Cancer Chemother Pharmacol (1998) 41: 275–280

ORIGINAL ARTICLE

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Effect of 5-aza-2′-deoxycytidine and vitamin D_3 analogs on growth and differentiation of human myeloid leukemic cells

Received: 18 February 1997 / Accepted: 3 June 1997

Abstract *Purpose*: The object of this study was to investigate the antineoplastic action of 5-aza-2′-deoxycytidine (5-AZA) in combination with vitamin D analogs on HL-60 and NB-4 myeloid leukemic cells. The vitamin D analogs chosen for this investigation were 1,25-$(OH)_2$-23-yne-cholecalciferol (23-D) and 1,25-$(OH)_2$-Δ16-23-yne-cholecalciferol (16-23-D) since they have the potential to be used clinically owing to their minimal action on calcium metabolism. *Methods*: HL-60 and NB-4 leukemic cells were incubated with different concentrations of 5-AZA and either 23-D or 16-23-D and their antineoplastic action determined by inhibition of DNA synthesis and growth, induction of differentiation and colony assay. *Results*: 5-AZA in combination with either vitamin D analog produced a greater growth inhibition and induction of differentiation than either agent alone. For HL-60 leukemic cells the combination of 5-AZA with either analog produced a synergistic loss of clonogenicity. These effects on clonogenicity correlated with the effects of the combination on inhibition of growth and DNA synthesis. *Conclusion*: These results suggest that vitamin D analogs may enhance the antileukemic action of 5-AZA and that it may be interesting to test these agents in combination in patients with myeloid leukemia.

Key words 5-Aza-2′-deoxycytidine · Vitamin D analogs · Differentiation · Myeloid leukemia · Chemotherapy

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Abbreviations *5-AZA* 5-aza-2′-deoxycytidine, *23-D* 1,25-$(OH)_2$-23-yne-cholecalciferol, *16-23-D* 1,25-$(OH)_2$-Δ^{16}-23-yne-cholecalciferol, *D* 1,25-$(OH)_2$-cholecalciferol (vitamin D_3), *MEM* minimal essential medium, *NBT* nitroblue tetrazolium, *PMA* phorbol myristate acetate

Introduction

5-AZA is a cytosine nucleoside analog that shows interesting antineoplastic activity in patients with leukemia [1–4] and myelodysplastic syndrome [5]. Interest in this analog has increased following recent reports on its activation of several different types of tumor suppressor genes [6–10]. 5-AZA is a potent inhibitor of DNA methylation [11, 12] which can result in the transcription of genes that were silenced by the methylation of cytosine residues in their promoter region [13, 14]. This novel way of activating gene expression by 5-AZA can also lead to induction of differentiation of neoplastic cell lines [11, 15] and primary leukemic blasts from patients [16].

Most therapeutic regimens for the clinical treatment of leukemia use drug combinations in order to overcome the problem of drug resistance that occurs with single-agent therapy and to increase therapeutic efficacy. Our laboratory has investigated different types of agents that could be used in combination with 5-AZA for the therapy of leukemia. The interesting report that retinoic acid can induce complete remissions in patients with acute promyelocytic leukemia [17–19] led us to investigate the use of retinoids in combination with 5-AZA. An additional rationale for our investigation was the rapid development of resistance to retinoic acid in this type of leukemia [20–22]. We reported that this cytosine analog in combination with retinoic acid produces a synergistic antineoplastic effect on HL-60 leukemic cells [23] and DLD-1 colon carcinoma cells [10]. These promising results stimulated us to investigate the action of other vitamin agents in combination with 5-AZA.

An interesting agent to investigate is vitamin D since it has been reported to induce the in vitro differentiation of human myeloid leukemic cell lines [24]. Since the calcium toxicity produced by vitamin D limits its clinical use for leukemia therapy [24], several analogs of vitamin D have been synthesized which have reduced activity on calcium metabolism, but still retain the ability to induce leukemic cell differentiation [25, 26]. Some of these vitamin D analogs show interesting in vivo activity in mice with myeloid leukemia [27]. Our initial investigations led us to report that retinoic acid in combination with vitamin D analogs shows synergistic antineoplastic activity against HL-60 leukemic cells [28]. In addition, we observed that a clone of HL-60 leukemic cells, resistant to retinoic acid, is more sensitive to the inhibitory effects of vitamin D analogs [29]. These investigations provided additional rationale for the investigation of the effects of vitamin D analogs in combination with 5-AZA on leukemic cells.

The objective of this study was to investigate the antineoplastic activity of the combination of 5-AZA with vitamin D analogs in HL-60 leukemic cells at concentrations of this cytosine analog attainable in patients during therapy [1, 2]. We observed that the combination showed an additive to a synergistic antileukemic activity in this cell line. We also observed that the combination showed additive growth inhibition on NB4 human promyelocytic leukemic cells.

Materials and methods

Materials

MEM and nonessential amino acids were obtained from GIBCO/BRL (Burlington, Ontario). Fetal calf serum was obtained from Flow Laboratories (Mississauga, Ontario). 5-AZA was obtained from Mack Co. Jllertissen (Germany), and was dissolved in 0.45% NaCl containing 10 m*M* sodium phosphate (pH 6.8) and stored at −70 °C. 16-23-D, 23-D and D were kindly provided by Dr. M.R. Uskokovic of Hoffman-LaRoche (Nutley, N.J.), and were dissolved in absolute ethanol at a concentration of 10^{-3} *M* and stored at −20 °C. NBT and PMA were obtained from Sigma Chemical Co. (St Louis, MO.). (^{3}H-methyl)-Thymidine was obtained from DuPont Mississauga Canada.

Cell growth and colony assays

Human HL-60 myeloid leukemic cells were obtained from Dr. R. Gallo, National Cancer Institute, Bethesda, Md. The cells were cultured in MEM containing nonessential amino acids and 10% heat-inactivated fetal calf serum, and had a doubling time of 20–24 h. NB4 human promyelocytic leukemic cells were obtained from Dr. N. Lanotte [30] and were maintained in RPMI 1640 medium containing 10% fetal calf serum.

For growth assays, the cells were removed at indicated times during drug exposure and counted with a ZBI Coulter Counter or with a hemocytometer. For colony assays, after drug treatment, the cells were centrifuged at 1200 *g* for 5 min, placed in drug-free medium and suspended in 0.15% agar in MEM containing 15% serum. After incubation at 37 °C in an atmosphere containing 5% CO_2 for 14 days the colonies (> 500 cells) were counted.

Biochemical and histochemical assays

In order to measure DNA synthesis after drug exposure the cells were centrifuged at 1200 *g* for 5 min and 10^5 cells placed in 2 ml of MEM containing 5% dialyzed serum (GIBCO/BRL) and 1 μCi [^{3}H-methyl]-thymidine (20 Ci/mmol). The cells were incubated in a shaker bath at 37 °C for 4 h and placed on Whatman GF/C glass fiber filters (2.4 cm diameter) previously washed with 0.9% NaCl. The filters were then washed with 0.9% NaCl, cold 5% trichloroacetic acid, absolute ethanol, dried and placed in scintillation fluid for the determination of radioactivity incorporation into DNA.

In differentiated myeloid cells, PMA is capable of inducing the production of superoxide which can be detected by the reduction of NBT to a black precipitate of formazan. For the NBT reduction test, 2×10^5 cells were placed in 1 ml MEM and mixed with 1 ml NBT (1.0 mg/ml). After the addition of PMA (0.25 μg/ml), the cells were placed in a shaker bath at 37 °C for 40 min and the fraction of NBT-positive cells determined microscopically using a hemocytometer.

Drug interaction analysis

The method of Valeriote and Lin [31] was used to quantity the interaction between 5-AZA and the vitamin D analogs. In this analysis the survival fraction produced by each agent alone is determined and the product of these two survival factions calculated. If the calculated survival fraction is equal to the observed survival fraction produced by the combination of agents, the drug interaction is classified as additive. If the observed survival fraction is less than the calculated product, the drug interaction is classified as synergistic.

Results

The effects of 5-AZA, 16-23-D, 23-D and D, alone or in combination, on the growth of HL-60 leukemic cells are shown in Table 1. A 96-h exposure to 5-AZA (25 n*M*), 16-23-D (100 n*M*), 23-D (100 n*M*) and D (100 n*M*) resulted in a 28%, 17%, 18% and 18% cell growth inhibition, respectively. The cotreatment of HL-60 leukemic cells with 5-AZA and 16-23-D, 23-D or D produced a growth inhibition of 46%, 49% and 50%, respectively.

The effects of 5-AZA, 16-23-D, 23-D and their combination on DNA synthesis are shown in Table 2. The treatment of the leukemic cells with 5-AZA (50 n*M*) and 16-23-D (10 and 100 n*M*) decreased the level of radioactive thymidine incorporation by 31%, 5% and 15%, respectively, whereas the combination of 5-AZA

Table 1 Effect of 5-AZA and 16-23-D, 23-D or D on cell growth of HL-60 myeloid leukemic cells. Cells (10^4 cells/ml) were exposed to the indicated concentrations of drugs. Cells were counted after a 96-h drug exposure. The cell count for untreated control cells was 755 640 ± 1890. Values are means of three experiments (SE < 5%)

Treatment	Concentration (n*M*)	Growth inhibition (%)
5-AZA	25	27.5
16-23-D	100	16.9
23-D	100	18.0
D	100	18.0
5-AZA + 16-23-D	25 + 100	46.0
5-AZA + 23-D	25 + 100	49.0
5-AZA + D	25 + 100	50.0

Table 2 Effect of 5-AZA and 16-23-D or 23-D on DNA synthesis in HL-60 myeloid leukemic cells. DNA synthesis (4 h incubation; 100 000 cells/2 ml) was assayed after a 96-h drug treatment

Treatment	Concentration (n*M*)	^{3}H-Thymidine incorporation (cpm)[a]	Inhibition DNA synthesis (%)
Control	–	41103 ± 411	–
5-AZA	50	28238 ± 367	31
16-23-D	10	39048 ± 820	5
16-23-D	100	34938 ± 1607	15
23-D	5	38226 ± 1223	7
23-D	10	36582 ± 878	11
5-AZA + 16-23-D	50 + 10	22483 ± 67 ***	45
5-AZA + 16-23-D	50 + 100	23305 ± 1375 *	43
5-AZA + 23-D	50 + 5	23305 ± 769 **	43
5-AZA + 23-D	50 + 10	21785 ± 152 ***	47

*$P \leq 0.1$, **$P \leq 0.025$, ***$P \leq 0.005$ vs 5-AZA alone
[a] Values are means ± SE, $n = 4$

with 16-23-D produced a 45% and 43% reduction. A 96-h treatment of the HL-60 cells with 23-D reduced the level of DNA synthesis by 7% and 11%. Its combination with 5-AZA decreased the level of radioactive thymidine incorporation by 43% and 47%, respectively.

The effects of 5-AZA, 16-23-D, 23-D and D alone or in combination on NBT reduction are summarized in Fig. 1. The treatment of the HL-60 leukemic cells with 5-AZA (50 n*M*) or 16-23-D (5 and 10 n*M*) for 96 h increased the percentage of NBT-positive cells from 3.3% in the control to 12.7%, 30.7% and 27.3%, respectively, in the single drug-treated cells. The cotreatment of the leukemic cells with 5-AZA and the different amounts of 16-23-D increased the level of differentiated cells to 43.3% and 42.3% (Fig. 1A). The treatment of the leukemic cells with 5-AZA (50 n*M*) or 23-D (5 and 10 n*M*) increased the percentage of differentiated cells from 3.9% in the controls to 17.1%, 33.7% and 37.3%, respectively. The combination of 5-AZA with 23-D resulted in 59.7% and 67.0% NBT-positive cells (Fig. 1B). A 96-h treatment of the HL-60 leukemic cells with 5-AZA (50 n*M*) or D (5 and 10 n*M*) increased the percentage of NBT-positive cells by 9.1%, 19.0% and

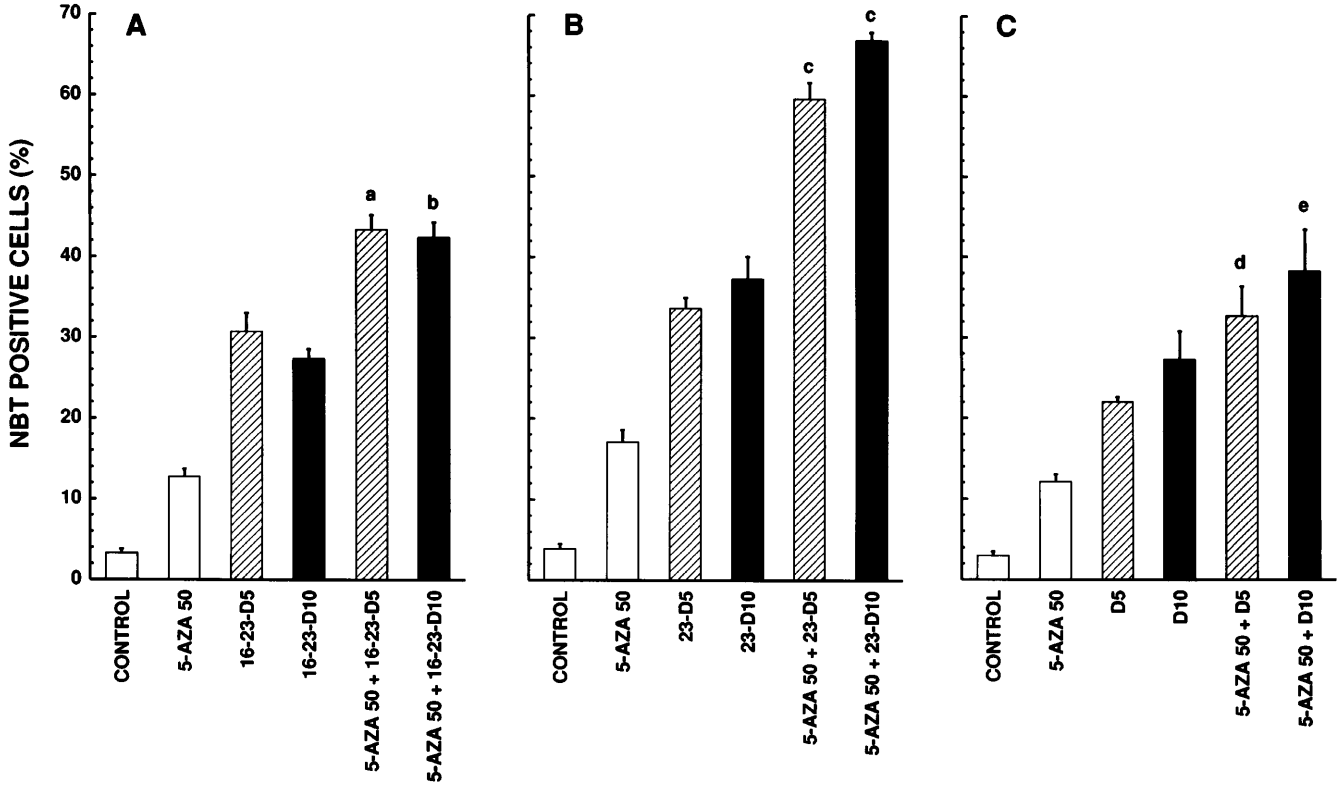

Fig. 1 A–C Effect of 5-AZA and vitamin D analogs on NBT reduction in HL-60 myeloid leukemic cells. NBT was assayed after a 96-h drug treatment. Values shown are means ± SE, $n = 3$. **A** 5-AZA (50 n*M*) and 16-23-D (5 and 10 n*M*); **B** 5-AZA (50 n*M*) and 23-D (5 and 10 n*M*); **C** 5-AZA (50 n*M*) and D (5 and 10 n*M*). [a] $P \leq 0.01$ vs 16-23-D5 alone; [b] $P \leq 0.005$ vs 16-23-D10 alone; [c] $P \leq 0.0005$ vs 23-D5 or 23-D10 alone; [d] $P \leq 0.025$ vs D5 alone; [e] $P \leq 0.1$ vs D10 alone

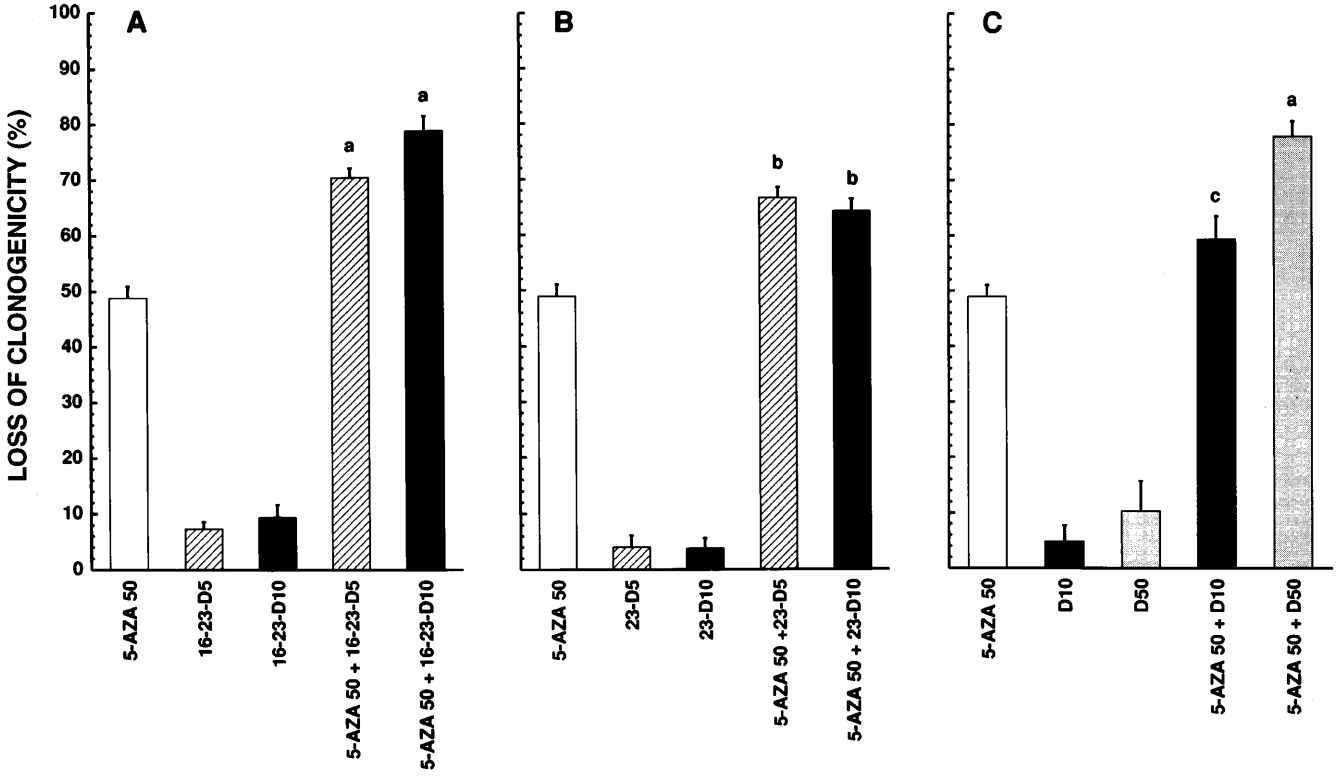

Fig. 2 A–C Effect of 5-AZA and vitamin D analogs on loss of clonogenicity in HL-60 myeloid leukemic cells. The cells were placed in soft agar after a 96-h drug treatment and the colonies counted after 14 days. Values shown are means ± SE, $n = 4$. **A** 5-AZA (50 n*M*) and 16-23-D (5 and 10 n*M*); **B** 5-AZA (50 n*M*) and 23-D (5 and 10 n*M*); **C** 5-AZA (50 n*M*) and D (10 and 50 n*M*). [a] $P \leq 0.0005$ vs 5-AZA50 alone; [b] $P \leq 0.005$ vs 5-AZA50 alone; [c] $P \leq 0.025$ vs 5-AZA50 alone

24.3%, respectively. The combination resulted in a 29.7% and 35.3% increase (Fig. 1C).

The loss in clonogenicity produced by 5-AZA, 16-23-D, 23-D, D and their combinations in HL-60 leukemic cells was evaluated with a colony assay. A 96-h exposure to 5-AZA (50 n*M*) or 16-23-D (5 and 10 n*M*) resulted in a 48.9%, 7.3% and 9.3% reduction in clonogenicity, respectively. The combination of 5-AZA with 16-23-D (5 and 10 n*M*) produced a loss in clonogenicity of 70.3% and 78.7%, respectively (Fig. 2A). A 96-h treatment with 23-D (5 and 10 n*M*) produced a 3.9% and 3.7% reduction in clonogenicity, respectively. The combination of 5-AZA (50 n*M*) with 23-D (5 and 10 n*M*) increased the loss in clonogenicity to 66.3% and 64.7% (Fig. 2B). A 96-h treatment with D (10 and 50 n*M*) produced a 4.8% and 10.3% loss in clonogenicity, respectively. The combination of 5-AZA with D increased the loss in clonogenicity to 59.2% and 77.7% (Fig. 2C).

The inhibition of growth of the NB4 promyelocytic leukemic cells by treatment with 5-AZA and 16-23-D alone or in combination is shown in Table 3. 5-AZA alone at a concentration of 200 n*M* produced a 33.5% growth inhibition of the NB4 leukemic cells. Alone, 16-23-D at a concentration of 10 n*M* produced a 28.9% growth inhibition of these cells. The combination of these two agents resulted in a 53.9% inhibition of growth.

Table 3 Effect of 5-AZA and 16-23-D on growth of NB4 myeloid leukemic cells. Assays were performed after a 96-h drug exposure. Values are means ± SE, $n = 3$

Treatment	Concentration (n*M*)	Growth inhibition(%)
5-AZA	200	33.5 ± 8.4
16-23-D	10	28.9 ± 3.8
5-AZA + 16-23-D	200 + 10	53.9 ± 3.5

Discussion

5-AZA is an active agent for the clinical therapy of leukemia [1–4]. However, dose intensification with 5-AZA is limited by its hematopoietic toxicity. One approach to increase the efficacy of treatment of leukemia with 5-AZA is to use this analog in combination with an antineoplastic agent that produces no hematopoietic toxicity, such as a differentiating agent. In our previous investigations we have demonstrated that 5-AZA in combination with retinoic acid produces a synergistic loss of clonogenicity of HL-60 myeloid leukemic cells [23], suggesting that this combination may merit investigation in patients with myeloid leukemia that relapses after conventional chemotherapy. In the study reported here we studied the interaction of 5-AZA

with vitamin D analogs since these latter agents show in vitro inhibition of the proliferation of human myeloid leukemic cells [24, 25] and antineoplastic activity in mice with myeloid leukemia [27]. These analogs have the potential for clinical use since they do not produce the unacceptable calcium toxicity reported for vitamin D [24–26].

We evaluated the in vitro antineoplastic activity of 5-AZA and vitamin D analogs in human myeloid leukemic cell lines using assays of growth, DNA synthesis, differentiation and colony formation. The combination of 5-AZA with the different vitamin D analogs produced an additive inhibition of growth of the HL-60 leukemic cells (Table 1). The drug concentrations were modified because 5-AZA (50 n*M*) produced too large an inhibition of growth and the vitamin D analogs (5 and 10 n*M*) too small an effect. A second assay which measured the incorporation of radioactive thymidine into DNA was also used to evaluate the loss of DNA synthesis activity produced by these agents after a 96-h exposure (Table 2). In this assay, as single agents, the vitamin D analogs at the concentrations used produced a weak inhibition of DNA synthesis (< 20%). 5-AZA at 25 n*M* produced a significant inhibition of DNA synthesis (31%). The concentration of 16-23-D was adjusted in order to get a level of inhibition similar to that obtained with 23-D. The combination of 5-AZA with the vitamin D analogs produced a greater inhibition of DNA synthesis than either agent alone. The reduction of DNA synthesis produced by these agents correlated with their inhibition of growth of the HL-60 leukemic cells.

The effects of 5-AZA and vitamin D analogs on differentiation of HL-60 myeloid leukemic cells were evaluated by the NBT test which measures superoxide production, a characteristic of differentiated myeloid cells (Fig. 1). The vitamin D analogs produced a greater number of NBT-positive HL-60 cells than 5-AZA. The combination of these agents produced significantly more NBT-positive cells than either agent alone, suggesting an additive interaction.

The clonogenic assay gave the best assessment of the in vitro antineoplastic activity of 5-AZA. Owing to the novel mechanism of action of this analog [13, 14], there is a delay in the onset of its antileukemic action. This was observed in our phase I study in children with leukemia in which we observed the presence of large numbers of leukemic blasts in the bone marrow 3 weeks after therapy with 5-AZA, yet some patients still went into complete remission [1, 2]. In the clonogenic assay we measured colony formation 2 weeks after treatment of the leukemic cells with 5-AZA, which allows adequate time for this analog to exert its action. As shown in Fig. 2, for the HL-60 leukemic cells, the combination of 5-AZA with the different vitamin D analogs (16-23-D, 23-D and D) resulted in a synergistic loss of clonogenicity as defined by Valeriote and Lin [31, 32]. The combination of these agents also showed signs of an additive growth inhibition in another myeloid leukemic cell line, NB4 (Table 3). For the NB4 cells, the concentration of 5-AZA was increased to 200 n*M* in order to obtain growth inhibition similar to that obtained in the HL-60 cells.

The mechanism for this interesting additive or synergistic antileukemic activity of 5-AZA in combination with vitamin D analogs is unknown. 5-AZA has been reported to activate the expression of different tumor suppressor genes that are silenced by aberrant methylation [6–10]. In hematopoietic cells, it is possible that this analog activates one or several "growth suppressor" genes that still remain to be identified. The action of vitamin D is mediated by its binding to the vitamin D receptor. This receptor binds to specific DNA sequences in the promoter region to activate transcription of specific genes, such as osteocalcin [33]. The vitamin D analogs also activate unidentified genes involved in cellular differentiation. The identification of the key genes activated by 5-AZA and vitamin D analogs would clarify the mechanism of interaction of these agents on leukemic cells and provide a rational basis for their effective use in the treatment of leukemia.

Acknowledgement This work was supported by a grant from the Cancer Research Society. We thank M.R. Uskokovic for providing the vitamin D analogs. RLM received a scientific exchange grant from INSERM/FRSQ.

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