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Review

# BRAF signals to pro-apoptotic BIM to enhance AraC cytotoxicity induced in AML cells by Vitamin D-based differentiation agents

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ABSTRACT

Vitamin D has so far not fulfilled its early promise as an antineoplastic agent, in spite of compelling in vitro data. With the aim of bringing vitamin D or its derivatives (VDDs) effectively to the clinic, we developed a two-pronged approach. First, by adding the plant-derived Carnosic Acid (CA) to a vitamin D2 derivative Doxercalciferol we increased its differentiation potency without increasing it hypercalcemic properties. Second, we added these two agents together to AML cells already treated with Cytarabine (AraC), the standard drug for the treatment of patients with AML. We now report that BRAF, a part of the MAPK signaling pathway, is required for the optimally increased cell death in this system and acts upstream of BIM, the regulator of the caspase cascade that leads to cell death by apoptosis. It is proposed that this therapeutic regimen should be tested in a clinical trial.



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## 1. Introduction

The original enthusiasm for the use of vitamin D derivatives (VDDs) for the treatment of neoplastic diseases was greatly

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diminished by the publication of the Institute of an Medicine (IOM) report, which concluded that in contrast to the positive effect of vitamin D on bone health, there is no credible evidence of a similar effect on malignant cells [1]. This opinion was largely based on the published record indicating that although there is clear evidence of an "anti-cancer" effect of VDDs in preclinical studies (eg [2–5]), multiple attempts to demonstrate such an effect in the clinic were in almost all cases negative (eg. [6–10] also www.clinicaltrials.gov). The few publications with positive results were based on small numbers of patients, and there remains the concern that there are confounding factors in the interpretation of the results (eg [11–13]).

However, others in the field of vitamin D and cancer have suggested that the clinical trials performed so far were not performed in a manner that guarantees detection of positive effects of VDDs on cancer treatment (eg; [14–16]), and this suggests that future trials, well designed, are warranted. This seems to be particularly true regarding the use of VDDs in the treatment of Acute Myeloid Leukemia (AML). The prognosis for most AML patients is abysmal, but while it is fortunate that the disease has low incidence, this makes it difficult to accrue patients to large clinical trials. Thus, an understanding of the mechanistic basis of the actions of VDDs in the proposed trials seem essential in order to structure such trials to detect small differences between the results of therapeutic regimens offered to patients with AML, other than the subtype APL, where there has been significant success [17].

It is arguable that it will not be possible to use VDDs alone in the clinic, as the concentrations of these compounds, even the currently available vitamin D analogs with claimed low calcemic activity, are still hypercalcemic in vivo if used alone as anti-cancer treatment. Thus, numerous attempts have been made to combine VDDs such as Calcitriol (1,25D), or analogs, with either toxic or non-toxic compounds, but to date these combinations have not resulted in clinically demonstrated advances of the field [5,14]. Recently, we presented in vitro studies of human AML blasts, which demonstrated a selective increase in cytotoxicity of Arabinocytosine (AraC, Cytarabine), the key therapeutic for AML [18,19], when the exposure of the AML cells to AraC was followed by a combination of differentiating agents [20,21]. The combination consisted of the vitamin D2 analog Doxercalciferol (D2) already approved for human administration, together with a plant-derived anti-oxidant Carnosic Acid (CA), currently used as a food flavoring agent [22], and previously reported to enhance1,25-dihydroxyvitamin D3-induced differentiation of AML cells [23]. Our studies were conducted using patient blasts ex vivo as well as in established culture, and the selectivity to malignant blasts was demonstrated by the finding that the addition of the D2/CA combination to normal bone marrow (NBM) cells treated with AraC did not kill more cells than the treatment with AraC alone [20].

While our initial studies of the mechanisms responsible for the effect of differentiating agents on cells with AraC-induced DNA damage showed that the Vitamin D Receptor (VDR), needed for the induction of monocytic differentiation by VDDs, and the BCL2 interacting mediator of apoptosis (BIM, BCL2L11) were required for the optimal enhancement of AraC cytotoxicity [20] it was not clear how BIM was upregulated in this "enhancement of cytotoxicity" model. Here we report that BRAF is a part of the signaling pathway from VDR to BIM, which then activates the caspase cascade.

## 2. Materials and methods

### 2.1. Cell lines and chemicals

Two AML cell lines, HL60 (cultured from a patient with acute promyeloblastic leukemia) [24], and U937 (monocytes from histiocytic lymphoma) [25], were cultured as previously described [21].

Arabinocytosine (AraC) and Doxercalciferol (1α-hydroxyvitamin $D_2$; D2) were purchased from Sigma-Aldrich (St. Louis, MO). Carnosic acid (CA) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). BRAF inhibitors SB590885 and TAK632 were purchased from Selleck Inc., stated to inhibit both wild type and mutated BRAF. The antibodies used for Western blots were: BRAF, CRAF, BIM (sc-8625), VDR (sc-1008), and CRK-L, the V-Crk Avian Sarcoma Virus CT10 Oncogene Homolog-Like (sc-319), were obtained from Santa Cruz Biotechnology (Dallas, TX). Phospho-H2AX-Ser139 (#9718), phospho-BIM-Ser69 (#4581) and HRP-linked anti-rabbit (#7074) antibodies were from Cell Signaling Technologies (Danvers, MA). The siRNA transfection reagents were from Santa Cruz Biotechnology (Dallas, TX).

### 2.2. Isolation of mononuclear cells from peripheral blood or bone marrow samples and their culture

Peripheral blood specimens from patients with AML, and a bone marrow sample from a volunteer, were obtained with written consent according to an IRB approved protocol. Mononuclear cells were isolated from these samples by using Histopaque-1077 (Sigma-Aldrich) gradient centrifugation, as previously described [26]. Briefly, isolated mononuclear cells were divided into a group exposed for 72 h to 100 nM AraC or the vehicle (0.1% DMSO). The cells were washed with control medium after this 72 h pretreatment, and each of these groups was further divided for addition of D2 (100 nM), or CA (10 μM), or both, for 96 h. Cell viability was determined by Trypan blue (TB) exclusion using a Neubauer hemocytometer as described before [21], and apoptosis, and necrosis by Annexin V as described below.

### 2.3. Knockdown of VDR or BRAF expression

AML blasts or HL60 cells were transfected with 10 nM of VDR siRNA or BRAF siRNA or scrambled Control siRNA, using Endo-Porter delivery reagent from Gene Tools Inc (Philomath, OR) before exposure to other agents. The cells were allowed to recover in RPMI 1640 medium with 10% FCS for 24 h, and then were exposed to the indicated compounds for times indicated in the individual experiments. The reduction of target protein or mRNA by siVDR or siBRAF transfection in groups designed to show cell death enhancement changes was approximately 40%-50%. We transfected cells with siRNA (20 nM) for 72 h before adding other agents, then we replenished with half of the original amount of siRNA every 48 h throughout the whole experiment.

For inhibition of BRAF kinase activity, AML cells were pretreated with 100 nM AraC for 72 h, and then incubated with either of the BRAF inhibitors for 1 h before adding other agents. However, it is possible that knockdown of BRAF was incomplete under our conditions which were not toxic to the cells.

### 2.4. Flow cytometry analysis for Annexin V and propidium iodide staining

Cells were washed twice with 1×PBS, then re-suspended in the binding buffer, containing 0.14 M NaCl and 2.5 mM $CaCl_2$, pH 7.5, and incubated with 50 μg/ml Annexin V-FITC (Kit from Sigma) and 20 μg/ml propidium iodide in 1× binding buffer at room temperature in the dark for 15 min, then immediately analyzed by flow cytometry (EPICS XL). Annexin V-positive/PI-negative cells were considered as early apoptotic, cells both Annexin V and PI positive, as late apoptotic, and Annexin negative but PI positive as "necrotic", likely a variety of caspase independent modes of cell death [27].

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