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Antileukemia activity of the combination of 5-aza-2′-deoxycytidine with valproic acid

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Abstract

Aberrant DNA methylation of promoter associated CpG islands is a common phenomenon in human leukemias and cooperates with histone code changes in the control of gene expression. 5-Aza-2′-deoxycytidine (DAC) is a hypomethylating agent with significant antileukemia activity in humans. Recently, valproic acid (VPA) has been shown to be a histone deacetylase inhibitor and to have potential antineoplastic activity. In this report, we study the in vitro effects of the combination of DAC and VPA on the leukemic cell lines HL-60 and MOLT4. DAC alone induced growth inhibition and apoptosis at doses of 1 μM, an effect observed with VPA at doses of 1 mM. Each drug alone had the capacity to induce the expression of p57KIP2 and p21CIP1. DAC mediated hypomethylation of p57KIP2 was not required to induce p57KIP2 gene expression, and treatment with DAC resulted in the induction of p21CIP1. VPA induced global histone acetylation, an effect enhanced by the addition of DAC. The combination of DAC and VPA had a synergistic effect in terms of growth inhibition, induction of apoptosis and reactivation of p57KIP2 and p21CIP1. These results suggest that the combination of DAC and VPA could have significant antileukemia activity in vivo.

Keywords: DNA methylation; Histone acetylation; 5-Aza-2′-deoxycytidine; Valproic acid; Leukemia

1. Introduction

DNA methylation consists in the addition of a methyl group to the cytosine (C) in a cytosine–guanine (CpG) pair. CpG pairs are underrepresented in the human genome and cluster together in the so-called CpG islands. These islands are generally located in the proximity of gene promoters and non-coding repetitive DNA elements [1]. DNA methylation of promoter CpG islands (herein DNA methylation) is associated with gene silencing. Aberrant DNA methylation is frequently observed in different human malignancies [2], including acute leukemias [3,4], and is considered a functional equivalent to the effect of inactivating mutations or deletions of tumor suppressor genes [5]. Beyond its importance in the control of gene expression, specific patterns of aberrant DNA methylation have been shown to identify patients with leukemia and poor prognosis [6], and to be the target for the clinical activity of hypomethylating agents such as 5-aza-2′-deoxycytidine (DAC) [7,8]. Recently, a low-dose schedule of DAC has been reported to have significant clinical activity in patients with advanced leukemia [9].

Changes in the biochemical composition of nucleosome-associated histone tails are associated with specific gene expression states. These biochemical changes include acetylation, methylation, and phosphorylation of several residues both in histones 3 and 4, and have received the term of histone code [10]. This histone code is a highly dynamic process [11], and responds to gene regulatory signals [11]. Acetylation of several residues on histone H3 and H4 is associated with an open chromatin configuration and active gene transcription, whereas deacetylation of those same residues results in gene repression. These changes in acetylation are catalytically me-

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diated by enzymes with acetylase (histone acetyltransferases) [12], and deacetylase activity (histone deacetylases) [13], respectively. Several compounds with histone deacetylase inhibitory (HDI) activity are being studied that induce global and gene specific hyperacetylation, and associated changes in gene expression patterns [14]. These compounds have been shown to have preclinical in vitro anticancer activity in different models, and are currently being studied in humans. Valproic acid (VPA) is a short-chained fatty acid [15] that is clinically used as an antiepileptic agent. Recently, VPA has been shown to have HDI activity at achievable concentrations in humans [16,17]. VPA is an attractive alternative as an HDI in humans as its safety profile is well known and is widely available.

Both DNA methylation and changes in histone code are known to cooperate in the control of gene activation repression [18]. Whether one epigenetic change leads to the other, or whether they respond to the level of gene activation itself is currently not clearly known, with different systems providing quite distinct models [18–20]. Regardless of the basic association between DNA methylation and histone deacetylation, multiple investigators have shown that the combined use of a hypomethylating agent and an HDI results in more potent gene reactivation than that observed by each agent alone [19].

Based on the known hypomethylating activity and clinical antileukemia activity of DAC [9], and the recently reported HDI activity of VPA [16,17], we decided to study the effect of this combination in two leukemia cell line systems, in an attempt to preclinically model this combination.

2. Materials and methods

2.1. Cell culture and drug treatment

The human T cell leukemia cell line MOLT4, and the human acute myeloid leukemia cell line HL-60 were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 (Gibco BRL, Crand Island, NY) supplemented with 10% fetal calf serum (Gemini Bio-Products, Woodland, CA) and penicillin-streptomycin (Gibco BRL, Grand Island, NY) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were plated at low density 6–8 h before drug treatment, and were subsequently treated with DAC, VPA, or the combination. Media was changed daily and both DAC and VPA, or the same volume of vehicle, were freshly added every 24 h.

2.2. Determination of cell viability

Cell viability was assessed using trypan blue staining (Sigma, St. Louis, MO). After culture, cells were harvested and stained with 0.4% trypan blue solution. Stained cells were counted immediately using conventional microscopy. Stained black cells were considered as non-viable cells, and unstained bright cells as viable.

2.3. Measurement of induction of apoptosis

Induction of apoptosis was determined using the TACS^{TM} Annexin V-FITC Kit (Trevigen, Gaithbersburg, MD). Cells were washed with PBS, diluted in 100 μl of annexin V incubation reagent, and incubated in the dark for 15 min at room temperature. Subsequently, 400 μl of 1× binding buffer was added, and cells were analyzed by flow cytometry.

2.4. RNA extraction and RT-PCR

Total cellular RNA was extracted with TriZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was re-suspended in DEPC-treated water and was quantitated by spectrophotometry. Three micrograms of total RNA were used for reverse transcription (RT) reactions. RT reactions were performed using Moloney murine leukemia virus RT enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and it was followed by PCR of the target gene. Glyceraldehyd-3-phospate dehydrogenase (GAPDH) was used as control. All reactions were performed with RT-negative controls. PCR primers and reaction conditions are summarized in Table 1. PCR products were separated in agarose gels that were stained with ethidium bromide. Images were captured with a digital image analyzer (BioRad Geldoc 2000, Hercules, CA).

2.5. Analysis of gene expression with real-time PCR

TaqMan probes and the Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) were used in our experiments. PCR reactions were performed using 20× Assays-On-Demand^{TM} Gene Expression Assay Mix (contained unlabeled PCR primers and TaqMan probe) and TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PCR conditions were 95 °C for 10 min, then 95 °C for 15 s and 60 °C for 1 min repeated for 40 cycles. Experiments were performed in triplicate for each data point. For RNA internal control, expression of GAPDH was examined. Quantitative values were obtained from the cycle number (C_T value) at which the increment in fluorescent signal associated with an exponential growth of PCR products started to be detected. The amount of target gene was normalized to the endogenous level of GAPDH. This was done obtaining the relative threshold cycle (ΔC_T), in relation to the C_T of the control gene in order to measure the relative expression level ($2^{-\Delta\Delta C_T}$) of the target gene.

2.6. Cell cycle analysis

Cellular DNA content was analyzed using propidium iodide staining followed by flow cytometry. Briefly, cells were washed with PBS, then fixed by incubation in 70% ethanol at room temperature at least for 30 min, and then at −20 °C overnight. After washing twice, cells were stained with pro-