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Experimental Hematology

Experimental Hematology 2017;50:1–12

REVIEW

Application of vitamin D and vitamin D analogs in acute myelogenous leukemia

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(Received 16 December 2016; revised 16 January 2017; accepted 26 January 2017)

Acute myeloid leukemia (AML) is characterized by the accumulation of malignant, transformed immature hematopoietic myeloid precursors that have lost their ability to differentiate and proliferate normally. Current treatment for AML requires intensive cytotoxic chemotherapy and results in significant morbidity and mortality, especially in older patients. Effective and better-tolerated treatment is urgently needed. Studies have shown that 1α,25-dihydroxyvitamin D3 (1,25-D3, active VD3) or vitamin D analogs (VDAs) can potently differentiate AML cells in vitro and ex vivo, which led to early clinical trials in AML and myelodysplastic syndrome patients. However, one major limiting factor in the clinical application of active VD3 or VDAs is the supraphysiologic dose required, which results in systemic hypercalcemia. Several important questions (i.e., dosage, method of delivery, metabolism of 1,25-D3 in situ, systemic hypercalcemia, and mechanisms of action of combination treatment) have to be addressed before vitamin D treatment can be applied to the clinical setting. This review focuses on 1,25-D3's mechanism of action in AML, preclinical data, and clinical trial outcomes, with an emphasis on major roadblocks to successful trials and suggestions for future directions.

Acute myeloid leukemia (AML) is characterized by the accumulation of malignant, transformed immature hematopoietic myeloid precursors that have lost their ability to differentiate and proliferate normally [1]. The original French–American–British system, first introduced in 1976, classified AML into eight subtypes based on morphologic and cytogenetic abnormalities. However, in the past 15 years, the molecular heterogeneity of the disease has become increasingly apparent as advanced genomic techniques are more readily available. In view of these new discoveries, in 2008, the World Health Organization published the classification guidelines, grouping AML into several major categories based on recurrent genetic abnormalities, myelodysplasia-related changes, prior-therapy-related changes, and AML not otherwise specified. The identified molecular features have yielded further perspective regarding diagnostic and prognostic markers in the updated classification for AML [2].

The treatment outcome for AML depends on two major aspects: patient-related factors (i.e., age, performance status, and comorbidities) and disease-related factors (i.e., white cell count, prior myelodysplastic syndrome [MDS] or cytotoxic therapy for another cancer, and leukemic-cell genetic aberrations). Thus far, three molecular markers, NPM1 and CEBPA mutations (associated with

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0301-472X/
http://dx.doi.org/10.1016/j.exphem.2017.01.007

good outcome) and FLT3 internal tandem duplications (associated with adverse outcome), have been applied in clinical practice as recommended by the European LeukemiaNet. Other markers, such as RUNX1, ASXL1, and TP53 mutations, have been consistently associated with inferior outcome; however, they have not been widely used as prognostic indicators [3]. In the near future, these molecular markers are anticipated to play a more significant role in helping clinicians stratify AML patients into different risk categories and guide their treatment accordingly.

Despite advancements in cancer treatment, the AML survival rate remains abysmal, with an overall survival at 4 years of 49%, 25%, and 9% for patients with favorable, intermediate, and unfavorable cytogenetics, respectively [4]. The main reason is that the general therapeutic approach has not changed substantially in the past 30 years despite numerous attempts with various chemotherapy regimens. Induction chemotherapy, which consists of either daunorubicin or idarubicin in combination with cytarabine, is the initial treatment modality for young and healthy patients. However, not everyone tolerates this standard, intensive regimen. Often, patients suffer severe adverse effects and complications requiring prolonged hospitalization. Palliative and hospice care are typically offered to older, debilitated patients. Therefore, scientists have been vigorously seeking new treatment approaches to improve cure rate and tolerability at the same time. One of those treatments is differentiation therapy, which takes into consideration the maturation arrest of AML cells.

Leukemia cells are well characterized by their "maturation arrest" at a given stage of differentiation. Differentiation therapy has shown extraordinary success in treating acute promyelocytic leukemia (APL or M3), a subtype of AML, with retinoic acid (RA; a vitamin A metabolite) and arsenic trioxide. APL involves a balanced translocation of the promyelocytic leukemia (PML) gene on chromosome 15 and the RA receptor alpha gene (RAR-α) on chromosome 17. The resultant fusion protein acts as a negative inhibitor of normal PML and RAR-α function. It also recruits nuclear corepressors and histone deacetylase (HDAC), inhibiting the transcription of genes needed for myeloid differentiation. All-*trans* RA reverses the process by binding to the fusion protein, changing its configuration and allowing release of the nuclear corepressor and HDAC complexes, thus permitting resumption of transcription. In this era, APL can be cured without using cytotoxic chemotherapy [5]. Such revolutionary clinical findings provided a foundation for research and application of other differentiation therapies, such as VD3, as treatments for non-M3 AML.

The concept of inducing leukemic cells to undergo differentiation with 1,25-D3 and VDAs was made popular during the 1980s based on several in vitro studies [6–8]. These compounds demonstrated antiproliferative and prodifferentiating properties toward AML cells. The major function of vitamin D in the human body is maintenance of calcium and phosphate homeostasis; however, it was also found to have other nonclassical properties: regulation of the immune response and influence on the proliferation, maturation, and apoptosis of normal and neoplastic cells [9–13].

In this article, we review current knowledge on vitamin D metabolism and its effect on leukemic cells, its underlying mechanisms in the preclinical setting, and the major roadblocks to successful clinical trials. We also discuss potential solutions to overcoming difficulties in translating vitamin D into therapeutic strategies for AML.

Vitamin D metabolism: Local and systemic regulation

Systemic function of 1,25-D3 through renal 1α-hydroxylase expression

The term "vitamin D" refers to a group of lipid-soluble compounds with a four-ringed cholesterol backbone. In humans, the major source of vitamin D is dermal synthesis, with a small proportion coming from consumed food. To become biologically active, vitamin D undergoes serial enzymatic conversions through the hydroxylation process. It is first converted in the liver to 25-hydroxyvitamin D (25-D3), which is the main circulating form. This is followed by 1α-hydroxylation in the kidney to produce the active form, 1,25-D3 [14]. The 25-D3-1α-hydroxylase is a tightly regulated enzyme in the proximal convoluted tubule cells of the kidney. It is upregulated by parathyroid hormone (PTH) and hypophosphatemia and suppressed by 1,25-D3 and calcium. An increase in 1,25-D3 level will induce vitamin D 24-hydroxylase, an enzyme involved in the additional hydroxylation step that inactivates 1,25-D3. Vitamin D 24-hydroxylase is expressed in most tissues [15].

Systemically, 1,25-D3 and its metabolites play a critical role in calcium homeostasis and bone metabolism through interaction with different organs. It exerts biologic effects by binding to a member of the nuclear receptor superfamily, the vitamin D receptor (VDR), which is a ligand-induced transcription factor and a major regulator of 1,25-D3 [16]. PTH, the key hormone in calcium homeostasis, preserves tight control of the systemic calcium levels through its regulation of renal 1α-hydroxylase. A low serum calcium level prompts the body to increase secretion of PTH, which in turn promotes the synthesis of 1,25-D3 in the kidney. The active vitamin D then stimulates the mobilization of calcium from bone and intestine and regulates the synthesis of PTH by negative feedback [15]. Use of 1,25-D3 for various therapeutic purposes, especially at a supraphysiologic dose, requires special consideration of the potential disturbance to the calcium system, which could cause severe side effects to patients. Below, we discuss in detail some issues with therapeutic utilization of 1,25-D3 in AML and suggest possible solutions (see

"Clinical experience of 1,25-D3 and its VDAs in AML" and "Future directions and possible solutions" sections).

Autocrine/paracrine function of 1,25-D3 through extrarenal 1α-hydroxylase expression

VDR is expressed in a wide range of cells and tissues, including small intestine, colon, osteoblasts, parathyroid gland, skin, uterus, ovary, breast, and prostate. This widespread distribution has raised the possibility that 1,25-D3 is involved in multifaceted cellular functions in addition to bone and mineral metabolism [17]. There is recent evidence indicating that 1,25-D3 also plays significant autocrine/paracrine functions through the expression of 1α-hydroxylase in extrarenal tissues. With the development of new tools, the detection of 1α-hydroxylase expression in extrarenal tissues (skin, placenta, colon, pancreas, vasculature, and parts of the brain) and cells (macrophages, monocytes, and dendritic cells) further confirmed the active autocrine and paracrine function of 1,25-D3 in local tissues. Hsu et al. found that there was a significant decreased activity of 1α-hydroxylase in human cells derived from prostatic adenocarcinomas compared with cells derived from normal tissues or benign prostatic hyperplasia [17]. This and other studies showing varying levels of 1α-hydroxylase expression in malignant colon tissue and parathyroid tissue versus normal tissues demonstrated the importance of 1,25-D3's autocrine/paracrine function in maintaining normal proliferation and differentiation of local tissues [18–21]. The local tissue concentration of 1,25-D3 depends on several factors: (1) local expression of 25-D3-24α-hydroxylase, which is overexpressed in cancer tissues; (2) extrarenal local expression of 1α-hydroxylase, which could increase or decrease depending on the cancer type; and (3) 25-D3 substrate level. Therefore, to exploit fully 1,25-D3's properties as an anticancer agent for AML, one needs to understand the native environments of leukemic patients' bone marrow (BM), which has not been well studied.

Role of 1,25-D3 in epigenetic modification and cancer treatment

There is a mutual interaction between the 1,25-D3 system and epigenetic mechanisms. VDR interacts with chromatin modifiers and remodelers directly or indirectly to fine-tune gene expression, whereas VDR and 1,25-D3 target genes can be silenced by DNA methylation or histone modification [22]. 1,25-D3 has been reported to be able to alter methylation of DNA in the promoters of some genes [23]. Conversely, 1,25-D3 insensitivity is related to methylation of the VDR promoter, which impairs 1,25-D3 regulation of tumor suppressor genes [24,25]. In addition, aberrant upregulated HDAC activity can interfere with calcitriol-induced AML differentiation [26].

Interestingly, epigenetic modification drugs have the potential to reverse 1,25-D3 insensitivity. VDR reactivation can be induced by HDAC inhibitors (vorinostat or trichostatin A) in combination with vitamin D, which has been shown to upregulate uniquely a group of suppressed gene targets in control of proliferation and induction of apoptosis [27,28]. Further study also indicates a synergistic role of DNA demethylation in 1,25-D3 metabolism and enhancement of 1,25-D3 efficiency for monocytic differentiation by a DNA hypomethylating agent (5-aza-2-desoxycytidine, decitabine) [29]. Therefore, with possible mutual interactions with a specific set of genes, combination treatment of epigenetic modification drugs and 1,25-D3 might act additively or synergistically in the treatment of cancer, specifically AML.

Vitamin D and AML

Preclinical experience with 1,25-D3 and its VDAs in AML

In vitro studies

General mechanism. To appreciate the concept of using vitamin D therapeutically for leukemia, one needs to understand its basic mechanism. As a steroid hormone, 1,25-D3 binds to the VDR, which is a ligand-induced transcription factor and a major regulator of 1,25-D3 [16]. Once this interaction occurs, the VDR becomes protected from degradation and translocates from cell cytosol to the nucleus [30–32]. In this activated state, the VDR heterodimerizes with the retinoid X receptor (RXR) and attaches to the promoter regions of target genes (osteocalcin, 24-hydroxylase of 1,25-D3, kinase suppressor of Ras-1, p27, and CD14) that play significant roles in the regulation of calcium and phosphate homeostasis, vitamin D metabolism, cellular differentiation, and cell cycle [33–39]. It was shown in experiments by Gocek et al. that AML cell lines express a very low constitutive level of the VDR protein, which increases significantly after cell exposure to 1,25-D3 [40]. The mRNA levels for the VDR remained unchanged after 1,25-D3 exposure, so the mechanism of VDR accumulation must be at the posttranscriptional level. It was also observed that, once the VDR forms a ligation complex with 1,25-D3 and translocates to the cell nuclei, the VDR degradation process is slower [40,41].

Role of 1,25-D3 in cellular differentiation. A number of important pathways have been elucidated to play critical roles in 1,25-D3-stimulated monocytic differentiation. The mitogen-activated protein kinase (MAPK) signaling pathway involves a family of serine threonine kinases that play an active part in coupling cell surface receptors to changes in transcriptional programs. The MAPKs are grouped into three main families: the extracellular signal-regulated protein kinases (ERKs), the p38 MAPKs, and the c-Jun N-terminal kinases (JNKs) [42]. This pathway involves the creation of a

multiprotein signaling complex that ultimately targets multiple transcription factors, resulting in cellular differentiation [43,44]. Gocek et al. discovered that 1,25-D3-stimulated monocytic differentiation was associated with increased ERK and JNK activity and was augmented by p38 MAPK inhibition [45]. Another key pathway central to vitamin D's function is the phosphatidylinositol 3-kinase (PI3K)–protein kinase B (Akt-1) signaling pathway. The PI3Ks are a family of related intracellular signal transducer enzymes that are involved in many essential cellular functions such as cell growth, proliferation, metabolism, survival, motility, and differentiation [46,47]. They are a lipid kinase family that have the ability to phosphorylate the inositol ring 3′-OH group in inositol phospholipids to generate the second messenger, phosphatidylinositol-3,4,5-triphosphate [46]. Interaction with these phospholipids is required for the translocation of Akt to the inner side of plasma membrane, where it is activated by sequential phosphorylation by phosphoinositide-dependent kinase-1 (PDK-1) and mammalian target of rapamycin complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK) [48–50]. Increased Akt activity promotes neutrophil and monocyte development, whereas reducing its activity results in eosinophil differentiation [51]. Prior studies demonstrated that PI3K and Akt activation are critical to 1,25-D3-stimulated induction of monocytic differentiation and protection from apoptosis [52–56]. Dysregulation of the genes in the PI3K/Akt signaling pathway is commonly involved in AML [3].

In addition to the two aforementioned pathways, 1,25-D3 also increases the activities of lipid signaling pathways, affecting multiple protein kinases and the phospholipase family in such a way that drives cellular differentiation. After exposure to 1,25-D3, protein kinase C (PKC) activity is increased, specifically PKCα and PKCβ. These two PKCs play critical roles in 1,25-D3-induced monocytic differentiation and maintenance of terminal differentiation [57]. Prior studies showed that increased activity of several phospholipase C (PLC) isoforms and phospholipase D (PLD) are observed during monocytic differentiation of leukemic cells [58,59]. It appears that PLC and PLD mediate the reaction that breaks down membrane phospholipids to generate diacylglycerol, which is required for the activation of the classical PKC isoforms [60–62]. 1,25-D3 causes phospholipase A2-mediated release of arachidonic acid from leukemic cells, which in turn affects downstream signals, leading to monocytic differentiation [63]. Finally, several ceramide derivatives (sphingolipid breakdown products) were shown recently to potentiate 1,25-D3-stimulated monocytic differentiation via modulation of the activity of the mentioned pathways involving PI3K, PKC, JNK, and ERK [64].

The interaction between 1,25-D3 and various cellular pathways is complex and has not been fully elucidated. A literature search did not reveal any relationship between genetic mutations in the production of calcitriol or VDR signaling pathway to the leukemogenesis or maintenance of AML. However, as described above in the "Autocrine/Paracrine function of 1,25-D3 through extra-renal 1α-hydroxylase expression" section, it is possible that the local BM concentration of 1,25-D3 is significantly reduced due to the overexpression of 25-D3-24α-hydroxylase, which involves in the degradation of 1,25-D3. Therefore, by supplementing AML patients with a high dose of 1,25-D3, this local effect of 24α-hydroxylase could be overcome. Because it is a powerful differentiator, 1,25-D3 can be reasonably applied to the treatment of AML to promote differentiation and maturation of cells arrested in different stages of myeloid development.

Role of 1,25-D3 in cell cycle arrest and apoptosis. Many studies have demonstrated the involvement of cell cycle regulatory molecules in 1,25-D3-stimulated growth inhibition and cell differentiation in human leukemic cells. Liu et al. showed that p21, a cyclin-dependent kinase (CDK) inhibitor (CKI) and a member of the CDK-interacting protein/kinase inhibitory protein family (CIP/KIP) is a target gene for 1,25-D3 [65]. Exposure of different human leukemic cell lines in vitro to various VDAs led to increased expression of p21, which is involved in the regulation of both growth arrest and induction of differentiation [65–67]. Another important CKI in the CIP/KIP family is p27, which is overexpressed when leukemic cells are exposed to 1,25-D3, resulting in induction of expression of monocyte/macrophage specific markers [65,68]. CKIs of the inhibitors of CDK (INK) family, such as p15, p16, and p18, are mainly involved in inhibition of cell proliferation. Although the pro-apoptotic effect of 1,25-D3 on leukemic cells has not been studied directly, this property has been well documented in other cell lines such as breast and colon cancers [69–72]. 1,25-D3 upregulates the pro-apoptotic protein Bax and downregulates the anti-apoptotic protein BCL-2, causing cancer cell growth inhibition and eventual cancer cell death.

The distinctive properties of 1,25-D3 as a powerful differentiator, cell cycle inhibitor, and pro-apoptotic agent make it an attractive therapeutic option for AML treatment. Figure 1 summarizes the general schematic interaction of 1,25-D3 with various pathways in vitro.

Selective sensitivities of AML subtypes to 1,25-D3 and VDA-induced differentiation in vitro and ex vivo. In vitro studies indicate that 1,25-D3 and VDAs induce differentiation on a variety of AML subtypes (HL60, AML-193, U937, HL90, THP-1, NB-4, KG-1, and MOLM-13), with HL60 being the most well-studied cell line [73]. The variable responses of AML subtypes to VDAs are consistent with current genomic data showing that AML is a heterogenous disease involving mutations from multiple cellular processes. Furthermore, AML subtypes express different VDR protein variants. Marchiwicka et al. discovered that

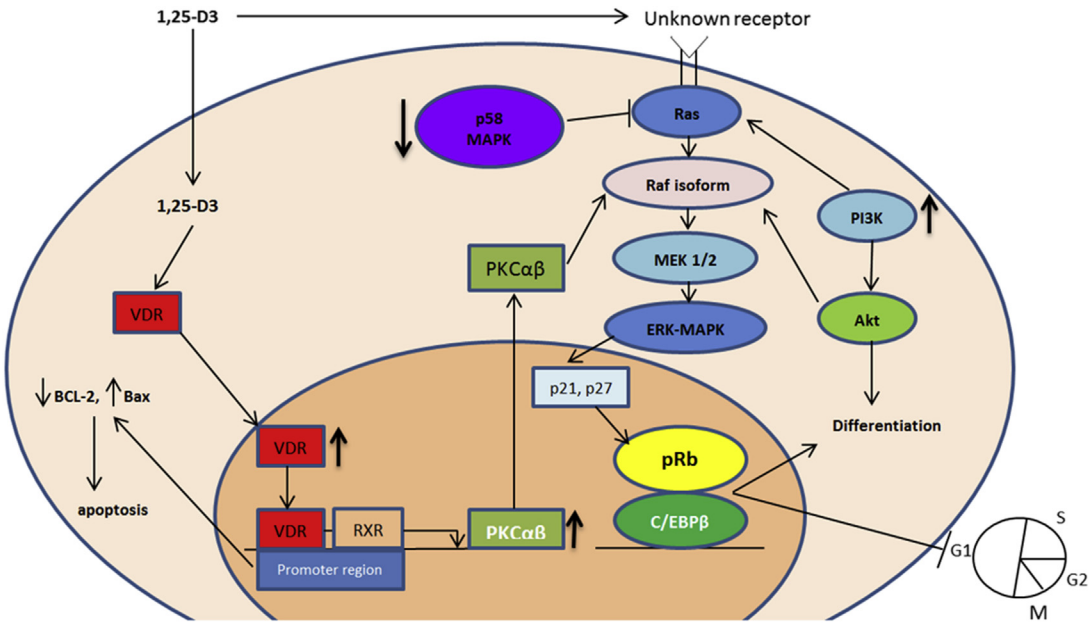

Figure 1. General schematic of the interaction of 1,25-D3 with various pathways in vitro. C/EBP = CCAAT-enhancer-binding protein; Raf = rapidly accelerated fibrosarcoma; Rb = retinoblastoma.

HL60, a highly responsive cell line, expressed a shorter VDR variant than KG-1, a less responsive cell line [33]. Studies of enhancers/silencers in VDR regular region and their interactions with the VDR promoters in different cell lines might further our understanding this wide spectrum of responses. Unlike RA, which works specifically on the AML M3 subtype with a reciprocal t(15;17)(q22;q12) chromosomal translocation, none of the in vitro studies on VDAs have identified a specific mutation or translocation. Therefore, further deep-gene-sequencing studies corresponding to VDA targeting are needed to address this issue.

An ex vivo study from Baurska et al. found that samples from patients with an AML M5 subtype underwent the most differentiation with VDA treatment [74]. Further, samples with normal karyotype responded with more efficient differentiation than samples with abnormal karyotypes. FLT-3 mutation samples were more resistant to the VDA treatment than NPM1 mutation samples. Therefore, future studies should focus on elucidating the molecular mechanism of selectivity of VDAs in AML subtypes.

In vivo studies. VDAs have also been tested in animal models. The earliest studies performed in a mouse model can be traced back to the late 1980s. Specifically, Zhou et al. used 1,25-dihydroxy-16-ene-23-yne-vitamin D3 to treat leukemia-induced BALB/c mice [75]. This agent is a VDA that is very potent in inhibiting proliferation, inducing differentiation of myeloid leukemic cells in vitro, but is 300 times less active in mediating intestinal calcium absorption and bone calcium mobilization compared with conventional 1,25-D3. The investigators explored the therapeutic potential of this VDA using three different leukemia models that differed in disease burden. In the first, BALB/c mice were injected with 2.5×10^5 myeloid leukemic cells in combination with no treatment, 1,25-D3, or the VDA. Death secondary to leukemia occurred in all mice that received no treatment or 1,25-D3 by day 26, whereas mice that received VDA had a significantly longer survival, with the last mouse dying of leukemia on day 50. In the second model, injecting 1×10^5 leukemic cells into BALB/c mice with no treatment resulted in 86% dead of leukemia at 51 days. BALB/c mice that received the same amount of leukemic cells and treatment with VDA had a significantly longer survival than controls; only 53% of the mice were dead by day 100. In the third model, injecting a lower amount of leukemic cells (1.5×10^4) into the control group (no treatment) and the treatment group (VDA) resulted in significantly different survival (13% vs. 43% disease free at day 180) [75]. The study demonstrated that this particular VDA could significantly increase the survival of mice with myeloid leukemia.

The essential role of vitamin D and its receptor in hematologic dysplasia was further demonstrated by Erben et al. [76]. By gene targeting, they were able to generate mice expressing functionally inactive mutant VDR. After a change in environmental conditions from specific-pathogen-free conditions to a modified barrier system, a high percentage of mutant, but not wild-type, mice developed a hematological disorder characterized by splenomegaly, granulocytosis, thrombocytosis, and dysplastic changes with displacement of erythropoiesis in BM during subsequent months. All cases were associated with high serum levels of acute-phase reaction protein serum amyloid A and

antibodies against murine hepatitis virus; however, electron microscopy of spleen and BM cells did not reveal virus particles [76]. This study illustrated an important association between VDR and the regulation of myeloid cell differentiation and proliferation, especially under conditions of environmental stress.

Clinical experience with 1,25-D3 and its VDAs in AML

Although vitamin D's mechanism of action and its effect on leukemic cells have been elucidated extensively in the preclinical setting, its translation into clinical practice has been challenging. There have been >20 clinical trials using 1,25-D3 or its analogs for both MDS and AML patients (Table 1). The majority of trials were small and enrolled MDS or AML patients who were not ideal candidates for traditional 7 + 3 regimens (anthracycline + cytarabine) or had exhausted other options (i.e., failed hypomethylating agents). The outcomes were mixed, ranging from no objective response in one study up to 79% in another study with patients who failed prior chemotherapy. These differences reflect the heterogeneity of the studied populations, assortments of VDAs and different dosages being utilized, and different definitions of response from earlier studies (survival, remission rates, objective response from BM, and hematologic response) [77,78,81–98]. As summarized in Table 1, the largest number of patients in a single study was 63, with many studies reporting findings in groups of <20 patients. Treatment options were heterogeneous, with either single-agent vitamin D/VDAs or combinations of VDAs, cytarabine, and other agents (alpha-interferon, prednisone, RA, hydroxyurea, thioguanine, valproic acid, vitamin K, and deferasirox [DFX]). There was no consensus among researchers in the type and dosage of VDAs used. Some of the common VDAs used were vitamin 1,25-D3, 1 alpha-hydroxyvitamin D3, calcifediol, paricalcitol, and doxercalciferol. To summarize, the clinical outcomes of 1,25-D3 and VDAs are rather disappointing due to the limitations and inconsistencies between studies.

Combination treatment with 1,25-D3 and low-dose cytarabine generally tended to have better responses. Unfortunately, there are no recent clinical trials that further explore the option of combined therapy of VDAs with low-dose cytarabine. The latest study that involved combination treatment with vitamin D and cytarabine was in 2004 [90]. It enrolled 26 AML patients and four high-risk MDS patients who were ineligible for intensive chemotherapy because of age (average 70 years), poor clinical conditions, or treatment refusal. They were treated with a combination of 13-cis RA (20–40 mg/d), 1,25-D3 (1 μg/d) for 5 weeks, with the addition of 6-thioguanine (6-TG, 40 mg/d) and cytosine arabinoside (Ara-C, 8 mg/m^2 × 2/d by subcutaneous injection) during the first 2–3 weeks. The 5-week course was repeated if there was no disease progression observed. After two courses of treatment, patients who had at least a partial response (PR) continued a maintenance therapy with RA + 1,25-D3 + intermittent 6-TG or Ara-C + 6-mercaptopurine (50 mg/d) for 14 days every 5–6 weeks until disease progression. Eight patients (27%) achieved a complete response (CR) and seven (23%) had a PR. The median response duration and survival were both 7.5 months and the 2-year survival was 17%. The patients who responded (CR + PR) lived significantly longer (median of 16.5 months, 2-year survival of 34%). Considering their age and poor prognosis, most patients tolerated the therapy well, with grade 4 neutropenia and thrombocytopenia observed in 28 patients (94%) during the first two courses of low-dose chemotherapy. Thirteen patients completed the induction treatment as outpatients, whereas 17 patients were hospitalized for a median time of 18 days. Eleven patients required intravenous antibiotics and two required granulocyte colony-stimulating factor for prolonged neutropenia. This study highlighted an alternative treatment option for AML patients, especially those of advanced age and poor clinical status.

Akiyama et al. in Japan conducted an interesting, open-labeled single-arm prospective phase II clinical trial of menatetrenone (a vitamin K2 analog) and alfacalcidol (1-alpha-hydroxyvitamin D3) for patients with MDS [96]. The efficacy of oral menatetrenone therapy in MDS has been reported in Japan, with improvement of cytopenias ranging from 20% to 75% in clinical pilot studies [99–101]. Although the underlying mechanism of action remains unknown, vitamin K2 (VK2) has been reported to induce apoptosis and differentiation in some leukemic cell lines in vitro [102–104]. Further, there are reports of combination of VK2 plus either 22-oxa-1,25-D3 or 1,25-D3 synergistically, enhancing the induction of cellular differentiation in HL-60 cells along with inhibition of VK2-induced apoptosis in vitro [105,106]. In the first part of this study, 38 patients received VK2 monotherapy (45 mg/d) [96]. After 16 weeks of therapy, based on the International Working Group criteria, 5 of 38 patients responded, including four cases with improvement of both anemia and thrombocytopenia and one case with thrombocytopenia. Of the 33 nonresponders, 23 patients subsequently received combination treatment of VK2 and alfacalcidol (0.75 μg/d) for 16 weeks. Of the 20 evaluable cases, six patients showed a response (30%). The investigators noticed that higher International Prognostic Scoring System scores and absolute neutrophil counts were positively correlated with the response to VK2 and alfacalcidol combination therapy.

In a retrospective case–control study, Paubelle et al. enrolled 17 elderly AML patients who had failed prior demethylating agents [98]. A combination of DFX (1–2 g/d) and 25-hydroxycholecalciferol (VD) (100,000 IU/wk) was given to these patients, who were not eligible to receive any other treatments. During the same period, 13 matched-control patients received best supportive care (BSC). Baseline characteristics in terms of blood tests and AML prognostic factors were not different between the two

Table 1. Summary of clinical trials using vitamin D or its analogs to treat MDS or AML

Study	Disease	No. of patients	Treatment	Result
Koeffler et al. [77]	MDS	18	Vitamin 1,25-D3	Seven patients developed leukemia by end of 12 weeks; eight patients developed hypercalcemia; no enduring therapeutic effect
Hellstrom et al. [78]	MDS, AML	62	Different combinations of low-dose Ara-C, alpha-interferon (IFN), 1-alpha-hydroxyvitamin D3, RA (IDR)	Overall response rate 44%; 50% responded favorably to the combination of IFN, vitamin D3, and IDR
Masuda et al. [79]	AML	8	1-alpha-hydroxyvitamin D3	Four patients with CR, one PR, and two minor responses (MR); concentrations of 25-OH-D and 1,25-D3 in the BM had very similar values to those in serum
Takahashi et al. [80]	MDS, AML, chronic myeloid leukemia	8, 2, 1	0.25–10 μg/d of vitamin 1(OH)D3	Three patients with PR, three patients with minor response, and five with no response; hematological improvement of responders lasted 1–2 months
Hellstrom et al. [81]	MDS, AML	63, 15	Low-dose Ara-C, 13-cis-RA (13-CRA), vitamin 1(OH)D3	18 (26.1%) responded to therapy; 12/27 patients progressed from MDS to AML; 6/29 patients progressed receiving 13-CRA and 1(OH)D3
Blazsek et al. [82]	MDS		Prednisone, vitamin 1,25-D3, 13-CRA	Long-lasting hematological remission
Motomura et al. [83]	MDS	30	15 patients treated with 4–6 μg/d of 1-hydroxyvitamin D3 (D group) vs. 15 patients with no treatment (N group)	Leukemic transformation free survival of D group had significant advantage over N group; seven of 15 patients in N group developed acute leukemia; one of 15 patients in D group developed leukemia
Petrini et al. [84]	Acute non-lymphoid leukemia		Low-dose Ara-C and 1(OH)D3	17% with CR; 45% with PR; seven of 11 showed monocytic/monoblastic shift
Slapak et al. [85]	AML	29	Cytarabine, hydroxyurea, 1,25-D3	79% response rate: 45% with CR, 34% with PR; three early deaths; median remission duration was 9.8 months; overall median survival was 12 months for all patients
De Rosa et al. [86]	MDS	44	Low-dose Ara-C, RA, and vitamin 1,25-D3	Response rate 50%; survival in responders statistically better than in nonresponders; toxicity acceptable
Ferrero et al. [87]	MDS	53	Low-dose combination of CRA (20–40 mg/d) and 1,25 alpha(OH)2 (1–1.5 μg/d) ± 6-thioguanine (30 mg/m^2/d)	In 25 patients with BM blasts $<5\%$, response rate was 52% with median response duration of 8 months and median survival of 76 months; in 31 patients with BM blast excess $>5\%$, response rate was 61% with median response duration of 6 months; reduction in transfusion need observed
Mellibovsky et al. [88]	MDS	19	Calcifediol 266 μg 3×/wk; calcitriol 0.25–0.75 μg/d	11/19 patients responded
Hirri et al. [89]	MDS, BM fibrosis	1	Vitamin 1,25-D3 0.75 μg/d × 12 weeks	CR in hemoglobin level; complete reversal of fibrosis
Ferrero et al. [90]	AML, MDS (all old/poor prognosis patients)	26, 4	13-CRA (20–40 mg/d) + 1,25-D3 (1 μg/d) for 5 weeks; 6-TG (40 mg/d) and Ara-C 8 mg/m^2 twice a day for 2–3 weeks	Response rate of 50% with 27% CR; overall median survival of 7.5 months and 16.5 months in responders
Koeffler et al. [91]	MDS	12	Paricalcitol starting at 8 μg and increasing in increments of 8 μg/d	No responses observed
Yamada et al. [92]	Relapsed AML	2	Patient 1: cytarabine 0.6 mg/kg × 14 days + calcitriol 0.25 μg twice a day; Patient 2: low-dose cytarabine + calcitriol + aclarubicin 20 mg × 4 days	Remission observed in both patients with approximate duration of 6 months
Siitonen et al. [93]	MDS, chronic myelomonocytic leukemia	19	Valproic acid dose escalation + 13-cis RA (10 mg twice a day) + 1,25-D3 (1 μg/d)	Eight patients discontinued treatment due to toxicity; three patients (16%) responded to treatment
Petrich et al. [94]	MDS	15	Doxercalciferol 12.5 μg/d for 12 weeks	One patient removed from study due to hypercalcemia; 9 of 15 patients completed treatment course and six of nine patients experienced stable disease; eight patients had progressive disease

(*continued*)

Table 1. (*continued*)

Study	Disease	No. of patients	Treatment	Result
Ferrero et al. [95]	MDS	63	Combination of 13-CRA, dihydroxylated vitamin D3 ± 6-TG in addition to recombinant erythropoietin	60% erythroid response rate; Median response duration was 16 months; median survival was 14 months for refractory anemia with excess blasts type 1 (RAEB1) and 55 months for non-RAEB patients
Akiyama et al. [96]	MDS	38	Part 1: VK2 (45 mg/d) only; part 2: nonresponders in part 1 received VK2 + 1 alpha-hydroxyvitamin D3 (VD3) (0.75 μg/d)	Overall response rate of 13% (5 of 38 patients) in VK2 monotherapy; 30% response rate (six of 20 patients) with VK2 and VD3 combination therapy
Crisa et al. [97]	MDS	63	Combination of 13-CRA, dihydroxylated vitamin D3 ± 6-TG in addition to recombinant erythropoietin	Updates of 7-year follow-up of previous study by Ferro et al.; 49 patients died during follow-up; leukemic evolution occurred in 11 patients, five (31%) from RAEB1 and six (13%) from non-RAEB; causes of death were infections (17%), cardiovascular events (14%), second tumors (8.5%), ischemic stroke (6%), hepatic failure, transfusion reaction, autoimmune hemolytic anemia, and unknown cause (nine patients)
Paubelle et al. [98]	AML patients who failed demethylating agents	17 (treatment) vs. 13 (BSC)	Combination of DFX (1–2 g/d) + 25-hydroxycholecalciferol (100,000 IU/wk) vs. BSC	Median survival was significantly increased in the treatment group (10.4 vs. 4 months)

AML = Acute myeloid leukemia; BM = bone marrow; MDS = myelodysplastic syndrome; PR = partial response.

groups. At the 6-month evaluation, four treated patients had significantly increased monocyte numbers, but the transfusion requirement did not decrease in the DFX/VD group. However, the median survival of patients treated with the DFX/VD combination was significantly increased compared with matched patients receiving BSC alone (10.4 vs. 4 months). The investigators found that the only factor associated with an increased overall survival in the treatment group was normal serum vitamin D levels (≥50 nmol/L).

To summarize, clinical application of 1,25-D3 has resulted in inconclusive data due to the aforementioned limitations, but most importantly, the major obstacle of this therapy was adverse events secondary to systemic hypercalcemia [77,94,107]. The concentrations of 1,25-D3 required to induce differentiation in vitro are typically in the range of 10–100 nmol/L [108]; however, a serum level of such a concentration would result in hypercalcemia in humans, in whom the typical concentration of 1,25-D3 is ∼0.1 nmol/L. In the next section, we will discuss new findings and possible solutions to this limitation.

Future directions and possible solutions

As mentioned in the "Clinical experience of 1,25-D3 and its VDAs in AML" section, one of the major obstacles to clinically meaningful application of 1,25-D3 to AML treatment is systemic hypercalcemia. One strategy is to synthesize a VDA that has enhanced antitumor activities but limited systemic interaction. The ideal VDA would be characterized by reduced gastrointestinal calcium absorption while maintaining other properties; patients receiving this VDA should be instructed to maintain a low-calcium diet. In this way, patients could be treated with a much higher dose of VDA without incurring systemic hypercalcemia. Thus far, several VDAs were tested in clinical trials, but the results were rather disappointing (Table 1). It is possible that, due to concerns about systemic side effects, the doses given in those trials did not reach the local therapeutic level required to achieve antitumor properties. Another potential strategy is to deliver high-dose 1,25-D3 locally to the BM utilizing either cell surface marker (α4 integrin) or substrate (bisphosphonates) with a strong affinity for bone to guide vehicle cells, as described by Yao and Kumar [109,110].

The impact of 1,25-D3 in the maintenance of the normal epigenetic landscape underlines its potential role in AML epigenetic regulation and prognosis. Puccetti et al. reported that AML-associated chromosomal translocated fusion proteins (PML/RARα, PLZF/RARα, and AML-1/ETO) block blast differentiation process by sequestering VDR, which is involved in the differentiation signaling pathways [26]. These fusion proteins inhibit downstream transcriptional signaling of VDR through aberrant recruitment of HDAC activity by binding to corepressors. They found that inhibition of HDAC activity increases 1,25-D3-induced leukemic differentiation of HL60 cells. In addition, overexpression of VDR in the U93 cells expressing the AML/ETO fusion protein overcomes differentiation blockage. Combination therapies of 1,25-D3 with HDAC inhibitors or hypomethylating drugs might provide new treatments for AML [111]. One such promising combination is 1,25-D3 and 5-azacytidine (AZA). Because both AZA and 1,25-D3 demonstrate the ability to affect gene expression and induce leukemic differentiation, it is possible that their combined treatment might

result in synergistic effects. AZA is a ring analog of cytidine that possesses cytotoxic activity through incorporation into DNA [112]. In addition, it induces leukemic cell differentiation in vitro and inhibits DNA methyltransferase, which results in synthesis of hypomethylated DNA and changes in gene transcription and expression [113]. AZA received approval from the Food and Drug Administration in 2004 for the treatment of MDS, but in the clinical setting, it is often used as a palliative treatment for unfit patients with AML. Our postulation of combination treatment is further strengthen by a recent retrospective review study in which Radujkovic et al. reported that MDS and oligoblastic AML patients who were treated with AZA had a significantly worse 2-year overall survival (14% vs. 40%, $p < 0.05$) if their vitamin D level (25[OH]-D3) was <32.8 nmol/L [114]. They also found that AZA and active VD3 worked synergistically to inhibit growth of AML cell lines in vitro. Therefore, oral supplementation with 1,25-D3 or VDAs in combination with systemic AZA are both reasonable options to be explored further in AML mouse models and in the clinical setting. This could potentially lower the dose of 1,25-D3/VDAs required to achieve clinical benefits.

Conclusion

As scientists make great strides in the molecular genetic front, major breakthroughs in treatment for both hematologic and oncologic diseases have been achieved. However, such is not the case with AML because the standard regimens have remained the same and the overall survival rate continues to be abysmal for elderly and frail patients. This underscores the complexity of the disease and also highlights the fact that standard treatment is too toxic for elderly or unfit patients. Based on the available data, it is likely that vitamin D therapy would not be a "one size fit all" treatment for AML. Therefore, it is critical that we try to define a subtype of AML that would be most responsive to vitamin D. Nevertheless, using vitamin D and VDAs alone as an alternative, palliative treatment for elderly/unfit patients or in combination with low-dose cytotoxic chemotherapy with potential for long-term remission is an appealing concept and has the possibility of limiting the systemic side effects. However, several important questions (i.e., dosage, method of delivery, systemic hypercalcemia, and mechanisms of action of combination treatment) must be addressed before vitamin D treatment can be applied to the clinical setting.

Acknowledgments

The authors thank the Loma Linda University Cancer Center for support of this project.

All costs associated with the study design, collection, analysis, interpretation of data, and writing the manuscript were funded by the Loma Linda University School of Medicine, Division of Medical Oncology/Hematology Internal Research Fund.

Conflict of interest disclosure

The authors declare no competing financial interests.

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