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Cooperative anti-leukemic effects of vitamin D derivatives and plant polyphenols in AML models: the role of the interplay between NRF2, AP-1, and VDR

Treatment of acute myeloid leukemia (AML) has not significantly improved in the past 40 years. The active form of vitamin D, 1.25-dihydroxyvitamin D3 (1,25D3), has strong in-vitro antileukemic effects at toxic concentrations in vivo. Several low-calcemic vitamin D analogs (VDAs) have been synthesized to reduce 1,25D3 toxicity. We have previously shown that plant polyphenolic antioxidants (PAOx) synergistically enhance the effects of 1,25D3 and VDAs at non-toxic doses. These effects were mediated by the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and activator protein-1 (AP-1) transcription factors. The study aimed to 1) Characterize the combined effects of novel highly potent vascular disrupting agents with peroxisomal N(1)–acetylspermine/spermidine oxidase on differentiation and growth of acute myeloid leukemia cells; 2) Elucidate the role of the interplay between nuclear factor erythroid 2, activator protein-1, and vitamin D receptor (VDR) in the combined antileukemic effects of vascular disrupting agents and peroxisomal N (1)–acetylspermine/spermidine oxidase. This article presents the treatment of AML with pharmaceutical plants and vitamin D. Such techniques as culture dilution (HL60, U937) and Western Blotting were used. We determined the in-vitro antileukemic effects of novel plant vitamin D2-based VDAs with removed C-19 (19-nor analogs; PRI-5201 and PRI-5202) or with a side-chain modification at C-24 (24-cis analogs; PRI-1916 and PRI-1917) on HL60, U937, and MOLM-13 human AML cells. The differentiation potency of PRI-5201 and PRI-5202 was 1-2 orders of magnitude higher than that of 1,25D2 or 1,25D3, while the 24-cis modification was almost ineffective. The 19-nor VDA/CA combinations are promising for treating AML. In vivo, testing of these combinations is in progress. We suggest that direct or indirect Nrf2-mediated up regulation of AP-1 and VDR by PAOx increases the sensitivity of AML cells to low doses of 1,25D3 or VDAs.

Keywords: acute myeloid leukemia (AML), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), activator protein-1 (AP1), polyphenolic antioxidants (PAOx), vitamin D analogs (VDAs).

Introduction

Leukemia is represented by a variety of malignant diseases that are characterized by the uncontrolled proliferation of white blood cells (WBC) [1–3]. These actively dividing, immature, functionless cells accumulate in the bone marrow and peripheral blood of a patient with leukemia [4]. The bone marrow can no longer produce enough normal erythrocytes, WBC, and platelets. As a result, leukemia patients suffer from anemia, cytopenia, increased risk of infections, and bleeding. Additionally, malignant hematological cells circulating in the peripheral blood may reach the lymph nodes, liver, spleen, central nervous system, testicles, and other organs [5-6].

There are about 300,000 leukemia new cases over the world each year and 222,000 deaths. The five-year relative survival rate for patients with leukemia has more than tripled in the past 47 years [3]. In 1960–1963, a leukemia patient had a 14 percent chance of living for five years when compared to a person

without leukemia. Gradually, the overall relative survival rate increased to almost 50 percent in 1996–2003. However, the five-year survival improved mainly for patients with acute lymphocytic leukemia (60 percent) and chronic lymphocytic leukemia (70 percent), whereas the same five-year survival rate for patients diagnosed with acute myelogenous leukemia (AML) remained low, at approximately 20 percent [7].

To date the main treatment for acute myeloid leukemia AML, chemotherapy is very ineffective and toxic. Following the treatment 80 percent of AML patient will dye of the disease or due to cytotoxic effects [8]. Consequently, new therapies are necessary to improve survival of AML patients. Differentiation therapy is used as an alternative or complement to conventional chemotherapy for the treatment of cancer. Several compounds are known to induce differentiation of neoplastic cells. For example, all-trans retinoic acid (ATRA) has proven extremely valuable in the treatment of acute promyelocytic leukemia (APL), a subtype of AML. The serendipitous introduction of ATRA for the treatment of APL turned out to be the first truly effective targeted therapy for the treatment of APL and has had a profound effect on increasing the cure rate of this disease. Similarly, vitamin D3 is currently under investigation as a differentiating agent in a variety of tumor types. It is well-known that the therapeutic use of the most biologically active vitamin D metabolite, 1,25D3, is limited by its hypercalcemic effect. Synergistically acting drugs that can potentiate the differentiating effect of 1,25D3 at low, non-toxic concentrations may present a solution to this challenge [9-10].

This research is a continuation of our studies demonstrating that the plant-derived polyphenol carnosic acid (CA) enhances myeloid leukemia cell differentiation induced by nearly physiologic concentrations of 1,25D3 and ATRA (10). The aim of the present study is to investigate the molecular mechanism of potentiation of 1,25D3-induced myeloid leukemia cell differentiation by CA.

Tasks: 1) To characterize combined effects of novel highly potent vascular disrupting agents with peroxisomal N (1)-acetyl-spermine/spermidine oxidase on differentiation and growth of acute myeloid leukemia cells; 2) To elucidate the role of the interplay between nuclear factor erythroid 2, Activator protein 1 and vitamin D receptor in the combined anti-leukemic effects of vascular disrupting agents and peroxisomal N (1)-acetyl-spermine/spermidine oxidase.

Experimental

Reagents and antibodies. Carnosic acid was purchased from Alexis Biochemicals (Läufenfingen, Switzerland). 1,25D3 was a gift from Dr. Milan Uskokovic (BioXell, Inc., Nutley, NJ). 12-O-tetradecanoylphorbol 13-acetate (TPA), tert-butylhydroquinone (tBHQ), and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO). The antibodies against VDR (C-20), Nrf2 (C-20 and H-300), c-Jun (H-79), c-Fos (4-10G), ATF2 (C-19X), and NQO1 (C-19), were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-GCS antibody (RB-1697-P) was obtained from NeoMarkers (Fremont, CA). Anti-calreticulin antibody (PA3-900) was obtained from Affinity BioReagents (Goden, CO). Peroxidase-conjugated AffiniPure donkey anti-rabbit and donkey anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Stock solutions of CA (10 mM), 1,25D3 (0.25 mM), and tBHQ (30 mM) were prepared in absolute ethanol. TPA (1 mM) and (MG132 25 mM) stock solutions were prepared in DMSO. The precise concentration of 1,25D3 in stock solutions was verified spectrophotometrically at 264 nm.

Cell Culture and Treatment. HL-60 human myeloblastic leukemia cells and U937 human myelomonocytic leukemia cells were obtained from Dr. R. Levy (Ben-Gurion University). Cells were grown in RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 10% FCS. Cells were plated in 6-well plates (4 × 104 cells/ml) unless stated otherwise and incubated with the indicated concentrations of CA and/or 1,25D3 for various periods of time. All procedures were done under dim light.

Western Blotting. Cells were washed twice with ice-cold PBS and resuspended 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 TM protease inhibitor cocktail. The lysates were incubated for 10 min on ice, and the cellular debris were cleared by centrifugation (20,000 x g, 10 min, 4°C). The protein content of the samples was determined by the Bradford method using a protein assay kit (Bio-Rad, Richmond. CA). Equal amounts of protein (30 μg) were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked with 5% nonfat milk and probed with one of the abovementioned primary antibodies. Protein bands were visualized using the Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, MA) after exposure of the membrane to a corresponding secondary antibody coupled to horseradish peroxidase (Promega, Madison, WI). Protein abundance

was quantitated by densitometric analysis using an Image Master VDS-CL imaging system (Amersham Pharmacia Biotech).

Statistical Analysis. The significance of the differences between the means of the various subgroups was assessed by the two-tailed Student's t-test. Two compounds (A and B) were considered to show enhancement in the 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. Data are presented as mean±SE. P value less than 0.05 was statistically significant. The computations were performed using Microsoft EXCEL and GraphPad Prism software (GraphPad Software, San Diego, CA) programs.

Results

Figure 1 illustrates that, while not affect the differentiation of U937 cells, CA (10μM) strongly potentiated the ability of a low concentration of 1,25D3 (2.5 nM) to induce expression of an early monocytic surface marker, CD14, following treatment for 60 h. Cells were incubated with vehicle (A) or indicated compounds (B, D, E) for 60 h, followed by expression analysis of the surface differentiation markers CD11b and CD14, as described in "Materials and Methods". 1,25D3 (100 nM) was used as a positive control (F). Panel C summarizes the data of two independent experiments (means ± the upper and lower limits of experimental values).

The expression of a general myeloid differentiation marker, CD11b, was also enhanced. Importantly, the differentiation effect of combination treatment was like that induced by a high (100 nM) concentration of 1,25D3 alone.

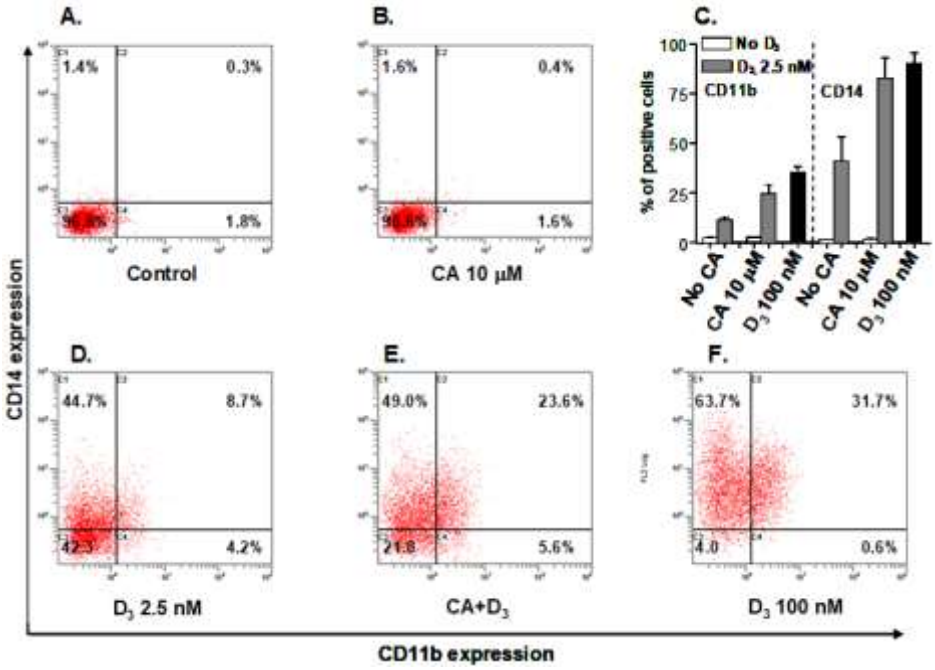

Figure 1. CA potentiate U937cell differentiation induced by 1,25D3 (D3)

Since, it was recently demonstrated in our laboratory that CA acts as an antioxidant in HL60G leukemia cells, we first determined whether potentiation of $1,25D_3$–induced differentiation by CA is accompanied by the activation of the Nrf2/ARE system. Therefore, we set out to investigate whether CA induces any detectable changes in the mRNA and protein levels of Nrf2, the key transcription factor of the ARE. U937 cells were incubated with 10 μM CA or 30 μM tBHQ (the classic Nrf2/ARE inducer) for 1 h, followed by semi-quantitative RT-PCR to measure mRNA levels. As shown in Figure 2 (A, B), these antioxidants did not significantly change Nrf2 expression. However, Nrf2 protein levels were increased in U937 cells treated for 1 to 6 h with CA or tBHQ (Fig. 2C), as compared to control cells. Cells were treated with CA or tBHQ for the indicated times, followed by determination of Nrf2 mRNA and protein levels by semi-quantitative RT-PCR (A) and western blotting (C), respectively. Whole cell lysate of tBHQ-treated T47D breast cancer cells was

used as a positive control (C). 28S (A) and calreticulin (C) were used as loading controls. In A and C, one representative of two similar experiments is shown. In B, the quantification of these experiments is presented. Data shown represent the average of two experiments. Error bars correspond to the variance of the means.

The total protein extracts from tBHQ-treated T47D breast cancer cells were used as a positive control for Nrf2 protein expression.

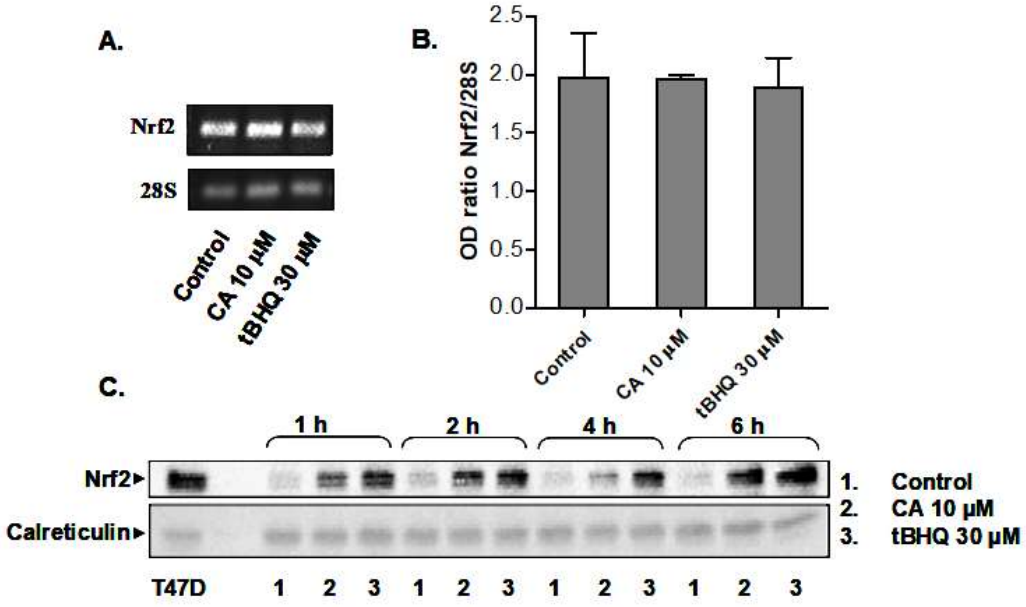

Figure 2. Induction of Nrf2 in U937 cells

In order to evaluate whether CA is able to transactivate the ARE, we measured its effects on the NQO1-ARE4-luc reporter gene system in transiently transfected U937 cells. As shown in Figure 3A, CA induced transactivation of the ARE in a concentration-dependent manner. Concentrations higher than 20 μM were found to be cytotoxic (data not shown). Additionally, other plant-derived polyphenols — curcumin, carnosol, and silibinin — with known antioxidant property were able to reduce the levels of intracellular ROS in the HL60 leukemic cells (Gabi Izumchenko, unpublished data) and were evaluated as possible inducers of the NQO1-ARE4 reporter gene system in leukemic cells. We found that, unlike CA, neither silibinin nor rosmarinic acid transactivated the ARE, but carnosol and curcumin activated the ARE-luc reporter in a concentration-dependent manner, albeit to a lesser extent than CA (Fig. 3A, B). Accordingly, figure 3B demonstrates that CA and curcumin, but not silibinin, transactivated the ARE×4-luc reporter gene system, which contains four tandem synthetic ARE repeats.

In order to evaluate whether CA and $1,25D_3$ are cooperative in terms of ARE transactivation, we measured the effects of CA, $1,25D_3$, and their combination treatment on the NQO1-ARE4-luc (Fig. 4A) and ARE×4-luc (Fig. 4B) reporter gene systems in transiently transfected U937 cells. When added alone, $1,25D_3$ did not significantly affect the ARE activity but moderately enhanced CA-induced ARE transactivation (Fig. 4A, B) U937 (A, B) and WEHI-3B (C) leukemia cells (2×10^5) were co-transfected with NQO1-ARE4-luc (A) or ARE×4-luc reporter (B, C) and Renilla plasmids. Four hours later, cells were treated with CA or $1,25D_3$ (D_3) alone and in combination. Luciferase activity was determined after an additional 20 h. tBHQ was used as a positive control. The Firefly luciferase to Renilla luciferase ratios of a preliminary experiment (B) or of 2-4 experiments (A, C) are presented as the means ± SE of triplicate.* significant enhancement of CA effect by $1,25D_3$, as compared to CA alone ($P < 0.05$). To demonstrate that the CA-induced ARE transactivation is not unique to the U937 cell line, we transiently transfected WEHI-3B murine myelomonocytic leukemia cells with the ARE×4-luc reporter plasmid and evaluated ARE induction. In results similar to those obtained in U937 cells, CA transactivated the ARE in WEHI cells, and $1,25D_3$ increased CA-induced ARE transactivation; $1,25D_3$ alone, though, even at 100 nM concentration, did not affect ARE activity (Fig. 4C).

To assure that the results of the reporter gene assays reflect a physiological increase in ARE activity, we determined whether CA and $1,25D_3$ treatments induced the expression of the Nrf2/ARE-responsive proteins,

NQO1 and the catalytic subunit of γ-GCS, in untransfected U937 cells. As shown in Figure 5A, NQO1 was substantially induced in cells treated for 20 h with CA (10 μM).

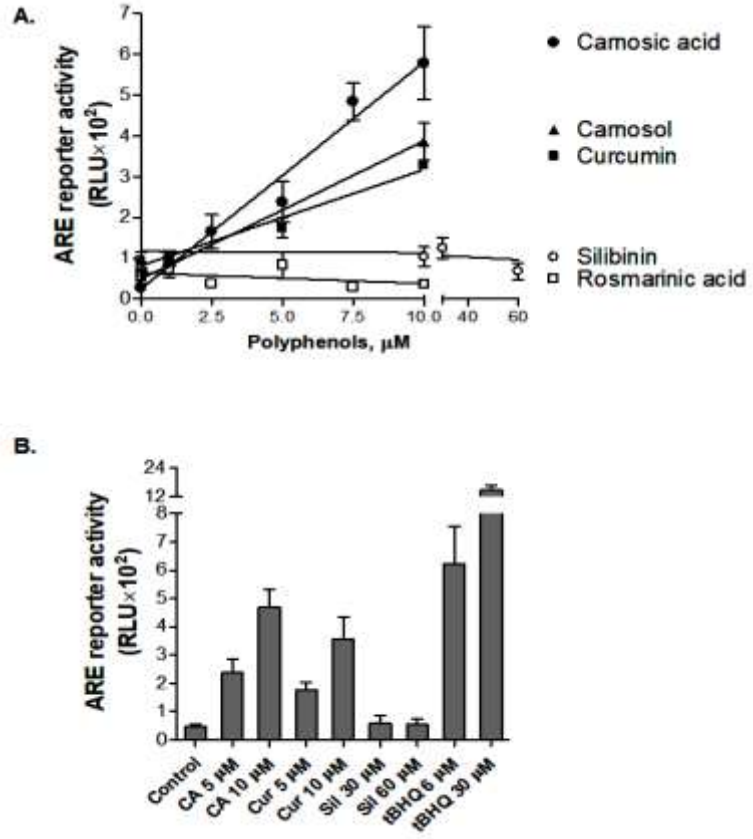

Figure 3. Effects of different polyphenols on the ARE transactivation. U937 cells (2×10^5) were co-transfected with NQO1-ARE4-luc (A) or ARE×4-luc (B) reporter and Renilla plasmids

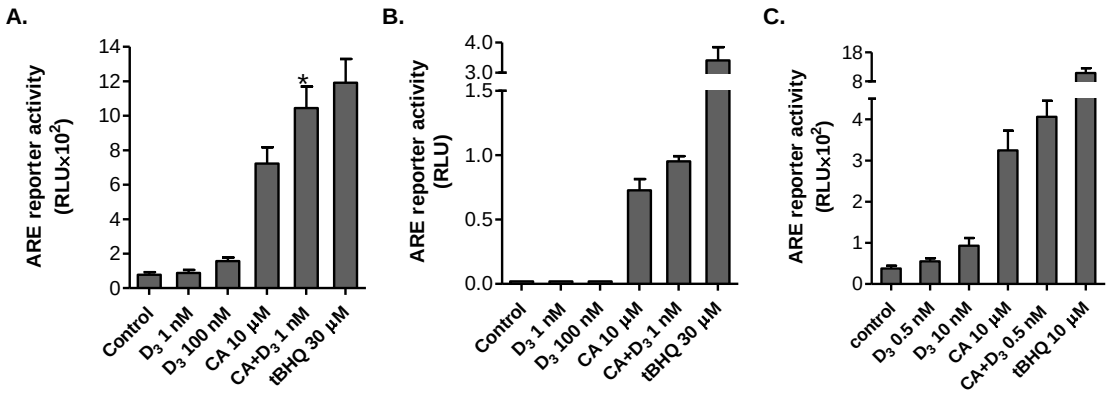

Figure 4. 1,25D_3 moderately increases CA-induced ARE activity

When added alone or in combination with CA, 1,25D_3 did not significantly affect NQO1 expression. γ-GCS was also induced following treatment with CA, and 1,25D_3 slightly enhanced this effect (Fig. 5A, B). tBHQ (30 μM) induced, as expected, the expression of both Nrf2/ARE-responsive proteins. Cells were treated with the indicated agents for 20 h for western blot analysis of NQO1 and γ-GCS levels (A). tBHQ was used as a positive control. Calreticulin was used as loading control. Representative blots of two similar experiments are shown (A). Densitometric analysis of protein bands presented in panel B (means ± the upper and lower limits of experimental values, n=2).

γ-GCS is responsible for glutathione synthesis [5-6]. Therefore, we determined whether the cooperation between CA and 1,25D_3 on ARE transactivation and CA-induced elevation of γ-GCS protein level was accompanied by changes in the intracellular glutathione levels in U937 cells. To this end, cells were incubated with CA (10 μM) 1,25D_3 (1 nM), by themselves and in combination or with tBHQ (30 μM) for 24 h, followed by measurement of total cellular glutathione. When added alone, CA and 1,25D_3 showed a moderate effect (Fig. 6).

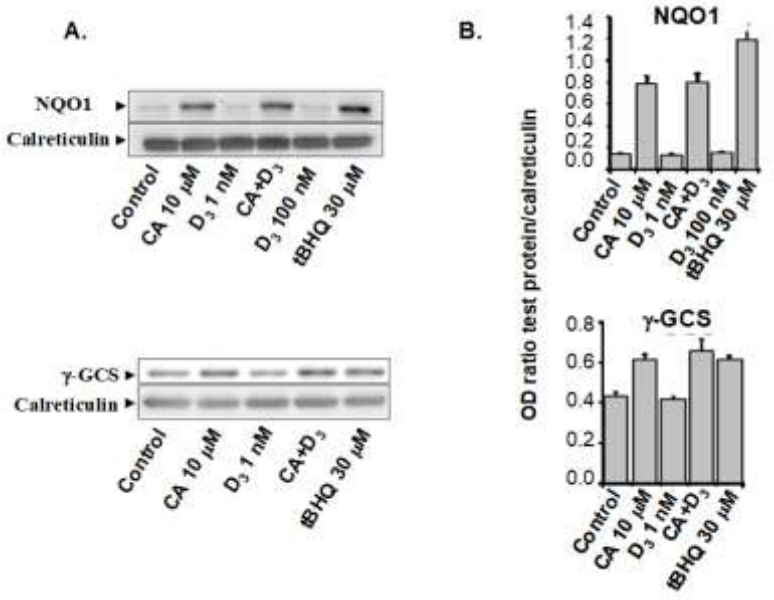

Figure 5**.** Effects of CA, 1,25D_3 (D_3) and tBHQ on NQO1 and γ-GCS protein expression

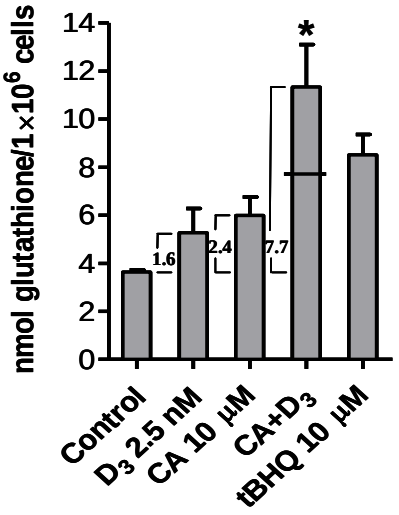

Figure 6. Effects of CA, 1,25D_3 (D_3) and tBHQ on total glutathione levels in U937 cells

Cells were incubated with the indicated compounds for 24 h followed by measurement of total glutathione by the glutathione reductase recycling assay described in "Materials and Methods". Data are the means ± SE of two independent experiments performed in triplicate. *CA/1,25D_3 combination-induced effect was significantly stronger than of the sum of each agent alone which is signed by a horizontal line ($P<0.05$). However, in combination, they produced a substantial synergistic elevation of glutathione levels.

To explore the role of the Nrf2/ARE system in the 1,25D_3/CA-induced differentiation, we employed Nrf2 over expression and down-modulation strategies. To this end, U937 cells were stably transfected with expression vectors for Nrf2, its dominant-negative mutant (dnNrf2), or empty vector (pEF) and were desig-

nated U937-Nrf2, U937-dnNrf2, or U937-pEF cells, respectively. The transfected cells were cloned by limiting dilution. Prior to determine the differentiation induction, the functional consequences of Nrf2 over expression or down-modulation were estimated by determining Nrf2 protein levels (Fig. 7A) and induction of ARE-responsive gene products (Fig. 7B). These assays were performed in two U937-Nrf2 clones (#18 and #20) and two U937-dnNrf2 clones (#13 and #17). Untransfected U937 cells, as well as a mixed U937-pEF clone culture and the isolated clone U937-pEF#3, were used as controls.

As shown in Figure 7A, in the absence of stimulation, untransfected U937 cells, control transfectants, and U937-dnNrf2 clones demonstrated a low expression level of a ~97-kDa Nrf2 polypeptide, whereas U937-Nrf2 clones had relatively higher Nrf2 levels. U937-Nrf2 #18 clone exhibited a slightly higher Nrf2 expression than U937-Nrf2 #20 clone. In both U937-dnNrf2 clones, we observed the appearance of a truncated dnNrf2 form (~22.5 kDa). Upon stimulation with tBHQ, the increase in Nrf2 levels was more pronounced in the Nrf2 transfected cells, U937-Nrf2#18 clone being more responsive than U937-Nrf2#20 clone. Cells were treated with CA or tBHQ for 24 h, followed by determination of protein levels by western blotting. The Nrf2 and dnNrf2 detecting strips were taken from the same blot. The γ-GCS and NQO1 levels were determined in the U937-Nrf2 #18 and U937-dnNrf2 #17 clones. Blots of one experiment are presented. The similar data for the NQO1 levels were obtained in 3 independent experiments performed with mixed (uncloned) dnNrf2 transfectants (data not shown).

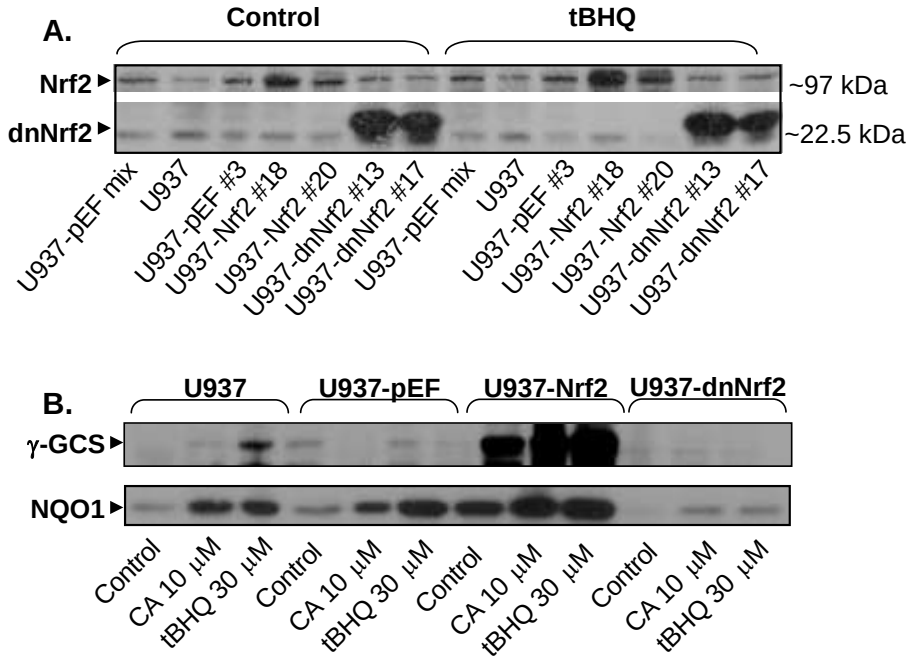

Figure 7. Induction of Nrf2 (A) and ARE-regulated gene products γ-GCS and NQO1 (B) in U937 cells stably transfected with Nrf2, dn-Nrf2, empty vector (pEF), or in untransfected cells (U937)

The enhancement or attenuation of the Nrf2/ARE transcriptional system activity due to Nrf2 modulation was accompanied by respective changes in the expression of the ARE-responsive gene products, NQO1 and γ-GCS. As demonstrated in Figure 7B, Nrf2 over expression resulted in an increase in both the basal and tBHQ-induced NQO1 and γ-GCS expression in the U937-Nrf2 cells, compared to untransfected U937 cells or U937-pEF cells. On the other hand, dnNrf2 expression led to a marked reduction in both the basal and induced NQO1 and γ-GCS levels.

Collectively, these results show that CA activates the Nrf2/ARE system and induces the ARE-regulated proteins, and that transfection of Nrf2 or dnNrf2 can increase or decrease, respectively, the levels and activity of Nrf2 in leukemic cells.

To elucidate the mode of the involvement of the Nrf2/ARE system in the induction of leukemia cell differentiation by $1,25D_3$ and CA, we first explored the idea that this system may participate in the regulation of cellular levels of VDR and RXR and, hence, cell sensitivity to $1,25D_3$. Furthermore, we addressed the role of

Nrf2/ARE system in the regulation of one of the major transcription factors implicated in the regulation of VDR expression, AP-1, in myeloid leukemia cells.

Vitamin D_3 induces leukemic cell differentiation acting via a nuclear VDR/RXR ligand-activated transcription complex. Previous studies in our laboratory have demonstrated that $1,25D_3$ and CA alone upregulates VDR and RXR protein levels in HL60-G cells and greatly synergize in this effect. In order to find whether CA enhances VDR transcriptional activity as well, we measured VDRE activation by a reporter gene assay in transiently transfected U937 cells. As expected, $1,25D_3$ induced a dose-dependent VDRE transactivation (Fig. 8A). CA significantly ($p<0.01$) increased $1,25D_3$-induced transactivation of VDRE-luc reporter system (Fig. 8B). U937 cells (2×10^5) were co-transfected with VDRE-luc reporter and Renilla plasmids. Four hours later cells were stimulated with the indicated compounds. Expression of firefly and Renilla luciferases was determined 24 h after the transfection. $1,25D_3$ (D_3) at 100 nM was used as the positive control. The Firefly luciferase to Renilla luciferase ratios of 7 (A) and 2 (B) experiments are presented as the means ± SE of triplicate. *P<.05, significant enhancement of $1,25D_3$-induced effect by CA, compared with $1,25D_3$ alone.

The effect of the CA/$1,25D_3$ combination treatment was comparable to that of a high concentration of $1,25D_3$ (100 nM). Treatment with the polyphenol alone slightly increased VDRE activity probably due to the presence of $1,25D_3$ traces in serum.

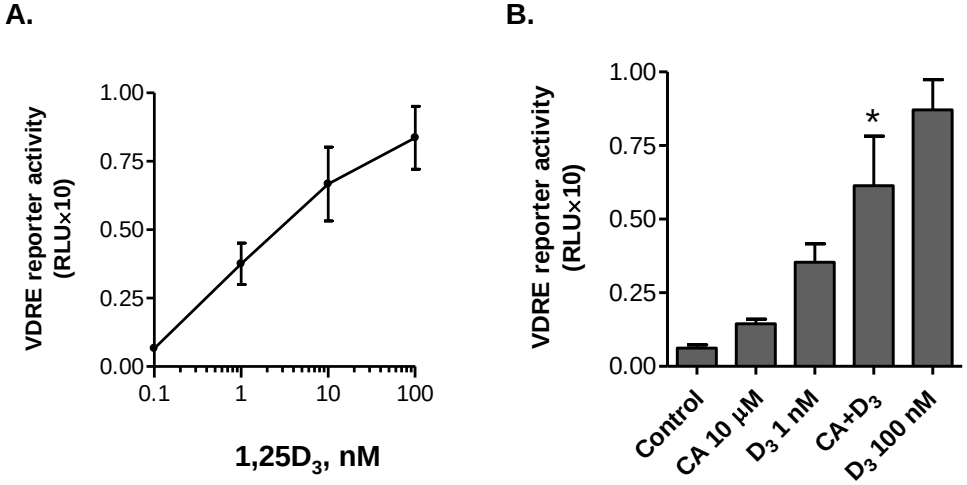

Figure 8. Carnosic acid enhances $1,25D_3$-induced VDRE transactivation

To determine whether the Nrf2/ARE system is involved in the regulation of VDR expression and activity we measured the effects of CA, $1,25D_3$ and their combination on VDR protein levels and VDRE activation in U937-pEF, U937-Nrf2, and U937-dnNrf2 cells. As shown in Figure 8 (A, B), treatment for 24 h with CA slightly increased the VDR protein levels in U937-pEF cells, and this effect seemed to disappear after 96h.$1,25D_3$ (2.5 nM) alone elevated VDR content in U937-pEF cells following incubation for 24 h or 96 h while the addition of CA did not further increase the receptor level. Importantly, Nrf2 over expression resulted in a marked upregulation of treatment induced VDR protein expression. This effect was more pronounced in the U937-Nrf2#18 clone, which had higher Nrf2 levels than the U937-Nrf2#20 clone (Fig. 6). The basal VDR levels also increased in the U937-Nrf2#18 clone (Fig. 8). Transfection with dnNrf2 strongly reduced VDR content in both U937-dnNrf2#13 and U937-dnNrf2#17 clones.

Discussions

Our laboratory focuses on studies of natural food derivatives and their anticancer effects [10]. Recently, we and others have shown that plant-derived polyphenolic antioxidants CA, curcumin, and silibinin markedly potentiate the differentiating action of low, near-physiological concentrations of $1,25D_3$ and ATRA in HL60 human leukemia cells. Similarly, we demonstrated that CA or the CA-rich ethanolic extract of rosemary leaves synergistically potentiated differentiation of WEHI-3B D(-)murine myelomonocytic leukemia cells induced by 1 nM 1,25D3 or its low-calcemic analogs, Ro25-4020 and Ro27-5646. Importantly, this treatment elicited strong cooperative antitumor effect, without inducing hypercalcemia in Balb/c mice bearing WEHI-3B D(-)tumors. In this study, we have investigated the molecular mechanism of synergy between

CA as a representative of ARE-activating plant-derived polyphenols and $1,25D_3$ at low doses. In general, antioxidants exert their anticancer therapeutic effects via two major mechanisms: 1) direct scavenging of harmful radicals and 2) induction of various defense systems, which depend on redox-sensitive transcription systems. One such transcription system is the ARE and its major activating transcription factor, Nrf2. The ARE is found in the promoters of detoxifying and redox-regulating enzymes. We hypothesized that polyphenolic antioxidants drive Nrf2/ARE activation, which, in turn, potentiates the vitamin D receptor activity, leading to an enhanced differentiation response in leukemia cells. Our hypothesis was supported by several lines of evidence:

First, we found that the phenolic diterpene CA and, to a lesser extent, some other polyphenols, induced ARE transactivation in leukemic cells. We demonstrated that CA strongly upregulated the Nrf2 protein levels without significant change of its mRNA levels. Since the mechanism of action of CA on Nrf2 expression/translation is not clear yet, we suggest that CA acts similarly to the other Nrf2/ARE inducing agents that increase the half-life of Nrf2 by stabilization of theNrf2 protein without affecting the steady-state level of mRNA. We found that CA promoted the expression of the Nrf2/ARE-responsive gene products (e.g., NQO1, γ-GCS) and de novo synthesis of glutathione in U937 cells (Fig. 2, 5). In support of our finding, Wei et al. recently reported that CA activated the Keap1/Nrf2 transcriptional pathway in monkey kidney COS7, murine hippocampal HT22, and rat adrenal pheochromocytoma PC12h cell lines by binding to specific Keap1 cysteine residues. Although the Nrf2/ARE system and its regulation by phytochemicals and other compounds have been well characterized in various cell and animal models, this system remains only scarcely investigated in leukemia cells. For example, synthetic triterpenoids, hemin, and the gold(I)-containing compound auranofin have been found to activate the Nrf2/ARE transcription system in U937 cells. Interestingly, in our study CA-induced ARE transactivation and increased total glutathione levels were augmented by $1,25D_3$, even though NQO1 and γ-GCS protein levels were not significantly further affected by the differentiation inducer. We suggest that $1,25D_3$ may potentiate the effects of CA on ARE activation by modulating Nrf2 function, e.g., via stimulation of signaling kinase pathways. Extracellular signal-regulated kinase (ERK), c-Jun terminal kinase (JNK), phosphoinositide 3-kinase (PI3-K), and protein kinase C (PKC), which are known to be activated by $1,25D_3$ in myeloid leukemia cells, may also positively regulate the Nrf2/ARE pathway. We propose two putative pathways mediating the effect of the CA/$1,25D_3$-combined treatment on the intracellular glutathione levels. We hypothesize that the CA/$1,25D_3$-induced enhancement of the γGCS complex enzymatic activity is the result of 1) enhanced expression of ARE-driven γGCS regulatory subunit, 2) posttranslational modifications of γGCS protein or the combination of both. This hypothesis requires further investigation and will be the subject of next study.

Second, we demonstrated that CA–triggered Nrf2/ARE activation potentiates $1,25D_3$-induced leukemia cell differentiation. We showed previously and in the present study (Fig. 1) that, while having almost no effect on leukemia cell differentiation, CA potentiates the ability of low, near-physiological concentrations of $1,25D_3$ and ATRA to induce differentiation of leukemia cells. Strikingly, the CA/$1,25D_3$-induced effect on leukemia cell differentiation was strongly enhanced by Nrf2 overexpression (Fig 8), but depletion of Nrf2 by siRNA or expression of a dominant-negative Nrf2 completely abolished this effect. This suggests a critical role for Nrf2 in CA-mediated potentiation of $1,25D_3$-induced leukemia cell differentiation. Importantly, the differentiation induced by a high concentration (100 nM) of $1,25D_3$ in leukemia cells was significantly enhanced by the Nrf2 overexpression and reduced by the expression of dnNrf2. These results imply that the Nrf2/ARE transcription system participates in the basic mechanism of the differentiation induction by vitamin D_3. Our findings demonstrated that the Nrf2/ARE transcription system plays a major role in myeloid leukemia cell differentiation. Furthermore, these findings are supported by recent publications reporting the role of Nrf2 in differentiation of hematopoietic cells. It has been shown that Nrf2 is upregulated during megakaryocytic and erythroid differentiation of human CD34+ cord blood cells induced by thrombopoietin and erythropoietin, respectively. Furthermore, hemin, the inducer of erythroid differentiation, activated ferritin H gene transcription in K562 human erythroleukemic cells through ARE activation. Generally, these findings suggest that the Nrf2/ARE system is essential in the maturation of hematopoietic progenitors and differentiation of leukemia cells.

Third, we demonstrated that the regulatory action of the Nrf2/ARE system in cell differentiation is mediated, at least in part, by the AP-1 and VDR transcriptional systems. We analyzed two potential mechanisms that may mediate the regulatory action of the Nrf2/ARE system during differentiation of myeloid leukemia cells: the activation of VDR/VDRE and AP-1/TRE transcriptional systems. Both systems have been shown to be involved in $1,25D_3$-induced differentiation. For example, El-Huneidi et al. found that inhi-

bition of VDR transcriptional activity by a VDR oligodeoxynucleotide (ODN) decoy markedly abrogated $1,25D_3$-induced differentiation in HL60 cells. The same group also showed that functional AP-1 is required for $1,25D_3$-induced monocytic differentiation. Our experiments clearly indicate that the component expression and activity of both the VDR/VDRE and AP-1/TRE transcriptional systems are enhanced or reduced by Nrf2 overexpression or downmodulation, respectively (Fig. 8). Of equal importance, is our finding that Nrf2 may regulate the basic mechanism of $1,25D_3$-induced differentiation, by controlling VDR and RXRα protein levels and VDRE transactivation, as was shown by positive or negative modulation of the Nrf2/ARE transcriptional system respectively. Our results demonstrated that the dnNrf2 expression reduced the basal expression of NQO1 and γGCS proteins and caused 22–49% reduction in $1,25D_3$-induced VDRE transactivation, therefore we suggest that the basal activity of Nrf2 and ARE-driven products is important for the $1,25D_3$-induced activation of the VDR/VDRE transcription system. To estimate the precise effect of $1,25D_3$ treatment we plan to use stripped serum in our future research.

Using Nrf2 and dnNrf2 stable transfections we showed a positive role of the Nrf2/ARE system in protein expression regulation of several major AP-1 components, such as c-Fos, c-Jun, and ATF2. We observed that regulation of AP-1 protein levels was accompanied by Nrf2/ARE-regulated AP-1 functional activity. These results are supported by recent data that describe a decrease in c-Fos and c-Jun mRNA levels and AP-1 activity in Nrf2 knockout mice. Using AP-1 decoy ODN we proved that the Nrf2/ARE system facilitates leukemia cell differentiation through the activation of the AP-1 transcriptional complex. In support of this finding, Trachtenberg et al. also found that AP-1 decoy ODN decreased $1,25D_3$-induced HL60 leukemia cell differentiation, which was associated with reduced VDR protein levels. The VDR promoter contains two AP-1 response elements, and the data from our laboratory demonstrated that polyphenols potentiated $1,25D_3$-induced AP-1 binding to them. In addition, AP1-mediated regulation of the VDR expression was demonstrated by application of truncated c-jun, and site-directed mutagenesis of the TRE inthe VDR promoter. Therefore, it is likely that Nrf2 acting via AP-1 regulates the expression of the VDR in myeloid leukemia cells, thereby increasing their responsiveness to $1,25D_3$.

The possibility of direct interaction between Nrf2 and VDR is supported by recent findings in our laboratory demonstrating co-recruitment of Nrf2 and estrogen receptor (ER) to the estrogen response element (ERE), using chromatin immunoprecipitation (ChIP) assays (Andrea Atzmon and Keren Hirsch, unpublished data). Furthermore, inhibitory function of Nrf2 on ER/ERE transcriptional activity in breast cancer cells was also demonstrated by stable expression of Nrf2, dnNrf2 or Nrf2 silencing. In contrast to the negative role of Nrf2 in the ER/ERE system, a possible physical and functional interaction between Nrf2 and VDR/RXR/VDRE is likely activating, as supported by our VDRE activation data.

Motif scanning results of the RXRα promoter suggest an additional level of the regulation vitamin D_3-induced cell differentiation by Nrf2. We found two Nrf2 binding sites in the RXRα promoter and showed that Nrf2 overexpression led to a marked upregulation in RXRα protein expression, while transfection with dnNrf2 decreased RXRα content. These results imply that CA may also potentiate $1,25D_3$-induced differentiation by upregulation of RXRα through activation of the Nrf2/ARE transcriptional system, but further studies are required to elucidate the possible direct induction of nuclear receptors by Nrf2/ARE.

Conclusions

In this study we have investigated the mechanism for enhancement of $1,25D_3$–induced differentiation of myeloid leukemia cell lines by antioxidant polyphenols, utilizing CA as the strong ARE activating phenolic compound. Our results suggest the following model of the functional role for the Nrf2/ARE system in differentiation enhancement (Fig. 1):

1. CA transactivates the Nrf2/ARE transcriptional system and induces the ARE-regulated genes, thereby encoding antioxidant and phase II detoxifying enzymes, such as, NQO1, and γ-GCS. γ-GCS is particularly interesting as it is a rate-limiting enzyme in the glutathione (GSH) synthesis.
2. Elevation of GSH content can facilitate the activation of the redox-sensitive transcription factor AP-1.
3. The activated AP-1 binds to two AP-1 response elements (TRE) located in the VDR promoter thus increasing the VDR expression. The VDR transcriptional system might also be activated directly via physical interaction with Nrf2.
4. The classical VDR/VDRE pathway activated by $1,25D_3$, leads to the expression of differentiation-related genes. We suggest that at least some polyphenols may enhance $1,25D_3$-induced leukemia cell differentia-

tion by activating Nrf2/ARE and AP-1/TRE, leading to the upregulation of the VDR/VDRE transcriptional system and thus to the sensitization of the leukemia cells to the low non-toxic concentrations of $1,25D_3$.

In conclusion, our findings demonstrate for the first time that the Nrf2/ARE transcriptional system plays a major role in myeloid leukemia cell differentiation and thus may represent a target for mechanism-based therapy of AML.

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ЖМЛ модельдеріндегі D дәрумені мен өсімдік полифенол туындыларының лейкозғақарсы бірлескен әсері: NRF2, AP-1 және VDR арасындағы өзара әрекеттесудің рөлі

Жедел миелоидты лейкозды (ЖМЛ) емдеу соңғы 40 жылда айтарлықтай жақсарған жоқ. D витаминінің 1,25-дигидроксивитамин D3 (1,25D3) белсенді түрі *in vivo* уытты концентрациясында *in vitro* күшті лейкозғақарсы әсерге ие. 1,25D3 уыттылығын төмендету үшін құрамында кальцийі төмен D дәруменінің (VDA) бірнеше аналогтары синтезделді. Бұрын біз өсімдік полифенолды антиоксиданттардың (PAOx) улы емес дозаларда 1,25D3 және VDA әсерін синергетикалық түрде күшейтетінін көрсеткен болатынбыз. Бұл әсерлер ядролық фактор (эритроид 2 туындысы)-2 (Nrf2) және активатор ақуызы-1 (AP-1) транскрипция факторлары арқылы жүзеге асырылды. Зерттеудің мақсаты: 1) пероксисомалық N (1) – ацетилспермин/спермидиноксидазасы бар жаңа күшті әсер ететін тамырды кеңейтетін агенттердің жедел миелоидты лейкоз жасушаларының дифференциациясы мен өсуіне біріктірілген әсерін сипаттау; 2) тамырды кеңейтуші агенттердің лейкозғақарсы және пероксисомальды N (1)-ацетилспермин/спермидиноксидазаның біріктірілген әсеріндегі эритроид 2 ядролық факторын, активатор ақуызы-1 және D дәрумені рецепторы (VDR) арасындағы өзара әрекеттесудің рөлін анықтау. Мақалада ЖМЛ-ны фармацевтикалық өсімдіктермен және D дәруменімен емдеу ұсынылған. Өсіндіні өсіру (HL60, U937) және вестерн-блоттинг сияқты әдістер қолданылған. Жойылған C-19 (19-nor аналогтары; PRE-5201 және pri-5202 аналогтары) өсімдік негізіндегі B2 дәрумені немесе HL 60, U937 және MOLM-13 адам жасушаларында C-24 (24-цис аналогтары; pri-1916 және PRI-1917) бүйірлік тізбекті модификациялануы жаңа VDA-да лейкозғақарсы әсері *in vitro* анықталды. PRI-5201 және PRI-5202 дифференциациясының тиімділігі 1, 25D2 немесе 1,25d3-тен 1-2 реттік жоғары болған, ал сол уақытта 24-цис модификациясы іс жүзінде тиімсіз болды. 19-nor VDA/CA комбинациялары ЖМЛ емдеу

үшін тиімді. Қазіргі уақытта бұл комбинацияларды *in vivo* тестілеу жүргізілуде. PAOx көмегімен AP-1 және VDR тікелей немесе жанама Nrf2 арқылы белсендірілуі ЖМЛ жасушаларының 1,25D3 немесе VDAs төмен дозаларына сезімталдығын арттырады деп болжаймыз.

Кілт сөздер: жедел миелоидты лейкемия (ЖМЛ), ядролық фактор (эритроид туындысы 2)-ақуызды белсендіретін 2 (Nrf2)-1 (AP1), полифенол антиоксиданттары (PAOx), D дәрумені аналогтары (VDAs).

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Совместные противолейкозные эффекты производных витамина D и растительных полифенолов в моделях острого миелоидного лейкоза: роль взаимодействия между NRF2, AP-1 и VDR

Лечение острого миелоидного лейкоза (ОМЛ) за последние 40 лет существенно не улучшилось. Активная форма витамина D, 1,25-дигидроксивитамин D3 (1,25D3), обладает сильным противолейкозным действием *in vitro* при токсичных концентрациях *in vivo*. Для снижения токсичности 1,25D3 было синтезировано несколько аналогов витамина D с низким содержанием кальция (VDA). Ранее мы показали, что растительные полифенольные антиоксиданты (PAOx) синергически усиливают действие 1,25D3 и VDA в нетоксичных дозах. Эти эффекты были опосредованы ядерным фактором (производным эритроида 2)-подобным 2 (Nrf2) и активаторным белком-1 (AP-1) факторами транскрипции. Целью исследования было: 1) охарактеризовать комбинированное воздействие новых сильнодействующих сосудорасширяющих агентов с пероксисомальной N (1)-ацетилспермин/спермидиноксидазой на дифференцировку и рост клеток острого миелоидного лейкоза; 2) выяснить роль взаимодействия между ядерным фактором эритроид 2, активаторным белком-1, и рецептор витамина D (VDR) при комбинированном антилейкозном воздействии сосудорасширяющих агентов и пероксисомальной N (1)-ацетилспермин/спермидиноксидазы. В настоящей статье представлено лечение ОМЛ фармацевтическими растениями и витамином D. Использовались такие методы, как разведение культуры (HL60, U937) и вестерн-блоттинг. Мы определили противолейкозные эффекты *in vitro* новых VDA на основе растительного витамина D2 с удалением C–19 (аналоги 19-nor; PRI-5201 и PRI-5202) или с модификацией боковой цепи на C-24 (24-цис-аналоги; PRI-1916 и PRI-1917) на человеческих клетках AML HL60, U937 и MOLM-13. Эффективность дифференцирования PRI-5201 и PRI-5202 была на 1–2 порядка выше, чем у 1,25D2 или 1,25D3, в то время как 24-цис-модификация была практически неэффективной. Комбинации 19-nor VDA/CA являются многообещающими для лечения ОМЛ. В настоящее время проводится тестирование этих комбинаций *in vivo.* Мы предполагаем, что прямая или непрямая Nrf2-опосредованная активация AP-1 и VDR с помощью PAOx повышает чувствительность клеток ОМЛ к низким дозам 1,25D3 или VDAs.

Ключевые слова: острый миелоидный лейкоз, ядерный фактор (производный эритроида 2)-подобный 2, активирующий белок-1, полифенольные антиоксиданты (PAOx), аналоги витамина D (VDAs).

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