Enhancement of antineoplastic action of 5-aza-2′-deoxycytidine by zebularine on L1210 leukemia

Maryse Lemaire[a], Louise F. Momparler[a], Mark L. Bernstein[b], Victor E. Marquez[c] and Richard L. Momparler[a]

Tumor suppressor genes that have been silenced by aberrant DNA methylation are potential targets for reactivation by novel chemotherapeutic agents. The potent inhibitor of DNA methylation and antileukemic agent, 5-aza-2′-deoxycytidine (5-AZA-CdR, Decitabine), can reactivate silent tumor suppressor genes. One hindrance to the curative potential of 5-AZA-CdR is its rapid *in vivo* inactivation by cytidine deaminase (CD). An approach to overcome this obstacle is to use 5-AZA-CdR in combination with zebularine (Zeb), a potent inhibitor of CD. Zeb also possesses independent antineoplastic activity due to its inhibition of DNA methylation. We tested the capacity of 5-AZA-CdR and Zeb alone and in combination to inhibit growth and colony formation of different leukemic cell lines. 5-AZA-CdR and Zeb in combination produced a greater inhibition of growth against murine L1210 lymphoid leukemic cells, and a greater reduction in colony formation by L1210 and human HL-60 myeloid leukemic cells, than either agent alone. The ability of these agents to reactivate the tumor suppressor gene, p57KIP2, was also tested using RT-PCR. The combination produced a synergistic reactivation of p57KIP2 in HL-60 leukemic cells. A methylation-specific PCR assay showed that this combination also induced a significantly greater demethylation level of the p57KIP2 promoter than either drug alone. The *in vivo* antineoplastic activity of the agents was evaluated in mice with L1210 leukemia. A greater increase in survival time of mice with L1210 leukemia was observed with the combination than with either agent alone using three different dose schedules. The enhanced activity observed with 5-AZA-CdR plus Zeb in both murine and human leukemic cells lines provides a rationale for the clinical investigation of these drugs in patients with advanced leukemia. The probable mechanism of this drug interaction involves inhibition of CD by Zeb and the complementary inhibition of DNA methylation by both agents. *Anti-Cancer Drugs* 16:301–308 © 2005 Lippincott Williams & Wilkins.

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[a]Département de Pharmacologie, Université de Montréal, Centre de Recherche Pédiatrique, [b]Service d'Hématologie–Oncologie, Hôpital Sainte-Justine, Montréal, Canada and [c]Laboratory of Medicinal Chemistry, Center for Cancer Research, National Cancer Institute, NIH, Fredrick, MD, USA.

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Correspondence to R. L. Momparler, Centre de Recherche, Hôpital Sainte-Justine, 3175 Côte Sainte-Catherine, Montréal, Québec H3T 1C5, Canada. Tel: +1 514 345-4931 extn 6140; fax: +1 514 345-4801; e-mail: richard.l.momparler@umontreal.ca

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Introduction

Aberrant DNA methylation in the promoter region has been shown to silence the expression of many genes that suppress the development of neoplasia [1]. The genes that are silenced by this epigenetic event are involved in all the major steps of cancer formation [2]. These silent cancer-related genes provide interesting targets for chemotherapeutic intervention by agents that can reverse this epigenetic change [3].

5-Aza-2′-deoxycytidine (5-AZA-CdR, Decitabine), a potent inhibitor of DNA methylation, has been reported to reactivate many of these silent genes in neoplastic cell lines [3]. 5-AZA-CdR is a pro-drug that requires conversion to its active phosphorylated form by deoxycytidine kinase [4,5] (Fig. 1). Phosphorylated 5-AZA-CdR is rapidly incorporated into DNA where it inhibits DNA methylation [6] after forming a covalent bond with DNA methyltransferase [7]. Cytidine deaminase (CD) is also a key enzyme in the metabolism of 5-AZA-CdR [8] (Fig. 1), since deamination of 5-AZA-CdR results in complete loss of its antineoplastic activity [9].

In preclinical studies, we observed that 5-AZA-CdR is a very potent antileukemic agent in the mouse model [10] and that its antileukemic activity correlated well with the extent of inhibition of DNA methylation [11]. These studies led us to initiate clinical trials in patients with advanced leukemia. We observed that intensive therapy of 5-AZA-CdR of 2–3 days duration could induce complete remissions in some patients with advanced leukemia [12]. However, the duration of remission was short. In one patient that relapsed after 5-AZA-CdR therapy, we detected an increase in CD activity [13], a sign of drug resistance.

Recently, low-dose therapy for 10 days duration with 5-AZA-CdR was shown to produce good responses in

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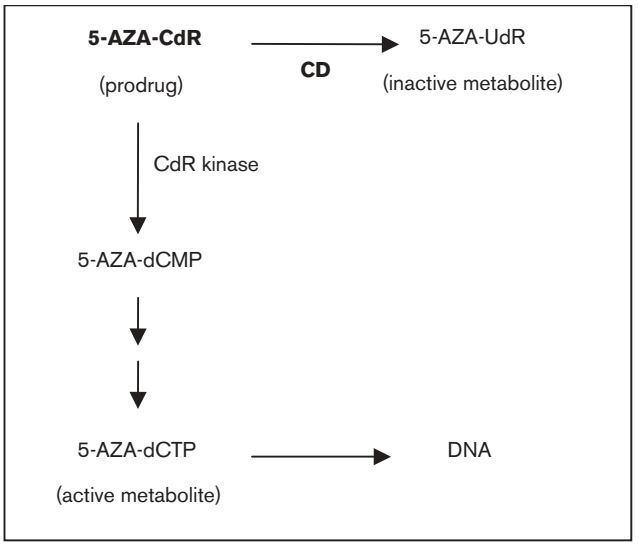
Fig. 1



Metabolism of 5-AZA-CdR. 5-AZA-CdR must be phosphorylated to 5-AZA-dCTP by deoxycytidine (CdR) kinase prior to incorporation into the DNA. The rate-limiting step in these reactions is the formation of 5-AZA-dCMP by CdR kinase. 5-AZA-CdR is deaminated by CD to 5-aza-2′-deoxyuridine (5-AZA-UdR), which is completely inactive.

patients with advanced leukemia [14]. This analog also showed effectiveness in patients with the pre-leukemic disease, myelodysplastic syndrome [15]. However, the optimal schedule for 5-AZA-CdR still remains to be determined. One potential obstacle for curative therapy with 5-AZA-CdR therapy is the presence of very high levels of CD in human liver and spleen [16]. These organs may act as 'biochemical sanctuaries' for leukemic cells by reducing the concentration of 5-AZA-CdR to levels below its minimal cytotoxic range due to rapid inactivation by CD [17].

One approach to overcome this problem is to administer an inhibitor of CD along with 5-AZA-CdR. Zebularine (Zeb) is a potent inhibitor of CD [18,19] capable of blocking the deamination of 5-AZA-CdR [20]. Zeb has been recently shown to have independent antineoplastic activity due to its inhibition of DNA methyltransferase [21–23]. In tumor cells, this CD inhibitor was shown to reactivate the expression of the tumor suppressor gene, p16CDKN2A [22,23]. Due to its chemical stability, Zeb can be administered orally [24,25], a major advantage for clinical use. These characteristics of Zeb make it an interesting agent to investigate in combination with 5-AZA-CdR.

In this report, we observed that Zeb produced an enhancement of the *in vitro* and *in vivo* antileukemic activity of 5-AZA-CdR. In addition, we observed that the combination produced an enhanced reactivation of the expression of the tumor suppressor gene p57KIP2 and a significant demethylation of its promoter. This gene, a cyclin-dependent kinase inhibitor, is frequently silenced by aberrant DNA methylation in leukemia [26,27]. A preliminary report of this work has been published in abstract form [28].

Materials and methods

Cell line

The mouse lymphocytic leukemia cell line L1210 was obtained from Dr T. Khwaja (University of Southern California, Los Angeles). Cells were maintained at 37°C with 5% CO_2 in RPMI 1640 medium (Life Technologies, Burlington, Ontario) with 5% heat-inactivated fetal calf serum (WISENT, St-Bruno, Quebec) and with 6 μM of 2-mercaptoethanol. The doubling time of the L1210 cells was 10–12 h. The human myeloid leukemia cell line HL-60 was obtained from ATCC (Manassas, VA). HL-60 cells were maintained at 37°C with 5% CO_2 in RPMI-1640 medium with 10% heat-inactivated fetal calf serum. The doubling time of HL-60 cells was approximately 20 h.

Chemicals

5-AZA-CdR (FW 228,2) and Zeb (FW 228,2) were obtained from Pharmachemie (Haarlem, Netherlands) and the Laboratory of Medicinal Chemistry at the NCI, respectively. 5-AZA-CdR was dissolved in sterile PBS and stored at –70°C to prevent decomposition. Zeb was dissolved in PBS for *in vitro* assays and in water for the *in vivo* assay.

Inhibition of growth and clonogenic assay

A 5-ml aliquot of L1210 or HL-60 cells in log-phase growth at a density of 1×10^4 cells/ml was placed in 25-cm^2 tissue culture flasks. The indicated concentrations of 5-AZA-CdR and/or Zeb were added. 5-AZA-CdR was added every 24 h. The flasks were incubated at 37°C and at 48 h an aliquot was removed for counting with a Model Z Coulter Counter. For the clonogenic assay, the drugs were removed by centrifugation and the cells were suspended in drug-free medium. An aliquot of 100 cells was placed in 2 ml of 0.3% soft agar RPMI 1640 medium containing 10% serum for L1210, and 20% serum for HL-60. After 10 days of incubation, the number of colonies (more than 200 cells) was counted. The cloning efficiency in soft agar of the cells without drug treatment was in the range of 50–60%.

Animals

BALB/c × DBA/2 (hereafter called $CD2F_1$) male mice (24–28 g) were obtained from Taconic Biotechnology (Germantown, NY). Mice were acclimatized to housing conditions for at least 2 weeks before experiments. They received food and water *ad libitum*. The animal committee approved the experimental protocol and animals were handled in accordance with institutional guidelines.

Transplantation and therapy of L1210 leukemia in mice

Transplantation of L1210 leukemic cells was performed by weekly i.p. injections of 1×10^4 cells in 0.1 ml of RPMI 1640 medium into $CD2F_1$ mice. Seven days later, the ascitic fluid was obtained from the mice and a cell count of the leukemic cells was performed with a hemocytometer prior to the subsequent transplantation. The mice were divided in four groups: control, 5-AZA-CdR, Zeb and 5-AZA-CdR + Zeb. The mice were injected i.v. with 0.1 ml of L1210 cells (1×10^4) [29]. Both drugs were sterilized by 0.2 μm filtration. The dose schedules for 5-AZA-CdR and Zeb is described in the legend of Table 2. For Schedule C, a Harvard infusion pump was used at a flow rate of 0.22 ml/h via a 25-gauge needle into the lateral tail vein. Mice were placed in a restrainer cage during drug treatment with access to food. Toxicity was evaluated by body weight loss as described previously [29]. The survival time of each group of mice was monitored and the increase in life span (ILS) calculated. Leukemic mice that survived more than 55 days after drug treatment were classified as long-term survivors.

Isolation of RNA and RT-PCR analysis

In order to study the reactivation of the p57KIP2 gene, HL-60 leukemic cells were treated with 5-AZA-CdR (25 and 50 ng/ml) and Zeb (100 ng/ml), alone or in combination, for 48 h. Cells were harvested 24 h after removal of the drugs. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Mississauga, Ontario, CA). For cDNA synthesis, total RNA was reverse-transcribed in a reaction mixture using Omniscript Reverse Transcriptase kit (Qiagen). The reaction was performed at 37°C for 60 min followed by 5 min at 93°C to inactivate the enzyme. PCR amplifications were performed using HotStar Taq Polymerase (Qiagen) and specific primers spanning different exons for human 18S ribosomal RNA and p57KIP2. The 18S RNA (GenBank accession number X03205) was amplified as an internal control using as sense primer 5′-TCG ATG GTA GTC GCC GTG CCT A-3′ and antisense primer 5′-CTG CTG CCT TCC TTG GAT GTG GTA-3′. The length of the PCR product of 18S cDNA was 110 bp. For p57KIP2 (GenBank accession number NM_000076), the primers were sense 5′-AGG AGC CTC TCG CTG ACC A-3′ and antisense 5′-ATC GCC CGA CGA CTT CTC A-3′ The length of the PCR product of p57KIP2 was 167 bp. Samples were amplified in a Whatman Biometra T gradient thermocycler (Göttingen, Germany) under the following conditions. For 18S and p57KIP2, the PCR conditions were 15 min at 95°C to activate Taq polymerase, denaturation at 94°C for 45 s, annealing at 60°C (18S) or 62°C (p57KIP2) for 30 s and extension at 72°C for 30 s, for 5 cycles. Then the annealing temperature was lowered by 2°C for an additional 10–15 cycles (18S) or for an additional 37–40 cycles (p57KIP2). For each gene, the number of cycles was terminated during the exponential phase of DNA amplification. The PCR products were electrophoresed on 2% agarose gel and detected by ethidium bromide staining. For quantitative detection of gene expression, we used the 18S as the reference standard to normalize the data. The amount of cDNA from the drug-treated samples that would amplify the identical amount of 18S DNA during the exponential phase for each sample was determined. The concentration of the amplified DNA was determined by Agilent 2100 Bioanalyzer (Palo Alto, CA). This instrument uses a very sensitive capillary electrophoresis and fluorescent detection to measure the concentration and size of DNA from a sample size of only 1 μl.

Isolation of DNA and bisulfite treatment

In order to study the methylation status of the promoter of the p57KIP2 gene, HL-60 leukemic cells were treated with 5-AZA-CdR (25 and 50 ng/ml) and Zeb (100 ng/ml), alone or in combination, for 48 h. Cells were harvested 24 h after removal of the drugs. Genomic DNA was isolated using DNeasy Tissue Kit (Qiagen). Genomic DNA was then treated with bisulfite [30]. This bisulfite treatment converts unmethylated cytosine residues to uracil, whereas 5-methylcytosine residues remain unchanged. Briefly, 1.5 ng of DNA was diluted in 20 μl of water and then denatured at 37°C for 30 min in 0.3 M NaOH followed by the addition of 278 μl of bisulfite (3.7 M) and 2 μl of 100 mM hydroquinone. The tubes were flushed with nitrogen gas to remove oxygen followed by an incubation period of 15 h at 50°C. Samples were diluted with water and transferred in a Microcon YM-100 filter (Millipore Amicon, Bedford, MA) to collect purified bisulfite-treated DNA.

Analysis of DNA methylation by the methylation-specific PCR (MSP) assay

The MSP assay [31] allows us to distinguished methylated from unmethylatated DNA, using specific primers for p57KIP2. PCR amplifications were performed using HotStar Taq Polymerase (Qiagen) and specific primers for unmethylated DNA (MSP-U, 5′-TTT GTT TTG TGG TTG TTA ATT AGT TGT-3′ [26] and MSP-A, 5′-AAA-CAC-AAC-ACA-CTT-AAC-CTA-TAA-AAC-3′). The length of the PCR product was 272 bp. For methylated DNA (MSP-M, 5′-CGC-GGT-CGT-TAA-TTA-GTC-GC-3′ [26] and MSP-A), the length of the PCR product was 265 bp. Samples were amplified under the following conditions: 5 min at 95°C to activate Taq polymerase, denaturation at 94°C for 45 s, annealing at 57°C (unmethylated) or 62°C (methylated) for 30 s and extension at 72°C for 30 s, for 5 cycles. Then the annealing temperature was lowered by 2°C for an additional 35 cycles. The number of cycles was terminated during the exponential phase of DNA amplification. The PCR products were electrophoresed on 2% agarose gel and detected by ethidium bromide staining. The concentration of the amplified DNA was determined using an Agilent 2100 Bioanalyzer. For quantitation of the

MSP reaction we determined the ratio of methylated/unmethylated DNA from the concentration obtained by Bioanalyzer analysis.

Statistical considerations

In order to evaluate whether the variations between groups were random, one-way ANOVA testing was performed. The p value was evaluated accordingly to Tukey's method. A value $p < 0.05$ was taken to indicate statistical significance. The data correspond to the mean values ± SD for $n \geq 4$. Valeriote and Lin's method [32,33] was used to determine if the interaction observed between drugs in the clonogenic assays was additive, synergistic or antagonistic.

Results

Effect of 5-AZA-CdR and Zeb on the growth of L1210 leukemic and HL-60 cells

The concentration of 5-AZA-CdR that produced 40% inhibition of growth after a 48-h exposure was in the range of 1 ng/ml for L1210 leukemic cells (Fig. 2A) and 1000 ng/ml for HL-60 leukemic cells (Fig. 2C). Under these conditions Zeb (1000 ng/ml) was a weak inhibitor of growth, producing less than 30% inhibition in both cell lines. The combination of these agents produced a greater inhibition of growth of L1210 cells than either agent alone, which was apparent at longer exposure times. At 48 h (Fig. 2B), 5-AZA-CdR (1 ng/ml) produced 43.7% inhibition and Zeb (100 ng/ml) produced 6.5% inhibition, whereas the combination produced 60% inhibition ($p < 0.01$). The co-incubation of both drugs on HL-60 did not produce significant inhibition of growth than either drug alone ($p > 0.05$).

Effect of 5-AZA-CdR and Zeb on colony formation of L1210 and HL-60 leukemic cells

In order to determine if a similar interaction can be demonstrated in a clonogenic assay, L1210 and HL-60 cells were exposed to a 48-h treatment with different concentrations of 5-AZA-CdR and Zeb (Table 1). The concentrations of 5-AZA-CdR that gave about 40% inhibition of growth (1 ng/ml for L1210 and 10 ng/ml for HL-60) were used to evaluate the efficacy of the combination with 100 ng/ml of Zeb (a concentration that, as a single agent, results in an approximate 10% inhibition of growth in both cell lines). For a 48-h exposure, 5-AZA-CdR plus Zeb produced a significantly greater loss of clonogenicity (Fig. 3) than either drug alone ($p < 0.05$ for L1210; $p < 0.01$ for HL-60).

Effect of 5-AZA-CdR and Zeb on survival time of mice with L1210 leukemia

In order to have an anatomical distribution of the leukemic cells similar to the human disease, the mice received an i.v. injection of 1×10^4 L1210 leukemic cells on day 1. The survival time of mice is directly proportional to the number of L1210 cells injected i.v. [29]. Chemotherapy was started on day 2, considered akin to an early leukemia. Three different schedules were evaluated (Table 2). As shown in Table 2, the mice received an i.p. injections of 5-AZA-CdR (1 mg/kg for Schedule A or 1.25 mg/kg for Schedule B) every 4 h for a total of five injections and/or an i.p. injection of Zeb. In Schedule A, Zeb (20 mg/kg) was administrated at the same time as the fourth injection of 5-AZA-CdR. In Schedule B, the mice received an i.p. injection of Zeb (10 mg/kg) every 4 h for a total of five injections (50 mg/kg total dose). The effects of these drugs on survival time of $CD2F_1$ mice with L1210 leukemia, the ILS and the number of long-term survivors (more than 55 days) were determined for each group.

For Schedule A, the combination produced a significantly greater ILS (244%) as compared to 5-AZA-CdR alone (156% ILS) or Zeb alone (7% ILS) ($p < 0.05$). One mouse treated with the combination survived more than 55 days. According to the method of Valeriote and Lin [32,33], the interaction of the drugs combination was defined as synergistic. In Schedule B, the combination produced a greater antileukemic effect with three out of 12 mice surviving more than 55 days, whereas none of those treated with 5-AZA-CdR alone survived more than 29 days.

For the Schedule C, the mice received a continuous 15-h i.v. infusion of 5-AZA-CdR (7 mg/kg) and/or Zeb (25 mg/kg). The combination produced a significantly greater ILS (267%) as compared to 5-AZA-CdR alone (146% ILS) or Zeb alone (1.3% ILS) ($p < 0.05$). According to the method of Valeriote and Lin [32,33], the interaction of the drugs combination was defined as synergistic. One mouse treated with 5-AZA-CdR alone survived more than 55 days. The toxicity produced by the combination was minimal for all three regimens (less than 3% loss in body weight).

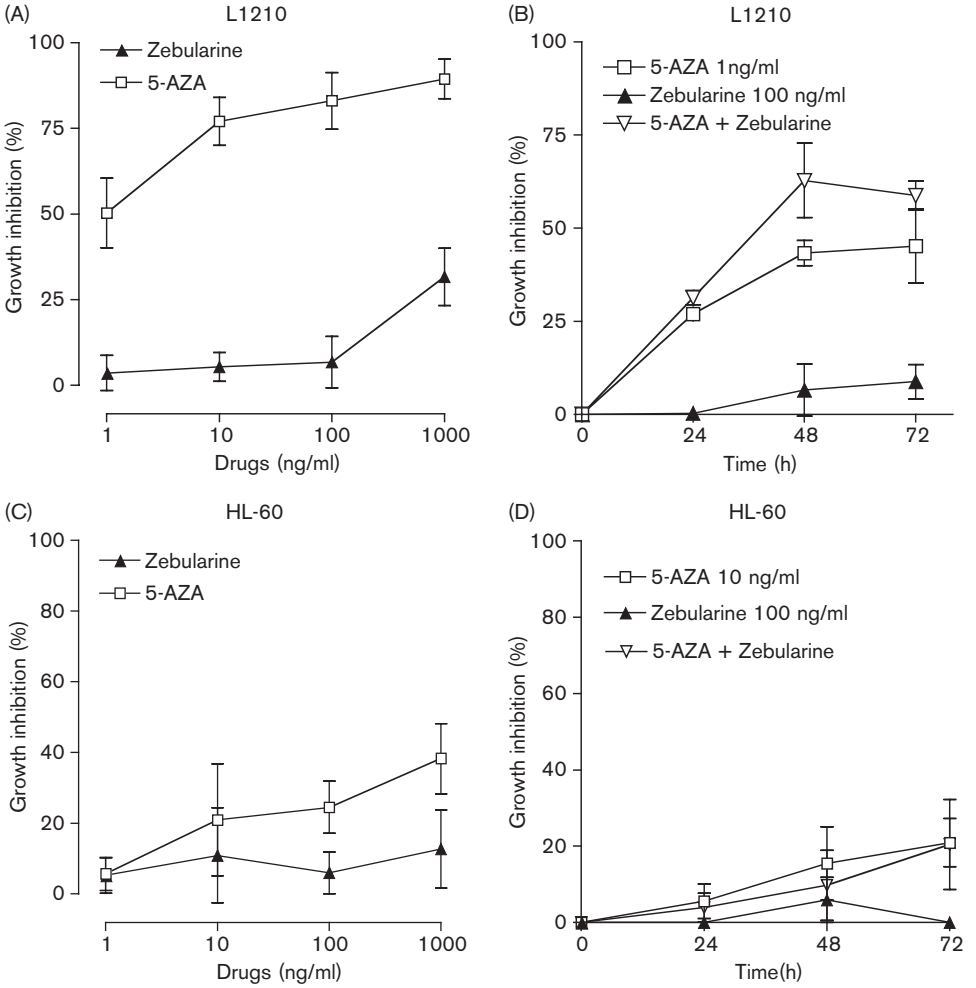
Effect of 5-AZA-CdR and Zeb on expression of p57KIP2

In order to evaluate the potential of the 5-AZA-CdR and Zeb combination in reactivating a gene that is silenced by epigenetic events in HL-60 leukemic cells, we investigated the effect of this combination on the activation of the tumor suppressor gene, p57KIP2, which is reported to be silenced in human leukemia [26,27]. Using RT-PCR, we observed a synergistic reactivation of p57KIP2 by 5-AZA-CdR plus Zeb as compared to either drug alone (Fig. 4).

Effect of 5-AZA-CdR and Zeb on the methylation status of the promoter of p57KIP2

In order to study the methylation status of the promoter of the p57KIP2 gene, HL-60 leukemic cells were treated with different concentrations of 5-AZA-CdR (25 and 50 ng/ml) and/or Zeb (100 ng/ml). Using MSP assay, we

Fig. 2



Effect of 5-AZA-CdR and/or Zeb on growth of L1210 or HL-60 leukemic cells. L1210 (A) or HL-60 (C) leukemic cells were treated with the indicated concentration of drugs and the growth inhibition (%) relative to control cells without treatment was evaluated by cell count. Data shown are mean values ± SD, $n \geq 4$. (B) 5-AZA-CdR and Zeb in combination produced a significantly greater growth inhibition than either drug alone ($p < 0.01$). (D) 5-AZA-CdR and Zeb in combination does not produce a significantly greater growth inhibition than either drug alone ($p > 0.05$).

observed that the combination of 5-AZA-CdR (25 ng/ml) and Zeb produced a greater promoter demethylation than either drugs alone ($p < 0.05$) (Fig. 5). 5-AZA-CdR (25 ng/ml) decreased the methylation level of the promoter of 54% compare to untreated cells. Zeb has no effect on this promoter methylation level, even at concentration up to 1000 ng/ml (data not shown). Values are not significant for 5-AZA-CdR 50 ng/ml, since this concentration demethylates around 80% of the promoter ($p > 0.05$). We were able to demethylated almost completely (more than 95%) the gene promoter with 5-AZA-CdR 1000 ng/ml (data not shown).

Discussion

Recent clinical studies of 5-AZA-CdR indicate that this potent inhibitor of DNA methylation has promising activity against hematological malignancies [14,15]. However, the curative potential of 5-AZA-CdR for the therapy of leukemia may be limited by its rapid *in vivo* inactivation by CD. In this study we have investigated the possibility of enhancing the antileukemic activity of 5-AZA-CdR by using it in combination with Zeb, a potent inhibitor of CD. An additional advantage in using Zeb is that this drug is also an inhibitor of DNA methylation [21–23], which may additionally enhance or complement the antineoplastic action of 5-AZA-CdR.

We investigated the growth inhibitory activity of 5-AZA-CdR and Zeb alone and in combination on murine L1210 lymphoid leukemic cells (Fig. 2). 5-AZA-CdR was a more potent inhibitor of growth than Zeb (Fig. 2A). The combination of these agents produced greater inhibition

than either agent alone (Fig. 2B). In the clonogenic assay, the combination also produced a greater reduction in colony formation by L1210 or HL-60 leukemic cells than either 5-AZA-CdR or Zeb alone (Fig. 3). The enhancement of the antineoplastic activity of 5-AZA-CdR by Zeb was much greater against HL-60 leukemic cells as compared to the L1210 leukemic cells. Perhaps this was due in part to the induction of CD in HL-60 leukemic cells following treatment with 5-AZA-CdR, as we have previously reported [34].

Table 1 Effect of 5-AZA-CdR or Zeb on loss of clonogenicity on L1210 and HL-60 leukemic cells

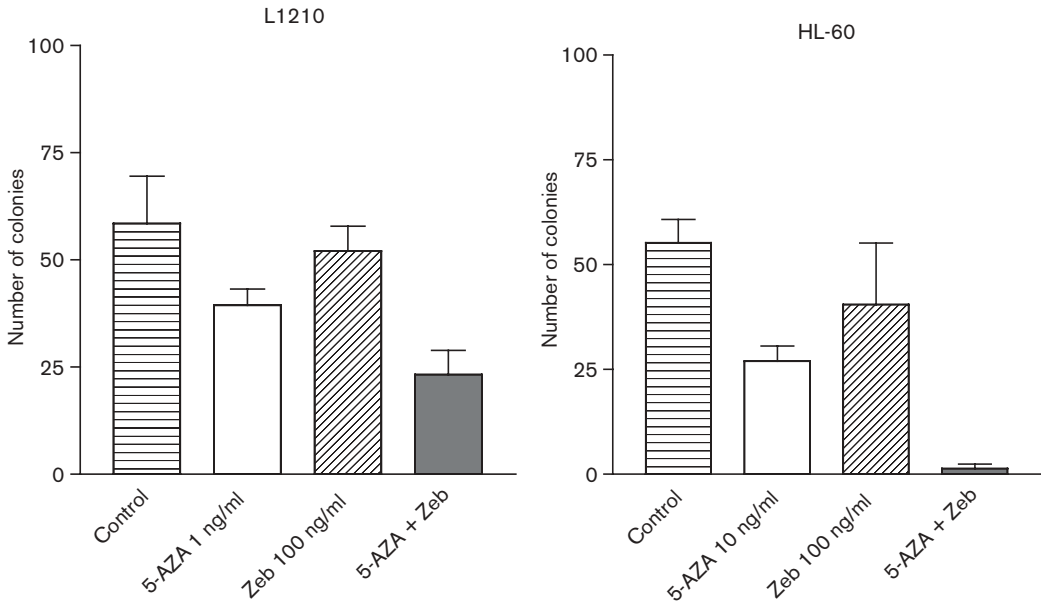
Drug concentration (ng/ml)	L1210		HL-60	
	No. of colonies[a]	Loss of clonogenicity (%)	No. of colonies[a]	Loss of clonogenicity (%)
5-AZA-CdR				
0	58.6 ± 11.0	–	55.2 ± 6.1	–
1	39.5 ± 3.8	32.6	46.0 ± 4.1	16.7
10	0.8 ± 0.8	>98	27.0 ± 3.6	51.1
100	0	100	0.3 ± 0.5	>99
1000	0	100	0	100
Zeb				
0	58.6 ± 11.0	–	55.2 ± 6.1	–
1	55.3 ± 9.9	5.6	46.8 ± 9.6	15.2
10	45.0 ± 4.5	23.2	38.2 ± 16.3	30.8
100	52.1 ± 5.8	11.0	40.5 ± 14.7	26.6
1000	50.3 ± 5.4	14.2	27.7 ± 12.3	49.8

L1210 and HL-60 leukemic cells were exposed to the indicated concentrations of 5-AZA-CdR or Zeb for 48 h. The number of colonies (greater than 200 cells) was determined on day 10 for L1210 cells and on day 15 for HL-60 cells. Loss of clonogenicity is relative to control cells without treatment.
[a]Mean values ± SD, $n \geq 4$.

The molecular mechanism responsible for the antineoplastic action of 5-AZA-CdR is probably related to its reactivation of tumor suppressor genes that are silenced by DNA methylation [1–3]. In order to determine if Zeb could influence this event, we investigated its effects, alone and in combination with 5-AZA-CdR, on the activation of p57KIP2 [26,27] and on its promoter methylation status. p57KIP2 is a negative regulator of cellular proliferation reported to be inactivated frequently by aberrant DNA methylation in leukemia. We observed that 5-AZA-CdR in combination with Zeb produced a synergistic reactivation of p57KIP2 in HL-60 leukemic cells (Fig. 4). This combination also produced a significant greater demethylation of the p57KIP2 promoter than either drug alone as demonstrated by MSP analysis (Fig. 5). In both these analyses Zeb alone did not produce a significant effect suggesting that its interaction with 5-AZA-CdR was primarily due to its inhibition of CD. However, further experimentation is required to clarify this hypothesis.

In order to facilitate the translation of the *in vitro* data into a clinical trial, it is important to confirm drug interactions in an *in vivo* model as the CD present in host cells can influence the response to chemotherapy. Mice were inoculated i.v. with L1210 leukemic cells and 24 h later injected i.p. (Table 2, Schedule A and B) or i.v. (Table 2, Schedule C) with 5-AZA-CdR, Zeb or with a combination of both these agents. In this model, if only one leukemic cell survives chemotherapy, the mice will

Fig. 3



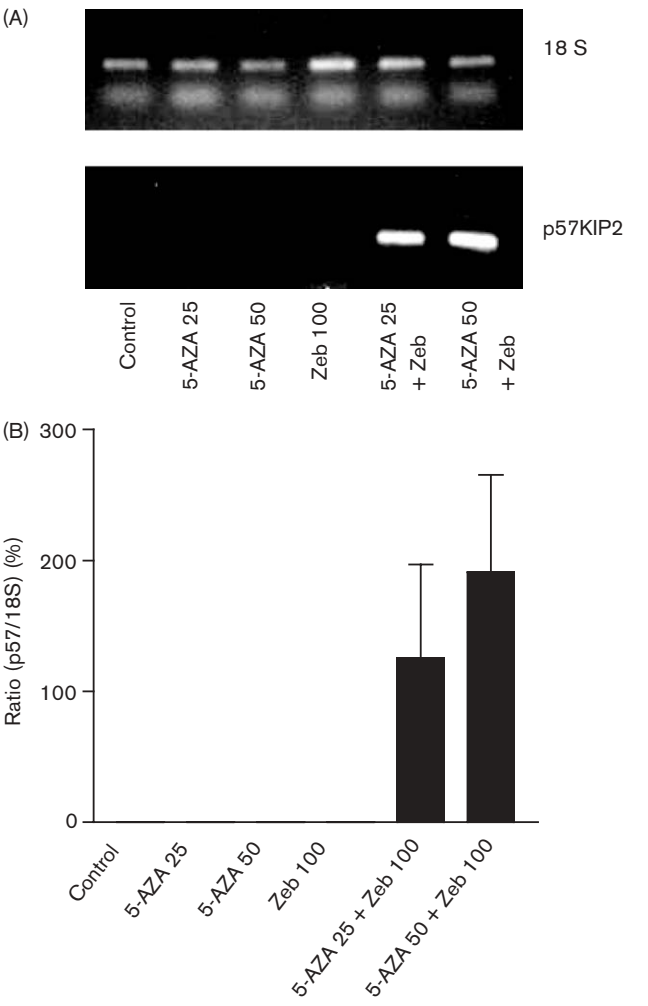
Effect of 5-AZA-CdR (5-AZA) and/or Zeb on loss of clonogenicity of L1210 and HL-60 leukemic cells. Cells were treated with the indicated concentration of drugs for 48 h and cell survival determined by colony count. Data shown are mean values ± SD, $n \geq 4$. 5-AZA-CdR and Zeb in combination produced a significantly greater loss in clonogenicity than either drug alone ($p < 0.05$ for L1210; $p < 0.01$ for HL-60).

Table 2 Effect of different dose schedules of 5-AZA-CdR and/or Zeb on survival time of $CD2F_1$ mice with L1210 leukemia

Drug	Total dose (mg/kg)	Mean survival time (days)[a]	ILS (%)	>55-day survivors	Weight loss (%)
Schedule A					
control	0	7.8 ± 0.5	–	0/10	–
5-AZA	5	19.9 ± 2.5	156	0/10	<3
Zeb	20	8.3 ± 0.6	7	0/7	<1
5-AZA + Zeb	5 + 20	26.8 ± 2.1[b]	244[b]	1/7	<3
Schedule B					
control	0	7.7 ± 0.4	–	0/10	–
5-AZA	6.25	24.6 ± 3.8	219	0/10	<3
Zeb	50	7.8 ± 0.4	2	0/11	<1
5-AZA + Zeb	6.25 + 50	25.8 ± 3.6[b]	235[b]	3/12	<2
Schedule C					
control	0	7.9 ± 0.1	–	0/5	–
5-AZA	7	19.4 ± 4.3	146[b]	1/5	<3
Zeb	25	8.0 ± 0.4	1.3	0/5	<1
5-AZA + Zeb	7 + 25	29.0 ± 7.8	267	0/5	<3

Mice were injected i.v. with 10^4 L1210 cells on day 1. Chemotherapy was started on day 2. For Schedule A, the mice received i.p. injections of 5-AZA-CdR (1mg/kg) every 4 h for a total of five injections. The last group received an injection of Zeb (20 mg/kg) with the fourth injection of 5-AZA-CdR. For Schedule B, the mice received i.p. injections of 5-AZA-CdR (1.25 mg/kg) and/or of Zeb (10 mg/kg) every 4 h for a total of five injections. For Schedule C, the mice received a 15-h i.v. infusion of 5-AZA-CdR (total dose 7 mg/kg) and/or Zeb (total dose 25 mg/kg). Weight loss was determined every 7 days.
[a]Mean values ± SD.
[b]Value does not include >55-day survivors.

Fig. 4

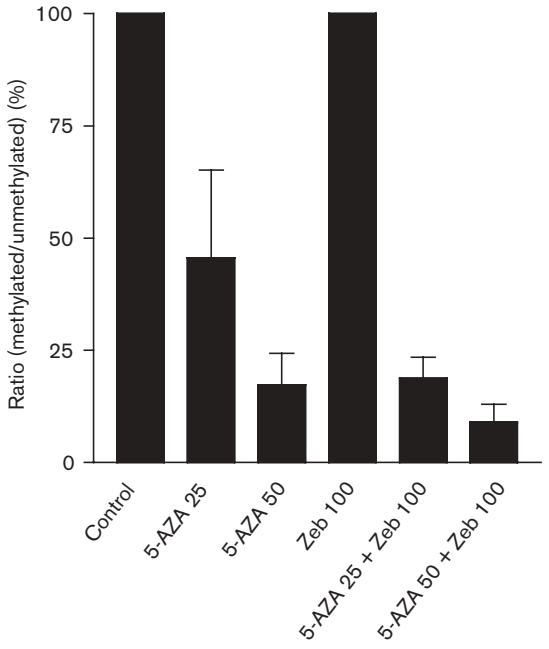


Effect of 5-AZA-CdR (5-AZA) and Zeb alone or in combination on the expression of the p57KIP2 gene in HL-60 leukemic cells. HL-60 cells were treated with the indicated concentrations of 5AZA-CdR and/or Zeb for 48 h. Total RNA was isolated at 72 h and 18 S ribosomal RNA gene and p57KIP2 gene expression analyzed by RT-PCR (A). The amount of cDNA amplified during the exponential phase of PCR was analyzed by quantification of amplified DNA (B) by an Agilent 2100 Bioanalyzer ($p < 0.05$). Data are mean values ± SD, $n \geq 4$. Control = cells with no drug treatment.

die from leukemia. In addition, there is an excellent correlation between the number of surviving leukemic cells and the survival time of the mice [29]. In all regimens, Zeb produced a very weak antileukemic effect as compared to 5-AZA-CdR. However, the combination showed a more potent antileukemic effect than either agent alone for the three different schedules. From a total of 24 mice, four mice treated with the combination survived more than 55 days, which in this model is classified as long-term survivors. The mouse model of leukemia confirms our *in vitro* data, which showed that Zeb enhanced the antineoplastic action of 5-AZA-CdR. The toxicity produced by the single agents or combination was minimal as evaluated by body weight loss (less than 3% change). Our data are in accord with the report that Zeb increased the antineoplastic activity of cytosine arabinoside in mice with L1210 leukemia [25]. Cytosine arabinoside is a cytosine analog whose metabolism is similar to 5-AZA-CdR. These investigators also observed no enhancement of the toxicity by the combination of Zeb with cytosine arabinoside.

In conclusion, our data indicate that Zeb can enhance the antileukemic activity of the deoxycytidine analog, 5-AZA-CdR. This interaction is most likely due to protection against inactivation of 5-AZA-CdR by CD. However, the inhibition of DNA methylation by Zeb may play some role in this process. This hypothesis is supported by our results that demonstrate that the combination of both drugs produces significantly greater demethylation of the p57KIP2 promoter than either drug alone and that Zeb (100 ng/ml) by itself is unable to achieve (Fig. 5). Cheng *et al.* also reported that Zeb hinders the remethylation of tumor suppressor genes in tumor cells following treatment with 5-AZA-CdR [23]. At the clinical level, the combination also has the potential to eradicate leukemic cells that show drug resistance to 5-AZA-CdR due to increased CD expression [13]. These points provide a rationale to support clinical trials with 5-AZA-CdR and Zeb in combination in patients with leukemia resistant to conventional chemotherapy.

Fig. 5



Effect of 5-AZA-CdR (5AZA) and Zeb alone or in combination on the methylation status of the promoter of the p57KIP2 gene in HL-60 leukemic cells. HL-60 cells were treated with the indicated concentrations of 5AZA-CdR and/or Zeb for 48 h. Genomic DNA was isolated at 72 h, treated with bisulfite and a MSP assay was performed. The amount of DNA amplified during the exponential phase of PCR was analyzed by quantification of amplified DNA by an Agilent 2100 Bioanalyzer ($p < 0.05$). Data are mean values ± SD, $n \geq 4$. Control = cells with no drug treatment.

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