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Effects of 5-aza-2′-deoxycytidine and interferon-α on differentiation and oncogene expression in HL-60 myeloid leukemic cells

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The antineoplastic effects of 5-aza-2′-deoxycytidine (5-AZA-CdR) and interferon-α (IFN-α) on human HL-60 myeloid leukemic cells were investigated. 5-AZA-CdR and IFN-α in combination produced a greater inhibition of DNA synthesis and cell growth than either agent alone. The co-treatment produced a synergistic reduction in the clonogenicity of the HL-60 leukemic cells. In addition, this combination also produced a greater reduction in mRNA expression for c-*myc* than each agent alone. This antileukemic action produced by 5-AZA-CdR and IFN-α in combination correlated with the increase in induction of differentiation of the HL-60 leukemic cells. These results suggest that 5-AZA-CdR and IFN-α in combination produces an enhancement of their antineoplastic action on HL-60 human myeloid leukemic cells.

Key words: 5-AZA-CdR, differentiation, IFN-α, oncogene expression.

Introduction

One of the major events in the pathogenesis of acute myeloid leukemia is a blockage of cell differentiation. The non-differentiated cells remain at the level of stem cell undergoing continuous cell proliferation. Sachs[1] proposed the use of inducers of differentiation as a new approach for the treatment of acute myeloid leukemia. This approach promotes the reactivation of the differentiation program in leukemic cells instead of the production of acute cell kill with a chemotherapeutic agent. 5-Aza-2′-deoxycytidine (5-AZA-CdR) is an experimental antileukemic agent which has the capacity to induce differentiation in leukemic cells.[2–4] The action of 5-AZA-CdR is mediated by the inhibition of DNA methylation[2,3,5,6], which results in the activation of specific genes.[7,8] Phase I trials have demonstrated the antileukemic activity of 5-AZA-CdR in man.[9,10] The combination of 5-AZA-CdR with a non-cytotoxic biochemical modifier may increase their chemotherapeutic potential in patients with malignant disease without increasing host toxicity.

An interesting biochemical modifier to use in combination with 5-AZA-CdR is interferon-α (IFN-α).[11] IFN-α has been demonstrated to inhibit *in vitro* growth of different human malignant cell lines.[12] IFN-α is also an effective chemotherapeutic agent in patients with hairy cell leukemia[13–15] and chronic myeloid leukemia.[16–18] However, IFN-α is not active against acute myeloid leukemia. *In vitro* studies showed that only 30% of leukemic cells from patients with acute myeloid leukemia were sensitive to the growth inhibitory effects of IFN-α.[19] The reason for this variability in the sensitivity of acute myeloid leukemia cells to IFN-α is unknown, but could be related to their degree of differentiation. Since 5-AZA-CdR can induce myeloid leukemic cell differentiation, the objective of this study was to determine if this agent could make these leukemic cells more sensitive to IFN-α.

In this report we investigate the effects of 5-AZA-CdR in combination with IFN-α on the proliferation and differentiation of HL-60 myeloid leukemic cells. We chose the HL-60 leukemic cells for our study because 5-AZA-CdR induces the differentiation of this cell line.[3,20] We have also investigated the effect of 5-AZA-CdR and IFN-α on the expression of the proto-oncogene c-*myc*. We studied c-*myc* because its gene is amplified in HL-60 leukemic cells.[21,22] In addition, many hematological malignancies show high expression of this oncogene[23] and terminal differentiation of HL-60 leukemic cells markedly reduces the expression of

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c-*myc*.[24] In this study we observed that the combination of 5-AZA-CdR with IFN-α interact in a synergistic manner with respect to their antineoplastic effect on human HL-60 myeloid leukemic cells.

Materials and methods

Materials

Minimal essential medium (MEM) and non-essential amino acids were obtained from GIBCO/BRL (Burlington, Ontario, Canada). Fetal calf serum (FCS) was obtained from Flow Laboratories (Mississauga, Ontario, Canada). IFN-α2b (Intron A) was obtained from Schering (Montreal, Canada), dissolved in sterile 0.9% NaCl and stored at 5°C. 5-AZA-CdR was obtained from Mack Co. (Germany), dissolved in 0.45% NaCl containing 10 mM sodium phosphate, pH 6.8 and stored at −70°C. Nitroblue tetrazolium (NBT), actinomycin D and phorbol myristate acetate (PMA) were obtained from Sigma (St Louis, MO, USA), [^{3}H-methyl]thymidine was obtained from DuPont Canada, c-*myc* (3rd exon) and N-*ras* DNA probes (Oncor) were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada).

Cell growth and colony assays

Human HL-60 myeloid leukemic cells were obtained from Dr R Gallo (National Cancer Institute, Bethesda, MD, USA). The cells were cultured in MEM medium containing non-essential amino acids and 10% heat-inactivated FCS, and had a doubling time of 20–24 h. For growth assays the cells were removed at indicated times during drug exposure and counted with a ZBI Coulter Counter. For colony assay, 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 medium containing 15% serum and 5000 U/ml of IFN-α. After incubation at 37°C in 5% CO_2 for 14 days the colonies (larger than 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 were placed in 2 ml of MEM medium containing 5% dialyzed serum (GIBCO/BRL) and 1 μCi of [^{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) that were washed previously with 0.9% NaCl. The filters were then washed with 0.9% NaCl, cold 5% trichloroacetic acid, 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[25] which can be detected by its reduction of NBT to a black precipitate of formazin. For the NBT reduction test, 2×10^5 cells were placed in 1 ml of MEM medium and mixed with 1 ml of 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.

Dot blot assay for oncogene expression

After drug exposure, total RNA was isolated from the cells by the method of Chomczynski and Sacchi.[26] Briefly, the cells were extracted with guanidinium thiocyanate containing Sarcosyl, phenol–chloroform and precipitated with isopropanol. The RNA was dissolved in diethyl pyrocarbonate-treated water and stored at −70°C. For use as a DNA probe, c-*myc* and N-*ras* were labeled to a specific activity above 1×10^9 c.p.m./μg by the random prime method of Feinberg and Vogelstein[27] using the kit obtained from Boehringer Mannheim (Dorval, Quebec, Canada) and [α-^{32}P]dCTP obtained from ICN Canada (Mississauga, Ontario, Canada). For hybridization experiments various dilutions of RNA in 20 × SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7) were heated at 60°C for 15 min and placed on a nitrocellulose filter (BA85) in a Minifold dot blot apparatus (Schleicher & Schuell, Inc.). The filter was previously soaked in water and 15 × SSC. The wells were rinsed with cold 20 × SSC under vacuum. The filter was then baked at 80°C for 2 h. The nitrocellulose filter containing the immobilized RNA was prehybridized in a solution of 5 × SSC containing 0.5% blocking reagent (Boehringer Mannheim), 0.1% Sarkosyl and 0.1% SDS at 65°C for 1 h. The filter was hybridized in 5 ml of prehybridization solution containing $1–3 \times 10^8$ c.p.m. of DNA probe (10 ng/ml) at 65°C for 3 h. The filter was then washed twice with 2 × SSC containing 0.1% SDS

at room temperature for 15 min followed by 2 washes with 0.1 × SSC containing 0.1% SDS at 65°C for 30 min. The filter was exposed to X-ray film (Kodak X-Omat) at −70°C for 72 h for autoradiographic analysis. Densitometric analysis of dot blots was performed with Pharmacia LKB Ultroscan XL densitometer.

Northern RNA analysis

After isolation of the total RNA as described above, the RNA was mixed with the loading buffer [50% formamide, 20 mM morpholinopropane sulfonic acid (MOPS), pH 7.0, 6.5% formaldehyde] and denatured by heating at 55°C for 15 min. The samples were then size fractionated in 20 mM MOPS (pH 7) on a 1.2% agarose–1.1% formaldehyde gel. After the electrophoresis in a 20 mM MOPS (pH 7), part of the gel was stained with ethidium bromide (0.5 μg/ml) to verify the relative amount, integrity and molecular size of the RNA using the 18S and 28S ribosomal RNA as reference standards. From the unstained gel, the RNA was transferred to nitrocelulose membrane using 20 × SSC by vacuum blotting technique (Vacugene Blotting System, Pharmacia). The membranes were baked in an oven at 80°C for 2 h. The same hybridization method as in the dot blot assay was used to measure c-*myc* and N-*ras* expression.

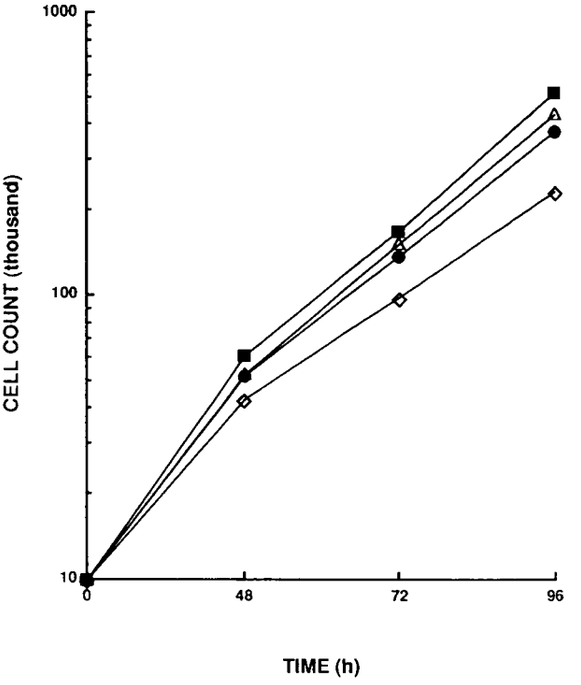


Figure 1. Effect of 5-AZA-CdR, IFN-α and these agents in combination on the growth of HL-60 myeloid leukemic cells. The cells (1 × 10^4/ml) were exposed to the drugs starting at time 0 h and cell counts were performed at 48, 72 and 96 h. Control, ■; 5-AZA-CdR, 0.02 μM, ●; IFN-α, 5000 U/ml, △; 5-AZA-CdR, 0.02 μM + IFN-α, 5000 U/ml, ◇. Experimental points represent mean value with SE < 10% ($n = 3$).

Results

The effect of 5-AZA-CdR, IFN-α alone or in combination on the growth of HL-60 leukemic cells is shown in Figure 1. After 96 h of treatment 5-AZA-CdR at 0.02 μM and IFN-α at 5000 U/ml produced a 27% and 17% inhibition of growth, whereas the combination of these agents produced a 55% inhibition. At shorter incubation times less growth inhibition was observed.

The effects of 5-AZA-CdR, IFN-α and their combination on DNA synthesis and NBT reduction are shown in Table 1. Treatment of the HL-60 leukemic cells with 5-AZA-CdR (0.05 μM) or IFN-α (5000 U/ml) for 96 h increased the percentage of NBT positive cells from 4.8% in the control to 14.9% and 6.3%, respectively, in the single drug-treated cells. The combination produced a 26.3% increase in the level of positive NBT cells. These results indicate that 5-AZA-CdR of IFN-α had the capacity to induce differentiation in HL-60 leukemic cells and that the combination was more effective than either agent alone. The treatment of HL-60 leukemic cells with 5-AZA-CdR (0.05 μM) or IFN-α (5000 U/ml) decreased the level of DNA synthesis by 13% and 5%, respectively. The combination of these agents produced a 49% reduction.

The cytotoxic action of 5-AZA-CdR, IFN-α and their combination on HL-60 leukemic cells was evaluated with a colony assay (Table 2). A 72 h pre-exposure to 5-AZA-CdR (0.05 μM) or IFN-α (5000 U/ml) produced 57% and less than 1% cell kill, respectively, whereas the combination of these agents produced a 73% cell kill. Analysis of this interaction by the method of Lin and Valeriote[28] showed that the interaction between the two agents was synergistic. When the pre-treated cells were placed in soft agar without IFN-α (5000 U/ml), the cytotoxic action in combination with 5-AZA-CdR was not greater than 5-AZA-CdR alone (BT Doré and RL Momparler, unpublished data), indicating that IFN-α required a longer exposure time interval to produce its biological effects.

Table 1. Effect of 5-AZA-CdR and IFN-α on DNA synthesis and NBT reduction in HL-60 myeloid leukemic cells

Treatment	Concentration	DNA synthesis (c.p.m.)	NBT (%)
Control	0	37774 ± 1440[a]	4.8 ± 0.6[c]
5-AZA-CdR	0.05 μM	32811 ± 869[b]	14.9 ± 0.7[b]
IFN-α	5000 U/ml	35764 ± 1416	6.3 ± 1.0
5-AZA-CdR + IFN-α	0.05 μM + 5000 U/ml	19399 ± 614[b]	26.3 ± 1.4[b]

DNA synthesis and NBT were assayed after a 96 h drug treatment.
[a] Mean value ± SE ($n = 3$).
[b] $p \leq 0.0005$
[c] Mean value ± S.E ($n = 5$).

The effects of 5-AZA-CdR and IFN-α on c-*myc* and N-*ras* expression in HL-60 cells are shown in Figure 2(A). In these experiments the concentration of 5-AZA-CdR was reduced from 0.05 to 0.02 μM because the higher concentration produced too large a decrease of c-*myc* expression (BT Doré and RL Momparler, unpublished data). The expression of c-*myc* was quantified by a densitometer tracing of the dot blot and Northern blot experiments (Figure 2B). For c-*myc* expression, the relative density (RD) was 34.5, 20.3, 34.3 and 10.9 for the control, 5-AZA-CdR (0.02 μM), IFN-α (5000 U/mol), and the combination of 5-AZA-CdR and IFN-α, respectively, in the HL-60 leukemic cells.

Since the half-life of the mRNA for c-*myc* is around 30 min, most c-*myc* mRNA should be eliminated after a 4 h treatment with actinomycin D, a potent inhibitor of DNA-dependent RNA synthesis.[20] The dot blot and Northern blot showed no signal with HL-60 cells treated with actinomycin D for 4 h, suggesting that the hybridization signal was specific for c-*myc* mRNA.[20] The spot in the actinomycin D lane is an artefact and was not present on dupicate blots.

The 18S and 28S ribosomal RNA in the stained gel (ethidium bromide) were used in the Northern blot analysis as reference markers for molecular size and RNA integrity (BT Doré and RL Momparler, unpublished data). The position of c-*myc* mRNA was just above the 18S ribosomal RNA (2.0 kb) and corresponded to a molecular size of about 2.4 kb, which is in agreement with the published literature.[20] The staining of the ribosomal RNA also revealed equal quantities of total RNA in each well. N-*ras* mRNA expression did not show significant changes in the drug-treated cells.

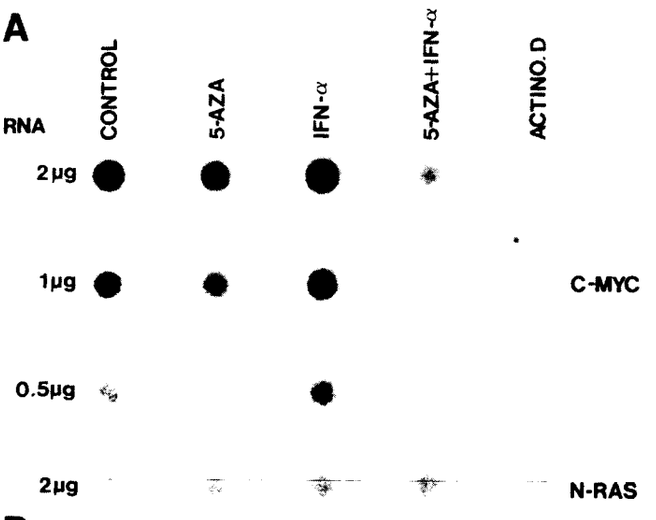
Table 2. Effect of 5-AZA-CdR and IFN-α on colony formation in HL-60 myeloid leukemic cells

Treatment	Concentration	Relative cell kill (%)
5-AZA-CdR	0.05 μM	57 ± 5[a,b]
IFN-α	5000 U/ml	<1
5-AZA-CdR + IFN-α	0.05 μM + 5000 U/ml	73 ± 4[b]

For colony survival the cells, after a 72 h drug exposure, were placed in soft agar containing IFN-α (5000 U/ml) for 17 days.
[a] Mean value ± SE ($n = 3$).
[b] $p \leq 0.0005$

Discussion

The proposal that malignancy could be reversed by inducing leukemic cells to differentiate and stop growing has opened new possibilities for therapy.[1] 5-AZA-CdR is a nucleoside analog which has been shown to induce the differentiation of myeloid leukemic cells through its block in DNA methylation.[2,3,5,8,20] Clinical trials with 5-AZA-CdR have shown that one of the major side effects produced by this analog is myelosuppression.[9,10] One approach to increase the effectiveness of this analog without increasing the toxicity of the chemotherapeutic regimen is to combine 5-AZA-CdR with a non-cytotoxic agent, such as a biological modifier. IFN-α, a biological modifier, is an interesting candidate to use in combination with 5-AZA-CdR.[11] IFN-α has already shown additive and synergic interactions when used in combination with cytotoxic agents.[29–31] In addition, IFN-α is an effective agent for the treatment of patients with hairy cell leukemia or chronic myeloid leukemia.[13–18]



B

Treatment	Concentration	c-myc (RD)[a]	Inhibition of expression[d]
Control	0	34.5 ± 0.8[b]	
5-AZA	0.02 μM	20.3 ± 0.7[c]	41
IFN-α	5000 U/ml	34.3 ± 1.0	<1
5-AZA + IFN-α	0.02 μM + 5000 U/ml	10.9 ± 1.1[c]	68

Figure 2. Effect of 5-AZA-CdR and IFN-α on c-*mys* or N-*ras* mRNA expression. HL-60 myeloid leukemic cells (10^4 cells/ml) were exposed to 5-AZA-CdR and/or IFN-α. 5-AZA-CdR, 0.02 μM; IFN-α, 5000 U/ml; 5-AZA-CdR + IFN-α, 0.02 μM + 5000 U/ml; Actino. D (actinomycin D), 5 μg/ml. (A) Dot blot for expression of c-*myc* and N-*ras* mRNA. (B) Quantification of c-*myc* relative expression after the 96 h drug treatment. The relative inhibition of c-*myc* expression was obtained by the scanning of dot blot and Northern blot experiments with the LKB Ultroscan XL densitometer. [a]RD, relative density. [b]Mean value ± SE ($n = 10$). [c]$P \leq 0.0005$. [d]Relative inhibition of c-*myc* mRNA expression.

Using HL-60 myeloid leukemic cells as an *in vitro* model for human acute myeloid leukemia, we observed that both 5-AZA-CdR and IFN-α inhibited the growth of this cell line (Figure 1). The combination of these agents resulted in a greater inhibition of growth than either agent alone. The induction of superoxide production and the reduction of DNA synthesis activity were used as markers to investigate the effects of 5-AZA-CdR and IFN-α on differentiation. This induction of differentiation by PMA is a characteristic of differentiated myeloid cells which is detected by a histochemical reaction involving NBT reduction. The treatment of HL-60 leukemic cells with 5-AZA-CdR produced an increase in the percentage of NBT positive cells whereas IFN-α had no significant effect. The combination of these agents resulted in a greater percentage of NBT positive cells compared with each agent alone. The modification of the cell differentiation level produced by 5-AZA-CdR and IFN-α correlated inversely with the level of DNA synthesis activity (Table 1).

We performed a colony assay in order to determine if the treatment of HL-60 leukemic cells with 5-AZA-CdR and IFN-α resulted in a loss of clonogenicity (Table 2). These studies have shown that 5-AZA-CdR produced a marked loss of cell clonogenicity while IFN-α had minimal effect. The combination of these agents resulted in a synergistic

effect. These observations suggest that the combination of 5-AZA-CdR and IFN-α produced a greater extent of differentiation than with either agent alone and that the induction of end stage differentiation is probably responsible for the loss of clonogenicity of these cells.

Several studies have demonstrated a correlation between the induction of differentiation by different agents and the reduction of c-*myc* oncogene expression in different leukemic cells. HL-60 leukemic cells are characterized by an amplification of the proto-oncogene c-*myc* and a decrease in its expression during the induction of this cell line to differentiate.[21,22,24] We investigated the effects of 5-AZA-CdR and IFN-α on c-*myc* expression in order to understand some of the molecular events occurring after treatment. In the dot blot and Northern blot analysis we observed a decrease in c-*myc* in the presence of 5-AZA-CdR. There was no detectable effect of IFN-α on c-*myc* expression as reported by other investigators.[20,32] The combination of these agents resulted in a greater reduction of c-*myc* expression (Figure 2). The level of N-*ras* expression was used as an RNA integrity control since other studies have reported that IFN-α had no effect on its expression and we showed that 5-AZA-CdR also had no effect on its expression.[33] These observations suggest that the inhibition produced by 5-AZA-CdR on c-*myc* oncogene expression is enhanced by IFN-α. The mechanism by which 5-AZA-CdR produces an enhancement of the antineoplastic action of IFN-α is unknown and may be related to the activation of specific genes required for IFN-α activity by this nucleoside analog. The downregulation of the oncogenes by IFN-α is probably due to the effects of this biological modifier on RNA degradation.[34] Further studies will be needed to elucidate the effect of 5-AZA-CdR on IFN-α action.

The results presented in this report suggest that 5-AZA-CdR can increase the sensitivity of acute myeloid leukemic cells to IFN-α. Since 5-AZA-CdR alone can induce complete remission in some patients with this disease,[9,10] its combination with IFN-α may be an interesting chemotherapeutic regimen for clinical trials in leukemic patients that fail conventional chemotherapy.

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