

[29] W. H. Miller, H. M. Schipper, J. S. Lee, J. Singer, and S. Waxman, "Mechanisms of action of arsenic trioxide," *Cancer Research*, vol. 62, no. 14, pp. 3893–3903, 2002.

[30] G. Q. Chen, X. G. Shi, W. Tang et al., "Use of arsenic trioxide (As^2O^3) in the treatment of acute promyelocytic leukemia (APL): I. As^2O^3 exerts dose-dependent dual effects on APL cells," *Blood*, vol. 89, no. 9, pp. 3345–3353, 1997.

[31] J. Ablain and H. De The, "Revisiting the differentiation paradigm in acute promyelocytic leukemia," *Blood*, vol. 117, no. 22, pp. 5795–5802, 2011.

[32] A. Ghavamzadeh, K. Alimoghaddam, S. H. Ghaffari et al., "Treatment of acute promyelocytic leukemia with arsenic trioxide without ATRA and/or chemotherapy," *Annals of Oncology*, vol. 17, no. 1, pp. 131–134, 2006.

[33] V. Mathews, B. George, K. M. Lakshmi et al., "Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: durable remissions with minimal toxicity," *Blood*, vol. 107, no. 7, pp. 2627–2632, 2006.

[34] E. Lengfelder, W.-K. Hofmann, and D. Novak., "Impact of arsenic trioxide in the treatment of acute promyelocytic leukemia," *Leukemia*, vol. 26, pp. 433–442, 2012.

[35] A. Glasow, N. Prodromou, K. Xu, M. Von Lindern, and A. Zelent, "Retinoids and myelomonocytic growth factors cooperatively activate RARA and induce human myeloid leukemia cell differentiation via MAP kinase pathways," *Blood*, vol. 105, no. 1, pp. 341–349, 2005.

[36] A. Glasow, A. Barrett, K. Petrie et al., "DNA methylation-independent loss of RARA gene expression in acute myeloid leukemia," *Blood*, vol. 111, no. 4, pp. 2374–2377, 2008.

[37] L. Berglund, E. Björling, P. Oksvold et al., "A genecentric human protein atlas for expression profiles based on antibodies," *Molecular and Cellular Proteomics*, vol. 7, no. 10, pp. 2019–2027, 2008.

[38] S. Lim, A. Janzer, A. Becker et al., "Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology," *Carcinogenesis*, vol. 31, no. 3, pp. 512–520, 2010.

[39] S. Hayami, J. D. Kelly, H. S. Cho et al., "Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers," *International Journal of Cancer*, vol. 128, no. 3, pp. 574–586, 2011.

[40] T. Schenk, W. C. Chen, S. Gollner et al., "Inhibition of the LSD1/KDM1 demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia," *Nature Medicine*, vol. 18, no. 4, pp. 605–611, 2012.

[41] M. G. Lee, C. Wynder, D. M. Schmidt, D. G. McCafferty, and R. Shiekhattar, "Histone H3 Lysine 4 Demethylation Is a Target of Nonselective Antidepressive Medications," *Chemistry and Biology*, vol. 13, no. 6, pp. 563–567, 2006.

[42] Y. Huang, E. Greene, T. M. Stewart et al., "Inhibition of lysine-specific demethylase 1 by polyamine analogues results in reexpression of aberrantly silenced genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 19, pp. 8023–8028, 2007.

[43] S. B. Baylin, "DNA methylation and gene silencing in cancer," *Nature Clinical Practice Oncology*, vol. 2, no. 1, pp. S4–S11, 2005.

[44] K. L. Rice, I. Hormaeche, and J. D. Licht, "Epigenetic regulation of normal and malignant hematopoiesis," *Oncogene*, vol. 26, no. 47, pp. 6697–6714, 2007.

[45] J. P. Issa, "Aging, DNA methylation and cancer," *Critical Reviews in Oncology/Hematology*, vol. 32, no. 1, pp. 31–43, 1999.

[46] P. Fenaux, G. J. Mufti, E. Hellstrom-Lindberg et al., "Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study," *The Lancet Oncology*, vol. 10, no. 3, pp. 223–232, 2009.

[47] P. Fenaux, G. J. Mufti, E. Hellstrom-Lindberg, V. Santini, N. Guttermann, and U. Germing, "Azacytidine prolongs overall survival compared with conventional care regimens in elderly patients with low bone marrow blast count acute myeloid leukemia," *Journal of Clinical Oncology*, vol. 28, pp. 562–569, 2010.

[48] N. Sudan, J. M. Rossetti, R. K. Shadduck et al., "Treatment of acute myelogenous leukemia with outpatient azacitidine," *Cancer*, vol. 107, no. 8, pp. 1839–1843, 2006.

[49] V. Kotla, S. Goel, S. Nischal et al., "Mechanism of action of lenalidomide in hematological malignancies," *Journal of Hematology and Oncology*, vol. 2, article 36, 2009.

[50] L. Escoubet-Lozach, I. L. Lin, K. Jensen-Pergakes et al., "Pomalidomide and lenalidomide induce p21WAF-1 expression in both lymphoma and multiple myeloma through a LSD1-mediated epigenetic mechanism," *Cancer Research*, vol. 69, no. 18, pp. 7347–7356, 2009.

[51] A. Pellagatti, M. Jädersten, A. M. Forsblom et al., "Lenalidomide inhibits the malignant clone and up-regulates the SPARC gene mapping to the commonly deleted region in 5q-syndrome patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 27, pp. 11406–11411, 2007.

[52] M. A. Sekeres, A. F. List, D. Cuthbertson et al., "Phase I combination trial of lenalidomide and azacitidine in patients with higher-risk myelodysplastic syndromes," *Journal of Clinical Oncology*, vol. 28, no. 13, pp. 2253–2258, 2010.

[53] D. A. Pollyea, H. E. Kohrt, L. Gallegos, M.E. Figueroa, O. Abdel-Wahab, and S. Bhattacharya, "Safety, efficacy and biological predictors of response to sequential azacitidine and lenalidomide for elderly patients with acute myeloid leukemia," *Leukemia*. In press.

[54] C. M. Bunce, P. J. French, J. Durham, R. A. Stockley, R. H. Michell, and G. Brown, "Indomethacin potentiates the induction of HL60 differentiation to neutrophils, by retinoic acid and granulocyte colony-stimulating factor, and to monocytes, by vitamin D3," *Leukemia*, vol. 8, no. 4, pp. 595–604, 1994.

[55] C. M. Bunce, J. C. Mountford, P. J. French et al., "Potentiation of myeloid differentiation by anti-inflammatory agents, by steroids and by retinoic acid involves a single intracellular target, probably an enzyme of the aldoketoreductase family," *Biochimica et Biophysica Acta*, vol. 1311, no. 2, pp. 189–198, 1996.

[56] K. I. Mills, A. F. Gilkes, M. Sweeney et al., "Identification of a retinoic acid responsive aldoketoreductase expressed in HL60 leukaemic cells," *FEBS Letters*, vol. 440, no. 1-2, pp. 158–162, 1998.

[57] J. C. Desmond, J. C. Mountford, M. T. Drayson et al., "The aldo-keto reductase AKR1C3 is a novel suppressor of cell differentiation that provides a plausible target for the non-cyclooxygenase-dependent antineoplastic actions of nonsteroidal anti-inflammatory drugs," *Cancer Research*, vol. 63, no. 2, pp. 505–512, 2003.

[58] T. M. Penning and M. C. Byrns, "Steroid hormone transforming aldo-keto reductases and cancer," *Annals of the New York Academy of Sciences*, vol. 1155, pp. 33–42, 2009.

[59] C. Y. Liu, Y. H. Hsu, P. C. Pan et al., "Maternal and offspring genetic variants of AKR1C3 and the risk of childhood leukemia," *Carcinogenesis*, vol. 29, no. 5, pp. 984–990, 2008.

[60] D. Mahadevan, J. DiMento, K. Della Croce et al., "Transcriptosome and serum cytokine profiling of an atypical case of myelodysplastic syndrome with progression to acute myelogenous leukemia," *American Journal of Hematology*, vol. 81, no. 10, pp. 779–786, 2006.

[61] J. Björk, B. Johansson, K. Broberg, and M. Albin, "Smoking as a risk factor for myelodysplastic syndromes and acute myeloid leukemia and its relation to cytogenetic findings: a case-control study," *Leukemia Research*, vol. 33, no. 6, pp. 788–791, 2009.

[62] J. Birtwistle, R. E. Hayden, F. L. Khanim et al., "The aldo-keto reductase AKR1C3 contributes to 7,12-dimethylbenz(a)anthracene-3,4-dihydrodiol mediated oxidative DNA damage in myeloid cells: implications for leukemogenesis," *Mutation Research*, vol. 662, no. 1-2, pp. 67–74, 2009.

[63] H. Asou, W. Verbeek, E. Williamson et al., "Growth inhibition of myeloid leukemia cells by troglitazone, a ligand for peroxisome proliferator activated receptor gamma, and retinoids.," *International Journal of Oncology*, vol. 15, no. 5, pp. 1027–1031, 1999.

[64] S. L. Fenton, M. T. Drayson, M. Hewison, E. Vickers, G. Brown, and C. M. Bunce, "Clofibric acid: a potential therapeutic agent in AML and MDS," *British Journal of Haematology*, vol. 105, no. 2, pp. 448–451, 1999.

[65] F. L. Khanim, R. E. Hayden, J. Birtwistle et al., "Combined bezafibrate and medroxyprogesterone acetate: potential novel therapy for acute myeloid leukaemia," *PLoS ONE*, vol. 4, no. 12, Article ID e8147, 2009.

[66] J. A. Murray, F. L. Khanim, R. E. Hayden et al., "Combined bezafibrate and medroxyprogesterone acetate have efficacy without haematological toxicity in elderly and relapsed acute myeloid leukaemia (AML)," *British Journal of Haematology*, vol. 149, no. 1, pp. 65–69, 2010.

[67] X. H. Tang and L. J. Gudas, "Retinoids, retinoic acid receptors, and cancer," *Annual Review of Pathology*, vol. 6, pp. 345–364, 2011.

[68] F. X. Ruiz, S. Porté, O. Gallego et al., "Retinaldehyde is a substrate for human aldo-keto reductases of the 1C subfamily," *Biochemical Journal*, vol. 440, pp. 335–344, 2011.

[69] E. Soppi, K. H. Robert, and G. Gahrton, "Clonal analysis of the response of HL-60 cells to retinoic acid and vitamin D in the presence or absence of leukocyte-conditioned medium," *European Journal of Haematology*, vol. 41, no. 1, pp. 66–73, 1988.

[70] M. Kizaki, Y. Ikeda, R. Tanosaki et al., "Effects of novel retinoic acid compound, 9-cis-retinoic acid, on proliferation, differentiation, and expression of retinoic acid receptor-α and retinoid X receptor-α RNA by HL-60 cells," *Blood*, vol. 82, no. 12, pp. 3592–3599, 1993.

[71] H. C. Hsu, W. H. Tsai, M. L. Hsu, C. H. Ho, and S. Y. Wang, "Effects of colony-stimulating factors on the all-trans retinoic acid-induced differentiation of acute promyelocytic leukemic cells," *Chinese Medical Journal*, vol. 57, no. 2, pp. 93–99, 1996.

[72] L. E. Purton, S. Dworkin, G. H. Olsen et al., "RARγ is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation," *Journal of Experimental Medicine*, vol. 203, no. 5, pp. 1283–1293, 2006.

[73] R. G. Keedwell, Y. Zhao, L. A. Hammond et al., "An antagonist of retinoic acid receptors more effectively inhibits growth of human prostate cancer cells than normal prostate epithelium," *British Journal of Cancer*, vol. 91, no. 3, pp. 580–588, 2004.

[74] S. E. Plyte, K. Hughes, E. Nikolakaki, B. J. Pulverer, and J. R. Woodgett, "Glycogen synthase kinase-3: functions in oncogenesis and development," *Biochimica et Biophysica Acta*, vol. 1114, no. 2-3, pp. 147–162, 1992.

[75] J. Huang, Y. Zhang, A. Bersenev et al., "Pivotal role for glycogen synthase kinase-3 in hematopoietic stem cell homeostasis in mice," *Journal of Clinical Investigation*, vol. 119, no. 12, pp. 3519–3529, 2009.

[76] S. M. Kornblau, R. Tibes, Y. H. Qiu et al., "Functional proteomic profiling of AML predicts response and survival," *Blood*, vol. 113, no. 1, pp. 154–164, 2009.

[77] Z. Wang, K. S. Smith, M. Murphy, O. Piloto, T. C. P. Somervaille, and M. L. Cleary, "Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy," *Nature*, vol. 455, no. 7217, pp. 1205–1209, 2008.

[78] Z. Wang, M. Iwasaki, F. Ficara et al., "GSK-3 promotes conditional association of CREB and its coactivators with MEIS1 to facilitate HOX-mediated transcription and oncogenesis," *Cancer Cell*, vol. 17, no. 6, pp. 597–608, 2010.

[79] T. Holmes, T. A. O'Brien, R. Knight et al., "Glycogen synthase kinase-3β inhibition preserves hematopoietic stem cell activity and inhibits leukemic cell growth," *Stem Cells*, vol. 26, no. 5, pp. 1288–1297, 2008.

[80] J. Bertrand, M. Despeaux, S. Joly et al., "Sex differences in the GSK3β-mediated survival of adherent leukemic progenitors," *Oncogene*, vol. 31, pp. 694–705, 2012.

[81] J. A. Sokoloski, J. Li, A. Nigam, and A. C. Sartorelli, "Induction of the differentiation of HL-60 and WEHI-3B D+ leukemia cells by lithium chloride," *Leukemia Research*, vol. 17, no. 5, pp. 403–410, 1993.

[82] A. M. Rice, J. Li, and A. C. Sartorelli, "Combination of all-trans retinoic acid and lithium chloride surmounts a retinoid differentiation block induced by expression of Scl and Rbtn2 transcription factors in myeloid leukemia cells," *Leukemia Research*, vol. 28, no. 4, pp. 399–403, 2004.

[83] A. M. Rice, K. M. Holtz, J. Karp, S. Rollins, and A. C. Sartorelli, "Analysis of the relationship between Scl transcription factor complex protein expression patterns and the effects of LiCl on ATRA-induced differentiation in blast cells from patients with acute myeloid leukemia," *Leukemia Research*, vol. 28, no. 11, pp. 1227–1237, 2004.

[84] E. Y. Song, P. Palladinetti, G. Klamer et al., "Glycogen synthase kinase-3β inhibitors suppress leukemia cell growth," *Experimental Hematology*, vol. 38, no. 10, pp. 908–921, 2010.

[85] M. R. Mirlashari, I. Randen, and J. Kjeldsen-Kragh, "Glycogen synthase kinase-3 (GSK-3) inhibition induces apoptosis in leukemic cells through mitochondria-dependent pathway," *Leukemia Research*, vol. 36, no. 4, pp. 499–508, 2011.

[86] J. Si, L. Mueller, and S. J. Collins, "GSK3 inhibitors enhance retinoic acid receptor activity and induce the differentiation of retinoic acid-sensitive myeloid leukemia cells," *Leukemia*, vol. 25, pp. 1914–1918, 2011.

[87] K. Gupta, F. Gulen, L. Sun et al., "GSK3 is a regulator of RAR-mediated differentiation," *Leukemia*. In press.

[88] R. A. Finch, J. Li, T. C. Chou, and A. C. Sartorelli, "Maintenance of retinoic acid receptor alpha pools by granulocyte colony-stimulating factor and lithium chloride in all-trans retinoic acid-treated WEHI-3B leukemia cells: relevance to the synergistic induction of terminal differentiation," *Blood*, vol. 96, no. 6, pp. 2262–2268, 2000.

[89] A. M. Rice and A. C. Sartorelli, "Inhibition of 20 S and 26 S proteasome activity by lithium chloride: impact on the differentiation of leukemia cells by all-trans-retinoic acid," *Journal of Biological Chemistry*, vol. 276, no. 46, pp. 42722–42727, 2001.

[90] N. Ahmed and M. J. Weidemann, "Biochemical effect of three different inhibitors of purine/pyrimidine metabolism on differentiation in HL60 cells," *Leukemia Research*, vol. 19, no. 4, pp. 263–273, 1995.

[91] R. D. Knight, J. Mangum, and D. L. Lucas, "Inosine monophosphate dehydrogenase and myeloid cell maturation," *Blood*, vol. 69, no. 2, pp. 634–639, 1987.

[92] B. M. Goldstein, J. F. Leary, B. A. Farley, V. E. Marquez, P. C. Levy, and P. T. Rowley, "Induction of HL60 cell differentiation by tiazofurin and its analogues: characterization and efficacy," *Blood*, vol. 78, no. 3, pp. 593–598, 1991.

[93] K. W. Pankiewicz, K. B. Lesiak-Watanabe, K. A. Watanabe et al., "Novel mycophenolic adenine bis(phosphonate) analogues as potential differentiation agents against human leukemia," *Journal of Medicinal Chemistry*, vol. 45, no. 3, pp. 703–712, 2002.

[94] R. Yazdanparast, M. A. Moosavi, M. Mahdavi, and M. H. Sanati, "3-Hydrogenkwadaphnin from Dendrostellera lessertii induces differentiation and apoptosis in HL-60 cells," *Planta Medica*, vol. 71, no. 12, pp. 1112–1117, 2005.

[95] M. A. Moosavi, R. Yazdanparast, and A. Lotfi, "3-Hydrogenkwadaphnin induces monocytic differentiation and enhances retinoic acid-mediated granulocytic differentiation in NB4 cell line," *Journal of Biochemistry and Molecular Biology*, vol. 39, no. 6, pp. 722–729, 2006.

[96] M. Meli, M. Tolomeo, M. Grifantini et al., "The synergistic apoptotic effects of thiophenfurin, an inosine monophosphate dehydrogenase inhibitor, in combination with retinoids in HL60 cells.," *Oncology Reports*, vol. 17, no. 1, pp. 185–192, 2007.

[97] S. H. Ghaffari, N. Shayan-Asl, A. H. Jamialahmadi, K. Alimoghaddam, and A. Ghavamzadeh, "Telomerase activity and telomere length in patients with acute promyelocytic leukemia: indicative of proliferative activity, disease progression, and overall survival," *Annals of Oncology*, vol. 19, no. 11, pp. 1927–1934, 2008.

[98] L. Liu, J. B. Berletch, J. G. Green, M. S. Pate, L. G. Andrews, and T. O. Tollefsbol, "Telomerase inhibition by retinoids precedes cytodifferentiation of leukemia cells and may contribute to terminal differentiation," *Molecular Cancer Therapeutics*, vol. 3, no. 8, pp. 1003–1009, 2004.

[99] E. Savoysky, K. Yoshida, T. Ohtomo et al., "Down-regulation of telomerase activity is an early event in the differentiation of HL60 cells," *Biochemical and Biophysical Research Communications*, vol. 226, no. 2, pp. 329–334, 1996.

[100] T. W. Reichman, J. Albanell, X. Wang, M. A. Moore, and G. P. Studzinski, "Downregulation of telomerase activity in HL60 cells by differentiating agents is accompanied by increased expression of telomerase-associated protein," *Journal of Cellular Biochemistry*, vol. 67, pp. 13–23, 1997.

[101] W. K. Love, J. B. Berletch, L. G. Andrews, and T. O. Tollefsbol, "Epigenetic regulation of telomerase in retinoid-induced differentiation of human leukemia cells," *International Journal of Oncology*, vol. 32, no. 3, pp. 625–631, 2008.

[102] O. Yamada, K. Ozaki, M. Nakatake, M. Akiyama, K. Kawauchi, and R. Matsuoka, "Multistep regulation of telomerase during differentiation of HL60 cells," *Journal of Leukocyte Biology*, vol. 83, no. 5, pp. 1240–1248, 2008.

[103] F. Pendino, C. Dudognon, F. Delhommeau et al., "Retinoic acid receptor α and retinoid-X receptor-specific agonists synergistically target telomerase expression and induce tumor cell death," *Oncogene*, vol. 22, no. 57, pp. 9142–9150, 2003.

[104] F. Pendino, J. Hillion, C. Dudognon et al., "Telomerase targeting by retinoids in cells from patients with myeloid leukemias of various subtypes, not only APL," *Leukemia*, vol. 20, no. 4, pp. 599–603, 2006.

[105] S. R. Frankel, A. Earley, G. Lauwers et al., "The "retinoic acid syndrome" in acute promyelocytic leukemia," *Annals of Internal Medicine*, vol. 117, no. 4, pp. 292–296, 1992.

[106] Y. C. Su, P. Dunn, L. Y. Shih et al., "Retinoic acid syndrome in patients following the treatment of acute promyelocytic leukemia with all-trans retinoic acid," *Chang Gung Medical Journal*, vol. 32, no. 5, pp. 535–542, 2009.

[107] P. Kastner, H. J. Lawrence, C. Waltzinger, N. B. Ghyselinck, P. Chambon, and S. Chan, "Positive and negative regulation of granulopoiesis by endogenous RARα," *Blood*, vol. 97, no. 5, pp. 1314–1320, 2001.

[108] P. J. Hughes, Y. Zhao, R. A. Chandraratna, and G. Brown, "Retinoid-mediated stimulation of steroid sulfatase activity in myeloid leukemic cell lines requires RARα and RXR and involves the phosphoinositide 3-kinase and ERK-MAP kinase pathways," *Journal of Cellular Biochemistry*, vol. 97, no. 2, pp. 327–350, 2006.

[109] C. Robert, L. Delva, N. Balitrand et al., "Apoptosis induction by retinoids in eosinophilic leukemia cells: implication of retinoic acid receptor-α signaling in all-trans-retinoic acid hypersensitivity," *Cancer Research*, vol. 66, no. 12, pp. 6336–6344, 2006.

[110] L. A. Hammond, C. H. V. Krinks, J. Durham et al., "Antagonists of retinoic acid receptors (RARs) are potent growth inhibitors of prostate carcinoma cells," *British Journal of Cancer*, vol. 85, no. 3, pp. 453–462, 2001.

[111] L. A. Hammond, G. Brown, R. G. Keedwell, J. Durham, and R. A. S. Chandraratna, "The prospects of retinoids in the treatment of prostate cancer," *Anti-Cancer Drugs*, vol. 13, no. 8, pp. 781–790, 2002.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 401784, 12 pages
doi:10.1155/2012/401784

Research Article

Cell-Type-Specific Effects of Silibinin on Vitamin D-Induced Differentiation of Acute Myeloid Leukemia Cells Are Associated with Differential Modulation of RXRα Levels

Rina Wassermann, Victoria Novik, and Michael Danilenko

Department of Clinical Biochemistry, Faculty of Health Sciences, Ben-Gurion University of the Negev, P.O. Box 653, 84105 Beer-Sheva, Israel

Correspondence should be addressed to Michael Danilenko, misha@bgu.ac.il

Received 26 February 2012; Accepted 15 March 2012

Academic Editor: George P. Studzinski

Plant polyphenols have been shown to enhance the differentiation of acute myeloid leukemia (AML) cells induced by the hormonal form of vitamin D_3 (1α,25-dihydroxyvitamin D_3; 1,25D). However, how these agents modulate 1,25D effects in different subtypes of AML cells remains poorly understood. Here, we show that both carnosic acid (CA) and silibinin (SIL) synergistically enhancd 1,25D-induced differentiation of myeloblastic HL60 cells. However, in promonocytic U937 cells, only CA caused potentiation while SIL attenuated 1,25D effect. The enhanced effect of 1,25D+CA was accompanied by increases in both the vitamin D receptor (VDR) and retinoid X receptor alpha (RXRα) protein levels and vitamin D response element (VDRE) transactivation in both cell lines. Similar increases were observed in HL60 cells treated with 1,25D + SIL. In U937 cells, however, SIL inhibited 1,25D-induced VDRE transactivation concomitant with downregulation of RXRα at both transcriptional and posttranscriptional levels. These inhibitory effects correlated with the inability of SIL, with or without 1,25D, to activate the Nrf2/antioxidant response element signaling pathway in U937 cells. These results suggest that opposite effects of SIL on 1,25D-induced differentiation of HL60 and U937 cells may be determined by cell-type-specific signaling and transcriptional responses to this polyphenol resulting in differential modulation of RXRα expression.

1. Introduction

Acute myeloid leukemia (AML) is a hematologic cancer which results from blocked differentiation of hematopoietic stem and/or progenitor cells due to various genetic and epigenetic errors and is characterized by the uncontrolled proliferation of myeloid blasts. The standard approach for AML treatment is chemotherapy with cytarabine and anthracyclines; however, even after aggressive chemotherapy about 75% of AML patients relapse within 2 years of remission [1, 2]. Recently, several targeted cytotoxic approaches to treat AML have been developed, for example, the use of kinase and histone deacetylase inhibitors [3], but such strategy is difficult to apply to AML, since the molecular lesions in AML are highly heterogeneous.

Differentiation therapy is an alternative or complementary treatment of AML which aims at inducing maturation of poorly differentiated leukemic blasts. The hormonal form of vitamin D_3 (1α,25-dihydroxyvitamin D_3) is a strong differentiation inducer which has potential for the treatment of AML [4]. However, at concentrations capable of differentiating AML cells in culture 1,25D may cause fatal hypercalcemia *in vivo*. Many low-calcemic vitamin D analogs have been synthesized to date [5], but none has yet been shown to be appropriate for human use at concentrations required to induce differentiation of AML blasts. One way to overcome this problem is to combine low concentrations of 1,25D with other compounds which would enhance its anticancer, but not toxic, effects [6]. We have previously shown that carnosic acid (CA), the major phenolic diterpene

of rosemary, potentiates the differentiation effects of 1,25D in AML cell lines representing different developmental blocks in myeloid differentiation, such as myeloblastic (HL60) [7–9], promye- locytic (NB4) [10], and (myelo)monocytic (U937, OCL- AML3, MOLM-13, THP-1) [9–12] leukemia cells, as well as in leukemic blasts derived from patients with AML [11]. Furthermore, combined treatments with either CA or CA-rich rosemary extract and different vitamin D derivatives resulted in enhanced differentiation and growth arrest of WEHI-3B D-murine myelomonocytic leukemia cells *in vitro* and—in case of rosemary extract—in cooperative antileukemic effects in syngeneic mouse AML models *in vivo*, without inducing hypercalcemia [13, 14]. Another plant polyphenol, the flavonolignan silibinin (SIL) found in milk thistle, was also demonstrated to potentiate the differentiation effect of 1,25D in HL60 and OCL-AML3 cells [7, 12, 15]; however, this polyphenol tended to attenuate differentiation in U937 and THP-1 cells [16]. In addition, SIL exerted both potentiating and inhibitory effects on the differentiation of leukemic blast samples obtained from different patients with AML [17].

In this study we attempted to clarify the nature of the opposite effects of SIL on 1,25D-induced differentiation of HL60 and U937 cells using CA for comparison. We found that the differentiation-enhancing effect of the 1,25D/SIL combination in HL60 cells was associated with upregulation of vitamin D receptor (VDR) and retinoid X receptor (RXRα) levels compatible with increased transactivation of the vitamin D response element (VDRE). On the other hand, the inhibitory effect of SIL on 1,25D-induced differentiation of U937 cells was accompanied by downregulation of RXRα expression and attenuated VDRE transactivation.

2. Materials and Methods

2.1. Chemicals, Antibodies, and Plasmids. Tissue culture media and reagents were from Invitrogen (Grand Island, NY), Biological Industries (Beit Ha'Emek, Israel), and HyClone (Logan, UT). Carnosic acid was purchased from Alexis Biochemicals (Läufenfingen, Switzerland). 1,25D was a gift from Dr. Andrzej Kutner (Pharmaceutical Research Institute, Warsaw, Poland). Silibinin, cycloheximide, tert-butylhydroquinone (tBHQ), and DMSO were from Sigma (Rehovot, Israel). Stock solutions of CA (10 mM), SIL (30 mM), 1,25D (0.25 mM), and tBHQ (30 mM) were prepared in absolute ethanol. The precise concentrations of 1,25D in stock solutions were verified spectrophotometrically at 264 nm (ε = 19,000). The antibodies against NQO1 (C-19), VDR (C-20), RXRα (D-20), and TrxR1 (H-270) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Calreticulin antibody (PA3-900) was from Affinity BioReagents (Goden, CO). Peroxidase-conjugated donkey anti-rabbit and donkey anti-goat IgG were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The 4xARE-Luc reporter construct containing four tandem repeats of the antioxidant response element (ARE) sequence from the glutathione S-transferase Ya subunit was a gift from Dr. M. Hannink (University of Missouri, Columbia, MO) [18]. The VDREx6-Luc reporter construct containing a 6-fold direct repeat 3 (DR3) sequence was kindly provided by Dr. L.P. Freedman (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). *Renilla* luciferase expression construct (pRL-null vector) was purchased from Promega (Madison, WI) and served as an internal transfection standard.

2.2. Cell Culture, Treatment, and Enumeration. HL60-G cells, subcloned from HL60 human myeloblastic leukemia cells [19], and U937 human myelomonocytic leukemia cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum or bovine serum in a humidified atmosphere of 95% air and 5% CO_2, at 37°C. Cell cultures were passaged two to three times weekly to maintain a log phase growth. For experiments, cells were seeded in 6-well plates (Greiner Bio-One GmbH, Solingen, Germany) at densities indicated in legends to figures followed by the addition of vehicle (0.1% ethanol), 1,25D (1 nM), polyphenols, or their combinations under dim lighting conditions. Cell cultures were then incubated, as described above, for the indicated time periods. 1,25D alone at the higher concentration of 100 nM was used as the positive control. Cell numbers and viability were estimated on the basis of trypan blue exclusion by counting in Vi-Cell XR cell viability analyzer (Beckman Coulter Inc., Fullerton, CA).

2.3. Determination of Markers of Differentiation. Aliquots of 1×10^6 cells were harvested, washed twice with phosphate-buffered saline (PBS), and suspended in 10 μL 1× PBS. The cell suspensions were incubated for 45 minutes at room temperature with 0.3 μL MO1-FITC and 0.3 μL MY4-RD-1 to analyze the expression of the cell surface markers CD11b and CD14, respectively. The cells were then washed three times with ice-cold 1× PBS and resuspended in 1 mL of PBS. Analysis was performed using Cytomics FC500 flow cytometer equipped with CXP software (Beckman Coulter). Isotypic mouse IgG1 was used to set threshold parameters.

2.4. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Total RNA was extracted from cells with the PerfectPure RNA Tissue Kit (5PRIME, Gaithers- burg, USA) and cDNA was prepared with Verso cDNA kit (ABgene, Epsom, UK), according to the manufacturer's instructions. The following primers were used for the determination of CD11b, CD14, and RXRα mRNA expression: CD11b, forward primer (5′-CTGTCTGCCAGAGAATCCAGTG-3′), reverse primer (5′-GAGGTGGTTATGCGAGGTCTTG-3′); CD14, forward primer (5′-GCCCTTACCAGCCTAGACCT-3′), reverse primer (5′-CCCGTCCAGTGTCAGGTTAT-3′); RXRα, forward primer (5′-CAAACATGGGGCTGAACC-3′), reverse primer (5′-AAGTGTGGGATCCGCTTG-3′); ARP0, forward primer (5′-AGATGCAGCAGATCCGCAT-3′), reverse primer (5′-GTGGTGATACCTAAAGCCTG-3′). cDNA samples (7 μL) were diluted ninefold, mixed with the specific primers (0.2 mM) and ABsolute Blue SYBR Green ROX Mix

(ABgene, Epsom, UK) was then added to the reaction mixture. Reactions were carried out in the Rotor-Gene Real-Time PCR machine (Corbett-Research, Northlake, Australia). Standard cycling conditions for this instrument were 15 min initial enzyme activation at 95°C then 35 cycles as follows: 10 sec at 95°C, 15 sec at the annealing temperature, and 15 sec at 72°C. The results were normalized by ARP0 mRNA content and quantified using the $2^{-\Delta\Delta C_T}$ method.

2.5. Preparation of Whole Cell Extracts and Western Blot Analysis. Cells were lysed in ice-cold lysis buffer containing 50 mM, HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM EGTA, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 50 mM NaF, 1 mM DTT, and 1 : 50 Complete Protease Inhibitors Cocktail (Roche Molecular Biochemicals, Mannheim, Germany) and centrifuged at 20,000 ×*g*, 10 min, 4°C. Supernatant samples (30 μg protein) were subjected to SDS-PAGE and then electroblotted into nitrocellulose membrane (Whatman, Dassel, Germany). The membranes were blocked with 5% milk for 2 h and incubated with primary antibodies overnight at 4°C followed by incubation with HRP-conjugated secondary antibodies for 2 h. The protein bands were visualized using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, MA). The blots were stripped and reprobed for the constitutively present protein, calreticulin, which served as the loading control. The optical density (OD) of protein bands was quantitated using Image Gauge 3.11 software (Fuji Photo Film Co., Tokyo, Japan). OD values for each protein were normalized to calreticulin and are displayed beneath each protein band.

2.6. Transient Transfection and Reporter Gene Assay. HL60 cells were transiently cotransfected with 4xARE-luc reporter plasmid (1.35 μg) and *Renilla* luciferase vector (0.15 μg) or VDREx6-luc reporter plasmid (0.95 μg) and *Renilla* luciferase vector (0.05 μg) using Microporator (Digital Bio Technology, Seoul, Korea) under the following conditions: 1 pulse, 1400 Volts, pulse width 30 msec. U937 cells were transiently cotransfected with 4xARE-luc or VDREx6-luc reporter plasmids (0.8 μg) and *Renilla* luciferase vector (0.2 μg) using JetPEI Reagent (POLYplus-Transfection, Illkirch Cedex, France), according to the manufacturer's instructions. Transfected cells were exposed to the indicated treatments for 24 h followed by measurement of luciferase activity using the Dual Luciferase Reporter Assay system (Promega, Medison, WI, USA). The data are presented as the normalized ratios of firefly luciferase to *Renilla* luciferase activity (relative luminescence units, RLU).

2.7. Statistical Analysis. The significance of the differences between the means of the various subgroups was assessed by unpaired two-tailed Student's *t*-test. Two compounds (*A* and *B*) were considered to show enhancement in the particular experiment if the effect of their combination (*AB*) was larger than the sum of their individual effects ($AB > A + B$), the data being compared after subtraction of the respective control values from *A, B,* and *AB* [8]. The statistical analysis was performed with the GraphPad Prism 5.0 Program (GraphPad Software, San Diego, CA). Data are presented as the mean ± SE. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Silibinin Potentiates 1,25D-Induced Differentiation in HL60 Cells While Attenuating It in U937 Cells. We first compared the effects of CA and SIL on the expression of two cell surface markers of myeloid differentiation, CD11b and CD14, induced by a low concentration of 1,25D (1 nM) in HL60 and U937 cells (Figure 1). To exclude potential interference of reagent cytotoxicity in the determination of the vitamin D receptor expression and activity, as described in the following sections, we used noncytotoxic concentrations of the two polyphenols: 10 μM CA for both cell lines [7, 8, 20], 60 μM SIL for HL60 cells [7, 16, 17, 20], and 30 μM SIL for U937 cells. Under these conditions, cell viability was maintained at 95–98% throughout the course of the experiments performed here. Concentrations of SIL higher than 30 μM caused a dose-dependent decrease in the viability of U937 cells (up to ~20% dead cells at 60 μM SIL; data not shown). The addition of CA to 1,25D not only synergistically increased the percentage of CD11b- and CD14-positive HL60 and U937 cells, as measured by flow cytometry following 96 h treatment (Figures 1(a), 1(b)), but also markedly enhanced the mRNA expression of these markers after 24 h (Figures 1(c), 1(d), 1(e), and 1(f)), as compared to 1,25D alone. These potentiating effects of CA were more pronounced in HL60 cells than in U937 cells. On the other hand, while being even a stronger differentiation enhancer than CA in HL60 cells (Figures 1(a),1(c), and 1(d)), SIL appreciably reduced 1,25D-stimulated expression of CD11b and CD14 in U937 cell, as determined by both flow cytometry (Figure 1(b)) and qRT-PCR (Figures 1(e), 1(f)).

3.2. Silibinin Cooperates with 1,25D to Induce Growth Arrest in HL60 Cells but Not in U937 Cells. In parallel with flow cytometric determination of CD11b and CD14 expression (Figures 1(a) and 1(b)), cells from the same wells were enumerated to examine the effects of 1,25D and polyphenols on cell proliferation and viability. As shown in Figure 2(a), both CA and SIL administered alone for 96 h significantly decreased the number of proliferating HL60 cells while 1 nM 1,25D alone had a minor effect. However, the addition of 1,25D to either of the two polyphenols resulted in a marked cooperative inhibition of cell growth comparable to that produced by high-dose 1,25D (100 nM). U937 cells exhibited lower sensitivity to the antiproliferative effect of 1,25D, as compared to HL60 cells. CA produced similar effects in both cell lines, whereas SIL caused somewhat stronger growth inhibition in U937 even at a 2-fold lower concentration than used in HL60 cells (Figure 2(b) *versus* Figure 2(a)). Interestingly, while treatment with the 1,25D/CA combination resulted in a more pronounced reduction in U937 cell numbers than that exerted by single compounds, the antiproliferative effect of SIL was not significantly altered by

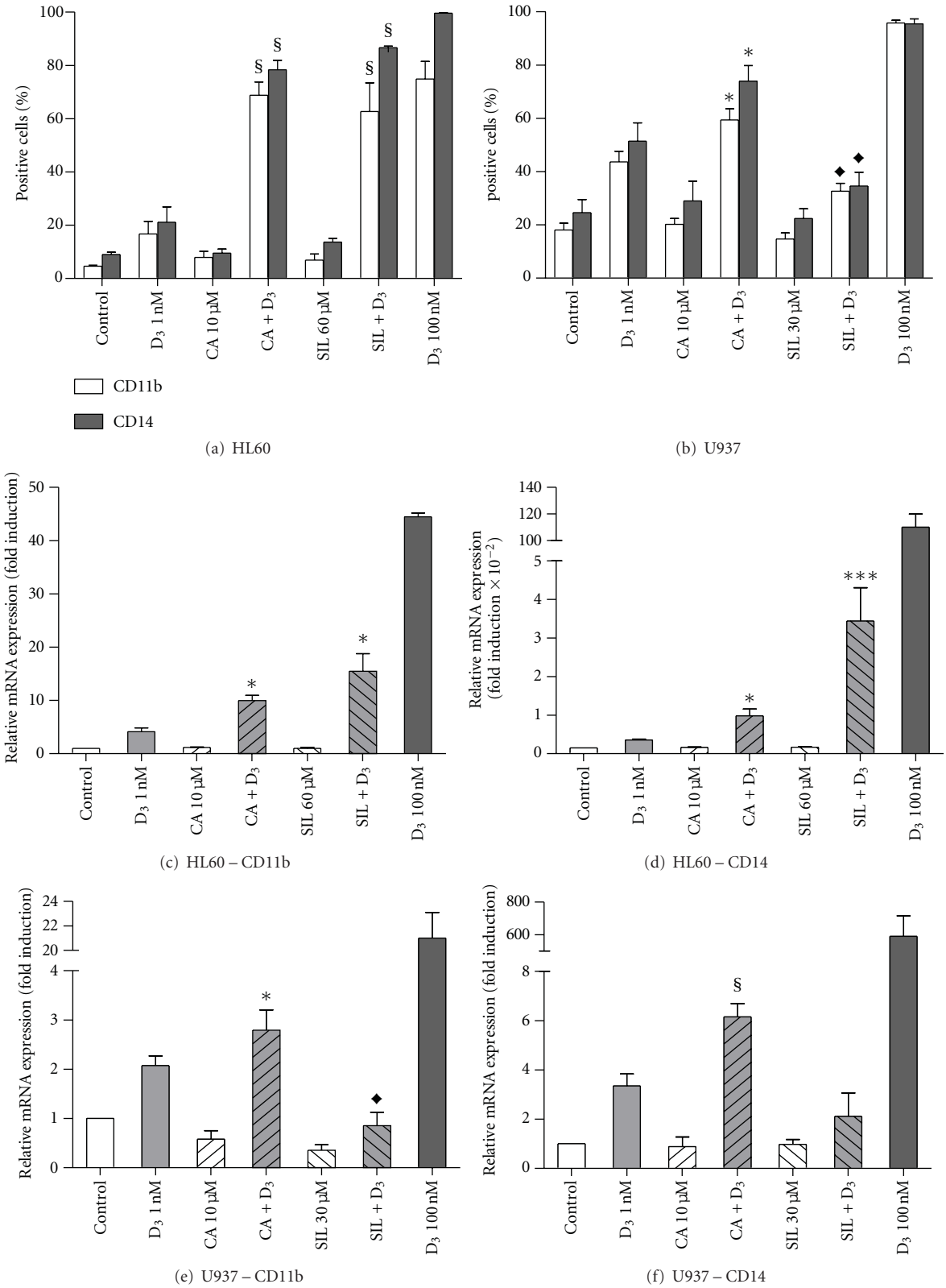
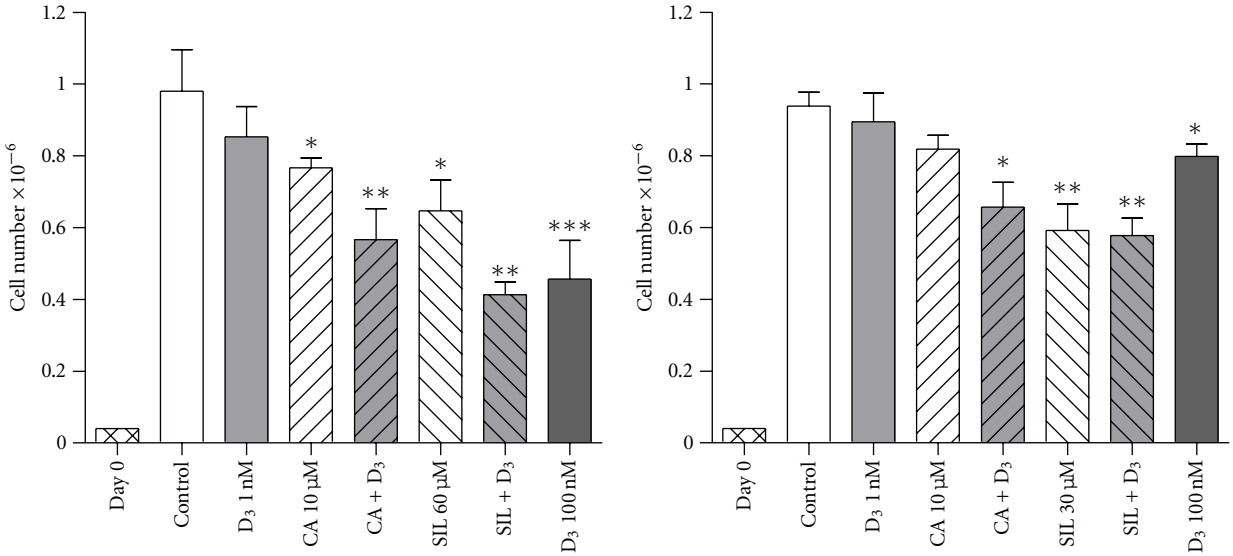


Figure 1: Carnosic acid enhances while silibinin differentially affects 1,25D-induced differentiation of HL60 and U937 cells. Cells were incubated at 4×10^4 cells/mL (a), (b) or 5×10^4 cells/mL (c-f) with 0.1% ethanol (vehicle control) or the indicated test agents, alone or in combination, for 96 h (a), (b) or 24 h (c-f). CD11b and CD14 expression was then determined by flow cytometry (a), (b) or qRT-PCR (c-f). The data are the means ± SE of 5 (a), (b) or 3 (c-f) independent experiments. $^*P < 0.05$; $^{\S}P < 0.01$, or $^{***}P < 0.001$, combination *versus* sum of single agents for enhancing effects. $^{\blacklozenge}P < 0.05$, combination versus 1 nM 1,25D alone for inhibitory effects.



(a) HL60 (b) U937

Figure 2: Carnosic acid and silibinin, alone and in combination with 1,25D, inhibit proliferation of HL60 and U937 cells. Cells were seeded at 4×10^4 cells/mL (day 0) and treated with test agents, for 96 h, as described in legend to Figure 1. Following incubation, cells were counted using the trypan blue exclusion assay. The numbers of viable cells are presented as the means ± SE of at least 5 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$, or $^{***}P < 0.001$, treatments *versus* control.

1,25D (Figure 2(b)). In the previous experiments, no treatment tested significantly affected cell viability (93–96% in all samples). These findings are consistent with our previous data showing antiproliferative effects of CA and SIL, alone and in combination with different vitamin D derivatives, in murine and human AML cell lines [8, 9, 13, 14, 20, 21].

3.3. Silibinin Potentiates 1,25D-Induced VDRE Transactivation in HL60 Cells While Inhibiting It in U937 Cells: Association with RXRα Protein Levels. To examine the mechanism underlying the differential effects of SIL on 1,25D-induced differentiation in HL60 and U937 cells we tested the hypothesis that, similar to CA, SIL promotes the functional activation of the vitamin D receptor by 1,25D in HL60 cells while, in contrast to CA, attenuating it in U937 cells. To this end, we first compared the effects of CA and SIL on 1 nM 1,25D-induced VDRE transactivation in transiently transfected HL60 and U937 cells using VDRE-Luc reporter gene assay. As expected, both CA and SIL markedly enhanced 1,25D-stimulated transcription from VDRE in HL60 cells (Figure 3(a)). A similar potentiating effect of CA was observed in U937 cells; however, SIL significantly reduced VDRE transactivation by 1,25D in this cell line (Figure 3(b)). The above differential effects of CA and SIL on VDRE activation correlated with their corresponding modulation of 1,25D-induced differentiation in the two cell lines (see Figure 1).

We then performed Western blot analysis of VDR and RXRα protein expression in untransfected HL60 and U937 cells following incubations with 1,25D, polyphenols, and their combinations for 48 h and 96 h. The data demonstrated that 1,25D alone induced a dose- and time-dependent elevation of VDR levels in both HL60 and U937 cells (Figures 4(a–d)) and of RXRα levels in HL60 cells (Figures 4(a) and 4(b)). When added alone, CA and SIL caused only modest increases in VDR levels in both cell lines but cooperated with 1 nM 1,25D in this effect. This cooperative VDR upregulation tended to strengthen with time and was much stronger in HL60 cells than in U937 cells (compare panels (a), (b) and (c), (d) in Figure 4). A similar, though less pronounced, positive cooperation between both polyphenols and 1,25D was also observed for RXRα levels in HL60 cells. However, in U937 cells, only CA, alone or in combination with 1,25D, positively affected RXRα expression. In contrast, treatment with SIL or 1,25D alone tended to decrease RXRα levels with time (Figures 4(c) and 4(d)) and their combination caused a marked time-dependent RXRα downregulation in these cells (Figures 4(c) and 4(d)). The latter inhibitory effect may, at least in part, account for the attenuated VDRE transactivation observed in 1,25D/SIL-treated U937 cells (Figure 3(b)).

3.4. Silibinin in Combination with 1,25D Downregulates RXRα mRNA Expression and Decreases RXRα Protein Stability in U937 Cells. To determine the mode by which treatments with SIL and its combination with 1,25D reduce RXRα protein levels in U937 cells, we first compared the effects of SIL, CA, 1,25D, alone and together, on RXRα mRNA expression in both HL60 and U937 cells. As shown in Figure 6(a), neither treatment significantly affected RXRα mRNA levels in HL60 cells following 24-h incubations. A similar pattern was observed in U937 cells for all of the treatments except for the 1,25D/SIL combination which markedly

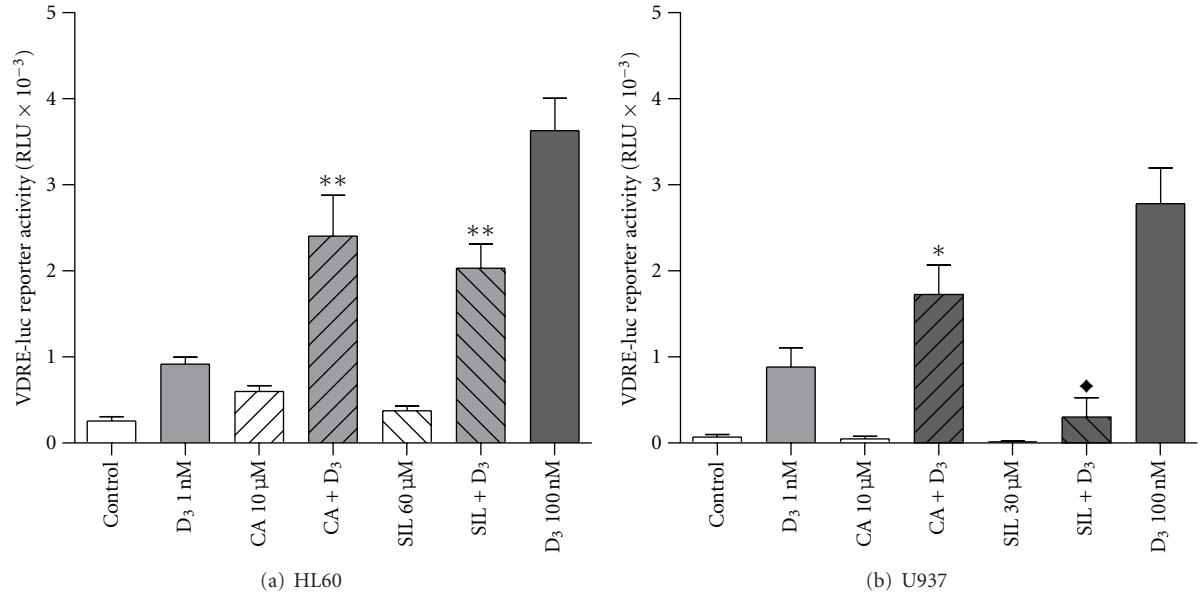


Figure 3: Carnosic acid enhances while silibinin differentially affects 1,25D-induced VDRE transactivation in HL60 and U937 cells. Cells (5×10^5 cells/ml) were transiently transfected with VDRE×6-luc and *Renilla* luciferase reporter constructs followed by treatment with 0.1% ethanol (control) or indicated test agents for 24 h. The relative VDREx6-luc activity (means ± SE) was calculated from the data of 3 individual experiments performed in triplicate. $^*P < 0.05$ and $^{**}P < 0.01$, combination *versus* sum of single agents for enhancing effects. $\blacklozenge P < 0.05$, combination versus 1 nM 1,25D alone for inhibitory effects.

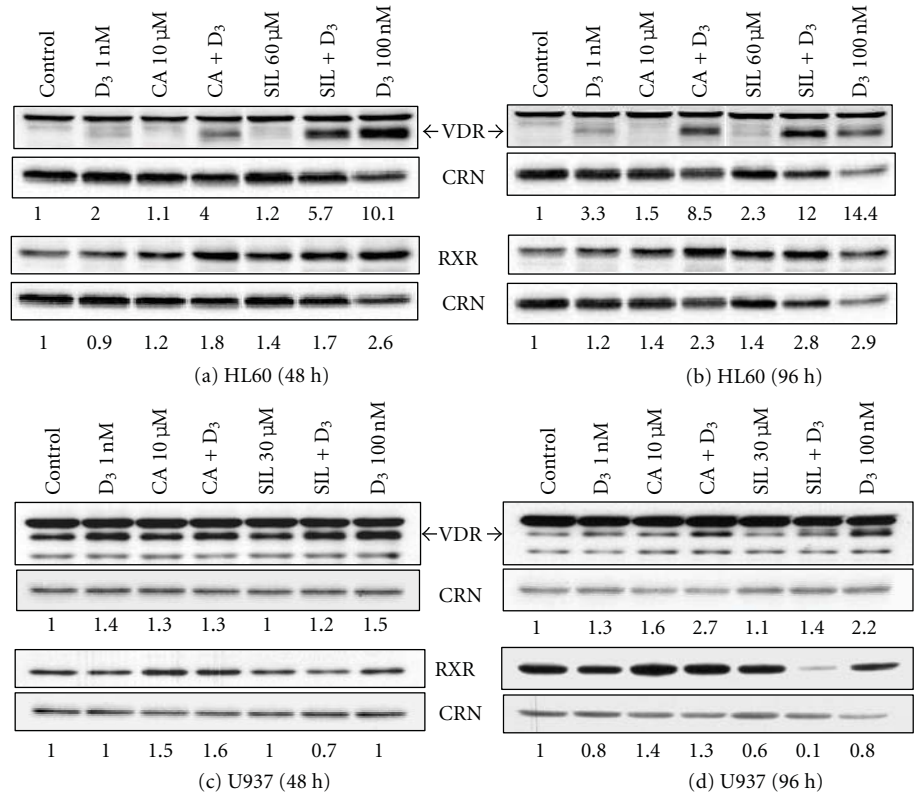


Figure 4: Effects of 1,25D, carnosic acid and silibinin, alone and in combination, on VDR and RXRα protein expression in HL60 and U937 cells. Cells were treated for 48 h (1×10^5 cells/mL; (a), (c)) or 96 h (4×10^4 cells/mL; (b), (d)) with 0.1% ethanol (control) or the indicated test agents. Protein expression was then determined by Western blotting. Calreticulin (CRN) was used as the protein loading control. Representative blots of at least 3 independent experiments are shown.

lowered RXRα mRNA expression levels compared to either untreated cells or treatments with single agents. The latter effect correlated with a strong reduction in RXRα protein levels following 1,25D/SIL treatment (e.g., Figure 4(d)). To examine if this reduction can also result from a decrease in RXRα protein stability, we performed cycloheximide chase experiments. U937 cells were preincubated for 24–72 h with or without SIL or SIL + 1,25D, washed, and exposed to 400 μM cycloheximide [22] to block protein synthesis. The time course of RXRα protein degradation in different samples was then followed for up to 8 h by Western blot analysis of relative protein levels. Cycloheximide did not significantly affect cell viability during the treatment. Interestingly, as shown in Figures 6(c) and 6(d), higher rates of reduction in RXRα levels were obtained in cells pretreated for 72 h with SIL alone (Figure 6(c), lanes 7–9) and, particularly, in combination with 1,25D (Figure 6(c), lanes 10–12), as compared to control cells (Figure 6(c), lanes 4–6). However, shorter preincubations revealed either less pronounced (48 h) or no (24 h) appreciable effects of SIL or SIL + 1,25D on RXRα degradation rates in the presence of cycloheximide (data not shown). Therefore, it appears that the time-dependent reduction in RXRα protein levels following treatment of U937 cells with SIL and, especially, its combination with 1,25D (Figures 4(c) and 4(d)), may result from both the earlier inhibition of mRNA expression (Figure 6(b)) and later decrease in protein stability (Figures 6(c) and 6(d)).

3.5. Silibinin Activates the Nrf2/Antioxidant Response Element (Nrf2/ARE) Signaling Pathway in HL60 Cells but Not in U937 Cells. Our recent data have demonstrated that CA and 1,25D can synergistically activate the Nrf2/ARE signaling pathway and that Nrf2 acts as an upstream positive regulator of VDR and RXRα expression in U937 cells [11]. We, thus, determined whether SIL, alone or together with 1,25D, is capable of activating this pathway in HL60 and U937 cells, as compared with CA ± 1,25D. Using ARE-Luc reporter gene assays in transiently transfected cells, we found that both CA and, to a lesser extent, SIL induced ARE transactivation in HL60 cells and that the addition of 1 nM 1,25D, which alone had only a slight effect, synergistically potentiated the effects of the two polyphenols (Figure 5(a)). Likewise, both CA and SIL as well as 1,25D/CA and 1,25D/SIL combinations induced the expression of the Nrf2/ARE-responsive gene products, NAD(P)H quinone oxidoreductase-1 (NQO1) and thioredoxin reductase-1 (TrxR1), in these cells to different extent (Figure 5(c)). On the other hand, only CA, alone or together with 1,25D, was capable of trasactivating the ARE reporter (Figure 5(b)) and inducing NQO1 and TrxR1 expression, whereas SIL ± 1,25D even tended to decrease the levels of these proteins (Figure 5(d)).

4. Discussion

In this study we characterized the distinct modulatory effects of SIL on myeloid differentiation of HL60 and U937 human AML cells induced by a low, near physiologic concentration of 1,25D. For comparison, we utilized another plant polyphenol, CA, which differentiation-enhancing activity has been consistently demonstrated in both cell lines [7–9, 11, 20]. SIL had also been first described as phytochemical which can synergistically potentiate 1,25D-induced differentiation of HL60 myeloblastic leukemia cells [15] and this finding was later confirmed in our studies [7, 20]. However, when testing the promonocytic leukemia cell lines U937 and THP-1, which can also be induced to differentiate by 1,25D, we observed a surprising inhibition of the 1,25D effect by this polyphenol ([16] and this study). This inhibition seems to be a rather unusual phenomenon in view of a number of reports consistently showing differentiation-enhancing activity of various plant-derived bioactive compounds. Among these are different polyphenols (besides CA and SIL) [7, 24–26], sesquiterpene lactones [27–29], carotenoids [30, 31], and other phytochemicals, such as genistein [32], capsaicin [33], or cotylenin A [34].

Mechanisms underlying the potentiating effects of the above compounds on 1,25D-induced differentiation of AML cells have been extensively studied and shown to involve the activation of various signaling kinases, such as protein kinase C [15, 28, 29], phosphatidylinositol 3-kinase [28]; MAPKs, including ERK [7, 15, 28, 29] and JNK [20], as well as the transcription factors AP-1 [7, 11, 20], Egr-1 [8, 20], and Nrf2 [11]. Furthermore, the differentiation-enhancing effects of some phytochemicals, for example, curcumin [24] and parthenolide [27], or unrelated antioxidants, for example, α-tocopherol succinate [30], were associated with their inhibition of NFκB, the transcription factor which has been reported to antagonize VDR-mediated 1,25D actions [35, 36].

Interestingly, activation of at least some of the above signaling and transcriptional pathways has been shown to promote VDR, and RXRα expression, which may eventually represent a major cause for the sensitization of cancer cells to lower doses of 1,25D in the presence of phytochemicals or other enhancers of 1,25D action. For instance, activation of the p38 and JNK MAPK pathways in breast cancer cells was found to increase VDR expression via upregulation of AP-1 which can bind to and transactivate the VDR gene promoter [37]. In addition, AP-1 was found to play an important role in regulation of RXRα expression in osteoblastic cells [38]. Our recent study has demonstrated that in U937 cells the expression of AP-1, VDR, and RXRα can be controlled by Nrf2 activity [11]. Correspondingly, we have previously shown that the synergistic differentiation effects of 1,25D and CA in AML cells correlated with a marked cooperative increase in VDR and RXRα levels [8, 11]. A similar VDR protein upregulation in leukemia cells was observed when 1,25D was combined with other inducers or enhancers of differentiation, such as all trans retinoic acid (ATRA) [39] and bufalin [40], a major digoxin-like component of toad venom. Likewise, enhanced antiproliferative effects of 1,25D combinations with the soy isoflavone genistein [41–43] or the *RRR* stereoisomer of α-tocopherol [44] in nonleukemic cancer cells were associated with elevated VDR expression.

In view of the above findings we hypothesized that the mechanism by which SIL attenuates 1,25D-induced

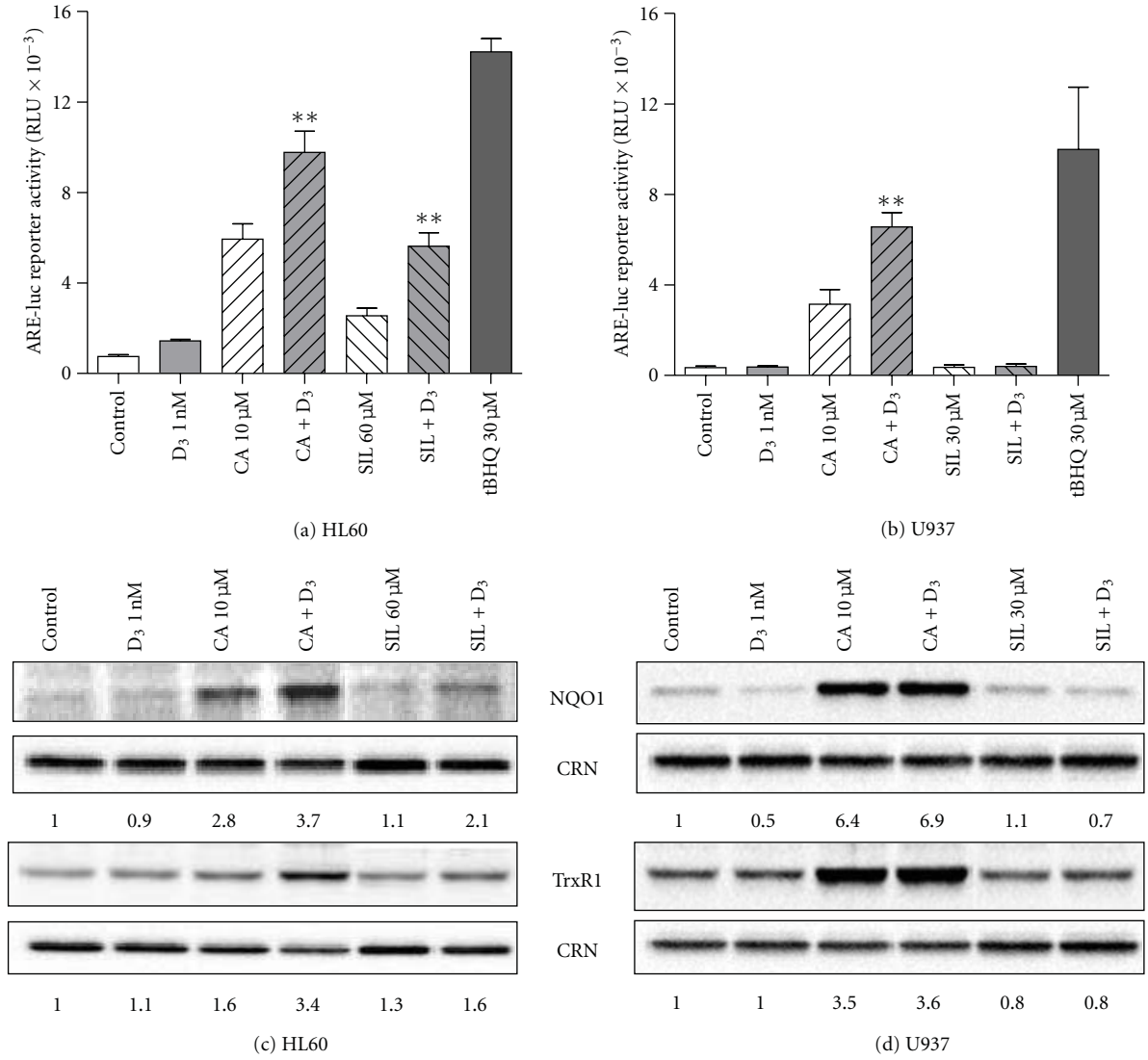


Figure 5: Effects of carnosic acid, silibinin, and 1,25D, alone and in combination, on the Nrf2/ARE signaling pathway in HL60 and U937 cells. (a), (b) Cells were transiently transfected with 4xARE-luc and *Renilla* luciferase reporter constructs followed by treatment with 0.1% ethanol (control) or indicated test agents for 24 h. *tert*-Butylhydroquinone (tBHQ), a classical Nrf2/ARE activator [23], was used as the positive control. The relative 4xARE-luc activity (means ± SE) was calculated from the data of 4 individual experiments performed in triplicate. $^{**}P < 0.01$, combination *versus* sum of single agents. (c), (d) Cells (1×10^5 cells/mL) were treated with 0.1% ethanol (control) or the indicated test agents, for 48 h. Protein expression was then determined by Western blotting. Calreticulin (CRN) was used as the protein loading control. Representative blots of at least 3 independent experiments are shown.

differentiation in U937 cells could result from a decrease in VDR/RXRα expression and/or activity. Indeed, we obtained several lines of evidence indicating a strong correlation between the attenuated response to 1,25D and reduced vitamin D receptor activity, namely, the SIL inhibition of 1,25D-induced VDRE transactivation that was consistent with a marked decrease in RXRα expression levels and protein stability. Of note, SIL also inhibited ATRA-induced differentiation of U937 cells in our experiments (manuscript in preparation). Since the retinoic receptor alpha (RARα) heterodimerizes with RXRα to produce a functionally active receptor for ATRA (e.g., [45]), these data suggest that RXRα can serve as a common target for the negative effect of SIL on maturation of these cells induced by both 1,25D and ATRA. Surprisingly, in contrast to HL60 cells, 1,25D itself tended to downregulate RXRα expression in U937 cells and, thus, the greatest reduction in RXRα levels was observed in cells treated with the 1,25D/SIL combination. One possible explanation for the "anti-differentiation" behavior of SIL in these cells is that it may activate some differentiation-antagonizing factor(s) and cooperate with 1,25D in this effect. Indeed, it has been shown that 1,25D and SIL can concurrently upregulate and activate ERK5 (big MAPK-1) and its upstream regulator, Cot1 kinase [16], which has a negative effect on 1,25D-induced differentiation of AML cells [46]. In addition, high expression levels of at least

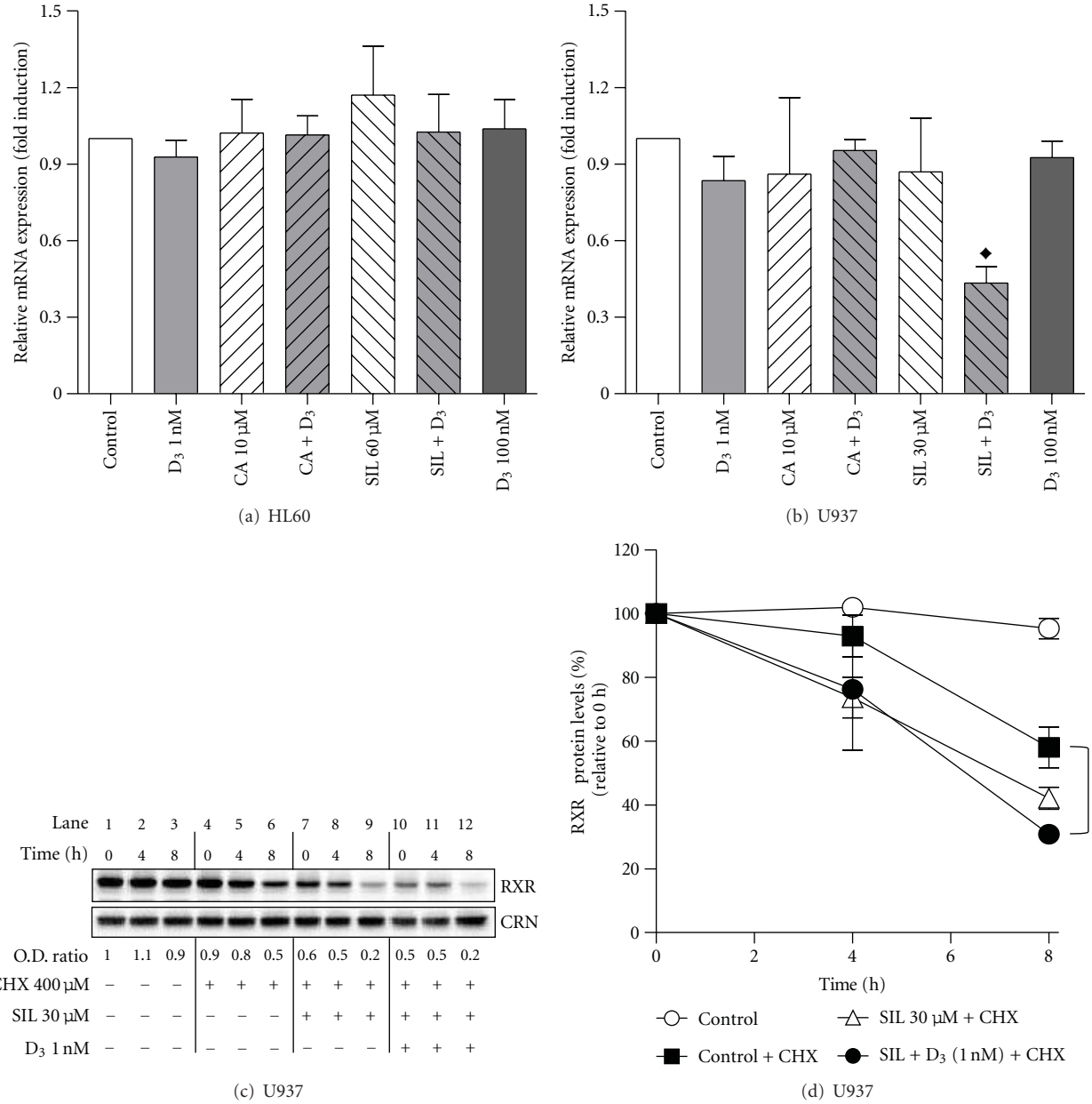


Figure 6: Silibinin promotes downregulation of RXRα in U937 cells but not in HL60 cells. (a), (b) HL60 and U937 cells (5×10^4 cells/mL) were treated with 0.1% ethanol (control) or the indicated test agents, for 24 h. RXRα mRNA expression was determined by quantitative RT-PCR. The data are the means ± SE of 3 independent experiments performed in duplicate. $^{\blacklozenge}P < 0.05$, combination versus control.(c) U937 cells (5×10^4 cells/mL) were preincubated for 72 h with 0.1% ethanol (lanes 1–6), silibinin alone (lanes 7–9), or in combination with 1,25D (lanes 10–12) followed by treatment with vehicle (H_2O, lanes 1–3) or cycloheximide (CHX, lanes 4–12) for 0–8 h, as indicated. Cells were then lysed and protein levels were determined by Western blotting. Calreticulin (CRN) was used as the protein loading control. Representative blots of 3 independent experiments are shown. (d) Graphical representation of changes in RXRα protein levels (determined as shown in panel (c)) relative to 0 h (lanes 1, 4, 7, 10, resp., for corresponding treatments); means ± SE; $n = 3$. $^{\blacklozenge}P < 0.05$.

a few transcription factors, for example, lymphoblastic-leukemia-derived sequence 1 (LYL1) [47] and homeobox B6 (HOXB6) [48], were associated with inhibition of monocytic differentiation of myeloid precursors. Although it was found that ERK5 does not directly phosphorylate RXRα in response to 1,25D [49], the relationship between the other negative regulators of monocytic differentiation and RXRα expression or function remains to be elucidated. Since Nrf2 may function as a positive regulator of RXRα expression [11], the failure of SIL to stimulate the Nrf2/ARE in U937 cells may contribute to its negative effect on RXRα levels and, thereby, on 1,25D-induced differentiation of these cells. The reason why, in contrast to CA, SIL can activate Nrf2/ARE in HL60 cells but not in U937 cells is unclear and may be related to

possible differences in the metabolic fate of SIL in the two cell types.

In conclusion, our results demonstrate that the contrasting effects of SIL on the differentiation of myeloblastic (HL60) and promonocytic (U937) AML cells are related, at least in part, to its opposing modulation of the VDR signaling pathway. We suggest that U937 cells may present a model for patient-derived leukemic blasts which respond to SIL [16, 17] and similarly acting compounds by inhibition of 1,25D-induced differentiation. SIL has been shown to inhibit proliferation of various types of cancer cells (see [50, 51] for recent reviews). Likewise, this polyphenol demonstrated a marked antiproliferative activity in both AML cell lines tested here, even though it negatively affected the differentiation of U937 cells. Therefore, further preclinical studies are required to determine the potential use of different plant polyphenols for AML treatment and/or prevention.

Acknowledgments

The authors thank Dr. Andrzej Kutner for the gift of 1,25D. This research was supported by the Israel Science Foundation grant 635/11.

References

[1] A. Redaelli, M. F. Botteman, J. M. Stephens, S. Brandt, and C. L. Pashos, "Economic burden of acute myeloid leukemia: a literature review," *Cancer Treatment Reviews*, vol. 30, no. 3, pp. 237–247, 2004.

[2] J. L. Shipley and J. N. Butera, "Acute myelogenous leukemia," *Experimental Hematology*, vol. 37, no. 6, pp. 649–658, 2009.

[3] A. T. Fathi, S. Grant, and J. E. Karp, "Exploiting cellular pathways to develop new treatment strategies for AML," *Cancer Treatment Reviews*, vol. 36, no. 2, pp. 142–150, 2010.

[4] P. J. Hughes, E. Marcinkowska, E. Gocek, G. P. Studzinski, and G. Brown, "Vitamin D_3-driven signals for myeloid cell differentiation—implications for differentiation therapy," *Leukemia Research*, vol. 34, no. 5, pp. 553–565, 2010.

[5] G. Jones, "Vitamin D analogs," *Endocrinology and Metabolism Clinics of North America*, vol. 39, no. 2, pp. 447–472, 2010.

[6] M. Danilenko and G. P. Studzinski, "Enhancement by other compounds of the anti-cancer activity of vitamin D_3 and its analogs," *Experimental Cell Research*, vol. 298, no. 2, pp. 339–358, 2004.

[7] M. Danilenko, Q. Wang, X. Wang, J. Levy, Y. Sharoni, and G. P. Studzinski, "Carnosic acid potentiates the antioxidant and prodifferentiation effects of 1α,25-dihydroxyvitamin D_3 in leukemia cells but does not promote elevation of basal levels of intracellular calcium," *Cancer Research*, vol. 63, no. 6, pp. 1325–1332, 2003.

[8] M. Danilenko, X. Wang, and G. P. Studzinski, "Carnosic acid and promotion of monocytic differentiation of HL60-G cells initiated by other agents," *Journal of the National Cancer Institute*, vol. 93, no. 16, pp. 1224–1233, 2001.

[9] M. Steiner, I. Priel, J. Giat, J. Levy, Y. Sharoni, and M. Danilenko, "Carnosic acid inhibits proliferation and augments differentiation of human leukemic cells induced by 1,25-dihydroxyvitamin D_3 and retinoic acid," *Nutrition and Cancer*, vol. 41, no. 1-2, pp. 135–144, 2001.

[10] J. Zhang, G. H. Posner, M. Danilenko, and G. P. Studzinski, "Differentiation-inducing potency of the seco-steroid JK-1624F2-2 can be increased by combination with an antioxidant and a p38MAPK inhibitor which upregulates the JNK pathway," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 105, no. 1–5, pp. 140–149, 2007.

[11] I. Bobilev, V. Novik, I. Levi et al., "The Nrf2 transcription factor is a positive regulator of myeloid differentiation of acute myeloid leukemia cells," *Cancer Biology and Therapy*, vol. 11, no. 3, pp. 317–329, 2011.

[12] T. Thompson, M. Danilenko, L. Vassilev, and G. P. Studzinski, "Tumor suppressor p53 status does not determine the differentiation- associated G_1 cell cycle arrest induced in leukemia cells by 1,25-dihydroxyvitamin D_3 and antioxidants," *Cancer Biology and Therapy*, vol. 10, no. 4, pp. 344–350, 2010.

[13] H. Sharabani, E. Izumchenko, Q. Wang et al., "Cooperative antitumor effects of vitamin D_3 derivatives and rosemary preparations in a mouse model of myeloid leukemia," *International Journal of Cancer*, vol. 118, no. 12, pp. 3012–3021, 2006.

[14] A. Shabtay, H. Sharabani, Z. Barvish et al., "Synergistic antileukemic activity of carnosic acid-rich rosemary extract and the 19-nor Gemini vitamin D analogue in a mouse model of systemic acute myeloid leukemia," *Oncology*, vol. 75, no. 3-4, pp. 203–214, 2008.

[15] S. N. Kang, M. H. Lee, K. M. Kim, D. Cho, and T. S. Kim, "Induction of human promyelocytic leukemia HL-60 cell differentiation into monocytes by silibinin: involvement of protein kinase C," *Biochemical Pharmacology*, vol. 61, no. 12, pp. 1487–1495, 2001.

[16] X. Wang, E. Gocek, V. Novik, J. S. Harrison, M. Danilenko, and G. P. Studzinski, "Inhibition of Cot1/Tlp2 oncogene in AML cells reduces ERK5 activation and upregulates p27Kip1 concomitant with enhancement of differentiation and cell cycle arrest induced by silibinin and 1,25-dihydroxyvitamin D_3," *Cell Cycle*, vol. 9, no. 22, pp. 4542–4551, 2010.

[17] J. Zhang, J. S. Harrison, M. Uskokovic, M. Danilenko, and G. P. Studzinski, "Silibinin can induce differentiation as well as enhance vitamin D_3-induced differentiation of human AML cells ex vivo and regulates the levels of differentiation-related transcription factors," *Hematological Oncology*, vol. 28, no. 3, pp. 124–132, 2010.

[18] S. B. Cullinan, D. Zhang, M. Hannink, E. Arvisais, R. J. Kaufman, and J. A. Diehl, "Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival," *Molecular and Cellular Biology*, vol. 23, no. 20, pp. 7198–7209, 2003.

[19] G. P. Studzinski, K. B. Reddy, H. Z. Hill, and A. K. Bhandal, "Potentiation of 1-β-D-arabinofuranosylcytosine cytotoxicity to HL-60 cells by 1,25-dihydroxyvitamin D_3 correlates with reduced rate of maturation of DNA replication intermediates," *Cancer Research*, vol. 51, no. 13, pp. 3451–3455, 1991.

[20] Q. Wang, H. Salman, M. Danilenko, and G. P. Studzinski, "Cooperation between antioxidants and 1,25-dihydroxyvitamin D_3 in induction of leukemia HL60 cell differentiation through the JNK/AP-1/Egr-1 pathway," *Journal of Cellular Physiology*, vol. 204, no. 3, pp. 964–974, 2005.

[21] S. Pesakhov, M. Khanin, G. P. Studzinski, and M. Danilenko, "Distinct combinatorial effects of the plant polyphenols curcumin, carnosic acid, and silibinin on proliferation and apoptosis in acute myeloid leukemia cells," *Nutrition and Cancer*, vol. 62, no. 6, pp. 811–824, 2010.

[22] A. Dimberg, F. Bahram, I. Karlberg, L. G. Larsson, K. Nilsson, and F. Öberg, "Retinoic acid-induced cell cycle arrest of human myeloid cell lines is associated with sequential down-regulation of c-Myc and cyclin E and posttranscriptional

up-regulation of $p27^{Kip1}$," *Blood*, vol. 99, no. 6, pp. 2199–2206, 2002.

[23] M. K. Kwak and T. W. Kensler, "Targeting NRF2 signaling for cancer chemoprevention," *Toxicology and Applied Pharmacology*, vol. 244, no. 1, pp. 66–76, 2010.

[24] J. A. Sokoloski, K. Shyam, and A. C. Sartorelli, "Induction of the differentiation of hl-60 promyelocytic leukemia cells by curcumin in combination with low levels of vitamin D_3," *Oncology Research*, vol. 9, no. 1, pp. 31–39, 1997.

[25] Y. Liu, R. L. Chang, X. X. Cui, H. L. Newmark, and A. H. Conney, "Synergistic effects of curcumin on all-trans retinoic acid-and 1α,25-dihydroxyvitamin D_3-induced differentiation in human promyelocytic leukemia HL-60 cells," *Oncology Research*, vol. 9, no. 1, pp. 19–29, 1997.

[26] K. H. Poon, J. Zhang, C. Wang, A. K. Tse, C. K. Wan, and W. F. Fong, "Betulinic acid enhances 1α,25-dihydroxyvitamin D_3-induced differentiation in human HL-60 promyelocytic leukemia cells," *Anti-Cancer Drugs*, vol. 15, no. 6, pp. 619–624, 2004.

[27] S. N. Kang, S. H. Kim, S. W. Chung, M. H. Lee, H. J. Kim, and T. S. Kim, "Enhancement of 1α,25-dihydroxyvitamin D_3-induced differentiation of human leukaemia HL-60 cells into monocytes by parthenolide via inhibition of NF-κB activity," *British Journal of Pharmacology*, vol. 135, no. 5, pp. 1235–1244, 2002.

[28] S. H. Kim, S. N. Kang, H. J. Kim, and T. S. Kim, "Potentiation of 1,25-dihydroxyvitamin D_3-induced differentiation of human promyelocytic leukemia cells into monocytes by costunolide, a germacranolide sesquiterpene lactone," *Biochemical Pharmacology*, vol. 64, no. 8, pp. 1233–1242, 2002.

[29] S. H. Kim, H. J. Kim, and T. S. Kim, "Differential involvement of protein kinase C in human promyelocytic leukemia cell differentiation enhanced by artemisinin," *European Journal of Pharmacology*, vol. 482, no. 1–3, pp. 67–76, 2003.

[30] J. A. Sokoloski, W. F. Hodnick, S. T. Mayne, C. Cinquina, C. S. Kim, and A. C. Sartorelli, "Induction of the differentiation of HL-60 promyelocytic leukemia cells by vitamin E and other antioxidants in combination with low levels of vitamin D_3: possible relationship to NF-κB," *Leukemia*, vol. 11, no. 9, pp. 1546–1553, 1997.

[31] H. Amir, M. Karas, J. Giat et al., "Lycopene and 1,25-dihydroxyvitamin D_3 cooperate in the inhibition of cell cycle progression and induction of differentiation in HL-60 leukemic cells," *Nutrition and Cancer*, vol. 33, no. 1, pp. 105–112, 1999.

[32] M. Makishima, Y. Honma, M. Hozumi et al., "Effects of inhibitors of protein tyrosine kinase activity and/or phosphatidylinositol turnover on differentiation of some human myelomonocytic leukemia cells," *Leukemia Research*, vol. 15, no. 8, pp. 701–708, 1991.

[33] S. N. Kang, S. W. Chung, and T. S. Kim, "Capsaicin potentiates 1,25-dihydoxyvitamin D_3 and all-trans retinoic acid-induced differentiation of human promyelocytic leukemia HL-60 cells," *European Journal of Pharmacology*, vol. 420, no. 2-3, pp. 83–90, 2001.

[34] K. Yamada, Y. Honma, K. I. Asahi, T. Sassa, K. I. Hino, and S. Tomoyasu, "Differentiation of human acute myeloid leukaemia cells in primary culture in response to cotylenin A, a plant growth regulator," *British Journal of Haematology*, vol. 114, no. 4, pp. 814–821, 2001.

[35] P. K. Farmer, X. He, M. L. Schmitz, J. Rubin, and M. S. Nanes, "Inhibitory effect of NF-κB on 1,25-dihydroxyvitamin D_3 and retinoid X receptor function," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 269, no. 1, pp. E213–E220, 2000.

[36] X. Lu, P. Farmer, J. Rubin, and M. S. Nanes, "Integration of the NfκB p65 subunit into the vitamin D receptor transcriptional complex: identification of p65 domains that inhibit 1,25-dihydroxyvitamin D_3-stimulated transcription," *Journal of Cellular Biochemistry*, vol. 92, no. 4, pp. 833–848, 2004.

[37] X. Qi, R. Pramanik, J. Wang et al., "The p38 and JNK pathways cooperate to trans-activate vitamin D receptor via c-Jun/AP-1 and sensitize human breast cancer cells to vitamin D_3-induced growth inhibition," *The Journal of Biological Chemistry*, vol. 277, no. 29, pp. 25884–25892, 2002.

[38] A. Takeshita, A. Watanabe, Y. Takada, and S. Hanazawa, "Selective stimulation by ceramide of the expression of the α isoform of retinoic acid and retinoid X receptors in osteoblastic cells. A role of sphingosine 1-phosphate-mediated AP-1 in the ligand-dependent transcriptional activity of these receptors," *The Journal of Biological Chemistry*, vol. 275, no. 41, pp. 32220–32226, 2000.

[39] J. Li, R. A. Finch, and A. C. Sartorelli, "Role of vitamin D_3 receptor in the synergistic differentiation of WEHI- 3B leukemia cells by vitamin D_3 and retinoic acid," *Experimental Cell Research*, vol. 249, no. 2, pp. 279–290, 1999.

[40] Y. Amano, Y. Cho, M. Matsunawa, K. Komiyama, and M. Makishima, "Increased nuclear expression and transactivation of vitamin D receptor by the cardiotonic steroid bufalin in human myeloid leukemia cells," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 114, no. 3–5, pp. 144–151, 2009.

[41] S. Swami, A. V. Krishnan, D. M. Peehl, and D. Feldman, "Genistein potentiates the growth inhibitory effects of 1,25-dihydroxyvitamin D_3 in DU145 human prostate cancer cells: role of the direct inhibition of CYP24 enzyme activity," *Molecular and Cellular Endocrinology*, vol. 241, no. 1-2, pp. 49–61, 2005.

[42] J. A. Wietzke and J. Welsh, "Phytoestrogen regulation of a Vitamin D_3 receptor promoter and 1,25-dihydroxyvitamin D_3 actions in human breast cancer cells," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 84, no. 2-3, pp. 149–157, 2003.

[43] L. A. Gilad, O. Tirosh, and B. Schwartz, "Phytoestrogen regulate transcription and translation of vitamin D receptor in colon cancer cells," *Journal of Endocrinology*, vol. 191, no. 2, pp. 387–398, 2006.

[44] Y. Yin, J. Ni, M. Chen, Y. Guo, and S. Yeh, "RRR-α-vitamin E succinate potentiates the antitumor effect of calcitriol in prostate cancer without overt side effects," *Clinical Cancer Research*, vol. 15, no. 1, pp. 190–200, 2009.

[45] S. Álvarez, W. Bourguet, H. Gronemeyer, and A. R. de Lera, "Retinoic acid receptor modulators: a perspective on recent advances and promises," *Expert Opinion on Therapeutic Patents*, vol. 21, no. 1, pp. 55–63, 2011.

[46] X. Wang and G. P. Studzinski, "Expression of MAP3 kinase COT1 is up-regulated by 1,25-dihydroxyvitamin D_3 in parallel with activated c-jun during differentiation of human myeloid leukemia cells," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 121, no. 1-2, pp. 395–398, 2010.

[47] Y. S. Meng, H. Khoury, J. E. Dick, and M. D. Minden, "Oncogenic potential of the transcription factor LYL1 in acute myeloblastic leukemia," *Leukemia*, vol. 19, no. 11, pp. 1941–1947, 2005.

[48] A. Giampaolo, N. Felli, D. Diverio et al., "Expression pattern of HOXB6 homeobox gene in myelomonocytic differentiation and acute myeloid leukemia," *Leukemia*, vol. 16, no. 7, pp. 1293–1301, 2002.

[49] P. P. Dwivedi, C. S. Hii, A. Ferrante et al., "Role of MAP kinases in the 1,25-dihydroxyvitamin D_3-induced transactivation of the rat cytochrome P450c24 (CYP24) promoter: specific functions for ERK1/ERK2 and ERK5," *The Journal of Biological Chemistry*, vol. 277, no. 33, pp. 29643–29653, 2002.

[50] C. W. Cheung, N. Gibbons, D. W. Johnson, and D. L. Nicol, "Silibinin—a promising new treatment for cancer," *Anti-Cancer Agents in Medicinal Chemistry*, vol. 10, no. 3, pp. 186–195, 2010.

[51] G. Deep and R. Agarwal, "Antimetastatic efficacy of silibinin: molecular mechanisms and therapeutic potential against cancer," *Cancer and Metastasis Reviews*, vol. 29, no. 3, pp. 447–463, 2010.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 713243, 11 pages
doi:10.1155/2012/713243

Review Article

Regulation of Leukemic Cell Differentiation through the Vitamin D Receptor at the Levels of Intracellular Signal Transduction, Gene Transcription, and Protein Trafficking and Stability

Elżbieta Gocek, Hanna Baurska, Aleksandra Marchwicka, and Ewa Marcinkowska

Faculty of Biotechnology, University of Wrocław, Tamka 2, 50-137 Wrocław, Poland

Correspondence should be addressed to Ewa Marcinkowska, ema@cs.uni.wroc.pl

Received 17 January 2012; Accepted 29 February 2012

Academic Editor: Michael Danilenko

1α,25-Dihydroxyvitamin D_3 (1,25$(OH)_2$D) exerts its biological activities through vitamin D receptor (VDR), which is a member of the superfamily of steroid receptors, that act as ligand-dependent transcription factors. Ligated VDR in complex with retinoid X receptor (RXR) binds to regulatory regions of 1,25$(OH)_2$D-target genes. 1,25$(OH)_2$D is able to induce differentiation of leukemic blasts towards macrophage-like cells. Many different acute myeloid leukemia (AML) cell lines respond to 1,25$(OH)_2$D by increasing CD14 cell surface receptor, some additionally upregulate CD11b and CD11c integrins. In untreated AML cells VDR protein is present in cytosol at a very low level, even though its mRNA is continuously expressed. Ligation of VDR causes protein stabilization and translocation to the cell nuclei, where it regulates transcription of target genes. Several important groups of genes are regulated by 1,25$(OH)_2$D in HL60 cells. These genes include differentiation-related genes involved in macrophage function, as well as a gene regulating degradation of 1,25$(OH)_2$D, namely CYP24A1. We summarize here the data which demonstrate that though some cellular responses to 1,25$(OH)_2$D in AML cells are transcription-dependent, there are many others which depend on intracellular signal transduction, protein trafficking and stabilization. The final effect of 1,25$(OH)_2$D action in leukemic cells requires all these acting together.

1. Introduction

The primary role of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D) is to maintain calcium and phosphate homeostasis in vertebrate organisms via actions in the intestine, bones, kidneys, and parathyroid glands. However, it is well known that physiological roles of 1,25$(OH)_2$D reach much beyond calcium and phosphate homeostasis. For example, 1,25$(OH)_2$D induces differentiation and inhibits proliferation of various normal and cancer cells, including osteoclasts, keratinocytes, and monocytes. In 1981 the group of Suda observed that 1,25$(OH)_2$D was able to induce differentiation in the M1 murine myeloid cell line [1] and that it extended the survival of mice inoculated with leukemia cells [2]. Since then many research projects have been performed in order to prepare ground for clinical use of 1,25$(OH)_2$D or of its low calcemic analogs in leukemia treatment [3–5].

There are two major signal transduction pathways activated by 1,25$(OH)_2$D in target cells. The most important and the best documented is the so-called "genomic pathway," with its most important player a vitamin D receptor (VDR). The less well described is "nongenomic pathway," which consists of intracellular signalling molecules, such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), and others, activated by mechanisms that are not fully understood now [6]. It is believed that both pathways need to be activated for full biological activity of 1,25$(OH)_2$D and that the most probable mediator of these actions is a putative membrane VDR (mVDR) [6].

VDR belongs to the superfamily of intracellular receptors for steroid and thyroid hormones. 48 members of the superfamily have been identified in humans; they act as ligand-induced transcription factors [7]. Most of the superfamily

members, in order to be biologically active, form homo- or heterodimers. For VDR, retinoid X receptor (RXR) is a dimerization partner. VDR upon ligation undergoes conformational changes that allow binding to specific sequences in promoter regions of target genes, called vitamin D response elements (VDREs). VDREs are composed of two repeated half-sites with the consensus sequence AGGTCAcagAGGTCA (VDRE-DR3). Binding of $1,25(OH)_2D$ to VDR enhances heterodimerization with RXR and allows binding of the coactivator complex, known as vitamin D receptor-interacting protein complex (DRIP) [8] and of other proteins, histone acetylase among them. Acetylated histones relax chromatin structure to make DNA accessible and permit initiation of transcription of target genes [9]. VDR may be ligated not only with $1,25(OH)_2D$ but also with other compounds such as lithocholic acid, docosahexaenoic acid, arachidonic acid, or curcumin [10]. Moreover, there are about 300 compounds closely related to $1,25(OH)_2D$, called $1,25(OH)_2D$ analogs, which can bind VDR and exert changed biological properties. Subtle conformational changes in VDR structure caused by analogs can produce antagonistic, agonistic, or even superagonistic effects. There are even some analogs that exert semiselective activities, with lowered calcemic and increased antiproliferative and cell differentiating effects [11].

The VDR protein is expressed at low concentrations in target tissues and cultured cells with the level of receptor expression ranging from a few copies of the VDR/cell to 25 000 copies/cell [7]. Among blood cells VDR is expressed in Tcells, Bcells, monocytes, neutrophils, platelets, macrophages, and dendritic cells. Also many different myeloid leukemia cell lines, blocked at various stages of maturation, expressed mRNA for VDR; however, the expression levels were variable [12]. Addition of $1,25(OH)_2D$ to certain acute myeloid leukemia (AML) cells induces dramatic changes in their phenotype and function; however, the extent of these changes is various in various cell lines.

The activation of MAPK/Erk1,2 signal transduction pathway in AML cells in response to $1,25(OH)_2D$ was for the first time reported in 1997 [13], and it was later shown to be important for the process of AML cell differentiation [14]. The exact mechanisms of how MAPK/Erk1,2 participate in the differentiation process are not known; however, they are being connected with a proliferative phase of AML cells differentiation [15]. MAPK/JNK pathway, whose activation was reported later [16], appeared to be involved in a subtle way in regulation of $1,25(OH)_2D$-dependent transcription factors [17]. Another MAP kinase, p38, has antagonistic effects to both MAPK/Erk1,2 and MAPK/JNK [16, 18]. Also activation of PI3K signal transduction pathway in AML cells exposed to $1,25(OH)_2D$ has been reported [19] and was later shown to be responsible for activation of myeloid zinc finger-1 (MZF-1) transcription factor, which in turn participates in regulation of proteins crucial for macrophage function [20].

2. Nuclear Trafficking of VDR

VDR to fulfil its function of nuclear receptor must be transported into the nucleus [21]. In eukaryotic cells nucleoplasm and genetic material are separated from the cytosol by a double membrane which contains highly selective, bidirectional transporter channel called nuclear pore complex (NPC) [22]. NPC is composed of nearly 30 proteins termed nuclear pore complex components or "nucleoporins" (NUPs) [22] which occur in multiple copies [23]. Nuclear import of proteins through NPC is mediated by transporter proteins, such as importinα and importinβ, which bind cargoes through nuclear localisation signal (NLS) and interact with NUPs. Complex cargo-importinα binds importinβ which interacts with Ran-GDP protein [21]. Ran-GDP exists mostly in cytoplasm, whereas Ran-GTP in nuclei and this GTP gradient ensures the right direction of nuclear transport [21]. High concentration of Ran-GDP promotes the formation of import complex, while high concentration of Ran-GTP dissociates them and promotes formation of export complexes [24]. To complete the cycle importinα and importinβ must be transported back to the cytoplasm. To ensure Ran-GTP gradient, in the cytosol Ran-guanosine triphosphatase activating protein (RanGAP) stimulates the intrinsic GTP-hydrolyzing activity of Ran to form Ran-GDP. Hydrolysis of Ran-GTP to Ran-GDP causes release of importinβ for the next cycle.

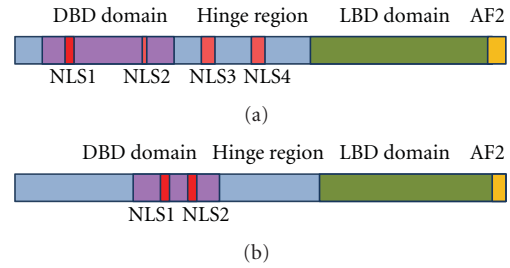


Figure 1: Localization of nuclear import domains (NLS) in VDR and RXR. NLS segments in VDR (a) and in RXR (b). Based on [26].

To overcome the NPC, large molecules, such as nuclear receptors, harbour NLSs recognized by the transporter proteins which interact with NUPs in NPC. In VDR four NLSs were indentified (Figure 1(a)). First (NLS1) is localized between two zinc fingers within DNA-binding domain (DBD) [29]. The second NLS is in the second zinc finger of the DBD but data show that NLS2 does not function as an obligatory NLS. The next two are localized in the hinge region of VDR. NLS3 (102–110) is important for ligand-induced nuclear localisation of VDR but has no effect on unligated VDR nuclear import. NLS4 (154–173) is a short segment without a confirmed function. The RXR which is a partner protein for VDR has two NLSs, the first localized between two zinc fingers in DBD and the second in the second zinc finger (Figure 1(b)) [26]. Some data demonstrated that VDR shuttles between nucleus and cytoplasm in the absence of ligand, but unligated VDR weakly interacts with importinα [27]. However, nuclear trafficking of unligated VDR involves importin 4 through the interaction with the aminoterminus of VDR [21]. Binding the ligand promotes heterodimerization with RXR and enhances nuclear localization. Whereas RXR is predominantly localized in the nuclei even in the absence

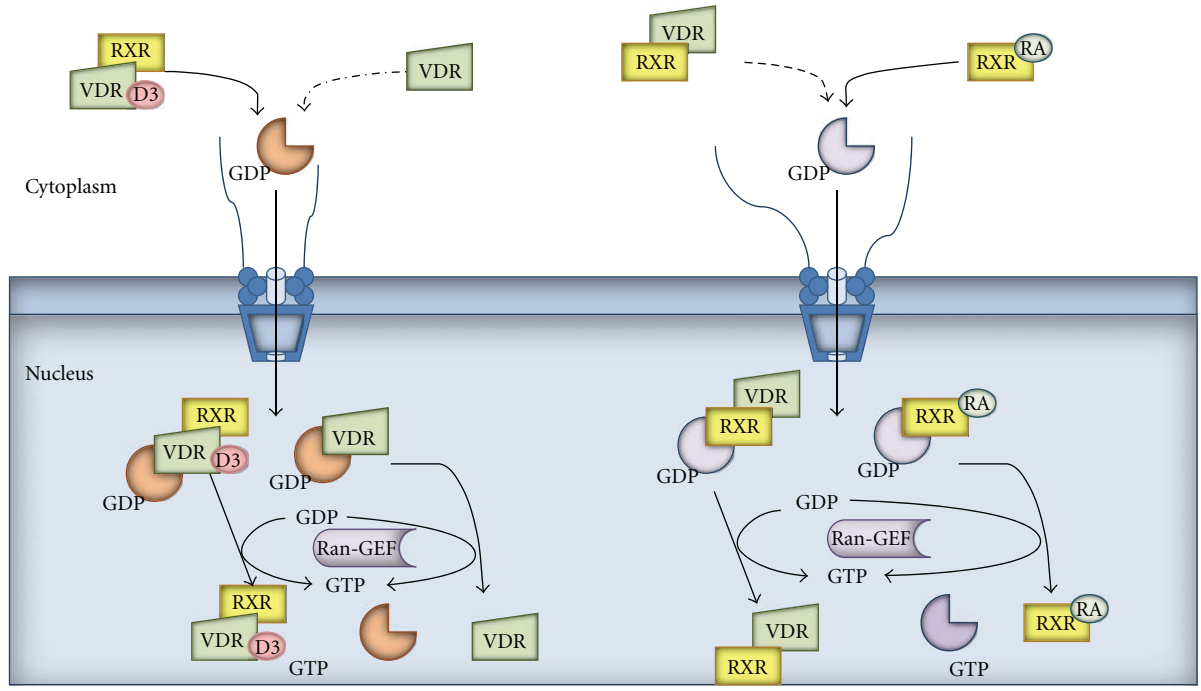


Figure 2: Nuclear import of VDR, RXR, and their heterodimer. GTP: guanosine 5′-triphosphate, GDP: guanosine diphosphate, α: importinα, β: importinβ, and RA: retinoic acid. Based on [27, 28].

of ligand, there is also a cytoplasmic fraction of RXR that is translocated to the nucleus in the presence of its ligand. The nuclear import of RXR is mediated through importinβ, while that of VDR is mediated through importinα (Figure 2) [27]. In their publication Prüfer and Barsony showed that VDR is imported using two different pathways: ligand-dependent and ligand-independent. In ligand independent pathway RXR is the dominant partner for nuclear translocation, whereas ligated VDR dominates the mobility of RXR [28]. Binding $1,25(OH)_2D$ stabilizes heterodimer VDR : RXR and its interaction with importinα but inhibits interaction of RXR with importinβ [27].

In order to let proteins out of the nucleus, exportin named the chromosomal region maintenance 1 protein (CRM1) recognizes proteins containing leucine-rich nuclear export signals (NESs). Export complex formation is favored by high concentrations of RanGTP in the nucleus, which facilitate specific interactions with nucleoporins at the nuclear basket for appropriate translocation through the NPC [25]. VDR utilizes two pathways of nuclear export: ligand dependent and ligand independent. Unligated VDR uses a CRM1 export mechanism using NES localized in position 320–325 in LBD [28]. Mechanism for liganded VDR is CRM1 indepentent and requires DBD which functions as NES [30].

3. Differentiation of Human AML Cell Lines in Response to $1,25(OH)_2D$

One of the major processes in the array of anticancer actions of $1,25(OH)_2D$ is differentiation of AML cells. Differentiation in AML cells consists of a G0/G1 cell cycle block [32], which is connected to an increase of proteins p21 and p27 [33], an increase in expression of antiapoptotic proteins [34], and acquisition of functional and phenotypic features characteristic for normal macrophages. Functional features are connected with an ability to phagocytose [35], with increased activity of monocyte specific esterase (MSE) [36], and with an ability to generate reactive oxygen species (ROS) and reduce nitroblue tetrazolium (NBT) [37]. Differentiation is also accompanied by upregulation of certain cell surface molecules, which are necessary for macrophage function, such as CD14, which is a coreceptor for lipopolysaccharide (LPS), as well as CD11b, which is a subunit of $\alpha_M\beta_2$ integrin or CD11c, an integrin αX, both involved in the cell adhesion [31, 38]. The process of $1,25(OH)_2D$-induced AML cell differentiation is not fast, it requires 3-4 days to reach plateau in expression of cell surface antigens, but differences in cell phenotype and function are spectacular (Figure 3). Since differentiation of blood cells may have beneficial effects, therapeutic applications for $1,25(OH)_2D$ have been postulated. However, a major limitation for therapeutic use of $1,25(OH)_2D$ is its potent calcemic and phosphatemic activity. The doses of $1,25(OH)_2D$, which are necessary to inhibit cell proliferation and induce differentiation, produce in vivo hypercalcemia and hyperphosphatemia that may be life threatening. Therefore there is a need for new $1,25(OH)_2D$ analogs that retain high differentiating and antiproliferative activities with minimal or tolerable calcemic and phosphatemic effects [39]. Many such analogs were tested in our laboratory and their activities in inducing differentiation of various AML cell lines, as well as differentiation of leukemic blasts from the peripheral blood of AML patients, were studied.

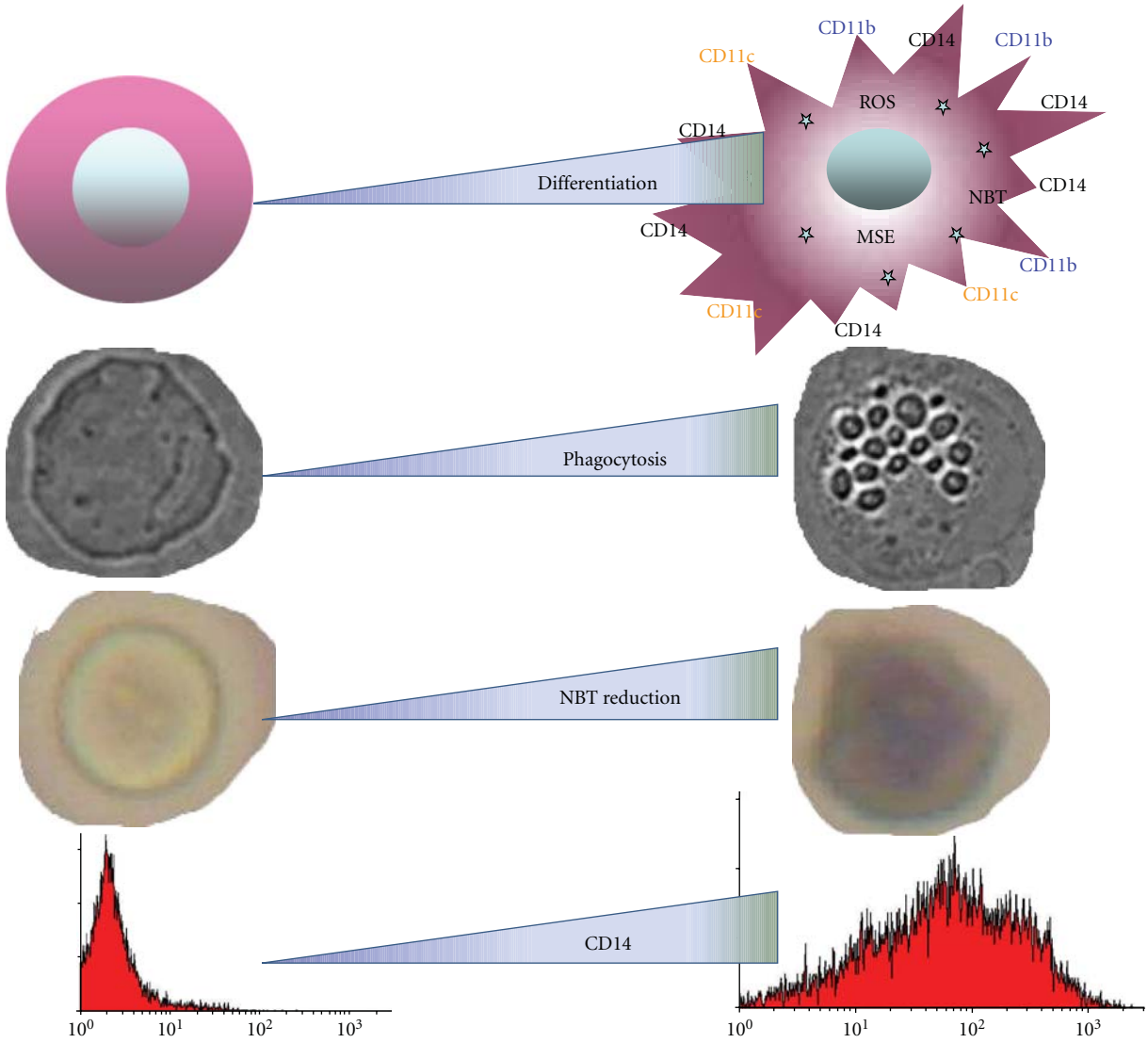


Figure 3: $1,25(OH)_2D$-induced changes in AML cells. MSE: monocyte specyfic esterase; ROS: reactive oxygen species; NBT: nitroblue tetrazolium, CD14: co-receptor for LPS, CD11b: subunit of $\alpha_M\beta 2$ integrin, CD11c: integrin αX. Based on [31].

4. Side-Chain Modified Analogs of Vitamin D_2 and Vitamin D_3

In our previous papers we described prodifferentiating activities of various side-chain modified analogs of vitamin D_3, PRI-2191, PRI-2201, PRI-2202, and PRI-2205 and of vitamin D_2, PRI-1906, PRI-1907 PRI-1908, and PRI-1909 [40, 41, 45–48]. We studied their pro-differentiating activities towards various human AML cell lines with genetic lesions characteristic for AML. Our studies indicated that some of the tested analogs were more potent than $1,25(OH)_2D$ in induction of cell differentiation. The most efficient were the two analogs of vitamin D_3, named PRI-2191 and PRI-2201, and the two analogs of vitamin D_2, PRI-1906 and PRI-1907 [40–42]. Interestingly, one of the most active analogs tested by our group was PRI-2191, which in fact is a $1,25(OH)_2D$ metabolite, already in use for treatment of psoriasis [41, 49] and a potential drug for vitiligo [50]. The four analogs mentioned above were tested in vivo for their calcemic activity. Results of these studies showed that PRI-2191, PRI-2201, and PRI-1906 were less calcemic than $1,25(OH)_2D$, while PRI-1907 was comparable to $1,25(OH)_2D$ [48, 51, 52].

The cell lines used in our studies were derived from various AML subtypes. HL60 cells originate from M2 subtype of AML and are the most sensitive to $1,25(OH)_2D$-induced differentiation out of all cell lines used in our tests. NB-4 cells carry the t(15;17) PML-RARA fusion gene, which is characteristic for AML M3, U937 cells carry translocation t(10;11) often seen in AML M5, MV4-11 cells express fusion gene MLL-AF4, and MOLM13 cells express fusion gene MLL-AF9. Moreover, MOLM13 and MV4-11 have an internal tandem duplication in Flt3 gene (Flt3-ITD), in one or in both alleles, respectively [53]. Our studies revealed that in all cell lines studied, $1,25(OH)_2D$ and its active analogs were

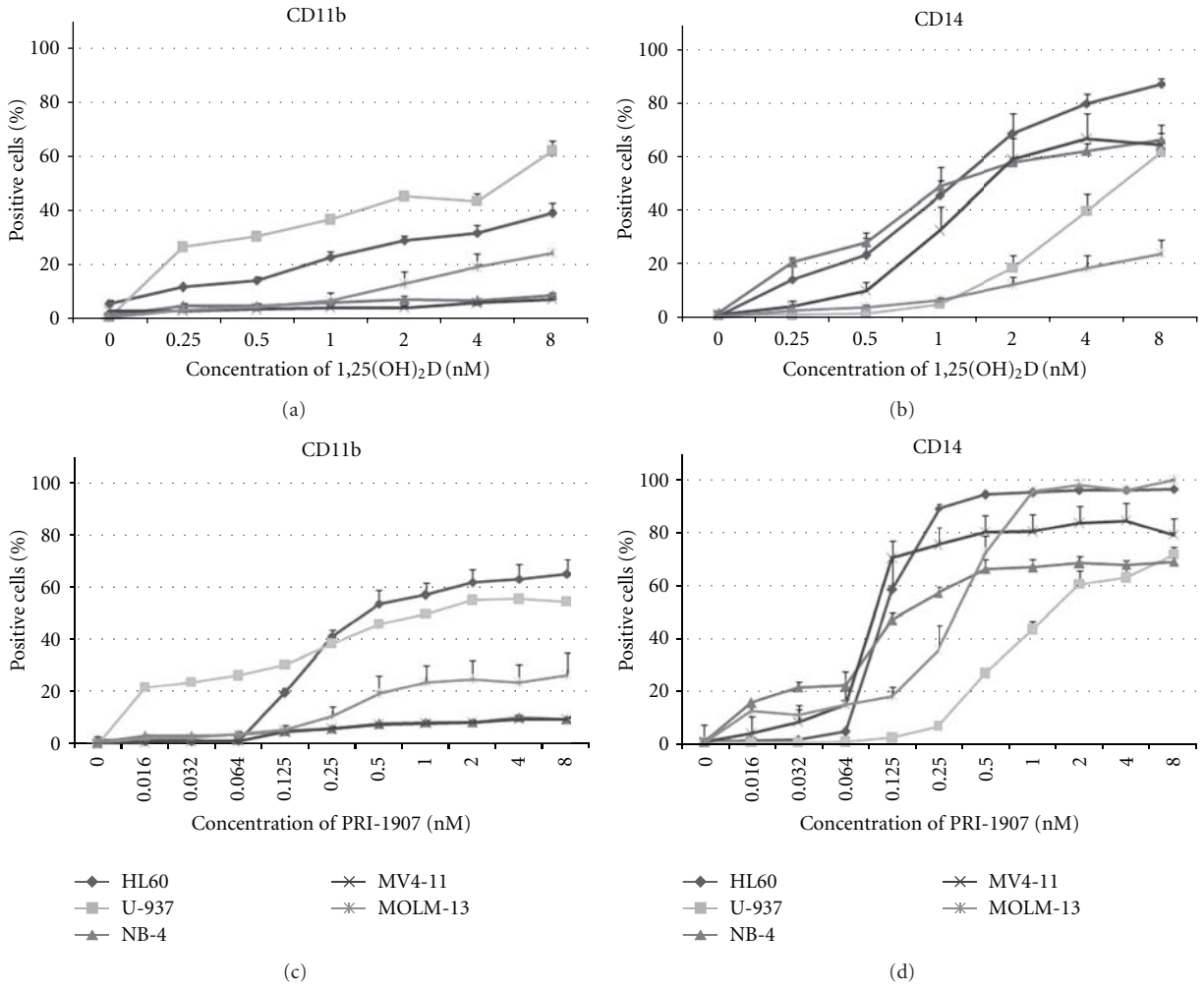


Figure 4: Expression of monocytic differentiation markers in AML cell lines exposed to either $1,25(OH)_2D$ or to PRI-1907. HL60, U-937, NB-4, MV4-11, and MOLM-13 cells were exposed either to $1,25(OH)_2D$ (a, b) or to PRI-1907 (c, d) for 96 h, and then the expression of CD11b and CD14 was tested in flow cytometry. The graphs show mean percentages (±SEM) of cells expressing cell differentiation markers. Based on [40].

able to upregulate CD14 cell surface antigen, while only in some cell lines CD11b integrin level was elevated (Figure 4). These data demonstrate that the expressions of CD14 and CD11b are controlled by two different signal transduction pathways, and in some AML cells CD11b pathway is blocked and cannot be overcome by $1,25(OH)_2D$.

5. VDR in Human AML Cell Lines

Experiments made by our group showed that AML cell lines have very low constitutive level of VDR protein, which increases significantly after exposure of the cells to $1,25(OH)_2D$. Therefore if the proposed nuclear import and export of unligated VDR exists in AML cells, it remains at a very low level, which is difficult to detect. $1,25(OH)_2D$-induced changes in nuclear trafficking of VDR could be observed using confocal microscopy, but western blotting of cell lysates fractionated into cytosol and nuclei appeared to be a much more sensitive method of VDR detection [54]. The kinetics of VDR accumulation in AML cells is surprisingly fast after exposure of the cells to $1,25(OH)_2D$. In HL60 cells, VDR starts to accumulate in the cell nuclei after few minutes from exposure to $1,25(OH)_2D$ [42] and after half of an hour the difference is significant (Figure 5(a)). However, most of our studies were performed using HL60 cell line; also in other cell lines (Figure 5(b)) and in AML blasts (Figure 5(c)) accumulation of VDR in cell nuclei was observed [42, 54]. Obviously, the increase has not been caused through transcription of new mRNA for VDR since mRNA levels remained almost unchanged by $1,25(OH)_2D$, as confirmed by real-time PCR (not shown). Therefore the mechanism of accumulation of VDR must be regulated at post transcriptional level, and according to our data it is caused by ligation-induced protection of VDR protein from degradation [42]. It seems that VDR is continuously produced in the cytosol, and as long as it is unligated, it undergoes degradation. Ligation of VDR with $1,25(OH)_2D$ induces rapid translocation of the receptor to the cell nuclei, where degradation process is slower. For effective nuclear trafficking, active p42,44/MAPK pathway and active PI3-K pathway are needed; however,

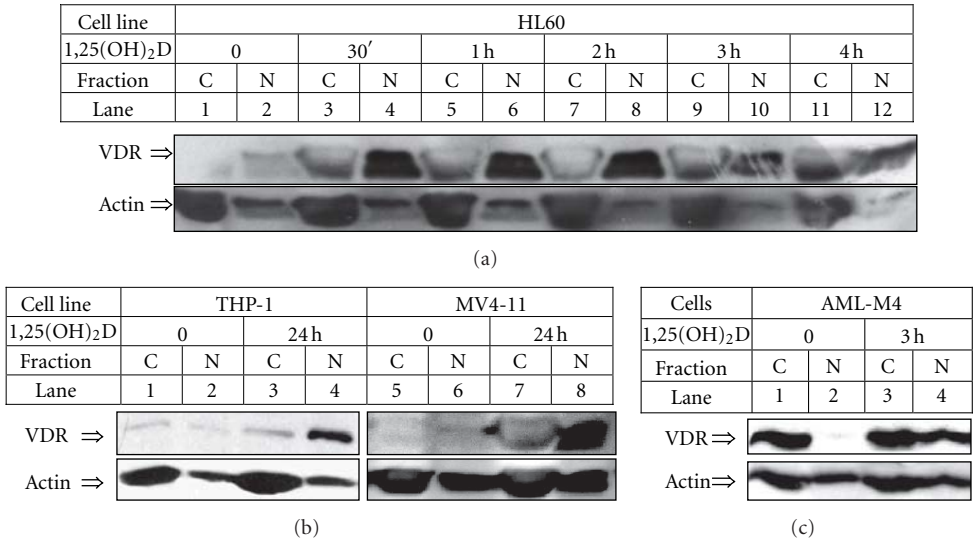


Figure 5: Expression of VDR protein in AML cells exposed to $1,25(OH)_2D$. AML cells after incubation for indicated times with 10 nM $1,25(OH)_2D$ were lysed and fractionated into the cytoplasmic (C) and nuclear (N) fractions. The lysates from equal numbers of cells were separated in SDS-PAGE and blotted to the membrane. The membrane was probed with anti-VDR. Actin was probed as a control of equal loading and transfer of proteins. (a) HL60 cells, (b) THP-1, MV4-11 cells, and (c) AML-M4 blasts from patient's peripheral blood. Based on [41, 42] and on unpublished data.

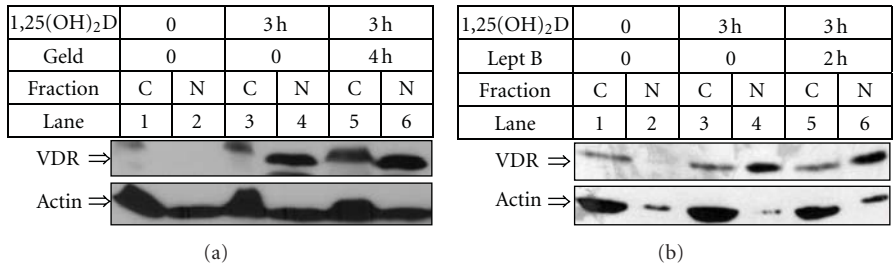


Figure 6: Nuclear trafficking of VDR in presence of geldanamycin (a) or leptomycin B (b). HL60 cells were exposed to 10 nM $1,25(OH)_2D$ for 3 h. One sample was pretreated with 1 μM geldanamycin (geld) for 1 h before exposure to $1,25(OH)_2D$ (a). Another sample was treated with 5 mg/ml of leptomycin B (lept B) for the last two hours of incubation (b). After incubation the cells were lysed and fractionated into the cytoplasmic (C) and nuclear (N) fractions. The lysates from equal numbers of cells were separated in SDS-PAGE and blotted to the membrane. The membrane was probed with anti-VDR. Actin was probed as a control of equal loading and transfer of proteins.

the mechanism of this effect is not known [54]. It was surprising that in most of freshly isolated AML cells from patients constitutive level of VDR in cytosol was higher than in established cell lines [41]. Unfortunately we were unable to test this phenomenon in a more detailed manner, because neither of the patient's samples was possible to be cultured in vitro for longer than two-three weeks. It is known; however, that other nuclear receptors become stabilized in the cytosol by heat shock proteins (Hsp) [55, 56], so the involvement of this class of proteins was addressed. We demonstrated recently that, in HL60 cells, VDR interacts with Hsp90 and that activation of Hsp90 is necessary for the differentiation process [57], but our new experiments documented that activation of Hsp90 is not necessary for nuclear translocation of VDR. Geldanamycin, which inhibits activity of Hsp90, was not able to block $1,25(OH)_2D$-induced nuclear accumulation of VDR (Figure 6(a)). VDR protein appears in the nuclei of $1,25(OH)_2D$-treated cells very fast but disappears slowly. Our experiments in which HL60 cells were exposed to $1,25(OH)_2D$ and an inhibitor of CRM1, namely, leptomycin B, confirmed that nuclear export of ligated VDR is CRM1 independent. As presented in Figure 6(b), presence of leptomycin B did not cause further accumulation of VDR in cells exposed to $1,25(OH)_2D$. As it was presented before, VDR protein levels remain elevated even after 4 days from exposure of HL60 cells to $1,25(OH)_2D$ [42]. During this time VDR activates transcription of its target genes. One of them is CYP24A1, which encodes an enzyme, 24-hydroxylase of $1,25(OH)_2D$, responsible for degradation of $1,25(OH)_2D$ to calcitrioic acid. As presented in our previous publication, $1,25(OH)_2D$ increased in HL60 cells levels of CYP24A1 mRNA significantly; however, kinetics of induction was very slow [40]. CYP24A1 protein is localized exclusively in an inner membrane of mitochondria, where its levels also increase slowly after exposure to $1,25(OH)_2D$ (not shown here).

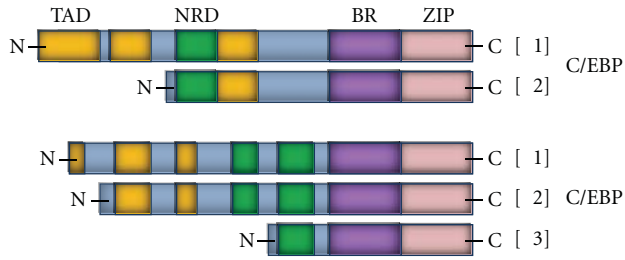


Figure 7: Schematic representation of the C/EBPα and C/EBPβ isoforms. TAD: transcription activation domain, NRD: negative regulatory domain, BR: basic region, and ZIP: leucine zipper. Based on [43, 44].

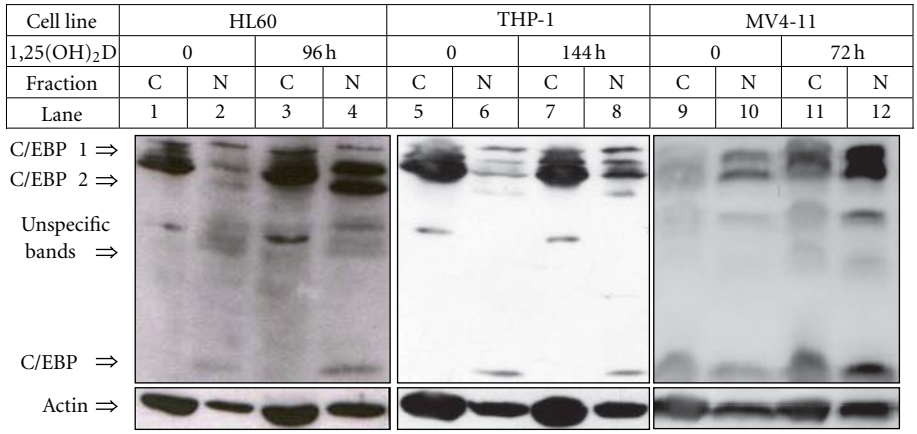


Figure 8: Subcellular localization of C/EBPβ isoforms in AML cells. The cells were exposed to 10 nM 1,25(OH)$_2$D for given times and then were fractionated into cytoplasmic (C) and nuclear (N) fractions. The lysates from equal numbers of cells were separated in SDS-PAGE and blotted to the membrane. The membranes were probed with anti-C/EBPβ antibody. Actin was probed as a control of equal loading and transfer of proteins.

6. Regulation of C/EBP Transcription Factors by 1,25(OH)$_2$D in AML Cells

There are several important groups of genes regulated by 1,25(OH)$_2$D in AML cells, including differentiation-related genes that encode proteins important for function of monocytes or macrophages. The examples of such are genes encoding CAAT-enhancer binding proteins (C/EBPs), belonging to the family of basic leucine zipper (bZIP) transcription factors [44, 58, 59]. There are six genes for different C/EBPs (α, β, γ, δ, ε, and ζ) which are expressed in hematopoietic cells, hepatocytes, adipocytes, spleen, kidney, brain, and others. They can form homodimers and heterodimers with other family members and with other transcription factors. The C/EBP proteins contain highly conserved bZIP domain at the C-terminus, an activation domain at the N-terminus and some other regulatory domains (Figure 7) [43]. In hematopoietic cells C/EBPα is necessary mainly for differentiation from lymphoid-myeloid progenitors to granulocytes [60], while C/EBPβ is crucial for specialization of normal and 1,25(OH)$_2$D-induced monocytes and macrophages, as well as for their proper functions [61–64], as it regulates transcription of many monocyte-specific proteins, such as CD14, lactoferrin, or lysozyme [43]. Recently an involvement of C/EBPβ in differentiation-related inhibition of proliferation was reported [65]. Because of alternative translation initiations sites, two different products of C/EBPα (42 kDa, 30 kDa) and three products of C/EBPβ genes are translated (55 kDa, 49 kDa, 20 kDa) [43, 66]. As presented in Figure 7, 30 kDa form of C/EBPα and 20 kDa form of C/EBPβ are devoid of portions of N-termini where transactivation domains are localized. They are, however, still able to dimerize, and therefore they can play an inhibitory function. Experiments done by groups of Studzinski and ours have shown that after exposure of HL60 to 1,25(OH)$_2$D C/EBPα was only transiently upregulated in an early phase of differentiation, whilst upregulation of C/EBPβ was strong, long-lasting and correlated with the differentiation process [67]. Specially two shorter C/EBPβ isoforms were abundant in differentiating cells and their increase correlated with acquisition of monocytic differentiation markers, such as CD11b, CD14 [67], or CD11c (not shown).

Transcriptional activity of C/EBP proteins is regulated not only by their length and dimerization but most importantly by their intracellular localization. As transcription factors, C/EBPs must enter the cell nuclei to bind CCAAT box motif in their target gene promoters [44, 66]. Therefore, cellular trafficking of C/EBPs was extensively studied by our group. Again, studied cells were fractionated into cytosol and nuclei. As shown in Figure 8, after exposure of the cells to 1,25(OH)$_2$D, the full length isoform of C/EBPβ-1 (55 kDa) is present in either cytoplasmic or nuclear fraction, whilst

Cell line	HL60				THP-1				AML-M4			
$1,25(OH)_2D$	0		96 h		0		96 h		0		96 h	
Fraction	C	N	C	N	C	N	C	N	C	N	C	N
Lane	1	2	3	4	5	6	7	8	9	10	11	12

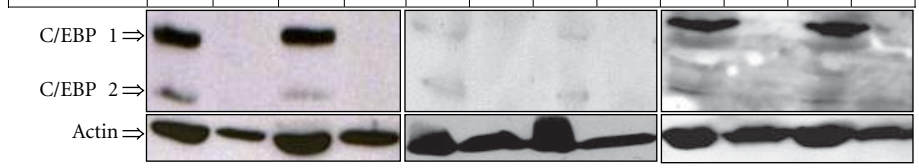


Figure 9: Subcellular localization of C/EBPα isoforms in AML cells. HL60 and AML-M4 blasts from patient's peripheral blood were exposed to 10 nM $1,25(OH)_2D$ for 96 h and then were fractionated into cytoplasmic (C) and nuclear (N) fractions. The lysates from equal numbers of cells were separated in SDS-PAGE and blotted to the membrane. The membrane was probed with anti-C/EBPα antibody. Actin was probed as a control of equal loading and transfer of proteins.

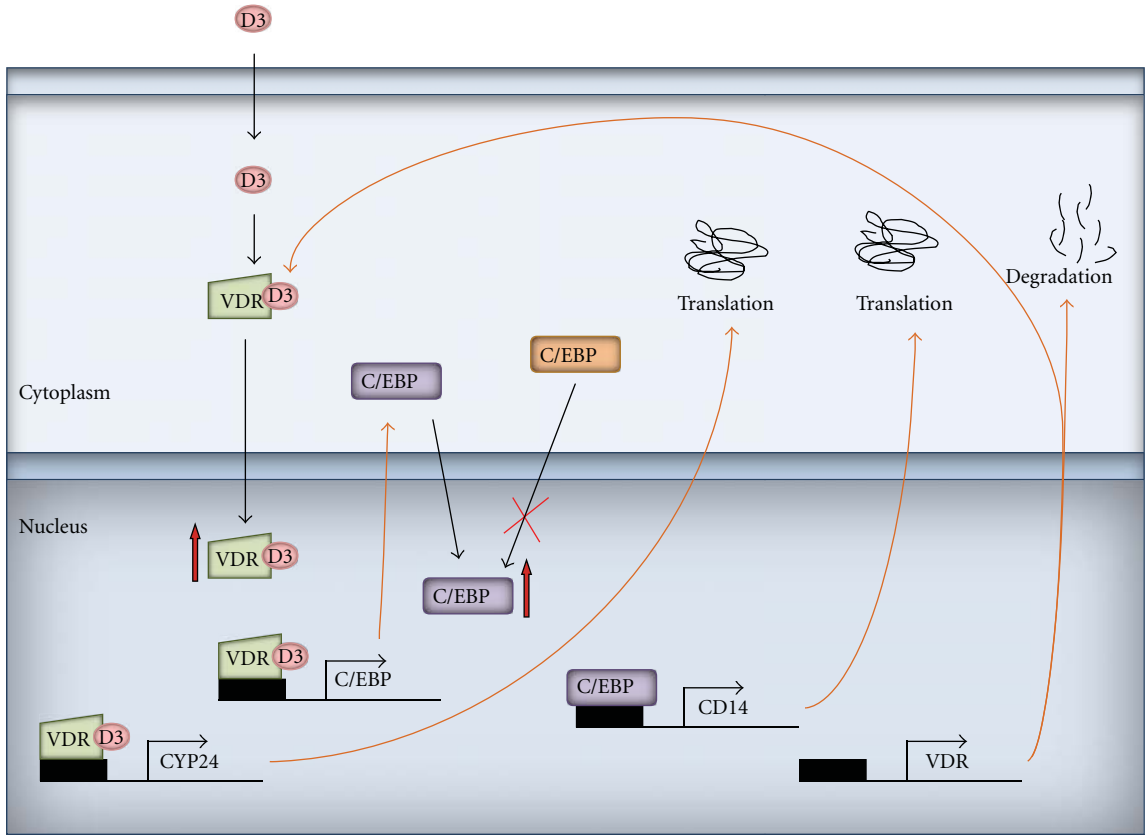


Figure 10: Overview of $1,25(OH)_2D$-induced intracellular events in AML cells. Description is in the text.

the majority of shorter isoforms C/EBPβ-2 (49 kDa) and C/EBPβ-3 (20 kDa) are placed in the nuclei of the cells. Similar results were observed in various AML cell lines, such as THP-1, MV4-11, or MOLM-13 and in some samples of AML blasts isolated from patients. But when localization of C/EBPα was tested in fractionated cells, it appeared to be cytoplasmic, and no translocation was noticed after various times of exposure of the cells to $1,25(OH)_2D$. As examples, HL60, THP-1 cell lines, and AML blasts from patient's peripheral blood are presented (Figure 9). These findings suggest that, in AML cells, even if not mutated, C/EBPα is transcriptionally inactive what leads to the disturbances in granulopoiesis. Elevated expression of C/EBPβ and its nuclear translocation induced by $1,25(OH)_2D$ can possibly allow the cells to bypass this block and switch differentiation into monocyte/macrophage pathway. The above hypothesis was presented before [68] and is further supported by the findings shown here, that in AML cells C/EBPα is localized in the cytosol, where it cannot exert its transcriptional activity.

7. Conclusions

Exposure of AML cells to $1,25(OH)_2D$ or to its analogs triggers a long series of events which eventually lead to

acquisition of monocyte/macrophage phenotype and function (some of them presented in Figure 10). The detailed description of $1,25(OH)_2D$-induced differentiation in cell line models, as well as in AML blasts isolated from patients might be important for future therapeutic applications of $1,25(OH)_2D$ analogs. It is especially significant to learn which differentiation pathways are blocked in certain AML subtypes and how they could be bypassed with help of pharmacological agents.

Acknowledgment

Part of the research described in this paper was supported by Wroclaw Research Center EIT+ within the project Biotechnologies and Advanced Medical Technologies—BioMed (POIG 01.01.02-02-003/08-00) financed by the European Regional Development Fund (Operational Program Innovative Economy, 1.1.2).

References

[1] E. Abe, C. Miamura, H. Sakagami et al., "Differentiation of mouse myeloid leukemia cells induced by 1-alpha,25-dihydroxyvitamin D_3," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, pp. 4990–4994, 1981.

[2] Y. Honma, M. Hozumi, E. Abe et al., "1-Alpha,25-dihydroxyvitamin D_3 and 1-alpha-hydroxyvitamin D_3 prolong survival time of mice inoculated with myeloid leukemia cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 80, pp. 201–204, 1983.

[3] R. Okamoto, T. Akagi, and P. Koeffler, "Vitamin D compounds and myelodysplastic syndrome," *Leukemia and Lymphoma*, vol. 49, no. 1, pp. 12–13, 2008.

[4] D. Nowak, D. Stewart, and H. P. Koeffler, "Differentiation therapy of leukemia: 3 Decades of development," *Blood*, vol. 113, no. 16, pp. 3655–3665, 2009.

[5] Q. T. Luong and H. P. Koeffler, "Vitamin D compounds in leukemia," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 97, no. 1-2, pp. 195–202, 2005.

[6] M. Mizwicki and A. Norman, "Vitamin D sterol/VDR conformational dynamics and nongenomic actions," in *Vitamin D*, D. Feldman, J. Pike, and J. Adams, Eds., Academic Press, 3rd edition, 2011.

[7] W. Pike, M. Meyer, and S. Lee, "The vitamin D receptor: biochemical, molecular, biological, and genomic era investigations," in *Vitamin D*, D. Feldman, J. Pike, and J. Adams, Eds., pp. 97–135, Academic Press, 3rd edition, 2011.

[8] C. Rachez, B. D. Lemon, Z. Suldan et al., "Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex," *Nature*, vol. 398, no. 6730, pp. 824–828, 1999.

[9] A. Aranda and A. Pascual, "Nuclear hormone receptors and gene expression," *Physiological Reviews*, vol. 81, no. 3, pp. 1269–1304, 2001.

[10] M. R. Haussler, C. A. Haussler, L. Bartik et al., "Vitamin D receptor: molecular signaling and actions of nutritional ligands in disease prevention," *Nutrition Reviews*, vol. 66, supplement 2, pp. S98–S112, 2008.

[11] N. Rochel and D. Moras, "Structural basis for ligand activity in VDR," in *Vitamin D*, D. Feldman, J. Pike, and J. Adams, Eds., pp. 171–191, Academic Press, 3rd edition, 2011.

[12] M. Kizaki, A. W. Norman, J. E. Bishop, C. W. Lin, A. Karmakar, and H. P. Koeffler, "1,25-dihydroxyvitamin D_3 receptor RNA: expression in hematopoietic cells," *Blood*, vol. 77, no. 6, pp. 1238–1247, 1991.

[13] E. Marcinkowska, A. Wiedlocha, and C. Radzikowski, "1,25-Dihydroxyvitamin D_3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL-60 cells," *Biochemical and Biophysical Research Communications*, vol. 241, pp. 419–426, 1997.

[14] E. Marcinkowska, "Evidence that activation of MEK1,2/erk1,2 signal transduction pathway is necessary for calcitriol-induced differentiation of HL-60 cells," *Anticancer Research*, vol. 21, no. 1, pp. 499–504, 2001.

[15] X. Wang and G. Studzinski, "Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1,25-dihydroxyvitamin D_3-induced differentiation of HL60 cells," *Journal of Cellular Biochemistry*, vol. 80, pp. 471–482, 2001.

[16] X. Wang, J. Rao, and G. P. Studzinski, "Inhibition of p38 MAP kinase activity up-regulates multiple MAP kinase pathways and potentiates 1,25-dihydroxyvitamin D_3-induced differentiation of human leukemia HL60 cells," *Experimental Cell Research*, vol. 258, no. 2, pp. 425–437, 2000.

[17] X. Chen-Deutsch, E. Garay, J. Zhang, J. S. Harrison, and G. P. Studzinski, "c-Jun N-terminal kinase 2 (JNK2) antagonizes the signaling of differentiation by JNK1 in human myeloid leukemia cells resistant to vitamin D," *Leukemia Research*, vol. 33, no. 10, pp. 1372–1378, 2009.

[18] M. P. Velders, M. Fatima Macedo, M. Provenzano et al., "Inhibition of p38MAP kinase potentiates the JNK/SAPK pathway and AP-1 activity in monocytic but not in macrophage or granulocytic differentiation of HL60 cells," *Journal of Cellular Biochemistry*, vol. 82, no. 1, pp. 68–77, 2001.

[19] Z. Hmama, D. Nandan, L. Sly, K. L. Knutson, P. Herrera-Velit, and N. E. Reiner, "1α,25-dihydroxyvitamin D_3-induced myeloid cell differentiation is regulated by a vitamin D receptor-phosphatidylinositol 3-kinase signaling complex," *Journal of Experimental Medicine*, vol. 190, no. 11, pp. 1583–1594, 1999.

[20] A. Moeenrezakhanlou, L. Shephard, L. Lam, and N. E. Reiner, "Myeloid cell differentiation in response to calcitriol for expression CD11b and CD14 is regulated by myeloid zinc finger-1 protein downstream of phosphatidylinositol 3-kinase," *Journal of Leukocyte Biology*, vol. 84, no. 2, pp. 519–528, 2008.

[21] Y. Miyauchi, T. Michigami, N. Sakaguchi et al., "Importin 4 is responsible for ligand-independent nuclear translocation of vitamin D receptor," *The Journal of Biological Chemistry*, vol. 280, no. 49, pp. 40901–40908, 2005.

[22] S. R. Wente, M. P. Rout, and G. Blobel, "A new family of yeast nuclear pore complex proteins," *Journal of Cell Biology*, vol. 119, no. 4, pp. 705–723, 1992.

[23] J. Yamada, J. L. Phillips, S. Patel et al., "A bimodal distribution of two distinct categories of intrinsically disordered structures with separate functions in FG nucleoporins," *Molecular and Cellular Proteomics*, vol. 9, no. 10, pp. 2205–2224, 2010.

[24] T. D. Allen, J. M. Cronshaw, S. Bagley, E. Kiseleva, and M. W. Goldberg, "The nuclear pore complex: mediator of translocation between nucleus and cytoplasm," *Journal of Cell Science*, vol. 113, no. 10, pp. 1651–1659, 2000.

[25] R. S. Faustino, T. J. Nelson, A. Terzic, and C. Perez-Terzic, "Nuclear transport: target for therapy," *Clinical Pharmacology and Therapeutics*, vol. 81, no. 6, pp. 880–886, 2007.

[26] J. Barsony and K. Prufer, "Vitamin D receptor and retinoid X receptor interactions in motion," *Vitamins and Hormones*, vol. 65, pp. 345–IN3, 2002.

[27] R. Yasmin, R. M. Williams, M. Xu, and N. Noy, "Nuclear import of the retinoid X receptor, the vitamin D receptor, and

their mutual heterodimer," *The Journal of Biological Chemistry*, vol. 280, no. 48, pp. 40152–40160, 2005.

[28] K. Prüfer and J. Barsony, "Retinoid X Receptor dominates the nuclear import and export of the unliganded vitamin D receptor," *Molecular Endocrinology*, vol. 16, no. 8, pp. 1738–1751, 2002.

[29] J. Hsieh, Y. Shimizu, S. Minoshima et al., "Novel nuclear localization signal between the two DNA-binding zinc fingers in the human vitamin D receptor," *Journal of Cellular Biochemistry*, vol. 70, pp. 94–109, 1998.

[30] B. E. Black, J. M. Holaska, F. Rastinejad, and B. M. Paschal, "DNA binding domains in diverse nuclear receptors function as nuclear export signals," *Current Biology*, vol. 11, no. 22, pp. 1749–1758, 2001.

[31] E. Gocek and G. P. Studzinski, "Vitamin D and differentiation in cancer Signaling differentiation E. Gocek and G. P. Studzinski," *Critical Reviews in Clinical Laboratory Sciences*, vol. 46, no. 4, pp. 190–209, 2009.

[32] Q. Wang, G. Studzinski, F. Chen, F. Coffman, and L. Harrison, "p53/56(lyn) antisense shifts the 1,25-dihydroxyvitamin D_3-induced G1/S block in HL60 cells to S phase," *Journal of Cellular Physiology*, vol. 183, pp. 238–246, 2000.

[33] F. D. Coffman and G. P. Studzinski, "Differentiation-related mechanisms which suppress DNA replication," *Experimental Cell Research*, vol. 248, no. 1, pp. 58–73, 1999.

[34] Y. Zhang, J. Zhang, and G. P. Studzinski, "AKT pathway is activated by 1,25-dihydroxyvitamin D_3 and participates in its anti-apoptotic effect and cell cycle control in differentiating HL60 cells," *Cell Cycle*, vol. 5, no. 4, pp. 447–451, 2006.

[35] E. Marcinkowska, A. Kutner, and C. Radzikowski, "Cell differentiating and anti-proliferative activity of side-chain modified analogues of 1,25-dihydroxyvitamin D_3," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 67, no. 1, pp. 71–78, 1998.

[36] C. C. Uphoff and H. G. Drexler, "Biology of monocyte-specific esterase," *Leukemia and Lymphoma*, vol. 39, no. 3-4, pp. 257–270, 2000.

[37] M. Steiner, I. Priel, J. Giat, J. Levy, Y. Sharoni, and M. Danilenko, "Carnosic acid inhibits proliferation and augments differentiation of human leukemic cells induced by 1,25-dihydroxyvitamin D_3 and retinoic acid," *Nutrition and Cancer*, vol. 41, no. 1-2, pp. 135–144, 2001.

[38] G. P. Studzinski, B. Rathod, Q. M. Wang, J. Rao, and F. Zhang, "Uncoupling of cell cycle arrest from the expression of monocytic differentiation markers in HL60 cell variants," *Experimental Cell Research*, vol. 232, no. 2, pp. 376–387, 1997.

[39] A. J. Brown and E. Slatopolsky, "Vitamin D analogs: therapeutic applications and mechanisms for selectivity," *Molecular Aspects of Medicine*, vol. 29, no. 6, pp. 433–452, 2008.

[40] H. Baurska, A. Klopot, M. Kielbinski et al., "Structure-function analysis of vitamin D_2 analogs as potential inducers of leukemia differentiation and inhibitors of prostate cancer proliferation," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 126, no. 1-2, pp. 46–54, 2011.

[41] E. Gocek, M. Kiełbiński, H. Baurska, O. Haus, A. Kutner, and E. Marcinkowska, "Different susceptibilities to 1,25-dihydroxyvitamin D_3-induced differentiation of AML cells carrying various mutations," *Leukemia Research*, vol. 34, no. 5, pp. 649–657, 2010.

[42] E. Gocek, M. Kiełbiński, P. Wyłób, A. Kutner, and E. Marcinkowska, "Side-chain modified vitamin D analogs induce rapid accumulation of VDR in the cell nuclei proportionately to their differentiation-inducing potential," *Steroids*, vol. 73, no. 14, pp. 1359–1366, 2008.

[43] D. P. Ramji and P. Foka, "CCAAT/enhancer-binding proteins: structure, function and regulation," *Biochemical Journal*, vol. 365, no. 3, pp. 561–575, 2002.

[44] J. Tsukada, Y. Yoshida, Y. Kominato, and P. E. Auron, "The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation," *Cytokine*, vol. 54, no. 1, pp. 6–19, 2011.

[45] A. Opolski, J. Wietrzyk, A. Chrobak et al., "Antiproliferative activity in vitro of side-chain analogues of calcitriol against various human normal and cancer cell lines," *Anticancer Research*, vol. 19, no. 6, pp. 5217–5222, 1999.

[46] M. Chodyński, J. Wietrzyk, E. Marcinkowska, A. Opolski, W. Szelejewski, and A. Kutner, "Synthesis and antiproliferative activity of side-chain unsaturated and homologated analogs of 1,25-dihydroxyvitamin D_2 (24E)-(1S)-24-Dehydro-24a-homo-1,25-dihydroxyergocalciferol and congeners," *Steroids*, vol. 67, no. 9, pp. 789–798, 2002.

[47] E. Marcinkowska and A. Kutner, "Side-chain modified vitamin D analogs require activation of both PI 3-K and erk1,2 signal transduction pathways to induce differentiation of human promyelocytic leukemia cells," *Acta Biochimica Polonica*, vol. 49, no. 2, pp. 393–406, 2002.

[48] J. Wietrzyk, D. Nevozhay, M. Milczarek, B. Filip, and A. Kutner, "Toxicity and antitumor activity of the vitamin D analogs PRI-1906 and PRI-1907 in combined treatment with cyclophosphamide in a mouse mammary cancer model," *Cancer Chemotherapy and Pharmacology*, vol. 62, no. 5, pp. 787–797, 2008.

[49] G. Marquez Balbas, M. Sanchez Regana, and P. Umbert Millet, "Tacalcitol ointment for the treatment of nail psoriasis," *Journal of Dermatological Treatment*, vol. 1, pp. 1–3, 2009.

[50] M. Rodríguez-Martín, M. García Bustínduy, M. Sáez Rodríguez, and A. Noda Cabrera, "Randomized, double-blind clinical trial to evaluate the efficacy of topical tacalcitol and sunlight exposure in the treatment of adult nonsegmental vitiligo," *British Journal of Dermatology*, vol. 160, no. 2, pp. 409–414, 2009.

[51] Y. Ji, A. Kutner, A. Verstuyf, L. Verlinden, and G. P. Studzinski, "Derivatives of vitamins D_2 and D_3 activate three MAPK pathways and upregulate pRb expression in differentiating HL60 cells.," *Cell Cycle*, vol. 1, no. 6, pp. 410–415, 2002.

[52] J. Wietrzyk, M. Chodyński, H. Fitak, E. Wojdat, A. Kutner, and A. Opolski, "Antitumor properties of diastereomeric and geometric analogs of vitamin D_3," *Anti-Cancer Drugs*, vol. 18, no. 4, pp. 447–457, 2007.

[53] H. Quentmeier, J. Reinhardt, M. Zaborski, and H. G. Drexler, "FLT3 mutations in acute myeloid leukemia cell lines," *Leukemia*, vol. 17, no. 1, pp. 120–124, 2003.

[54] E. Gocek, M. Kiełbiński, and E. Marcinkowska, "Activation of intracellular signaling pathways is necessary for an increase in VDR expression and its nuclear translocation," *FEBS Letters*, vol. 581, no. 9, pp. 1751–1757, 2007.

[55] D. Picard, "Chaperoning steroid hormone action," *Trends in Endocrinology and Metabolism*, vol. 17, no. 6, pp. 229–235, 2006.

[56] I. Grad and D. Picard, "The glucocorticoid responses are shaped by molecular chaperones," *Molecular and Cellular Endocrinology*, vol. 275, no. 1-2, pp. 2–12, 2007.

[57] E. Marcinkowska and E. Gocek, "Heat shock protein 90 interacts with vitamin D receptor in human leukemia cells," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 121, no. 1-2, pp. 114–116, 2010.

[58] D. G. Tenen, R. Hromas, J. D. Licht, and D. E. Zhang, "Transcription factors, normal myeloid development, and leukemia," *Blood*, vol. 90, no. 2, pp. 489–519, 1997.

[59] G. P. Studzinski, E. Garay, R. Patel, J. Zhang, and X. Wang, "Vitamin D receptor signaling of monocytic differentiation in human leukemia cells: role of MAPK pathways in transcription factor activation," *Current Topics in Medicinal Chemistry*, vol. 6, no. 12, pp. 1267–1271, 2006.

[60] A. D. Friedman, "Transcriptional control of granulocyte and monocyte development," *Oncogene*, vol. 26, no. 47, pp. 6816–6828, 2007.

[61] T. Nagamura-Inoue, T. Tamura, and K. Ozato, "Transcription factors that regulate growth and differentiation of myeloid cells," *International Reviews of Immunology*, vol. 20, no. 1, pp. 83–105, 2001.

[62] Y. Ji and G. P. Studzinski, "Retinoblastoma protein and CCAAT/enhancer-binding protein β are required for 1,25-dihydroxyvitamin D_3-induced monocytic differentiation of HL60 cells," *Cancer Research*, vol. 64, no. 1, pp. 370–377, 2004.

[63] B. Hackanson, K. L. Bennett, R. M. Brena et al., "Epigenetic modification of CCAAT/enhancer binding protein α expression in acute myeloid leukemia," *Cancer Research*, vol. 68, no. 9, pp. 3142–3151, 2008.

[64] T. Akagi, N. H. Thoennissen, A. George et al., "In vivo deficiency of both C/EBPβ and C/EBP results in highly defective myeloid differentiation and lack of cytokine response," *PLoS One*, vol. 5, no. 11, Article ID e15419, 2010.

[65] R. Gutsch, J. D. Kandemir, D. Pietsch et al., "CCAAT/enhancer-binding protein β inhibits proliferation in monocytic cells by affecting the retinoblastoma protein/E2F/cyclin E pathway but is not directly required for macrophage morphology," *The Journal of Biological Chemistry*, vol. 286, no. 26, pp. 22716–22729, 2011.

[66] C. Nerlov, "Transcriptional and translational control of C/EBPs: the case for "deep" genetics to understand physiological function," *BioEssays*, vol. 32, no. 8, pp. 680–686, 2010.

[67] E. Marcinkowska, E. Garay, E. Gocek, A. Chrobak, X. Wang, and G. P. Studzinski, "Regulation of C/EBPβ isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D_3," *Experimental Cell Research*, vol. 312, no. 11, pp. 2054–2065, 2006.

[68] E. Marcinkowska, X. Wang, and G. Studzinski, "C/EBPb: a candidate for a major player in vitamin D-induced monocytic differentiation of human leukemia cells," in *Vitamin D: New Research*, V. Stolzt, Ed., pp. 25–40, Nova Science, New York, NY, USA, 2006.

Hindawi Publishing Corporation
Leukemia Research and Treatment
Volume 2012, Article ID 671702, 19 pages
doi:10.1155/2012/671702

Review Article

The Interface between BCR-ABL-Dependent and -Independent Resistance Signaling Pathways in Chronic Myeloid Leukemia

Gabriela Nestal de Moraes, Paloma Silva Souza, Fernanda Casal de Faria Costas, Flavia Cunha Vasconcelos, Flaviana Ruade Souza Reis, and Raquel Ciuvalschi Maia

Laboratório de Hemato-Oncologia Celular e Molecular, Programa de Pesquisa em Hemato-Oncologia Molecular, Instituto Nacional de Câncer (INCA), Praça da Cruz Vermelha 23, 6° andar, Centro, 20230-130 Rio de Janeiro, RJ, Brazil

Correspondence should be addressed to Raquel Ciuvalschi Maia, rcmaia@inca.gov.br

Received 22 November 2011; Accepted 10 February 2012

Academic Editor: Philip J. Hughes

Chronic myeloid leukemia (CML) is a clonal hematopoietic disorder characterized by the presence of the Philadelphia chromosome which resulted from the reciprocal translocation between chromosomes 9 and 22. The pathogenesis of CML involves the constitutive activation of the BCR-ABL tyrosine kinase, which governs malignant disease by activating multiple signal transduction pathways. The BCR-ABL kinase inhibitor, imatinib, is the front-line treatment for CML, but the emergence of imatinib resistance and other tyrosine kinase inhibitors (TKIs) has called attention for additional resistance mechanisms and has led to the search for alternative drug treatments. In this paper, we discuss our current understanding of mechanisms, related or unrelated to BCR-ABL, which have been shown to account for chemoresistance and treatment failure. We focus on the potential role of the influx and efflux transporters, the inhibitor of apoptosis proteins, and transcription factor-mediated signals as feasible molecular targets to overcome the development of TKIs resistance in CML.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that results from the reciprocal translocation of the *ABL1* oncogene on chromosome 9 with the breakpoint cluster region (*BCR*) gene on chromosome 22 [$t(9; 22)$], leading to the formation of the BCR-ABL oncoprotein. The shortened chromosome 22 formed by this translocation is the Philadelphia (Ph) chromosome. The *BCR-ABL* fusion oncogene, which is responsible for the pathogenesis of CML, has greatly enhanced ABL1 tyrosine kinase constitutive activity [1]. CML is characterized by a biphasic evolutive course. Most patients are diagnosed in the chronic phase (CML-CP), which is characterized by the absence of symptoms in half of the patients. However, a prominent leukocytosis is frequently observed by routine testing. In the other half of patients, symptoms are common and include splenomegaly, weight loss, lethargy, and anemia [2]. The disease may progress either directly to blast phase (BP) or through an intermediate accelerated phase (AP). The time course for progression to BP is variable and the molecular mechanisms underlying disease progression are extremely complex. BCR-ABL-dependent pathways to blast transformation include an increase in genomic instability, telomere shortening, loss of tumor-suppressor function, and inhibition of tumor suppressors with cell regulatory functions [2, 3].

In order to identify prognostic factors for CML patients, many clinical and biological characteristics have been analyzed. Sokal risk score (based on spleen size, age, platelet count, and peripheral blood blast) is a prognostic factor widely used for prediction of cytogenetic response and of progression-free and overall survival in CML-CP with imatinib as front-line therapy. Other factor predictors for therapy response include OCT-1 activity, *ABCB1*/P-glycoprotein overexpression and polymorphisms, *in vivo* measurement of the Crkl phosphorylation, and molecular response [4].

The treatment of CML-CP can be divided into pre-imatinib and post-imatinib era. Prior to the imatinib era, busulphan and interferon-α recombinant [5, 6] were used to

control and to prolong CML survival in the CP phase, but allogenic stem-cell transplantation was, and is still, the only therapy with potential for curing CML patients [7]. After the introduction of imatinib, a potent tyrosine kinase inhibitor (TKI), there was a dramatic change in the CML outcome. Imatinib acts by binding to the BCR-ABL protein in the inactive conformation and is unable to bind to the active configuration [8]. The survival rate attributed to imatinib is arguably more elevated than interferon-based therapy [9]. In addition, imatinib is generally well tolerated [10]. Imatinib treatment is associated with high rates of complete cytogenetic and major molecular responses in patients with CML-CP. On the other hand, despite improvements related to survival by using imatinib or other TKIs, CML-BP prognosis remains disappointing [11].

Currently, imatinib is the standard therapy for all CML phases [12–14]. Despite the clinical success with imatinib demonstrating long-term survival for the majority of patients, one-third of patients need an alternative therapy, frequently a second-generation TKI, such as dasatinib and nilotinib. Patients who need second-line therapy include those with imatinib intolerance [10] or mainly primary or acquired imatinib resistance [15, 16].

The most common mechanism of resistance to imatinib is the development of point mutations or amplification of the *BCR-ABL* gene, which alters the kinase domain (KD) of BCR-ABL and is responsible for imatinib loss of efficacy [17]. KD mutations can be found at any phase of CML. Not all KD mutations are responsible for TKI resistance. However, T315I mutation is generally resistant to all TKIs [18].

BCR-ABL acts with other multiple cellular and genetic events that accumulate progressively to drive the disease into the blast phase. Therefore, additional mechanisms—dependent or independent to BCR-ABL—may also account for resistance to imatinib treatment and result in a poor outcome. In this review, the role of efflux and influx transporters, inhibitor of apoptosis proteins (IAP), and transcription factors as additional mechanisms responsible for chemoresistance in CML will be discussed.

1.1. Efflux and Influx Transporters. The multidrug resistance (MDR) phenotype related to increased expression of efflux pumps, such as ABCB1/P-glycoprotein (Pgp) and ABCG2/breast-cancer-related protein (BCRP), is one of the most studied mechanisms of resistance in CML. More recently, the decrease in influx transporters, such as the organic cation transporter-1 (Oct-1), has also emerged as a mechanism responsible for inefficient drug uptake and consequent treatment failure [16, 19].

1.1.1. ABCB1/P-Glycoprotein. The most common mechanism developed by tumor cells to escape a drug-induced death is displayed in intrinsic or acquired MDR phenotype by the overexpression of the drug-efflux protein ABCB1 [20, 21]. ABCB1, a product of the *ABCB1* gene, was first described in 1976 by Juliano and Ling, who observed a cell surface glycoprotein that altered drug permeability in hamster drug-resistant cells. Human cells also express ABCB1 on the cell surface, acting as a drug efflux pump and, consequently, decreasing intracellular drug concentration [22, 23]. Meanwhile, physiological ABCB1 expression has been identified in some tissues, particularly on the membranes of kidney tubules, in the canalicular membranes of hepatocytes, in the gastrointestinal tract, at blood tissue barriers, in the placenta, and in blood cells including $CD34^+$ hematopoietic stem cells, natural killer cells, antigen-presenting dendritic cells (DC), and T and B lymphocytes [24–28]. Its physiological function suggests a protection against potentially toxic compounds and harmful substances found in the blood stream. Studies on ABCB1 knockout mice showed no physiological abnormalities under normal conditions, although these animals display hypersensitivity to drugs and an increase in ABCB1 substrate accumulation [27, 29–31].

Clinical insensitivity to anticancer agents is mainly attributed to an elevated expression of ABCB1, which is related to treatment failure associated with lower remission and survival rates in some types of cancer, including leukemias [32–34]. Meanwhile, gene and protein expressions of ABCB1 are commonly acquired or increased during the course of chemotherapy, which make drug treatment a responsible factor for MDR [35, 36]. Other extrinsic factors may induce MDR by acquisition of ABCB1 expression. Levchenko et al. [37] showed that ABCB1, and, consequently, MDR are transferred by direct membrane contact of tumor cells. Moreover, resistant tumor cells may release membrane microparticles carrying surface ABCB1. The shared microparticles can bind to receptor cells, spread ABCB1 and, consequently, induce MDR phenotype [38].

Even though the ABCB1 efflux functions, other functions for this transporter have been studied. Studies have shown that the resistance induced by ABCB1 is also associated with the inhibition of cell death, and ABCB1 promotes additional protection to caspases-dependent apoptosis, UV radiation, serum starvation condition, and spontaneous apoptosis [39–42]. Recently, our group demonstrated that ABCB1 expression induced by drug treatment promotes resistance to apoptosis in BCR-ABL cells independently of its drug-efflux activity [43].

ABCB1 is related to resistance phenotype in some leukemias and it has been studied in advanced CML. A randomized trial evaluated the relevance of ABCB1 expression in CML patients. The authors observed that the response to cytarabine and daunorubicin was significantly related with both ABCB1 expression and function mainly in the blast phase. For this reason, chemotherapy resistance in CML-BP patients should be considered multifactorial and cannot be associated only with BCR-ABL [44–47]. Our group recently demonstrated that CML patients show high levels of ABCB1 expression independently of CML phases. Nevertheless, we showed that ABCB1 expression is more frequent than multidrug-resistant protein 1 (MRP1) in CML-BP [48].

In vitro data suggest that imatinib is able to induce ABCB1 in sensitive CML cell lines and, as a result, ABCB1 activity may confer resistance to this drug [49–51]. Mahon et al. [52] demonstrated that a multidrug-resistant CML cell line displayed resistance to many drugs including imatinib and the induced overexpression of *ABCB1* gene by retroviral

transduction in BCR-ABL cell line also leads to imatinib resistance. Moreover, Rumpold et al., [53] showed that a stable silencing of ABCB1 in imatinib-resistant CML cell lines abolished ABCB1-efflux substrates and induced sensibility to imatinib. Regardless of the *in vitro* data, there is no consistent evidence for this resistance *in vivo*, although several studies have discussed the role of ABCB1 in imatinib-resistant CML patients [54]. Zong et al. [55] demonstrated that bone marrow mice cells Mdr1a/1b-null transduced with *BCR-ABL* display a similar response to imatinib, which is related to increased peripheral white blood cells counts and marked hepatosplenomegaly, compared with *BCR-ABL*-transduced wild-type bone marrow. The authors concluded that the expression of ABCB1 in hematopoietic stem cells does not interfere with imatinib resistance. Another *in vivo* study revealed that imatinib treatment in CML patients in the accelerated phase induced an increase of ABCB1-positive cells with efflux activity. However, in imatinib-resistant CML patients, the efflux activity was independent of ABCB1 expression, suggesting participation of other ABC transporters [56]. Hatziieremia et al. [57] inhibited ABCB1 using PSC833 in $CD34^+$ cells from CML-CP patients and did not observe imatinib efficiency in eliminating CML cells.

Although these previously described works do not identify the role of ABCB1 in imatinib resistance, studies in polymorphisms of ABCB1 have shown the importance of ABCB1 in CML treatment resistance. Moreover, this kind of study may provide information for the prediction of drug disposition in a specific way and promote better response to imatinib in CML patients [58, 59]. Dulucq et al. [60] analyzed 1236C>T, 2677G>T/A, and 3435C>T ABCB1 single nucleotide polymorphisms (SNPs) in CML patients treated with imatinib. The authors observed that allele G in 2677G>T/A polymorphism was associated with the worst response to imatinib. In a Chinese population, Ni et al. [61] observed more imatinib resistance in patients homozygous for 1236T allele and 3435 TT/CT genotypes.

Studies have suggested that second- and third-generation TKIs can overcome imatinib resistance [62, 63]. There are studies suggesting that nilotinib does not induce resistance in CML cells through ABCB1 overexpression [64]. Nevertheless, Mahon et al. [65] developed nilotinib-resistant CML cell lines and observed that nilotinib is a substrate for ABCB1. Moreover, concomitant overexpression of ABCB1 and BCR-ABL provides nilotinib resistance in CML cells. Studies also revealed the interaction of dasatinib and ABCB1 efflux protein. Giannoudis et al. [66] showed that cell lines BCR-ABL (positive or not) are able to extrude dasatinib through ABCB1 activity. In concordance, Hiwase et al. [67] demonstrated that ABCB1 is able to transport dasatinib from CML cells. These studies show the importance of researching more about ABCB1 expression, function, and inhibition.

An important strategy to try reversing clinical MDR involves modulation or inhibition of ABCB1. The cyclosporine A (CsA) is capable of regulating the efflux function of ABCB1 dependently on its concentration in cancer cells [68, 69]. Some studies in hematological cancer have shown the benefits of CsA on reversing MDR or potentiating drug effects [70]. In a clinical study of our group, we evaluated the effect of CsA on the circumvention of leukemia patients MDR *in vitro*. Our data showed that combination of CsA and etoposide (VP-16) could induce a good response in ABCB1-positive CML patients [71]. In the same year, we also published a case report showing that the cytotoxic effect of VP-16 was enhanced in combination with CsA in blast cells of CML. Moreover, the patient returned from blast phase to chronic phase [72]. All these studies and others emphasized the importance of reversing the MDR phenotype.

1.1.2. BCRP/ABCG2. Another important efflux pump associated with chemotherapy resistance in CML is BCRP or ABCG2, coded by the gene *ABCG2*. ABCG2 is a 72-kDa protein composed of 665 amino acids. It has an N-terminal ATP-binding domain (NBF) and a C-terminal transmembrane domain (TMD), a structure half the size and in reverse configuration to most other ABC proteins comprising two NBFs and two TMDs. Because ABCG2 is a half-transporter, it is believed to homodimerize, or possibly oligomerize, in order to function [73].

Fetsch et al., [74] reported high levels of ABCG2 expression in normal placenta, interstitial cells of testes, endocervical cells of uterus, squamous epithelium of cervix, kidney, hepatocytes, pancreas, and small and large intestinal mucosa/epithelial cells. The first reported chemotherapy agent substrate of ABCG2 was mitoxantrone [75]. Other chemotherapeutic substrates include flavopiridol, topotecan, methotrexate, and the TKIs imatinib, gefitinib, and erlotinib [76]. If the amino acid at position 482 is mutated, mitoxantrone transport is more efficient and ABCG2 can additionally transport rhodamine 123 and anthracyclines such as doxorubicin and bisantrene [77, 78].

It was demonstrated that TKI had high-affinity interaction with ABCG2 and that it occurs at submicromolar concentrations [79, 80]. Although other TKIs promote ATPase activity, imatinib was the only one able to inhibit it, suggesting that this drug acts as a modulator agent. In addition, imatinib has promoted the accumulation of a fluorescent substrate inside the cells which reinforced its role as a modulator. Using a different methodology, Houghton et al. [81] demonstrated that overexpression of ABCG2 was not able to confer resistance to imatinib, suggesting that it is not a substrate for this transporter. In addition, imatinib promoted the accumulation of topotecan in functional ABCG2-expressing cell lines, indicating a role of imatinib as a modulator but not as a competitor. Conversely, Ko-143, an ABCG2 specific modulator, could increase the imatinib accumulation in ABCG2-overexpressing cell lines, suggesting its role as a competitor. Interestingly, mitoxantrone accumulation in the same cell lines was increased by the addition of imatinib, suggesting its role as a modulator. These findings suggest that imatinib can be both a substrate and a modulator [82]. Furthermore, Brendel et al. [83] confirmed that ABCG2 expression confers imatinib resistance and reduces imatinib accumulation in K562 cells, effects that are abrogated by the ABCG2 inhibitor fumitremorgin C (FTC). More importantly they observed that differences on imatinib accumulation were only seen when imatinib was used at low

concentrations but not at high concentrations. These data support the idea that imatinib may act as a modulator or a substrate depending on the concentration level. However, there is still no consensus on whether imatinib is a substrate or a modulator of ABCG2 transport. Regardless of its role as a substrate or modulator, imatinib interacts with ABCG2, which may have their effectiveness limited by the overexpression of this protein.

It is believed that CML is a clonal disorder originating from the hematopoietic stem cell (HSC). Graham et al. [84] demonstrated that primitive HSCs expressing BCR-ABL in CML-CP patients were resistant to imatinib. This discovery led to the hypothesis that the resistant population could contribute to the failure of treatment with imatinib. Nakanishi et al. [85] studied the interaction of ABCG2 and imatinib on a cell line expressing BCR-ABL. This cell line was resistant to substrates of ABCG2 as well as imatinib, and the resistance was reversed by inhibiting ABCG2. Another interesting finding was that the initial resistance to imatinib caused by ABCG2 was attenuated by the inhibition of BCR-ABL, suggesting that BCR-ABL regulates the expression of ABCG2 at a later stage of transcription.

Some authors have identified the presence of ABCG2 in a particular group of HSC called "side population" (SP) due to its efflux of the fluorochrome Hoechst 33342 and its ability to reconstitute bone marrow in irradiated mice [86]. Afterward, it was demonstrated that ABCG2 was responsible for the SP in mouse and human bone marrow [87, 88]. *ABCG2*-deficient mice are viable with normal numbers of stem cells. Despite the absence of SP, these data suggest that ABCG2 protein is not necessary for normal hematopoiesis [89]. However, ABCG2 may play a protective role for stem cells, because Zhou et al. [90] demonstrated that stem cells derived from *ABCG2*-deficient mice were more sensitive to cytotoxic substrates.

Once ABCG2 is expressed in the apical membrane of cells in the epithelium of the small intestine and colon, it is very likely that ABCG2 is involved in the active return of drug entering the intestine. This role would be important in reducing the systemic bioavailability of oral drugs such as imatinib. Studies in ABCG2 knockout mice indicate that ABCG2 and ABCB1 appear to regulate the penetration of imatinib into the brain tissue. Imatinib brain penetration in ABCG2 knockout mice was found to be increased [91].

The SNP 421C>A is responsible for decreased plasma membrane expression of ABCG2, reduced ATPase activity, or decreased drug transport [92–94]. Therefore, the daily imatinib dose for patients with the *ABCG2* 421C/C genotype might be higher than for those with the 421C/A or 421A/A genotype [95]. Knowledge of the *ABCG2* 421 genotype could be useful when making dosing decisions aimed at achieving the optimal imatinib exposure.

1.2. SLC22A1/OCT-1 Influx Transporter Protein. Members of the solute carriers (SLCs) superfamily of transporters are known as passive facilitator carriers that allow the passage of solute through the membrane without spending energy [96]. This superfamily is divided into 43 families according to the type of substrate transported and the type of transport. Some families carry specific substrates such as oligopeptides, sugars, phosphatases, or metals, whereas other families are polyspecific, transporting substrates with different sizes and structures [97].

SLC transporters are mostly expressed in the plasma membrane and play a critical role in a variety of physiological cellular processes such as import/export neurotransmitters, nutrients, or metabolites [96]. The family 22 of solute carrier proteins is composed of 12 members mostly of poly-specific transporters. Many members of this family are expressed in the intestine, liver, and kidney, indicating an important role in the absorption and excretion of drugs, xenobiotics, and endogenous compounds that exist as cations at physiological pH. The family is further divided into subgroups according to the substrate and the transport mechanism [96].

A growing number of scientific papers have shown that some chemotherapeutics are substrates for influx transporters. Recently, it was reported that imatinib is transported into the cell, preferably via SLC22A1 (also called OCT-1), and the expression of this transporter is predictive of achieving a complete cytogenetic remission after 6 months of treatment with imatinib [98].

It was reported that the influx of imatinib is temperature dependent, indicating the involvement of an active process of influence. When the cells were incubated with inhibitors of the transporter SLC22A1, the influx of imatinib was significantly reduced [99]. Since then, other studies have been published supporting the hypothesis that imatinib is a substrate for the transporter SLC22A1. White et al. [100] analyzed the activity of SLC22A1 in samples from CML patients before starting treatment with imatinib and compared this with getting a major molecular response at 24 months. In this study, the activity of SLC22A1 was an important determinant of molecular response to imatinib with strong predictive value on the dose. The analysis of SLC22A1 activity before the start of treatment with imatinib was able to identify patients who would need a higher dose of the drug to respond to medical treatment with imatinib.

Besides the SLC22A1 activity, the levels of expression of SLC22A1 may be related to a decreased influx of imatinib. Crossman et al. [101] analyzed the expression of the *SLC22A1* gene in samples from CML patients before starting treatment and observed that the expression of *SLC22A1* was variable and did not differ significantly from levels found in samples of bone marrow healthy individuals. However, patients who responded to treatment with imatinib had significantly higher levels of expression of the *SLC22A1* gene as compared to the group of nonresponders. Despite this and other articles suggesting a direct correlation between SLC22A1 and response to treatment with imatinib, Hu et al. [102] believe that SLC22A1 *per se* is not able to influence the retention of imatinib, as this drug would be a poor substrate for SLC22A1. On the other hand, Wang et al. [98] suggested that clinical responses to imatinib could be affected by transporters SLC22A1, ABCB1, and ABCG2. Patients with high pretreatment SLC22A1 expression had a higher probability of achieving a cytogenetic response and a

superior progression-free and overall survival. The same was not observed when analyzing ABCB1 and ABCG2.

The contribution of the SLC22A1 transporter to the clinical response to imatinib has not yet been elucidated. Therefore, further studies are needed to evaluate the role of this influx transporter in the clinical outcome of imatinib treatment.

2. Inhibitor of Apoptosis Proteins (IAPs)

The IAP family members are characterized by a common baculoviral IAP repeat (BIR) domain [103] and by the ability to block apoptosis through the inhibition of both mitochondrial-dependent and -independent apoptotic pathways [104, 105]. Among IAPs, much attention has been focused on survivin and XIAP due to their potential role as therapeutic targets.

2.1. XIAP. XIAP (X-linked of inhibitor of apoptosis protein) is a singular IAP because it is the only member of the family known to directly inhibit caspases-3, -7, and -9 [106, 107]. XIAP is able to bind their target caspases by a two-site interaction mechanism, which inhibits the apoptotic pathway by blocking the active caspase site or by dissociating the dimer of caspases [108].

There are at least two proteins, Smac/DIABLO [109] and XAF1 (XIAP-associated factor 1) [110], known to interact with XIAP and modulate its antiapoptotic activity, which suggests a significant role of XIAP in the maintenance of the cellular homeostasis [111]. Other relevant XIAP properties are the involvement in copper metabolism [112] and the capacity of self-ubiquitination and of other targets involved or not in the control of the cell death [113], demonstrating its versatility in the cellular physiologic processes. Studies using knockout murine models for XIAP (*XIAP/BIRC4* $^{-/-}$) showed that its absence does not alter caspases-dependent or -independent apoptosis, but increases the expression of other IAPs, possibly as a compensatory mechanism [114].

XIAP is widely expressed in normal tissues [115]; however, its overexpression in cancer is usually associated with an unfavorable prognosis [116–119]. Although it has been demonstrated that the nuclear localization of XIAP is an independent prognostic marker in breast cancer [120], little is known about the expression and subcellular localization relevance of XIAP in CML patient samples.

Increasing evidence demonstrates that treatment of CML cells with chemotherapeutic agents can overcome resistance through negatively regulating XIAP levels. Fang et al. [121] have observed that one of the mechanisms involved in BCR-ABL-positive cells sensitivity to imatinib is XIAP downregulation. Corroborating this data, a study conducted in K562 cells and leukemic blasts obtained from patients with CML in blast crisis showed that apicidin, a histone deacetylase inhibitor, was able to potentiate imatinib effects on apoptosis through XIAP degradation and the release of the proapoptotic protein Smac/DIABLO into cytosol [122]. These events were associated with reduced BCR-Abl protein expression and decreased phosphorylated Akt levels and were caspase dependent [122]. Imatinib-induced apoptosis could also be potentiated when coadministered with ABT-737, a Bcl-2 and Bcl-xL inhibitor [123]. Cotreatment of K562 cells and primary CML samples led to caspase-3 activation and HtrA2/Omi-mediated decreased XIAP levels both in K562 cells and TKI-insensitive CML hematopoietic progenitors [123]. In addition to these findings, treatment of K562 cells with TRAIL led to an apoptosis-resistant phenotype through the upregulation of antiapoptotic proteins, including XIAP [124], further emphasizing its role in chemoresistance in CML.

Many strategies have been used to inhibit both the expression and function of XIAP and resensitize cancer cells to different cytotoxic stimuli [125–127]. One study demonstrated that the downregulation of XIAP expression using antisense oligonucleotides increased the sensitivity to cytotoxic stimuli, inducing apoptosis and decreasing cell viability in the K562 cell line [128]. Recently, the same group showed that the simultaneous inhibition of XIAP and P-glycoprotein in cells that overexpress this efflux pump decreases imatinib resistance [129]. Consistent with this, a recent work published by our group found that cyclosporine-A-mediated Pgp modulation was associated with XIAP inhibition and an increased apoptotic index as a response of resistant CML cells to vincristine [130]. Altogether, these findings point XIAP as an interesting therapeutic target and suggest that combining chemotherapeutic agents with XIAP-targeted therapy seems to represent a promising strategy in CML.

2.2. Survivin. Survivin, another IAP member, is an anti-apoptotic protein [131], which also regulates cell division by controlling mitotic spindle checkpoint [132]. *Survivin* gene generates five different splice variant mRNAs, which encodes different proteins: wild-type survivin, survivin-2B, survivin-3B, survivin-δEx3, and survivin-2α [133]. Compared to wild-type survivin, little prognostic information is known about the functions of alternative splicing forms, which are generally expressed at lower levels than the wild-type survivin. In a recent study, it was found that patients in blast and accelerated phases displayed significantly lower levels of survivin-2B and -δEx3, compared to patients in CML-CP. However, there was no correlation between the isoform expression and clinical parameters or response to imatinib treatment [134].

Undetectable in normal differentiated tissues, survivin is abundantly expressed in all the most common human cancers [131, 135], which makes this protein a potential target for drug discovery and new anticancer interventions. Survivin can also be found in normal tissues characterized by self-renewal and proliferation [136], but its expression is significantly lower than in tumor cells. In $CD34^+$ hematopoietic progenitor stem cells, survivin was found to be expressed and associated with the inhibition of apoptosis [137]. However, a recent report showed that despite survivin being quite expressed in $CD34^+$ cells, its levels are low in more precursor leukemia stem cells [138], indicating that survivin is not an optimal therapeutic target for CML stem cells compartment

and suggesting that it may not be the main factor accounting for resistance to targeted therapy in CML [139].

In CML patient samples, several studies have reported that survivin was expressed in the accelerated and blast phases but it was low or undetectable in the chronic phase [4, 140–143], suggesting that survivin may be involved in the pathogenesis of progression from the CML-CP to the CML-BP. In addition, survivin overexpression in CML patients was correlated with the percentage of Ph chromosome positive cells and BCR-ABL expression [142], indicating that it can be regulated by BCR-ABL tyrosine kinase. In fact, Carter et al. [144] demonstrated that BCR-ABL and its downstream effector mitogen-activated protein kinase (MAPK) could target survivin expression at both RNA and protein levels in cells derived from a patient with CML-BP Ph chromosome positive. Survivin downregulation resulted in reduced cell viability in imatinib-sensitive CML cells, but not in imatinib-resistant CML cells or Ph chromosome negative cells, showing that survivin is regulated by the BCR-ABL/MAPK cascade in Ph positive CML. The prognostic importance of survivin in CML was also evaluated in a study from our group, where a correlation between survivin highest levels and high/intermediate Sokal score patients could be observed [145]. In addition, it was reported that survivin overexpression at diagnosis correlated with a low probability to achieve an optimal response to imatinib [134]. These data suggest that survivin may be closely involved in a more aggressive evolution of CML.

Growing evidence suggests that survivin plays an important role in chemoresistance phenotype of human malignancies [146], including CML. It has been demonstrated by our group that treatment of K562 CML cells with imatinib resulted in survivin downregulation and cell death [147]. Consistent with this, imatinib-induced apoptosis was increased when survivin expression was disrupted in BCR-ABL cells, as shown by enhanced cytochrome *c* release, caspase-9 activity, and BCR-ABL cleavage 199, which indicate that targeting survivin might be a useful tool to sensitize BCR-ABL cells to imatinib. Survivin has also been shown to play a resistant factor to agents other than imatinib in CML cells. In a recent publication, our group showed that survivin overexpression was involved in the resistance to idarubicin, an anthracycline commonly used to treat acute leukemia. On the other hand, idarubicin could induce DNA fragmentation and caspase-mediated apoptosis in K562 cells when survivin levels were down-regulated [148]. In addition, other groups have demonstrated that survivin inhibition is a common mechanism of apoptosis induced in CML cells by different classes of anticancer agents such as aurora kinase inhibitors, histone deacetylase inhibitors [149], microtubule targeting agents (MTAs), and cyclin-dependent kinase (CDK1) inhibitors [150]. Altogether, this amount of data shows that the modulation of survivin expression seems to be an interesting approach to overcome resistance and induce cell death in CML cells.

In recent years, considerable efforts have been made to validate survivin as a new target in cancer therapy. YM-155, a small-molecule inhibitor of survivin, was the first survivin-targeted therapy to be developed and tested in clinical trials. In CML, YM-155 anticancer efficacy has been recently assessed in a preclinical study, where CML-derived cell lines showed great sensitivity to the molecule [151]. This effect has also been demonstrated for sheperdin, which is a novel antagonist of the interaction between hsp90 and survivin, known to be important for stabilizing survivin cytoprotective functions [152]. Although sheperdin did not decrease the viability of phytohemagglutinin-stimulated peripheral blood mononuclear cells or induced organ toxicity in a xenograft acute myeloid leukemia (AML) model, it could inhibit viability in K562 cells and in patient-derived AML peripheral blasts [153], demonstrating that it is a highly selective molecule. Antisurvivin therapies developed, to date, have not revealed major systemic toxicities in animal models and clinical trials and are extremely encouraging. Targeting survivin alone or in conjunction with chemotherapeutic agents has a great potential as a novel therapeutic regimen in CML.

3. Transcription Factors

Signal transduction pathways within the cell act by transmitting the extracellular signals to transcription factors, which result in changes in gene expression. However, it is well known that most key signaling pathways are deregulated in cancer, leading to altered expression and function of transcription factors. The constitutive activation of the nuclear factor kappa B (NFκB) [169] and the inactivation of the forkhead box O (FoxO) factors [170] were shown to be important steps in carcinogenic transformation. Therefore, modulating the activity of FoxO and NFκB seems to represent a reasonable therapeutic strategy.

3.1. NFκB. Nuclear Factor κB (NFκB) was discovered in 1986 as a factor in the nucleus of B cells that bind to the enhancer of the kappa light chain of immunoglobulin [171]. It has been shown to be expressed in the cytoplasm of all cell types and, once activated, it translocates to the nucleus, where it regulates the expression of over 200 genes [172]. NFκB is an important transcription factor typically activated by proinflammatory cytokines and other specific stimuli, and is involved in the regulation of a variety of biological responses, such as inflammatory, apoptotic, and immune processes. It achieves this by regulating the expression of proteins such as cytokines, chemokines, adhesion molecules, and the cellular death cascade [173]. The members of NFκB protein family form dimers (usually heterodimers of p50 and p65 subunits) that interact in the cytoplasm with inhibitor of NFκB (IκB) proteins. When IκB is phosphorylated by IκB kinases (IKKs), it is degraded by the ubiquitin-proteasome pathway, liberating NFκB dimers from their inhibition and allowing them to migrate to the nucleus and to activate NFκB target genes [174].

In addition to its function as a central mediator of human immune responses, NFκB plays a major role in

activating genes involved in cellular survival, transformation, and oncogenesis. Loss of the normal regulation of NFκB has become apparent as a major contributor to the deregulated growth, resistance to apoptosis, and propensity to metastasize observed in many cancers [175]. The overexpression of p65- or c-Rel-containing dimers can impair apoptosis, whereas the inhibition of NFκB/Rel activity can enhance death induced by TNF-alpha, ionizing radiations, or chemotherapeutic agents in many cell types. Aberrant activation of NFκB/Rel factors contributes to reduce the sensitivity to apoptosis in a vast range of hematologic malignancies. Although alterations in *NFκB* or *IκB* genes are documented in some neoplasms, in other cases, dysfunctions in components of the NFκB/Rel-activating signaling pathways or influences of other mutated proteins on NFκB/Rel can be recognized [176]. Constitutively active NFκB has been detected in malignant cells derived from patients with multiple myeloma, AML, ALL, CML, and, most recently, in myelodysplastic syndromes. Targeting NFκB in these hematopoietic malignancies leads to apoptosis, corroborating the role of NFκB in the survival and clonal expansion of malignant cells [174].

The expression of BCR-ABL leads to the activation of NFκB-dependent transcription by causing nuclear translocation of NFκB and by increasing the transactivation function of the RelA/p65 subunit of NFκB. Importantly, this activation is dependent on the tyrosine kinase activity of BCR-ABL that partially requires Ras. It has also been demonstrated that NFκB is required for BCR-ABL-mediated tumorigenicity in nude mice and for transformation of primary bone marrow cells [169]. This activation regulates the transcription of important genes, such as c-myc, which are necessary for the transformation of BCR-ABL$^+$cells, as well as surface molecules, which are necessary for cellular adhesion and interaction, giving advantages for cellular growth [161, 177]. In particular, the constitutive activation of NFκB exists selectively in leukemia stem cells but not in normal HSC [178].

Alterations in NFκB regulation and in the signaling pathways that control its activities are involved in cancer progression, as well as in the treatment resistance during chemo- and radiotherapy. NFκB blocking can stop the proliferation of tumor cells or cause the tumor cells to become more sensitive to antitumor agents. This way, drugs that are capable of suppressing NFκB activation have important therapeutic potential in the carcinogenesis inhibition [179]. Several studies have demonstrated that the expression of BCR-ABL kinase activity in CML cell lines leads to a constitutive activation of NFκB through IKKβ downstream of BCR-ABL and the suppression of NFκB activation by the expression of IκBα blocked BCR-ABL-dependent xenograft tumor formation [161–163].

Cilloni et al. [161] demonstrated that a selective inhibitor of the IκB kinase (IKK) was capable of reducing NFκB binding activity and proliferation, followed by induction of apoptosis in CML cell lines sensitive and resistant to imatinib, as well as in bone marrow cells from sensitive and resistant CML patients. Corroborating with these data, Duncan et al. [162] demonstrated that a selective IKKβ inhibitor strongly suppressed growth and viability and induced cell death of cell lines expressing either wild-type or mutant versions of BCR-ABL, including the T315I mutation. Following the same rationale, Lounnas et al. [163] used another IKKβ inhibitor to block NFκB pathway capable of reducing cell survival and inducing apoptosis of imatinib-sensitive and imatinib-resistant cell lines. This work also demonstrated that cells from patients with T315I mutation appeared sensitive to NFκB inhibition in terms of proliferation. Furthermore, *in vivo* experiments resulted in a significant regression of the tumors after the administration of the IKKβ-inhibitor in nude mice injected with *BCR-ABL* wild-type and T315I mutant cells. Taken together, these results indicate that NFκB/IKK is essential for BCR-ABL—induced cell growth and survival and that the kinase IKKβ represents an attractive therapeutic target in CML.

Among these compounds acting as NFκB inhibitors, proteasome inhibitors have been widely used. Recently, it has been shown that BCR-ABL induces the activity of the proteasome, supporting the idea of using the proteasome as a suitable target for BCR-ABL-expressing cells [180]. The proteasome inhibition results in the accumulation of IκB in the cytoplasm, leading to inhibition of NFκB translocation to the nucleus. The most used proteasome inhibitor is bortezomib/Velcade/PS341, inhibitor of the chymotrypsin-like activity of the β5 subunit of the proteasome. In several studies, proteasome inhibition induced proliferation arrest and apoptosis in imatinib-resistant cells, providing a rationale for the use of this drug in the subset of patients resistant to imatinib [164, 165]. Hu et al. [165] showed the combined effect of bortezomib and imatinib in CML. The combinatory regimens in CML murine models significantly reduced disseminated disease, decreased tumor growth, and induced apoptosis in tumor sections. In this work, the combination of bortezomib and imatinib repressed the DNA-binding activity of NFκB. Albero et al. [164] demonstrated that bortezomib reduces proliferation and survival of Bcr-Abl-expressing cells, regardless of their sensitivity to imatinib, and including the highly resistant mutant T315I. In both studies, bortezomib inhibited proteasomal degradation of IκB, leading to its accumulation. Taken together, these results suggest that an approach combining imatinib and proteasome inhibitors can be a therapeutic strategy in reducing relapse and overcoming imatinib resistance by inactivating the NFκB pathway.

3.2. FoxO. FoxO transcription factors belong to the forkhead family of proteins, which are characterized by a conserved DNA-binding domain termed forkhead box (Fox) [181]. The FoxO class contains four members: FoxO1, FoxO3a, FoxO4, and FoxO6, whose expression can be found in a variety of different tissues [182]. FoxO proteins are implicated in crucial cellular functions including cell cycle regulation, stress response, glucose metabolism, and apoptosis [183]. Accumulating evidence suggests that FoxO act as tumor suppressors, inhibiting tumor growth by the activation of genes such as Bim, FasL, p27kip, cyclin D, GADD45a,

glucose-9-phosphatase, and manganese dismutase [184]. Except for FoxO6, which is constitutively nuclear [185], phosphorylation by kinases, mainly Akt, ERK, (IκB kinase) IKK, and serum and glucocorticoid-regulated kinase (SGK), regulates FoxO nuclear/cytoplasmic shuttling [186], leading to its nuclear exclusion, retention in the cytoplasm, and subsequent proteasome degradation and inactivation [187]. FoxO transcription factors can also be regulated by other posttranslational modifications such as acetylation, methylation, ubiquitination, and glycosylation [188].

Because BCR/ABL activity requires an activated PI3K/Akt pathway [189] and the inactivation of FoxO transcription factors was shown to be essential for tumorigenesis and resistance to treatment [190], the activation of FoxO by chemotherapeutic drugs seems to be a great strategy to overcome resistance [191]. Komatsu et al. [157] showed that BCR-ABL-positive cells have FoxO3a in a constitutively phosphorylated status and p27/kip1 downregulated. In agreement, exposure of CML cells to imatinib inhibited FoxO3a phosphorylation and induced p27/kip1 expression and G0/G1 arrest, blocking cell cycle progression. Essafi et al. [156] also showed that BCR-ABL inhibition induced by imatinib in CML cells resulted in FoxO3a activation. As a consequence, the induction of the FoxO3a-direct transcriptional target Bim was observed concomitantly with increased apoptosis. More recently, it was demonstrated that BCR-ABL-mediated FoxO3a inactivation was proteasome dependent [160]. Bortezomib treatment was able to restore FoxO3a expression, sensitize BCR-ABL T315I expressing cells to apoptosis, and inhibit CML-like disease in leukemic mice [160]. Regulation of FoxO3a expression affects the expression not only of Bim and p27/kip1, but also of cyclin D [155]. Imatinib-mediated inhibition of BCR-ABL represses cyclin D4 expression, upon FoxO3a activation and binding to cyclin D4 promoter. However, this effect can be prevented after FoxO3a silencing, indicating that FoxO3a is a key signaling molecule for BCR-ABL pathway and a relevant factor for apoptosis and cell cycle arrest in CML cells [155]. Imatinib can also exert its antileukemic effects through the concomitant activation of FoxO3a and the down-regulation of the inhibitor of DNA binding 1 (Id1) in K562 cells [159]. This study demonstrated that Id1 promoter is transcriptionally inhibited by FoxO3a, leading to differentiation of BCR-ABL transformed cells [159], suggesting that Id1 is essential for maintaining the leukemia phenotype. Moreover, experimental data suggest that FoxO3a activation can overcome imatinib resistance by increasing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression and by inducing apoptosis [158], further emphasizing the importance of the FoxO pathway in determining drug sensitivity.

Although a great amount of evidence demonstrates that FoxO3a functions as a downstream factor for TKI-induced apoptosis, recent data suggest that FoxO3a has a crucial role in maintenance of CML stem cells. In a recent study, it was demonstrated that FoxO3a deficiency is associated with a decreased ability of leukemia-initiating cells (LICs) to provoke CML in FoxO3a$^{-/-}$ mice [192]. Moreover, in CML stem cells, FoxO3a is predominantly nuclear and plays a resistant factor against TKI therapy [192]. Corroborating these data, the transcription factor Bcl-6 was identified as a target for the FoxO family, responsible for CML stem cells' self-renewal, repression of p53, leukemia initiation and resistance to TKI treatment [193]. As previously discussed [194], these findings reflect a "stem cell paradox" and may explain, in part, why CML stem cells persist after TKI treatment. The mechanisms and implications of these unexpected results regarding differential FoxO dynamics in CML stem cells still remain to be elucidated.

In conclusion, various findings have found that the activation of FoxO3a and its downstream genes are of clinical importance in diverse anticancer therapeutics, including in CML treatment. Different from p53 [17], FoxO mutation has not yet been found in human cancer, favoring FoxO targeted therapy. Clinical drugs which activate FoxO transcription factors can be used in combination with therapeutic agents for sensitizing CML malignant cells to therapy.

4. Molecular Interactions in Chemoresistance

Growing evidence has demonstrated that the development of the MDR phenotype arises as a result of a complex network involving multiple cellular and molecular mechanisms. It is a multifactorial process rather than a consequence of a single and isolated mechanism (Figure 1). As the problem of drug resistance cannot be solved by circumventing only an individual protein, many efforts have been made in order to target diverse mechanisms and enhance cell sensitivity to antineoplastic therapy (Table 1).

Wang et al. [98] had suggested that clinical responses to imatinib treatment could be affected by transporters SLC22A1, ABCB1, and ABCG2; however, a recent work showed no significant differences between *ABCB1, ABCG2, and SLC22A1* genotypes and imatinib plasma or intracellular concentrations [195].

These data indicate that other transporters may be crucial for determining imatinib intracellular and plasma concentrations in CML patients. By contrast, in experiments using *in vitro* models of acquired resistance, K562 cells displayed upregulated levels of *ABCB1* and *ABCG2* genes, after exposure to increasing concentrations of imatinib [167, 168], which would imply the involvement of these transporters in resistance to TKIs [168]. However, different from the ABCG2 inhibitor, the ABCB1 inhibitor was able to restore imatinib sensitivity, indicating that only ABCB1 is essential for the development of acquired resistance in CML. Regarding the expression of SLC22A1 gene, contradictory data show that K562 resistant-cells had an increased [167] or similar [168] expression compared to their parental ones. Another work has demonstrated that imatinib and nilotinib are capable of inhibiting ABCB1 and ABCG2 and may overcome resistance, despite high levels of these transporters [63].

Current studies have proposed the role of IAPs in MDR phenotype promotion in association with ABCB1 expression [196]. Recently, we evaluated the resistance induced by the overexpression of both ABCB1 and survivin proteins [43].

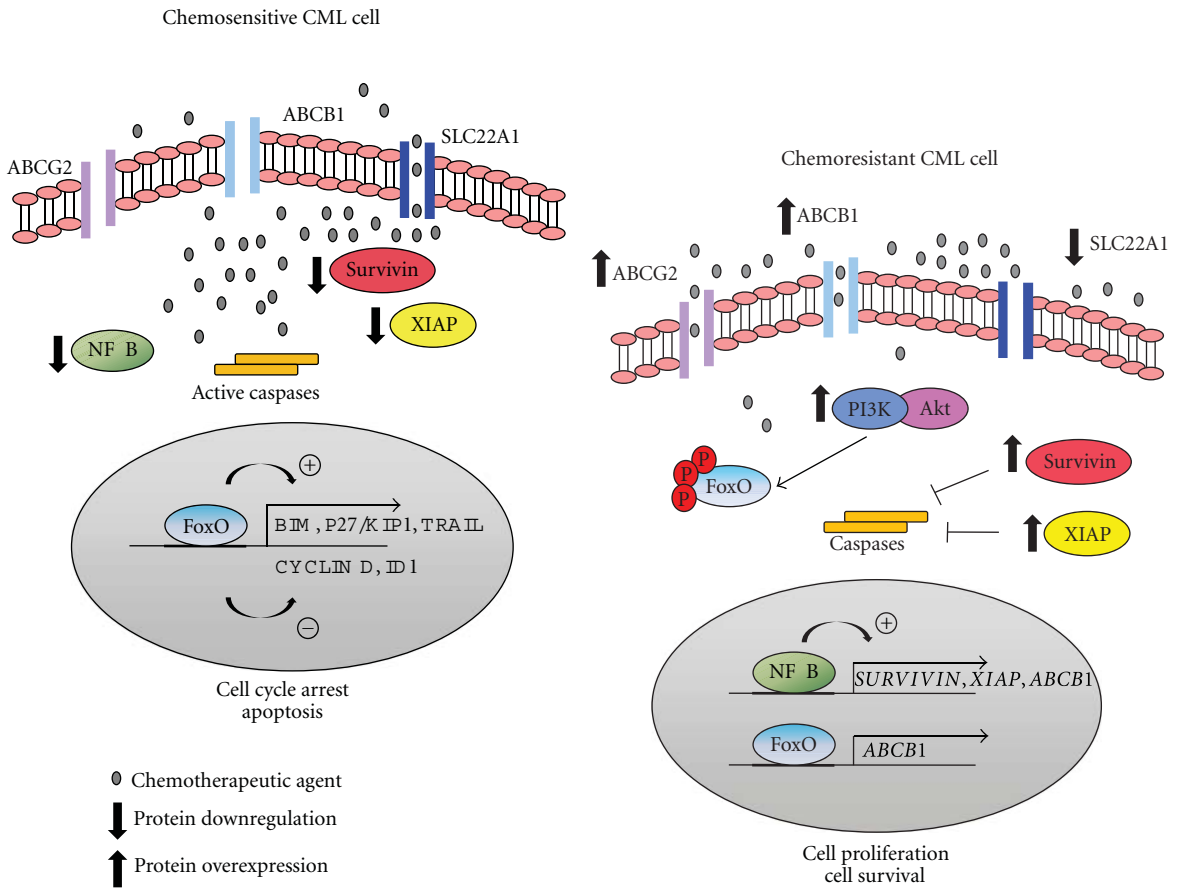


Figure 1: Molecular interactions in chemoresistance. Chemoresistant chronic myeloid leukemia (CML) cells display a multifactorial resistance phenotype characterized by deregulation of diverse signaling pathways which may act in concert or individually to prevent chemotherapy sensitivity (b). Resistant cells display constitutively active nuclear expression of NFκB which contributes to stimulate transcription of the inhibitor of apoptosis proteins (IAPs) survivin and XIAP and also the efflux drug transporter ABCB1. The transcription factor FoxO3a, which usually acts as an apoptosis mediator, may also lead to enhanced ABCB1 transcription when chronically activated. In addition, chemoresistant CML cells display an overexpression of the efflux pump ABCG2 and reduced levels of the influx drug transporter SLC22A1. By contrast, many chemotherapeutic agents may overcome resistance and sensitize cells to apoptosis by modulating these pathways (a). Drug-mediated down-regulation of NFκB, survivin, XIAP, and ABCB1 is associated with increased apoptotic levels, emphasizing their role as resistance factors. In addition, chemotherapy-induced FoxO3a activation results in cell cycle arrest and apoptosis by up-regulating *BIM, P27/KIP1, and TRAIL* and inhibiting *CYCLIN D* and *ID1* genes.

In this work, we showed that K562 cells (ABCB1-negative) progressively became resistant to vincristine treatment by simultaneous overexpression of ABCB1 and survivin. We also showed that ABCB1 promoted resistance to cell death independently of its membrane expression. Besides that, we could observe that ABCB1 and survivin colocalize in the cytoplasmatic compartment, suggesting a common regulatory pathway of apoptosis resistance control [43]. In another work, we observed that both ABCB1 and survivin protein expressions are associated in CML patients [145]. We could establish a positive correlation between ABCB1 and survivin expression, but not with ABCB1 activity in samples from late-phase CML-CP patients. These data suggest that ABCB1 and survivin may act in promoting resistance in CML patients and, thus, reinforce the hypothesis that ABCB1 is able to induce resistance independently of its activity function [145]. As discussed above, CML patients usually develop imatinib resistance, and, therefore, new treatment approaches are necessary to overcome CML resistance. Netto et al. [197] showed that a new compound named LQB-118 was effective against leukemia cell lines with low toxicity to peripheral blood cells. Recently, we evaluated the effect of LQB-118 on CML cell lines and observed that this compound was able to induce apoptosis in both sensitive and resistant CML cells [166]. Moreover, cells treated with LQB-118 also presented decreased levels of survivin, XIAP, and ABCB1 expression. We also analyzed the LQB-118 effect in CML patient samples and observed that this compound was effective in inducing apoptosis in patients displaying the MDR phenotype [166]. Corroborating these data, Seca et al. [129] showed that the simultaneous inhibition of XIAP and ABCB1 in cells overexpressing ABCB1 could decrease imatinib resistance.

Table 1: Anticancer drugs sensitize CML cells by targeting IAPs, drug transporters, NFκB and FoxO proteins.

Drug or therapy	Protein(s) targeted	Signaling pathways affected
Imatinib, idarubicin	Survivin	Imatinib and idarubicin inhibited viability and induced apoptosis in cells derived from a Ph^+ patient in blast crisis and K562 cells, respectively, through survivin downregulation [144].
Imatinib	Survivin	Enhanced imatinib-mediated apoptosis by modulating reactive oxygen species [147] and using antisense oligonucleotide or dominant-negative survivin [154] in CML cell lines.
Microtubule stabilizing agents and flavopiridol vorinostat, MK0457	Survivin	The combination of microtubule stabilizing agents and the cyclin-dependent kinase inhibitor flavopiridol [149] as well as the cotreatment with vorinostat and the aurora kinase inhibitor [155] led to survivin inhibition and increased apoptosis levels in K562 cells.
Sheperdin	Survivin	The survivin inhibitor molecule showed great toxicity against CML and AML cells, with no decrease in viability of phytohemagglutinin-stimulated peripheral blood mononuclear cells [153].
Imatinib	FoxO3a	Imatinib-mediated BCR-ABL inhibition resulted in FoxO3a activation, induction of Bim [156], p27/kip1 [157] and tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) [158], repression of cyclin D4 expression [156] and inhibitor of DNA binding 1 (Id1) [159], and consequent increased apoptosis in CML cell lines.
Bortezomib	FoxO3a	Bortezomib treatment was able to restore FoxO3a expression, sensitize imatinib-resistant T315I expressing cells to apoptosis, and inhibit CML-like disease in leukemic mice [160].
IKKB inhibitors	NFκB	The IKKB inhibitors led to the induction of apoptosis in cell lines (K562 and KCL) and bone marrow cells sensitive and resistant to imatinib [161], induced cell death in cell lines BaF3 BCR-ABL wild-type or mutant, including T315I mutation [162], suppressed proliferation of cells from patients with T315I mutation and *in vivo* experiments resulted in a regression of the tumors in nude mice [163].
Bortezomib	NFκB	Bortezomib reduced proliferation and survival of BCR-ABL-expressing cells, regardless of their sensitivity to imatinib and including the mutant T315I [164], and the combinatory effect with imatinib in CML led to reduced disseminated disease, decreased tumor growth and induced apoptosis in tumor sections [165].
Vincristine	ABCB1 and survivin	Overexpression of ABCB1 and survivin were associated with low apoptosis index induced by vincristine treatment [43].
LQB-118	ABCB1, survivin and XIAP	LQB-118 overcome resistance phenotype through ABCB1, survivin and XIAP downregulation [166].
Imatinib and nilotinib	*SLC22A1*, *ABCB1* and *ABCG2*	K562 cells displayed upregulated levels of *SLC22A*1, *ABCB1,* and *ABCG2* genes, after exposure to increasing concentrations of imatinib and nilotinib, respectively [167].
Imatinib	SLC22A1, ABCB1 and ABCG2	Chronic exposure to imatinib increased ABCB1 and ABCG2 at the protein and gene levels, but SLC22A1 expression remained unaltered [168].
Imatinib and vincristine	XIAP and ABCB1	Simultaneous inhibition of XIAP and ABCB1 in cells that overexpress this efflux pump decreases the resistance to imatinib [129] and vincristine [130].
Imatinib, apicidin and EBT-737	XIAP	Imatinib-induced apoptosis was found to be associated with XIAP downregulation [121] and could be potentiated when combined with apicidin [122] and EBT-737 [123] in K562 cells and CML progenitors.
Etoposide and doxorubicin	XIAP	The downregulation of XIAP expression with antisense oligonucleotides increased apoptosis and enhanced the effects of doxorubicin in K562 cells [128].

AML: acute myeloid leukemia, CML: chronic myeloid leukemia; IAPs: inhibitor apoptosis proteins.

Recent studies reported that *ABCB1* expression can be regulated by the NFκB transcription factor in hepatocytes and in drug-resistant cells. Moreover, the inhibition of NFκB activity sensitizes resistant colon cancer cells through a decreased *ABCB1* expression, providing a link between NFκB and resistance to chemotherapy through the regulation of human *ABCB1* gene expression [198]. In CML, Assef et al. [51] demonstrated that the resistance to imatinib exhibited in multidrug-resistant human leukemic K562 cells mediated by ABCB1 was reversed by the blockade of the NFκB pathway using a specific NFκB inhibitor [51]. Moreover, experimental evidence demonstrated the enhanced binding of NFκB to

the promoter region of *ABCB1* after K562 treatment with doxorubicin [199], further confirming the regulation of *ABCB1* by NFκB in the promotion of chemoresistance. In accordance to that, FoxO3a may also interact with ABCB1 gene and decrease cell sensitivity. Some reports have postulated that chronic induction of Foxo3a expression and nuclear localization may activate mechanisms of resistance in CML cells. By using doxorubicin-sensitive and resistant K562 CML cells, Hui et al. [200, 201] have demonstrated that resistance to doxorubicin is associated with increased activity of PI3K/Akt, through a mechanism of feedback and with the *ABCB1* gene induction. In contrast, it was recently demonstrated that FoxO3a is able to inhibit survivin expression while inducing cell death in melanoma [202] and neuroblastoma-derived cell lines [203]. Moreover, FoxO3a and FoxO1 were able to physically interact and inhibit survivin promoter, confirming the interaction between FoxO transcription factors and the antiapoptotic protein survivin [204]. However, the interaction between survivin and FoxO proteins, and its role in imatinib sensitivity, has not been investigated yet in CML-derived cells.

Survivin can also be targeted by NFκB [205], although it remains unclear how this interaction occurs. It was reported that inhibitors of the NFκB pathway, such as the natural compounds triptolide [206] and berbamine [207], have been shown to induce apoptosis in CML imatinib-resistant cells by down-regulating survivin levels. XIAP is another identified NFκB target, which is also implicated in modulating NFκB activation, through a feedback loop mechanism, in response to DNA damage and bacterial infection [208]. Studies suggest that XIAP recruits TAK1 in order to achieve NFκB activation and can mediate NFκB activation by promoting degradation of COMMD1, a negative regulator of NFκB [208]. As survivin, the interaction of XIAP and NFκB in CML remains unclear.

5. Conclusions

Although the introduction of imatinib and other TKIs in CML therapy has brought improvements in survival, CML prognosis still remains unfavorable for a group of patients. In addition to mutations found in the *BCR-ABL* gene, which alter the BCR-ABL kinase domain, there are currently identified secondary mechanisms of TKIs resistance. Multiple factors, such as inhibition of apoptotic signaling pathways, reduction in drug accumulation, and alterations in transcription factors, are known to contribute to the development of MDR and treatment failure in CML. These mechanisms usually act in concert in a multifactorial resistance context and play their role independent of or downstream BCR-ABL tyrosine kinase. Because the inhibition of only one mechanism is not effective enough to overcome clinical TKIs resistance, suppressing simultaneously several proteins must be required to increase the efficacy of the treatment in CML patients. Several questions remain to be answered to understand the interplay between these modes of resistance. For instance, how these proteins interact with each other to promote resistance and which one must be completely suppressed to antagonize malignancy? Regardless, what we know is that chemoresistance in CML is a multifactorial phenomenon and targeting these molecules seems to represent an interesting and feasible approach to overcome the development of TKIs-resistance in CML.

Abbreviations

ALL:	Acute lymphoid leukemia
AML:	Acute myeloid leukemia
BCR-ABL:	Breakpoint cluster region/V-abl Abelson murine leukemia viral oncogene homolog 1
BCRP:	Breast-cancer-related protein
BIR:	Baculoviral IAP repeat
BP:	Blast phase of chronic myeloid leukemia
CDK1:	Cyclin-dependent kinase 1
CLL:	Chronic lymphoid leukemia
CML:	Chronic myeloid leukemia
FOX:	Forkhead box
HSC:	Hematopoietic stem cell
IAP:	Inhibitor of apoptosis proteins
Id1:	Inhibitor of DNA binding 1
IκB:	Inhibitor of NFκB
IKK:	IκB kinase
KD:	Kinase domain
LIC:	Leukemia initiating cells
MAPK:	Mitogen-activated protein kinase
MDR:	Multidrug resistance
MRP1:	Multidrug resistance protein 1
MTA:	Microtubule targeting agents
NFκB:	Nuclear factor kappa B
OCT-1:	Organic cation transporter-1
Pgp:	P-glycoprotein
Ph:	Philadelphia
SGK:	Serum and glucocorticoid-regulated kinase
Si-RNA:	Small interfering RNA
SP:	Side population
TKI:	Tyrosine kinase inhibitors
TRAIL:	Tumor-necrosis-factor-related apoptosis-inducing ligand
XIAP:	X-linked of inhibitor of apoptosis protein.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This study was supported by research grants from CNPq, FAPERJ, INCT para Controle do Câncer, CNPq 573806/2008-0, FAPERJ EE26/170.026/2008, Programa de Oncobiologia (UFRJ/Fundação do Câncer), and FAPERJ-PPSUS.

References

[1] J. M. Goldman and J. V. Melo, "Mechanisms of disease: chronic myeloid leukemia—advances in biology and new

approaches to treatment," *New England Journal of Medicine*, vol. 349, no. 15, pp. 1451–1464, 2003.

[2] D. Perrotti, C. Jamieson, J. Goldman, and T. Skorski, "Chronic myeloid leukemia: mechanisms of blastic transformation," *Journal of Clinical Investigation*, vol. 120, no. 7, pp. 2254–2264, 2010.

[3] J. V. Melo and D. J. Barnes, "Chronic myeloid leukaemia as a model of disease evolution in human cancer," *Nature Reviews Cancer*, vol. 7, no. 6, pp. 441–453, 2007.

[4] J. C. Hernández-Boluda, B. Bellosillo, M. C. Vela, D. Colomer, A. Alvarez-Larrán, and F. Cervantes, "Survivin expression in the progression of chronic myeloid leukemia: a sequential study in 16 patients," *Leukemia and Lymphoma*, vol. 46, no. 5, pp. 717–722, 2005.

[5] R. Hehlmann, H. Heimpel, J. Hasford et al., "Randomized comparison of busulfan and hydroxyurea in chronic myelogenous leukemia: prolongation of survival by hydroxyurea," *Blood*, vol. 82, no. 2, pp. 398–407, 1993.

[6] F. Bonifazi, A. de Vivo, G. Rosti et al., "Chronic myeloid leukemia and interferon-α: a study of complete cytogenetic responders," *Blood*, vol. 98, no. 10, pp. 3074–3081, 2001.

[7] A. Gratwohl, R. Brand, J. Apperley et al., "Allogeneic hematopoietic stem cell transplantation for chronic myeloid leukemia in Europe 2006: transplant activity, long-term data and current results. An analysis by the chronic leukemia working party of the European Group for Blood and Marrow Transplantation (EBMT)," *Haematologica*, vol. 91, no. 4, pp. 513–521, 2006.

[8] T. Schindler, W. Bornmann, P. Pellicena, W. T. Miller, B. Clarkson, and J. Kuriyan, "Structural mechanism for STI-571 inhibition of abelson tyrosine kinase," *Science*, vol. 289, no. 5486, pp. 1938–1942, 2000.

[9] H. M. Kantarjian, J. E. Cortes, S. O'Brien et al., "Long-term survival benefit and improved complete cytogenetic and molecular response rates with imatinib mesylate in Philadelphia chromosome-positive chronic-phase chronic myeloid leukemia after failure of interferon-α," *Blood*, vol. 104, no. 7, pp. 1979–1988, 2004.

[10] J. Pinilla-Ibarz, J. Cortes, and M. J. Mauro, "Intolerance to tyrosine kinase inhibitors in chronic myeloid leukemia," *Cancer*, vol. 117, no. 4, pp. 688–697, 2011.

[11] M. Baccarani, G. Saglio, J. Goldman et al., "Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet," *Blood*, vol. 108, no. 6, pp. 1809–1820, 2006.

[12] G. Saglio, H. Kantarjian, T. Holyoake, A. Ranganathan, and J. E. Cortés, "Proceedings of the third global workshop on chronic myeloid leukemia," *Clinical Lymphoma, Myeloma and Leukemia*, vol. 10, no. 6, pp. 443–451, 2010.

[13] M. Breccia, F. Efficace, and G. Alimena, "Imatinib treatment in chronic myelogenous leukemia: what have we learned so far?" *Cancer Letters*, vol. 300, no. 2, pp. 115–121, 2011.

[14] A. M. Eiring, J. S. Khorashad, K. Morley, and M. W. Deininger, "Advances in the treatment of chronic myeloid leukemia," *BMC Medicine*, vol. 9, article 99, 2011.

[15] J. V. Melo and C. Chuah, "Resistance to imatinib mesylate in chronic myeloid leukaemia," *Cancer Letters*, vol. 249, no. 2, pp. 121–132, 2007.

[16] D. Bixby and M. Talpaz, "Mechanisms of resistance to tyrosine kinase inhibitors in chronic myeloid leukemia and recent therapeutic strategies to overcome resistance," *Hematology*, pp. 461–476, 2009.

[17] A. Quintás-Cardama and J. Cortes, "Molecular biology of BCR-ABL1-positive chronic myeloid leukemia," *Blood*, vol. 113, no. 8, pp. 1619–1630, 2009.

[18] S. Roychowdhury and M. Talpaz, "Managing resistance in chronic myeloid leukemia," *Blood Reviews*, vol. 25, no. 6, pp. 279–290, 2011.

[19] K. Eechoute, A. Sparreboom, H. Burger et al., "Drug transporters and imatinib treatment: Implications for clinical practice," *Clinical Cancer Research*, vol. 17, no. 3, pp. 406–415, 2011.

[20] S. V. Ambudkar, S. Dey, C. A. Hrycyna, M. Ramachandra, I. Pastan, and M. M. Gottesman, "Biochemical, cellular, and pharmacological aspects of the multidrug transporter," *Annual Review of Pharmacology and Toxicology*, vol. 39, pp. 361–398, 1999.

[21] G. D. Kruh, "Introduction to resistance to anticancer agents," *Oncogene*, vol. 22, no. 47, pp. 7262–7264, 2003.

[22] N. Kartner, D. Evernden-Porelle, G. Bradley, and V. Ling, "Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies," *Nature*, vol. 316, no. 6031, pp. 820–823, 1985.

[23] M. M. Gottesman, T. Fojo, and S. E. Bates, "Multidrug resistance in cancer: role of ATP-dependent transporters," *Nature Reviews Cancer*, vol. 2, no. 1, pp. 48–58, 2002.

[24] P. M. Chaudhary and I. B. Roninson, "Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells," *Cell*, vol. 66, no. 1, pp. 85–94, 1991.

[25] W. T. Klimecki, B. W. Futscher, T. M. Grogan, and W. S. Dalton, "P-glycoprotein expression and function in circulating blood cells from normal volunteers," *Blood*, vol. 83, no. 9, pp. 2451–2458, 1994.

[26] A. H. Schinkel, U. Mayer, E. Wagenaar et al., "Normal viability and altered pharmacokinetics in mice lacking MDR1-type (drug-transporting) P-glycoproteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 8, pp. 4028–4033, 1997.

[27] M. F. Fromm, "Importance of P-glycoprotein at blood-tissue barriers," *Trends in Pharmacological Sciences*, vol. 25, no. 8, pp. 423–429, 2004.

[28] G. Luurtsema, J. Verbeek, M. Lubberink et al., "Carbon-11 labeled tracers for in vivo imaging of P-glycoprotein function: kinetics, advantages and disadvantages," *Current Topics in Medicinal Chemistry*, vol. 10, no. 17, pp. 1820–1833, 2010.

[29] K. S. Lown, R. R. Mayo, A. B. Leichtman et al., "Role of intestinal P-glycoprotein (MDR1) in interpatient variation in the oral bioavailability of cyclosporine," *Clinical Pharmacology and Therapeutics*, vol. 62, no. 3, pp. 248–260, 1997.

[30] A. H. Schinkel, "P-glycoprotein, a gatekeeper in the blood-brain barrier," *Advanced Drug Delivery Reviews*, vol. 36, no. 2-3, pp. 179–194, 1999.

[31] B. Sarkadi, L. Homolya, G. Szakács, and A. Váradi, "Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system," *Physiological Reviews*, vol. 86, no. 4, pp. 1179–1236, 2006.

[32] I. Svoboda-Beusan, R. Kusec, K. Bendelja et al., "The relevance of multidrug resistance-associated P-glycoprotein expression in the treatment response of B-cell chronic lymphocytic leukemia," *Haematologica*, vol. 85, no. 12, pp. 1261–1267, 2000.

[33] M. Ohsawa, Y. Ikura, H. Fukushima et al., "Immunohistochemical expression of multidrug resistance proteins as a predictor of poor response to chemotherapy and prognosis

in patients with nodal diffuse large B-cell lymphoma," *Oncology*, vol. 68, no. 4–6, pp. 422–431, 2005.

[34] J. J. Lourenço, R. C. Maia, M. A. M. Scheiner, F. C. Vasconcelos, and M. A. M. Moreira, "Genomic variation at the MDR1 promoter and P-glycoprotein expression and activity in AML patients," *Leukemia Research*, vol. 32, no. 6, pp. 976–979, 2008.

[35] T. M. Grogan, C. M. Spier, S. E. Salmon et al., "P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy," *Blood*, vol. 81, no. 2, pp. 490–495, 1993.

[36] S. V. Ambudkar, Z. E. Sauna, M. M. Gottesman, and G. Szakacs, "A novel way to spread drug resistance in tumor cells: functional intercellular transfer of P-glycoprotein (ABCB1)," *Trends in Pharmacological Sciences*, vol. 26, no. 8, pp. 385–387, 2005.

[37] A. Levchenko, B. M. Mehta, X. Niu et al., "Intercellular transfer of P-glycoprotein mediates acquired multidrug resistance in tumor cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 6, pp. 1933–1938, 2005.

[38] M. Bebawy, V. Combes, E. Lee et al., "Membrane microparticles mediate transfer of P-glycoprotein to drug sensitive cancer cells," *Leukemia*, vol. 23, no. 9, pp. 1643–1649, 2009.

[39] L. J. Robinson, W. K. Roberts, T. T. Ling, D. Lamming, S. S. Sternberg, and P. D. Roepe, "Human MDR 1 Protein overexpression delays the apoptotic cascade in chinese hamster ovary fibroblasts," *Biochemistry*, vol. 36, no. 37, pp. 11169–11178, 1997.

[40] M. J. Smyth, E. Krasovskis, V. R. Sutton, and R. W. Johnstone, "The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 12, pp. 7024–7029, 1998.

[41] R. W. Johnstone, E. Cretney, and M. J. Smyth, "P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death," *Blood*, vol. 93, no. 3, pp. 1075–1085, 1999.

[42] M. Pallis, J. Turzanski, M. Grundy, C. Seedhouse, and N. Russell, "Resistance to spontaneous apoptosis in acute myeloid leukaemia blasts is associated with P-glycoprotein expression and function, but not with the presence of FLT3 internal tandem duplications," *British Journal of Haematology*, vol. 120, no. 6, pp. 1009–1016, 2003.

[43] P. S. Souza, F. C. Vasconcelos, F. R. de Souza Reis, G. N. De Moraes, and R. C. Maia, "P-glycoprotein and survivin simultaneously regulate vincristine-induced apoptosis in chronic myeloid leukemia cells," *International Journal of Oncology*, vol. 39, no. 4, pp. 925–933, 2011.

[44] Y. Kuwazuru, A. Yoshimura, S. Hanada et al., "Expression of the multidrug transporter, P-glycoprotein, in chronic myelogenous leukaemia cells in blast crisis," *British Journal of Haematology*, vol. 74, no. 1, pp. 24–29, 1990.

[45] A. Stavrovskaya, A. Turkina, N. Sedyakhina et al., "Prognostic value of P-glycoprotein and leukocyte differentiation antigens in chronic meloid leukemia," *Leukemia and Lymphoma*, vol. 28, no. 5-6, pp. 469–482, 1998.

[46] A. F. List, K. J. Kopecky, C. L. Willman et al., "Cyclosporine inhibition of P-glycoprotein in chronic myeloid leukemia blast phase," *Blood*, vol. 100, no. 5, pp. 1910–1912, 2002.

[47] E. Weisberg and J. D. Griffin, "Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines," *Blood*, vol. 95, no. 11, pp. 3498–3505, 2000.

[48] F. C. Vasconcelos, K. L. Silva, P. S. Souza et al., "Variation of MDR proteins expression and activity levels according to clinical status and evolution of CML patients," *Cytometry, B*, vol. 80, no. 3, pp. 158–166, 2011.

[49] F. X. Mahon, M. W. Deininger, B. Schultheis et al., "Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the signal transduction inhibitor STI571: diverse mechanisms of resistance," *Blood*, vol. 96, pp. 1070–1079, 2000.

[50] N. Widmer, H. Rumpold, G. Untergasser, A. Fayet, T. Buclin, and L. A. Decosterd, "Resistance reversal by RNAi silencing of MDR1 in CML cells associated with increase in imatinib intracellular levels," *Leukemia*, vol. 21, no. 7, pp. 1561–1562, 2007.

[51] Y. Assef, F. Rubio, G. Coló, S. del Mónaco, M. A. Costas, and B. A. Kotsias, "Imatinib resistance in multidrug-resistant K562 human leukemic cells," *Leukemia Research*, vol. 33, no. 5, pp. 710–716, 2009.

[52] F. X. Mahon, F. Belloc, V. Lagarde et al., "MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models," *Blood*, vol. 101, no. 6, pp. 2368–2373, 2003.

[53] H. Rumpold, A. M. Wolf, K. Gruenewald, G. Gastl, E. Gunsilius, and D. Wolf, "RNAi-mediated knockdown of P-glycoprotein using a transposon-based vector system durably restores imatinib sensitivity in imatinib-resistant CML cell lines," *Experimental Hematology*, vol. 33, no. 7, pp. 767–775, 2005.

[54] T. Illmer, M. Schaich, U. Platzbecker et al., "P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate," *Leukemia*, vol. 18, no. 3, pp. 401–408, 2004.

[55] Y. Zong, S. Zhou, and B. P. Sorrentino, "Loss of P-glycoprotein expression in hematopoietic stem cells does not improve responses to imatinib in a murine model of chronic myelogenous leukemia," *Leukemia*, vol. 19, no. 9, pp. 1590–1596, 2005.

[56] T. P. Stromskaya, E. Y. Rybalkina, S. S. Kruglov et al., "Role of P-glycoprotein in evolution of populations of chronic myeloid leukemia cells treated with imatinib," *Biochemistry*, vol. 73, no. 1, pp. 29–37, 2008.

[57] S. Hatziieremia, N. E. Jordanides, T. L. Holyoake, J. C. Mountford, and H. G. Jørgensen, "Inhibition of MDR1 does not sensitize primitive chronic myeloid leukemia $CD34^+$ cells to imatinib," *Experimental Hematology*, vol. 37, no. 6, pp. 692–700, 2009.

[58] H. Gurney, M. Wong, R. L. Balleine et al., "Imatinib disposition and ABCB1 (MDR1, P-glycoprotein) genotype," *Clinical Pharmacology and Therapeutics*, vol. 82, no. 1, pp. 33–40, 2007.

[59] C. Kimchi-Sarfaty, J. M. Oh, I. W. Kim et al., "A "silent" polymorphism in the MDR1 gene changes substrate specificity," *Science*, vol. 315, no. 5811, pp. 525–528, 2007.

[60] S. Dulucq, S. Bouchet, B. Turcq et al., "Multidrug resistance gene (MDR1) polymorphisms are associated with major molecular responses to standard-dose imatinib in chronic myeloid leukemia," *Blood*, vol. 112, no. 5, pp. 2024–2027, 2008.

[61] L. N. Ni, J. Y. Li, K. R. Miao et al., "Multidrug resistance gene (MDR1) polymorphisms correlate with imatinib response in

chronic myeloid leukemia," *Medical Oncology*, vol. 28, no. 1, pp. 265–269, 2011.

[62] R. Bhatia, M. Holtz, N. Niu et al., "Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment," *Blood*, vol. 101, no. 12, pp. 4701–4707, 2003.

[63] M. Dohse, C. Scharenberg, S. Shukla et al., "Comparison of ATP-binding cassette transporter interactions with the tyrosine kinase inhibitors imatinib, nilotinib, and dasatinib," *Drug Metabolism and Disposition*, vol. 38, no. 8, pp. 1371–1380, 2010.

[64] C. Hegedus, C. Özvegy-Laczka, A. Á. Apáti et al., "Interaction of nilotinib, dasatinib and bosutinib with ABCB1 and ABCG2: implications for altered anti-cancer effects and pharmacological properties," *British Journal of Pharmacology*, vol. 158, no. 4, pp. 1153–1164, 2009.

[65] F. X. Mahon, S. Hayette, V. Lagarde et al., "Evidence that resistance to nilotinib may be due to BCR-ABL, Pgp, or Src kinase overexpression," *Cancer Research*, vol. 68, no. 23, pp. 9809–9816, 2008.

[66] A. Giannoudis, A. Davies, C. M. Lucas, R. J. Harris, M. Pirmohamed, and R. E. Clark, "Effective dasatinib uptake may occur without human organic cation transporter 1 (hOCT1): implications for the treatment of imatinib-resistant chronic myeloid leukemia," *Blood*, vol. 112, no. 8, pp. 3348–3354, 2008.

[67] D. K. Hiwase, V. Saunders, D. Hewett et al., "Dasatinib cellular uptake and efflux in chronic myeloid leukemia cells: therapeutic implications," *Clinical Cancer Research*, vol. 14, no. 12, pp. 3881–3888, 2008.

[68] N. L. Bartlett, B. L. Lum, G. A. Fisher et al., "Phase I trial of doxorubicin with cyclosporine as a modulator of multidrug resistance," *Journal of Clinical Oncology*, vol. 12, no. 4, pp. 835–842, 1994.

[69] A. Garrigues, J. Nugier, S. Orlowski, and E. Ezan, "A high-throughput screening microplate test for the interaction of drugs with P-glycoprotein," *Analytical Biochemistry*, vol. 305, no. 1, pp. 106–114, 2002.

[70] I. Frydrych, P. Mlejnek, and P. Dolezel, "Cyclosporin A sensitises Bcr-Abl positive cells to imatinib mesylate independently of P-glycoprotein expression," *Toxicology in Vitro*, vol. 23, no. 8, pp. 1482–1490, 2009.

[71] R. C. Maia, M. K. Carriço, C. E.N.P. Klumb et al., "Clinical approach to circumvention of multidrug resistance in refractory leukemic patients: association of cyclosporin A with etoposide," *Journal of Experimental and Clinical Cancer Research*, vol. 16, no. 4, pp. 419–424, 1997.

[72] R. C. Maia, H. Noronha, F. C. Vasconcelos, and V. M. Rumjanek, "Interaction of cyclosporin A and etoposide. Clinical and in vitro assessment in blast phase of chronic myeloid leukaemia," *Clinical and Laboratory Haematology*, vol. 19, no. 3, pp. 215–217, 1997.

[73] Z. Ni, M. E. Mark, X. Cai, and Q. Mao, "Fluorescence resonance energy transfer (FRET) analysis demonstrates dimer/oligomer formation of the human breast cancer resistance protein (BCRP/ABCG2) in intact cells," *International Journal of Biochemistry and Molecular Biology*, vol. 1, no. 1, pp. 1–11, 2010.

[74] P. A. Fetsch, A. Abati, T. Litman et al., "Localization of the ABCG2 mitoxantrone resistance-associated protein in normal tissues," *Cancer Letters*, vol. 235, no. 1, pp. 84–92, 2006.

[75] M. Nakagawa, E. Schneider, K. H. Dixon et al., "Reduced intracellular drug accumulation in the absence of P-glycoprotein (MDR1) overexpression in mitoxantrone-resistant human MCF-7 breast cancer cells," *Cancer Research*, vol. 52, no. 22, pp. 6175–6181, 1992.

[76] C. Lemos, G. Jansen, and G. J. Peters, "Drug transporters: recent advances concerning BCRP and tyrosine kinase inhibitors," *British Journal of Cancer*, vol. 98, no. 5, pp. 857–862, 2008.

[77] Y. Honjo, C. A. Hrycyna, Q. W. Yan et al., "Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells," *Cancer Research*, vol. 61, no. 18, pp. 6635–6639, 2001.

[78] M. Miwa, S. Tsukahara, E. Ishikawa, S. Asada, Y. Imai, and Y. Sugimoto, "Single amino acid substitutions in the transmembrane domains of breast cancer resistance protein (BCRP) alter cross resistance patterns in transfectants," *International Journal of Cancer*, vol. 107, no. 5, pp. 757–763, 2003.

[79] C. Özvegy-Laczka, T. Hegedus, G. Várady et al., "High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter," *Molecular Pharmacology*, vol. 65, no. 6, pp. 1485–1495, 2004.

[80] S. Shukla, Z. E. Sauna, and S. V. Ambudkar, "Evidence for the interaction of imatinib at the transport-substrate site(s) of the multidrug-resistance-linked ABC drug transporters ABCB1 (P-glycoprotein) and ABCG2," *Leukemia*, vol. 22, no. 2, pp. 445–447, 2008.

[81] P. J. Houghton, G. S. Germain, F. C. Harwood et al., "Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro," *Cancer Research*, vol. 64, no. 7, pp. 2333–2337, 2004.

[82] H. Burger, H. van Tol, A. W. M. Boersma et al., "Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump," *Blood*, vol. 104, no. 9, pp. 2940–2942, 2004.

[83] C. Brendel, C. Scharenberg, M. Dohse et al., "Imatinib mesylate and nilotinib (AMN107) exhibit high-affinity interaction with ABCG2 on primitive hematopoietic stem cells," *Leukemia*, vol. 21, no. 6, pp. 1267–1275, 2007.

[84] S. M. Graham, H. G. Jørgensen, E. Allan et al., "Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro," *Blood*, vol. 99, no. 1, pp. 319–325, 2002.

[85] T. Nakanishi, K. Shiozawa, B. A. Hassel, and D. D. Ross, "Complex interaction of BCRP/ABCG2 and imatinib in BCR-ABL-expressing cells: BCRP-mediated resistance to imatinib is attenuated by imatinib-induced reduction of BCRP expression," *Blood*, vol. 108, no. 2, pp. 678–684, 2006.

[86] M. A. Goodell, K. Brose, G. Paradis, A. S. Conner, and R. C. Mulligan, "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo," *Journal of Experimental Medicine*, vol. 183, no. 4, pp. 1797–1806, 1996.

[87] S. Zhou, J. D. Schuetz, K. D. Bunting et al., "The ABC transporter BCRP1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype," *Nature Medicine*, vol. 7, no. 9, pp. 1028–1034, 2001.

[88] C. W. Scharenberg, M. A. Harkey, and B. Torok-Storb, "The ABCG2 transporter is an efficient hoechst 33342 efflux pump and is preferentially expressed by immature human

hematopoietic progenitors," *Blood*, vol. 99, no. 2, pp. 507–512, 2002.

[89] J. W. Jonker, M. Buitelaar, E. Wagenaar et al., "The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15649–15654, 2002.

[90] S. Zhou, J. J. Morris, Y. Barnes, L. Lan, J. D. Schuetz, and B. P. Sorrentino, "Bcrp1 gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 19, pp. 12339–12344, 2002.

[91] P. Breedveld, D. Pluim, G. Cipriani et al., "The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients," *Cancer Research*, vol. 65, no. 7, pp. 2577–2582, 2005.

[92] Y. Imai, M. Nakane, K. Kage et al., "C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance," *Molecular Cancer Therapeutics*, vol. 1, no. 8, pp. 611–616, 2002.

[93] A. Tamura, M. Watanabe, H. Saito et al., "Functional validation of the genetic polymorphisms of human ATP-binding cassette (ABC) transporter ABCG2: identification of alleles that are defective in porphyrin transport," *Molecular Pharmacology*, vol. 70, no. 1, pp. 287–296, 2006.

[94] S. Mizuarai, N. Aozasa, and H. Kotani, "Single nucleotide polymorphisms result in impaired membrane localization and reduced atpase activity in multidrug transporter ABCG2," *International Journal of Cancer*, vol. 109, no. 2, pp. 238–246, 2004.

[95] N. Takahashi and M. Miura, "Therapeutic drug monitoring of imatinib for chronic myeloid leukemia patients in the chronic phase," *Pharmacology*, vol. 87, no. 5-6, pp. 241–248, 2011.

[96] H. Koepsell, K. Lips, and C. Volk, "Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications," *Pharmaceutical Research*, vol. 24, no. 7, pp. 1227–1251, 2007.

[97] M. A. Hediger, M. F. Romero, J. B. Peng, A. Rolfs, H. Takanaga, and E. A. Bruford, "The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins," *Pflugers Archiv European Journal of Physiology*, vol. 447, no. 5, pp. 465–468, 2004.

[98] L. Wang, A. Giannoudis, S. Lane, P. Williamson, M. Pirmohamed, and R. E. Clark, "Expression of the uptake drug transporter hOCT1 is an important clinical determinant of the response to imatinib in chronic myeloid leukemia," *Clinical Pharmacology and Therapeutics*, vol. 83, no. 2, pp. 258–264, 2008.

[99] J. Thomas, L. Wang, R. E. Clark, and M. Pirmohamed, "Active transport of imatinib into and out of cells: implications for drug resistance," *Blood*, vol. 104, no. 12, pp. 3739–3745, 2004.

[100] D. L. White, V. A. Saunders, P. Dang et al., "Most CML patients who have a suboptimal response to imatinib have low OCT-1 activity: higher doses of imatinib may overcome the negative impact of low OCT-1 activity," *Blood*, vol. 110, no. 12, pp. 4064–4072, 2007.

[101] L. C. Crossman, B. J. Druker, M. W. N. Deininger, M. Pirmohamed, L. Wang, and R. E. Clark, "hOCT 1 and resistance to imatinib," *Blood*, vol. 106, no. 3, pp. 1133–1134, 2005.

[102] S. Hu, R. M. Franke, K. K. Filipski et al., "Interaction of imatinib with human organic ion carriers," *Clinical Cancer Research*, vol. 14, no. 10, pp. 3141–3148, 2008.

[103] N. E. Crook, R. J. Clem, and L. K. Miller, "An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif," *Journal of Virology*, vol. 67, no. 4, pp. 2168–2174, 1993.

[104] Q. L. Deveraux and J. C. Reed, "IAP family proteins—suppressors of apoptosis," *Genes and Development*, vol. 13, no. 3, pp. 239–252, 1999.

[105] A. Wrzesień-Kuś, P. Smolewski, A. Sobczak-Pluta, A. Wierzbowska, and T. Robak, "The inhibitor of apoptosis protein family and its antagonists in acute leukemias," *Apoptosis*, vol. 9, no. 6, pp. 705–715, 2004.

[106] Q. L. Deveraux, R. Takahashi, G. S. Salvesen, and J. C. Reed, "X-linked IAP is a direct inhibitor of cell-death proteases," *Nature*, vol. 388, no. 6639, pp. 300–304, 1997.

[107] B. P. Eckelman, G. S. Salvesen, and F. L. Scott, "Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family," *EMBO Reports*, vol. 7, no. 10, pp. 988–994, 2006.

[108] F. L. Scott, J. B. Denault, S. J. Riedl, H. Shin, M. Renatus, and G. S. Salvesen, "XIAP inhibits caspase-3 and -7 using two binding sites: evolutionary conserved mechanism of IAPs," *EMBO Journal*, vol. 24, no. 3, pp. 645–655, 2005.

[109] A. M. Verhagen, P. G. Ekert, M. Pakusch et al., "Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins," *Cell*, vol. 102, no. 1, pp. 43–53, 2000.

[110] P. Liston, W. G. Fong, N. L. Kelly et al., "Identification of XAF1 as an antagonist of XIAP anti-Caspase activity," *Nature Cell Biology*, vol. 3, no. 2, pp. 128–133, 2001.

[111] M. Holcik and R. G. Korneluk, "XIAP, the guardian angel," *Nature Reviews Molecular Cell Biology*, vol. 2, no. 7, pp. 550–556, 2001.

[112] A. R. Mufti, E. Burstein, and C. S. Duckett, "XIAP: cell death regulation meets copper homeostasis," *Archives of Biochemistry and Biophysics*, vol. 463, no. 2, pp. 168–174, 2007.

[113] S. Galbán and C. S. Duckett, "XIAP as a ubiquitin ligase in cellular signaling," *Cell Death and Differentiation*, vol. 17, no. 1, pp. 54–60, 2010.

[114] H. Harlin, S. B. Reffey, C. S. Duckett, T. Lindsten, and C. B. Thompson, "Characterization of XIAP-deficient mice," *Molecular and Cellular Biology*, vol. 21, no. 10, pp. 3604–3608, 2001.

[115] B. Vischioni, P. van der Valk, S. W. Span, F. A. E. Kruyt, J. A. Rodriguez, and G. Giaccone, "Expression and localization of inhibitor of apoptosis proteins in normal human tissues," *Human Pathology*, vol. 37, no. 1, pp. 78–86, 2006.

[116] S. S. Liu, B. K. Tsang, A. N. Cheung et al., "Anti-apoptotic proteins, apoptotic and proliferative parameters and their prognostic significance in cervical carcinoma," *European Journal of Cancer*, vol. 37, no. 9, pp. 1104–1110, 2001.

[117] B. Z. Carter, S. M. Kornblau, T. Tsao et al., "Caspase-independent cell death in AML: caspase inhibition in vitro

with pan-caspase inhibitors or in vivo by XIAP or Survivin does not affect cell survival or prognosis," *Blood*, vol. 102, no. 12, pp. 4179–4186, 2003.

[118] I. Tamm, S. Richter, F. Scholz et al., "Xiap expression correlates with monocytic differentiation in adult de novo AML: impact on prognosis," *Hematology Journal*, vol. 5, no. 6, pp. 489–495, 2004.

[119] I. Tamm, S. Richter, D. Oltersdorf et al., "High expression levels of x-linked inhibitor of apoptosis protein and survivin correlate with poor overall survival in childhood de novo acute myeloid leukemia," *Clinical Cancer Research*, vol. 10, no. 11, pp. 3737–3744, 2004.

[120] Y. Zhang, J. Zhu, Y. Tang et al., "X-linked inhibitor of apoptosis positive nuclear labeling: a new independent prognostic biomarker of breast invasive ductal carcinoma," *Diagnostic Pathology*, vol. 6, no. 1, article 49, 2011.

[121] G. Fang, C. N. Kim, C. L. Perkins et al., "CGP57148B (STI-571) induces differentiation and apoptosis and sensitizes Bcr-Abl-positive human leukemia cells to apoptosis due to antileukemic drugs," *Blood*, vol. 96, no. 6, pp. 2246–2253, 2000.

[122] J. S. Kim, H. K. Jeung, J. W. Cheong et al., "Apicidin potentiates the imatinib-induced apoptosis of Bcr-Abl-positive human leukaemia cells by enhancing the activation of mitochondria-dependent caspase cascades," *British Journal of Haematology*, vol. 124, no. 2, pp. 166–178, 2004.

[123] K. Airiau, F.-X. Mahon, M. Josselin, M. Jeanneteau, B. Turcq, and F. Belloc, "ABT-737 increases tyrosine kinase inhibitor-induced apoptosis in chronic myeloid leukemia cells through XIAP downregulation and sensitizes $CD34^+$ $CD38^-$ population to imatinib," *Experimental Hematology*. In press.

[124] X. S. Hao, J. H. Hao, F. T. Liu, A. C. Newland, and L. Jia, "Potential mechanisms of leukemia cell resistance to TRAIL-induced apopotosis," *Apoptosis*, vol. 8, no. 6, pp. 601–607, 2003.

[125] D. C. McManus, C. A. Lefebvre, G. Cherton-Horvat et al., "Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics," *Oncogene*, vol. 23, no. 49, pp. 8105–8117, 2004.

[126] A. D. Schimmer, S. Dalili, R. A. Batey, and S. J. Riedl, "Targeting XIAP for the treatment of malignancy," *Cell Death and Differentiation*, vol. 13, no. 2, pp. 179–188, 2006.

[127] A. D. Schimmer, E. H. Estey, G. Borthakur et al., "Phase I/II trial of AEG35156 X-linked inhibitor of apoptosis protein antisense oligonucleotide combined with idarubicin and cytarabine in patients with relapsed or primary refractory acute myeloid leukemia," *Journal of Clinical Oncology*, vol. 27, no. 28, pp. 4741–4746, 2009.

[128] R. T. Lima, L. M. Martins, J. E. Guimarães, C. Sambade, and M. H. Vasconcelos, "Chemosensitization effects of XIAP downregulation in K562 leukemia cells," *Journal of Chemotherapy*, vol. 18, no. 1, pp. 98–102, 2006.

[129] H. Seca, R. T. Lima, J. E. Guimarães, and M. H. Vasconcelos, "Simultaneous targeting of P-gp and XIAP with siRNAs increases sensitivity of P-gp overexpressing CML cells to imatinib," *Hematology*, vol. 16, no. 2, pp. 100–108, 2011.

[130] P. S. de Souza, F. da Cunha Vasconcelos, L. F. R. Silva, and R. C. Maia, "Cyclosporine A enables vincristine-induced apoptosis during reversal of multidrug resistance phenotype in chronic myeloid leukemia cells," *Tumor Biology*. In press.

[131] G. Ambrosini, C. Adida, and D. C. Altieri, "A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma," *Nature Medicine*, vol. 3, no. 8, pp. 917–921, 1997.

[132] F. Li, G. Ambrosini, E. Y. Chu et al., "Control of apoptosis and mitotic spindle checkpoint by survivin," *Nature*, vol. 396, no. 6711, pp. 580–584, 1998.

[133] F. Li and X. Ling, "Survivin study: an update of "what is the next wave?"," *Journal of Cellular Physiology*, vol. 208, no. 3, pp. 476–486, 2006.

[134] M. Speletas, N. Argentou, V. Karanikas et al., "Survivin isoform expression patterns in CML patients correlate with resistance to imatinib and progression, but do not trigger cytolytic responses," *Clinical Immunology*, vol. 139, no. 2, pp. 155–163, 2011.

[135] D. C. Altieri, "Validating survivin as a cancer therapeutic target," *Nature Reviews Cancer*, vol. 3, no. 1, pp. 46–54, 2003.

[136] S. Fukuda and L. M. Pelus, "Survivin, a cancer target with an emerging role in normal adult tissues," *Molecular Cancer Therapeutics*, vol. 5, no. 5, pp. 1087–1098, 2006.

[137] S. Fukuda and L. M. Pelus, "Regulation of the inhibitor-of-apoptosis family member survivin in normal cord blood and bone marrow $CD34^+$ cells by hematopoietic growth factors: Implication of survivin expression in normal hematopoiesis," *Blood*, vol. 98, no. 7, pp. 2091–2100, 2001.

[138] J. M. Gerber, L. Qin, J. Kowalski et al., "Characterization of chronic myeloid leukemia stem cells," *American Journal of Hematology*, vol. 86, no. 1, pp. 31–37, 2011.

[139] G. V. Helgason, G. A. R. Young, and T. L. Holyoake, "Targeting chronic myeloid leukemia stem cells," *Current Hematologic Malignancy Reports*, vol. 5, no. 2, pp. 81–87, 2010.

[140] A. Mori, H. Wada, Y. Nishimura, T. Okamoto, Y. Takemoto, and E. Kakishita, "Expression of the antiapoptosis gene survivin in human leukemia," *International Journal of Hematology*, vol. 75, no. 2, pp. 161–165, 2002.

[141] A. Badran, A. Yoshida, Yujiwano et al., "Expression of the antiapoptotic gene survivin in chronic myeloid leukemia," *Anticancer Research*, vol. 23, no. 1, pp. 589–592, 2003.

[142] E. Conte, F. Stagno, P. Guglielmo, A. Scuto, C. Consoli, and A. Messina, "Survivin expression in chronic myeloid leukemia," *Cancer Letters*, vol. 225, no. 1, pp. 105–110, 2005.

[143] M. Zhara, H. Mourad, G. Farouk, M. Elbatch, S. Ezzat, and W. Sami, "Molecular detection of survivin expression, antiapoptotic gene, and other prognostic markers, how they are correlated and how it could be of prognostic value in chronic myeloid leukemia patient," *The Egyptian Journal of Immunology*, vol. 14, no. 2, pp. 51–62, 2007.

[144] B. Z. Carter, D. H. Mak, W. D. Schober et al., "Regulation of survivin expression through BCR-Abl/MAPK cascade: targeting survivin overcomes imatinib resistance and increases imatinib sensitivity in imatinib-responsive CML cells," *Blood*, vol. 107, no. 4, pp. 1555–1563, 2006.

[145] F. R. D. S. Reis, F. D. C. Vasconcelos, D. L. Pereira, A. Moellman-Coelho, K. L. Silva, and R. C. Maia, "Survivin and P-glycoprotein are associated and highly expressed in late phase chronic myeloid leukemia," *Oncology Reports*, vol. 26, no. 2, pp. 471–478, 2011.

[146] H. Yamamoto, C. Y. Ngan, and M. Monden, "Cancer cells survive with survivin," *Cancer Science*, vol. 99, no. 9, pp. 1709–1714, 2008.

[147] J. K. Kwee, D. G. Luque, A. C. D. S. Ferreira et al., "Modulation of reactive oxygen species by antioxidants in chronic myeloid leukemia cells enhances imatinib sensitivity

through survivin downregulation," *Anti-Cancer Drugs*, vol. 19, no. 10, pp. 975–981, 2008.

[148] G. Nestal de Moraes, K. L. Silva, F. D. C. Vasconcelos, and R. C. Maia, "Survivin overexpression correlates with an apoptosis-resistant phenotype in chronic myeloid leukemia cells," *Oncology Reports*, vol. 25, no. 6, pp. 1613–1619, 2011.

[149] W. Fiskus, Y. Wang, R. Joshi et al., "Cotreatment with vorinostat enhances activity of MK-0457 (VX-680) against acute and chronic myelogenous leukemia cells," *Clinical Cancer Research*, vol. 14, no. 19, pp. 6106–6115, 2008.

[150] S. A. Bright, G. Campiani, M. W. Deininger, M. Lawler, D. C. Williams, and D. M. Zisterer, "Sequential treatment with flavopiridol synergistically enhances pyrrolo-1,5-benzoxazepine-induced apoptosis in human chronic myeloid leukaemia cells including those resistant to imatinib treatment," *Biochemical Pharmacology*, vol. 80, no. 1, pp. 31–38, 2010.

[151] T. Nakahara, A. Kita, K. Yamanaka et al., "Broad spectrum and potent antitumor activities of YM155, a novel small-molecule survivin suppressant, in a wide variety of human cancer cell lines and xenograft models," *Cancer Science*, vol. 102, no. 3, pp. 614–621, 2011.

[152] P. Fortugno, N. R. Wall, A. Giodini et al., "Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function," *Journal of Cell Science*, vol. 115, no. 3, pp. 575–585, 2002.

[153] B. Gyurkocza, J. Plescia, C. M. Raskett et al., "Antileukemic activity of shepherdin and molecular diversity of Hsp90 inhibitors," *Journal of the National Cancer Institute*, vol. 98, no. 15, pp. 1068–1077, 2006.

[154] Z. Wang, J. Sampath, S. Fukuda, and L. M. Pelus, "Disruption of the inhibitor of apoptosis protein survivin sensitizes BCR-ABL-positive cells to STI571-induced apoptosis," *Cancer Research*, vol. 65, no. 18, pp. 8224–8232, 2005.

[155] S. F. de Mattos, A. Essafi, I. Soeiro et al., "FOXO3a and BCR-ABL regulate cyclin D2 transcription through a STAT5/BCL6-dependent mechanism," *Molecular and Cellular Biology*, vol. 24, no. 22, pp. 10058–10071, 2004.

[156] A. Essafi, S. Fernández De Mattos, Y. A. M. Hassen et al., "Direct transcriptional regulation of Bim by FOXO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells," *Oncogene*, vol. 24, no. 14, pp. 2317–2329, 2005.

[157] N. Komatsu, T. Watanabe, M. Uchida et al., "A member of forkhead transcription factor FKHRL1 is a downstream effector of STI571-induced cell cycle arrest in BCR-ABL-expressing cells," *Journal of Biological Chemistry*, vol. 278, no. 8, pp. 6411–6419, 2003.

[158] S. Kikuchi, T. Nagai, M. Kunitama, K. Kirito, K. Ozawa, and N. Komatsu, "Active FKHRL1 overcomes imatinib resistance in chronic myelogenous leukemia-derived cell lines via the production of tumor necrosis factor-related apoptosis-inducing ligand," *Cancer Science*, vol. 98, no. 12, pp. 1949–1958, 2007.

[159] K. U. Birkenkamp, A. Essafi, K. E. van der Vos et al., "FOXO3a induces differentiation of Bcr-Abl-transformed cells through transcriptional down-regulation of Id1," *Journal of Biological Chemistry*, vol. 282, no. 4, pp. 2211–2220, 2007.

[160] Z. Jagani, K. Song, J. L. Kutok et al., "Proteasome inhibition causes regression of leukemia and abrogates BCR-ABL-induced evasion of apoptosis in part through regulation of forkhead tumor suppressors," *Cancer Research*, vol. 69, no. 16, pp. 6546–6555, 2009.

[161] D. Cilloni, F. Messa, F. Arruga et al., "The NF-κB pathway blockade by the IKK inhibitor PS1145 can overcome Imatinib resistance," *Leukemia*, vol. 20, no. 1, pp. 61–67, 2006.

[162] E. A. Duncan, C. A. Goetz, S. J. Stein et al., "IκB kinase β inhibition induces cell death in Imatinib-resistant and T315I Dasatinib-resistant BCR-ABL$^+$ cells," *Molecular Cancer Therapeutics*, vol. 7, no. 2, pp. 391–397, 2008.

[163] N. Lounnas, C. Frelin, N. Gonthier et al., "NF-κB inhibition triggers death of imatinib-sensitive and imatinib-resistant chronic myeloid leukemia cells including T315I Bcr-Abl mutants," *International Journal of Cancer*, vol. 125, no. 2, pp. 308–317, 2009.

[164] M. P. Albero, J. M. Vaquer, E. J. Andreu et al., "Bortezomib decreases Rb phosphorylation and induces caspase-dependent apoptosis in Imatinib-sensitive and-resistant Bcr-Abl1-expressing cells," *Oncogene*, vol. 29, no. 22, pp. 3276–3286, 2010.

[165] Z. Hu, X. F. Pan, F. Q. Wu et al., "Synergy between proteasome inhibitors and imatinib mesylate in chronic myeloid leukemia," *Plos ONE*, vol. 4, no. 7, Article ID e6257, 2009.

[166] R. C. Maia, F. C. Vasconcelos, T. de Sá Bacelar et al., "LQB-118, a pterocarpanquinone structurally related to lapachol [2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone]: a novel class of agent with high apoptotic effect in chronic myeloid leukemia cells," *Investigational New Drugs*, vol. 29, no. 6, pp. 1143–1155, 2011.

[167] M. Gromicho, J. Dinis, M. Magalhães et al., "Development of imatinib and dasatinib resistance: dynamics of expression of drug transporters ABCB1, ABCC1, ABCG2, MVP, and SLC22A1," *Leukemia and Lymphoma*, vol. 52, no. 10, pp. 1980–1990, 2011.

[168] C. Hirayama, H. Watanabe, R. Nakashima et al., "Constitutive overexpression of P-glycoprotein, rather than breast cancer resistance protein or organic cation transporter 1, contributes to acquisition of imatinib-resistance in K562 cells," *Pharmaceutical Research*, vol. 25, no. 4, pp. 827–835, 2008.

[169] O. Fuchs, "Transcription factor NF-κB inhibitors as single therapeutic agents or in combination with classical chemotherapeutic agents for the treatment of hematologic malignancies," *Current Molecular Pharmacology*, vol. 3, no. 3, pp. 98–122, 2010.

[170] K. C. Arden, "Multiple roles of FOXO transcription factors in mammalian cells point to multiple roles in cancer," *Experimental Gerontology*, vol. 41, no. 8, pp. 709–717, 2006.

[171] R. Sen and D. Baltimore, "Multiple nuclear factors interact with the immunoglobulin enhancer sequences," *Cell*, vol. 46, no. 5, pp. 705–716, 1986.

[172] T. Okamoto, T. Sanda, and K. Asamitsu, "Nk-κb signaling and carcinogenesis," *Current Pharmaceutical Design*, vol. 13, no. 5, pp. 447–462, 2007.

[173] A. K. Mankan, M. W. Lawless, S. G. Gray, D. Kelleher, and R. McManus, "NF-κB regulation: the nuclear response," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 4, pp. 631–643, 2009.

[174] T. Braun, G. Carvalho, C. Fabre, J. Grosjean, P. Fenaux, and G. Kroemer, "Targeting NF-κB in hematologic malignancies," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 748–758, 2006.

[175] T. Lu and G. R. Stark, "Cytokine overexpression and constitutive NFκb in cancer," *Cell Cycle*, vol. 3, no. 9, pp. 1114–1117, 2004.

[176] M. C. Turco, M. F. Romano, A. Petrella, R. Bisogni, P. Tassone, and S. Venuta, "NF-κB/Rel-mediated regulation of apoptosis in hematologic malignancies and normal hematopoietic progenitors," *Leukemia*, vol. 18, no. 1, pp. 11–17, 2004.

[177] D. Cilloni, G. Martinelli, F. Messa, M. Baccarani, and G. Saglio, "Nuclear factor κB as a target for new drug development in myeloid malignancies," *Haematologica*, vol. 92, no. 9, pp. 1224–1229, 2007.

[178] Z. Lu, Y. Jin, C. Chen, J. Li, Q. Cao, and J. Pan, "Pristimerin induces apoptosis in imatinib-resistant chronic myelogenous leukemia cells harboring T315I mutation by blocking NF-κB signaling and depleting Bcr-Abl," *Molecular Cancer*, vol. 9, article 112, 2010.

[179] S. Luqman and J. M. Pezzuto, "NFκB: a promising target for natural products in cancer chemoprevention," *Phytotherapy Research*, vol. 24, no. 7, pp. 949–963, 2010.

[180] L. J. Crawford, P. Windrum, L. Magill et al., "Proteasome proteolytic profile is linked to Bcr-Abl expression," *Experimental Hematology*, vol. 37, no. 3, pp. 357–366, 2009.

[181] K. H. Kaestner, W. Knöchel, and D. E. Martínez, "Unified nomenclature for the winged helix/forkhead transcription factors," *Genes and Development*, vol. 14, no. 2, pp. 142–146, 2000.

[182] E. L. Greer and A. Brunet, "FOXO transcription factors at the interface between longevity and tumor suppression," *Oncogene*, vol. 24, no. 50, pp. 7410–7425, 2005.

[183] H. Tran, A. Brunet, E. C. Griffith, and M. E. Greenberg, "The many forks in FOXO's road," *Science's STKE*, vol. 2003, no. 172, p. RE5, 2003.

[184] J. Y. Yang and M. C. Hung, "A new fork for clinical application: targeting forkhead transcription factors in cancer," *Clinical Cancer Research*, vol. 15, no. 3, pp. 752–757, 2009.

[185] L. P. van der Heide, F. M. Jacobs, J. P. Burbach, M. F. M. Hoekman, and M. P. Smidt, "FOXO6 transcriptional activity is regulated by Thr26 and Ser184, independent of nucleocytoplasmic shuttling," *Biochemical Journal*, vol. 391, no. 3, pp. 623–629, 2005.

[186] L. P. van der Heide, M. F. M. Hoekman, and M. P. Smidt, "The ins and outs of FOXO shuttling: mechanisms of FOXO translocation and transcriptional regulation," *Biochemical Journal*, vol. 380, no. 2, pp. 297–309, 2004.

[187] H. Huang and D. J. Tindall, "Regulation of FOXO protein stability via ubiquitination and proteasome degradation," *Biochimica et Biophysica Acta*, vol. 1813, no. 11, pp. 1961–1964, 2011.

[188] Y. Zhao, Y. Wang, and W.-G. Zhu, "Applications of post-translational modifications of FOXO family proteins in biological functions," *Journal of Molecular Cell Biology*, vol. 3, no. 5, pp. 276–282, 2011.

[189] T. Skorski, A. Bellacosa, M. Nieborowska-Skorska et al., "Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway," *EMBO Journal*, vol. 16, no. 20, pp. 6151–6161, 1997.

[190] J. Y. Yang and M. C. Hung, "Deciphering the role of forkhead transcription factors in cancer therapy," *Current Drug Targets*, vol. 12, no. 9, pp. 1284–1290, 2011.

[191] Z. Jagani, A. Singh, and R. Khosravi-Far, "FOXO tumor suppressors and BCR-ABL-induced leukemia: a matter of evasion of apoptosis," *Biochimica et Biophysica Acta*, vol. 1785, no. 1, pp. 63–84, 2008.

[192] K. Naka, T. Hoshii, T. Muraguchi et al., "TGF-β-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia," *Nature*, vol. 463, no. 7281, pp. 676–680, 2010.

[193] C. Hurtz, K. Hatzi, L. Cerchietti et al., "BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia," *Journal of Experimental Medicine*, vol. 208, no. 11, pp. 2163–2174, 2011.

[194] K. Naka, T. Hoshii, and A. Hirao, "Novel therapeutic approach to eradicate tyrosine kinase inhibitor resistant chronic myeloid leukemia stem cells," *Cancer Science*, vol. 101, no. 7, pp. 1577–1581, 2010.

[195] T. Nambu, A. Hamada, R. Nakashima et al., "Association of SLCO1B3 polymorphism with intracellular accumulation of imatinib in leukocytes in patients with chronic myeloid leukemia," *Biological and Pharmaceutical Bulletin*, vol. 34, no. 1, pp. 114–119, 2011.

[196] F. Liu, S. Liu, S. He, Z. Xie, X. Zu, and Y. Jiang, "Survivin transcription is associated with P-glycoprotein/MDR1 overexpression in the multidrug resistance of MCF-7 breast cancer cells," *Oncology Reports*, vol. 23, no. 5, pp. 1469–1475, 2010.

[197] C. D. Netto, A. J. M. da Silva, E. J. S. Salustiano et al., "New Pterocarpanquinones: synthesis, antineoplasic activity on cultured human malignant cell lines and TNF-α modulation in human PBMC cells," *Bioorganic and Medicinal Chemistry*, vol. 18, no. 4, pp. 1610–1616, 2010.

[198] M. Bentires-Alj, V. Barbu, M. Fillet et al., "NF-κB transcription factor induces drug resistance through MDR1 expression in cancer cells," *Oncogene*, vol. 22, no. 1, pp. 90–97, 2003.

[199] H. Shen, W. Xu, Q. Chen, Z. Wu, H. Tang, and F. Wang, "Tetrandrine prevents acquired drug resistance of K562 cells through inhibition of MDR1 gene transcription," *Journal of Cancer Research and Clinical Oncology*, vol. 136, no. 5, pp. 659–665, 2010.

[200] R. C. Y. Hui, A. R. Gomes, D. Constantinidou et al., "The forkhead transcription factor FOXO3a increases phosphoinositide-3 kinase/Akt activity in drug-resistant leukemic cells through induction of PIK3CA expression," *Molecular and Cellular Biology*, vol. 28, no. 19, pp. 5886–5898, 2008.

[201] R. C. Y. Hui, R. E. Francis, S. K. Guest et al., "Doxorubicin activates FOXO3a to induce the expression of multidrug resistance gene ABCB1 (MDR1) in K562 leukemic cells," *Molecular Cancer Therapeutics*, vol. 7, no. 3, pp. 670–678, 2008.

[202] C. Hilmi, L. Larribere, M. Deckert et al., "Involvement of FKHRL1 in melanoma cell survival and death," *Pigment Cell and Melanoma Research*, vol. 21, no. 2, pp. 139–146, 2008.

[203] P. Obexer, J. Hagenbuchner, T. Unterkircher et al., "Repression of BIRC5/survivin by FOXO3/FKHRL1 sensitizes human neuroblastoma cells to DNA damage-induced apoptosis," *Molecular Biology of the Cell*, vol. 20, no. 7, pp. 2041–2048, 2009.

[204] M. Guha, J. Plescia, I. Leav, J. Li, L. R. Languino, and D. C. Altieri, "Endogenous tumor suppression mediated by PTEN involves survivin gene silencing," *Cancer Research*, vol. 69, no. 12, pp. 4954–4958, 2009.

[205] L. Tracey, A. Pérez-Rosado, M. J. Artiga et al., "Expression of the NF-κB targets BCL2 and BIRC5/Survivin characterizes small B-cell and aggressive B-cell lymphomas, respectively," *Journal of Pathology*, vol. 206, no. 2, pp. 123–134, 2005.

[206] X. Shi, Y. Jin, C. Cheng et al., "Triptolide inhibits BCR-ABL transcription and induces apoptosis in STI571-resistant

chronic myelogenous leukemia cells harboring T315I mutation," *Clinical Cancer Research*, vol. 15, no. 5, pp. 1686–1697, 2009.

[207] Y. L. Wei, Y. Liang, L. Xu, and X. Y. Zhao, "The antiproliferation effect of berbamine on K562 resistant cells by inhibiting NF-κB pathway," *Anatomical Record*, vol. 292, no. 7, pp. 945–950, 2009.

[208] M. Gyrd-Hansen and P. Meier, "IAPs: from caspase inhibitors to modulators of NF-κB, inflammation and cancer," *Nature Reviews Cancer*, vol. 10, no. 8, pp. 561–574, 2010.