Journal of Steroid Biochemistry & Molecular Biology 164 (2016) 72–78

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

# Enhancement of arabinocytosine (AraC) toxicity to AML cells by a differentiation agent combination

Xuening Wang[a], Jonathan S. Harrison[b], George P. Studzinski[a,*]

[a] Department of Pathology & Laboratory Medicine, NJ Medical School, Rutgers, 185 South Orange Ave, Newark, NJ 07103, United States
[b] Department of Medicine, University of Missouri Medical School, Columbia, MO 65212, United States

ARTICLE INFO

*Article history:*
Received 12 June 2015
Received in revised form 28 July 2015
Accepted 20 August 2015
Available online 28 August 2015

*Keywords:*
Vitamin D analog
AraC
Plant antioxidant
Acute myeloid leukemia (AML)
Apoptosis
VDR
Chk1

ABSTRACT

Arabinocytosine (AraC, also known as cytarabine) is one of the mainstays of AML therapy, but like other DNA damaging therapeutic agents it is rarely curative by itself. There is an emerging realization that the therapeutic outcomes may be improved by combining AraC with other compounds. Here we report that the addition of a differentiating agent combination immediately following AraC damage to AML blasts, selectively increases the cell kill. The experiments were performed using cultured cells from established cell lines of AML (HL60 and U937). The cells were exposed to 100 nM AraC, a concentration which produced approximately 25–50% cell kill, followed by a combination of 100 nM 1alpha-hydroxyvitamin D2 (1-D2) and 10 μM carnosic acid (CA), which together can serve as a powerful differentiating agent combination for AML cells, but are not toxic alone. AraC-induced cell death, measured by annexin V/propidium iodide, was significantly ($p < 0.01$) increased by the 1-D2/CA combination in both cell lines, but not by 1-D2 or CA alone. The enhancement of cell death occurred by both apoptosis and necrosis, was associated with increased DNA damage and with higher levels of DNA damage response (DDR) activated marker Chk1, but the expression of p27, a cell cycle inhibitor protein, was not enhanced by 1-D2/CA. The principal finding is that a vitamin D analog 1-D2 combined with a plant-derived antioxidant CA can markedly augment the cytotoxic action of AraC, an anti-leukemia therapeutic agent.



**Contents**



* Corresponding author. Fax: +1 973 972 7293.
*E-mail address:* studzins@njms.rutgers.edu (G.P. Studzinski).

http://dx.doi.org/10.1016/j.jsbmb.2015.08.023
0960-0760/

## 1. Introduction

Poor long-term outcomes from standard therapy continue to be a major problem for patients with acute myeloid leukemia (AML). The physiologically active form of vitamin D3, 1α,25-dihydroxyvitamin D3 (calcitriol), has been known for over three decades to effectively overcome the blocked differentiation of AML cells. However, recent clinical trials of vitamin D derivatives (VDDs) showed unremarkable results, suggesting that approaches to the application of this knowledge to the clinic are currently sub-optimal, as recently articulated [1]. In addition to the problems cited in that review, it is also difficult to obviate the hypercalcemic effect of VDDs, the principal cause of patient toxicity [2,3]. Repeated attempts to synthesize and clinically test analogs of calcitriol, in which the hypercalcemic activity of the analog is dissociated from its differentiation-inducing activity, have as yet produced no tangible results [3,4]. An alternative approach to improve AML therapy is to combine VDDs, including calcitriol, with other compounds. Numerous preclinical studies, principally in vitro, have shown greater differentiation potency of VDDs combined with diverse compounds, when compared to VDDs as sole agents, and were often associated with cell death [5,6]. However, to date, most clinical trials of these combinations have not been encouraging [7,8]. Of note, most of these trials did not take into account the reported dependence on "time-sequencing", i.e., the time of exposure to the other agent relative to the VDD addition [9].

Our laboratories have shown that in order to obtain a potential beneficial therapeutic effect of calcitriol combined with a DNA damaging agent (DDA), arabinocytosine (AraC) or hydroxyurea on HL60 cells, an established AML-M2 cell line, calcitriol has to be administered after the DDA [9,10]. In contrast, if the exposure to calcitriol takes place before DNA damage is established, calcitriol can protect the cells from damage, possibly due to the up-regulation of hKSR-2 by calcitriol, which facilitates the functioning of survival-enhancing downstream targets of the MAPK pathway [11], and by the AKT pathway [12]. A recent report of survival improvement in patients with AML or MDS, who were in remission achieved with standard chemotherapy, and who then received maintenance treatment with agents that included differentiating agents [13], is consistent with the hypothesis that the exposure to a VDD after cellular DNA damage is necessary for improved outcome of therapy.

In the current study we found that doxercalciferol (1α-hydroxyvitamin $D_2$; 1-D2), a low calcemic analog of vitamin D2 [2,14], combined with carnosic acid (CA), a plant derived polyphenol with anti-oxidant properties [15,16], increases the cytotoxicity of AraC to AML cells.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

Arabinocytosine and doxercalciferol were purchased from Sigma–Aldrich (St. Louis, MO). Carnosic acid (CA) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). The following antibodies: Crk-L (sc-319), VDR (sc-1008), C/EBP beta (sc-150), and p27Kip1 (sc-528) were obtained from Santa Cruz Biotechnology (Dallas, TX). Phospho-Ser139-H2AX (#9718), phospho-Chk1 (#2348), and HRP-linked anti-rabbit (#7074) antibodies were purchased from Cell Signaling Technologies (Danvers, MA).

### 2.2. Cell culture and NSE staining

AML cell lines, HL60 (acute promyeloblastic leukemia) [17], and U937 (monocytes from histiocytic lymphoma) [18], were cultured in suspension with RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum in a humidified atmosphere at 37 °C. Cell cultures were passaged two to three times a week to maintain log phase growth. The absence of *Mycoplasma* was confirmed periodically in each cell line. For experiments, cells were seeded in 6-well plates at densities of $3 \times 10^5$/ml followed by the addition of combinations of the agents under study for the indicated times. For instance, to determine cell death, the cultures were divided into a group exposed for 72 h to only the vehicle for AraC (0.1% DMSO), and a group treated with 100 nM AraC. After 72 h the cells were washed with fresh medium, and each of these groups was further divided for treatment for 96 h with 1-D2 (100 nM), or CA (10 uM), or the combination of these two agents. Cell number and cell viability were determined using the Trypan blue exclusion counts in a Neubauer hemocytometer. Cell differentiation was assessed by non-specific esterase (NSE) staining of smears made by resuspending $1 \times 10^6$ cells in 100 μl 1xPBS and spreading on slides. The air-dried smears were fixed in formalin acetone mixture buffer for 30 s, then washed with distilled water and stained for 45 min at room temperature with the following solution: 67 mM phosphate buffer, pH 7.6, 8.9 ml, hexazotized pararosaniline, 0.6 ml, 10 mg alpha-naphtyl acetate, and 0.5 ml ethylene glycol monomethyl ether. The NSE-positive cells were enumerated by counting at least 500 cells in each group.

### 2.3. Annexin V and propidium iodide staining

Experimental samples were collected, washed twice with 1xPBS, then resuspended in the binding buffer, containing 0.14 M NaCl and 2.5 mM $CaCl_2$, pH 7.5, and stained using an Annexin V-FITC Kit (Sigma). The cells were incubated with 50 μg/ml annexin V and 20 μg/ml propidium iodide in 1x binding buffer at room temperature in the dark for 15 min, and immediately analyzed by flow cytometry (EPICS XL). Annexin V-positive/ PI-negative cells were considered as early apoptotic, cells with both annexin V and PI positive, as late apoptotic, and annexin negative but PI positive as "necrotic", most likely a variety of caspase independent modes of cell death [19].

### 2.4. Comet assay of DNA damage

HL60 and U937 cells were treated with AraC, 1-D2/CA or AraC/1-D2/CA for indicated times. DNA damage within cells was measured by comet assay [20]. This assay was performed according to the manufacturer's recommended procedure (Cell Biolabs, San Diego, CA). Briefly, AML cells were washed twice with 1xPBS, then 10,000 cells were mixed with low melting-point agarose gel and pipetted on the "Comet Slide". The cells transferred to the slide were maintained for 15 min at 4 °C in the dark. The slide was then immersed in the lysis buffer for 30 min at 4 °C in the dark, to relax and denature the nuclear DNA. Next, the slide was transferred into a horizontal electrophoresis tank in TBE buffer, and ran for 15 min at 30 V. The slide was rinsed three times with distilled water and once with 70% ethanol. Finally, the dried slide was stained with Vista Green DNA Dye for 15 min at room temperature. The cells on the slide were then visualized and photographed using a fluorescent microscope. The images were analyzed with the Opencomet Software, which was used as a plug-in for the image processing platform, ImageJ [21]. The percentage of DNA in tails was selected as a measure of DNA damage. In each slide, obtained from three or more independent experiments, at least 50 stained cells were measured and analyzed.

### 2.5. Western blotting

Western blotting was performed using 50 μg of whole cell extracts as described before [22]. Briefly, membranes were