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ORIGINAL ARTICLE

Inhibition of cytidine deaminase by zebularine enhances the antineoplastic action of 5-aza-2′-deoxycytidine

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Abstract Cytidine (CR) deaminase is a key enzyme in the catabolism of cytosine nucleoside analogues, since their deamination results in a loss of their pharmacological activity. In this report we have investigated the importance of CR deaminase with respect to the antineoplastic action of inhibitors of DNA methylation, 5-aza-2′-deoxycytidine (5-AZA-CdR) and zebularine. Zebularine has a dual mechanism of action, since it can also inhibit CR deaminase. The objective of our study was to investigate the importance of zebularine as an inhibitor of CR deaminase with respect to the antineoplastic action of 5-AZA-CdR. Using an in vitro clonogenic assay, we investigated the antineoplastic action of 5-AZA-CdR and zebularine, alone and in combination on wild type 3T3 murine fibroblasts and corresponding V5 cells transduced with CR deaminase gene to express a very high level of CR deaminase activity. The V5 cells were much less sensitive to 5-AZA-CdR than the wild type 3T3 cells. The addition of zebularine significantly enhanced the antineoplastic action of 5-AZA-CdR on V5 cells, but not 3T3 cells. Enzymatic analysis on CR deaminase purified from the V5 cells showed that zebularine is a competitive inhibitor of the deamination of 5-AZA-CdR. These in vitro observations are in accord with our in vivo study in mice with L1210 leukemia, which showed that zebularine increased the antileukemic activity of 5-AZA-CdR. Pharmacokinetic analysis also showed that zebularine increased the plasma level of 5-AZA-CdR during an i.v. infusion in mice. Our results indicate that the major mechanism by which zebularine enhances the antineoplastic action of 5-AZA-CdR is by inhibition of CR deaminase. These findings provide a rationale to investigate 5-AZA-CdR in combination with zebularine in patients with advanced leukemia.

Keywords 5-Aza-2′-deoxycytidine · Zebularine · Cytidine deaminase · Pharmacokinetics · Leukemia

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Introduction

Zebularine is a cytidine (CR) analogue reported to inhibit the DNA methyltransferase [1, 2] and consequently re-expressed genes silenced by hypermethylation of their promoter [3, 4]. Preclinical studies have shown that zebularine has both antitumor [3, 4] and antileukemic activity [5, 6]. Zebularine is also a potent competitive inhibitor of the enzyme CR deaminase [7, 8]. CR deaminase is a key enzyme in the catabolism of cytosine nucleoside analogs, such as 5-aza-2′-deoxycytidine (decitabine, 5-AZA-CdR) [4], since deamination results in a loss of its pharmacologic activity [9]. 5-AZA-CdR, a potent inhibitor of DNA methylation, has been shown to be an effective agent for treatment of the hematological malignancy myelodysplastic syndrome [10, 11]. It is reported that tumor cells and patient cancer cells increase their CR deaminase activity as a resistance mechanism to

antineoplastic drugs, such as 5-AZA-CdR [9, 12–14]. It is possible that the use of zebularine in combination with 5-AZA-CdR may be one approach to overcome this problem of drug resistance.

In a previous study, we observed that zebularine increased the curative action of high dose 5-AZA-CdR in mice with L1210 leukemia [15]. This result can be explained either by an additional de-methylation action of zebularine, by preventing 5-AZA-CdR degradation, or by a combination of these two mechanisms. In this report, we investigated the importance of zebularine as an inhibitor of CR deaminase with respect to the antineoplastic action of 5-AZA-CdR [16] using a cell line transduced with the CR deaminase gene [14]. We observed that zebularine enhanced the antineoplastic action of 5-AZA-CdR on cells containing high levels of CR deaminase.

Materials and methods

Cell lines

The mouse lymphocytic leukemia cell line L1210 was obtained from Dr. T. Khwaja (University of Southern California, Los Angeles). These cells were maintained in RPMI-1640 medium (Canadian Life Technologies, Burlington, ON) with 5% heat-inactivated fetal bovine serum (WISENT, St-Bruno, Quebec) and with 6 μM of 2-mercaptoethanol. The doubling time of the L1210 cells was 10–12 h. The mouse fibroblast NIH 3T3 was obtained from ATCC (ATCC, USA). 3T3-CD3-V5 cells (V5) transduced with CR deaminase gene was previously constructed in our laboratory [12, 14]. Both cell lines were maintained in Dulbecco's modified essential medium (Canadian Life Technologies) with 10% heat-inactivated fetal bovine serum (WISENT). The doubling time of the 3T3 and V5 cells was 30–35 h. All cell lines were maintained at 37°C with 5% CO_2.

Chemicals

5-Aza-2′-deoxycytidine (FW 228.2) was obtained from Pharmachemie (Haarlem, Netherlands). Zebularine (FW 228.2) was kindly provided by the Laboratory of Medicinal Chemistry (NCI, NIH, Frederick, MD, USA). Both agents were dissolved in sterile phosphate buffer saline solution (0.2 gm/l of KCl, 0.2 gm/l of KH2PO4, 8 gm/l of NaCl and 2.16 gm/l of $Na_2HPO_4{\cdot}7H_2O$) for in vitro and in vivo assays. 5-AZA-CdR was stored at −70°C to prevent its decomposition; whereas zebularine was stored at −20°C. CR and 5-[^{3}H]CR were obtained from Boehringer Mannheim (Laval, Canada) and ICN Biomedicals (Irvine, CA, USA), respectively.

Clonogenic assay

A 5 ml aliquot of L1210 (10^4 cells) in log growth phase was placed in 25 cm^2 tissue-culture flasks. The indicated concentrations of drugs were added. The flasks were incubated at 37°C and at the indicated times, an aliquot was removed for counting with a Model Z Coulter Counter. An aliquot of 100 cells was placed in 2 ml of 0.3% soft agar RPMI 1640 medium containing 11% serum. For 3T3 or V5 cells, 200 cells were plated in 6-well dishes and incubated for 24 h prior to addition of different concentrations of 5-AZA-CdR and/or zebularine. The drug was removed after the different exposure times and the cells were placed in drug-free medium. The number of colonies (>500 cells) was counted after 7 days of incubation for L1210 cells and after 14 days of incubation for both 3T3 and V5 cells. The cloning efficiency of L1210 cells was in the range of 60–70% whereas it was approximately 50% for 3T3 and V5 cells.

CR deaminase purification and quantification

A partial purification of CR deaminase was performed on 3T3 and on V5 cells as previously described [12, 14]. Briefly, 6×10^7 cells were suspended in 5 mM Tris–HCl (pH 7.4), then freeze–thawed three times and the buffer concentration adjusted to 100 mM Tris–HCl (pH 7.4). Samples were then centrifuged at 5°C to obtain the cytosolic extract. Streptomycin sulfate (65 mg/ml final concentration) was added followed by incubation for 10 min on ice and centrifugation. Ammonium sulfate, pH 6.8 (70% saturation final concentration) was then added to precipitate the enzyme on ice for 10 min. After centrifugation was performed, the precipitate was suspend in 50 mM Tris–HCl + 20% glycerol and stored at −70°C. A spectrophotometric assay was used for quantification of the CR deaminase activity [17].

CR deaminase assay

Cytidine deaminase activity was determined using radioactive CR. Diluted enzyme from both 3T3 and V5 cells was incubated with 10 μM of 5-[^{3}H]CR, 50 μCi/ml (Moraveck Biochemicals inc., CA) with 50 mM Tris–HCl (pH 7.4) for 10 min at 37°C [12, 13], with or without zebularine. The reaction was stopped by adding cold HCl 0.001 N and the mixture was placed on Whatman P-81 phosphocellulose discs. The amount of radioactive cytosine moiety bound to the disc was determined by scintillation counting. The amount of the enzyme that catalyzed the deamination of 1 nmol of CR per minute was defined as 1 unit (U) of enzyme activity. The rate of deamination of 5-AZA-CdR was determined by spectrophotometric assay at 245 nm using a Gilford 260 spectrophotometer [17]. The reaction mixture

(0.1 ml) contained 20 mM KH2PO4 (pH 7.5), 100 mM KCl, the indicated concentration of substrate, and about 2 U or 5.8 μg purified CR deaminase from V5 cells. The Michaelis–Menten constant (Km) for 5-AZA-CdR and inhibition constant (Ki) for zebularine were determined using the method of Lineweaver–Burk with different substrate concentrations and a constant inhibitor concentration [18].

Chemotherapy of leukemia in mice

BALB/c × DBA/2 (hereafter called $CD2F_1$) male mice weighing 24–28 g were obtained from Taconic Biotechnology (Germantown, NY, USA). Mice were acclimatized to housing conditions for at least 2 weeks before experiments. The mice received food and water ad libitum. The animal committee approved the experimental protocol and the animals were handled in accordance with institutional guidelines. Transplantation of L1210 leukemic cells was performed by weekly i.p. injections of 10^4 cells in 0.1 ml of RPMI-1640 medium into $CD2F_1$ mice. Seven days later, the ascitic fluid was obtained and a cell count of the leukemic cells was performed with a hemocytometer. For chemotherapy, the mice were injected i.v. into the lateral tail vein with 0.1 ml of L1210 (10^4 cells) on day 1 [15]. 5-AZA-CdR (2 mg/kg) and/or zebularine (20 mg/kg) were administered as a 15 h i.v infusion on day 2 using a Harvard infusion pump and flow rate of 0.22 ml/h via a 25-gauge needle attached to a butterfly tube into the lateral tail vein. Mice were placed in a restrainer cage during drug treatment. The survival time of each group of leukemic mice (11 mice per group) was monitored and the increase in life span (ILS) calculated.

Pharmacokinetic analysis of 5-AZA-CdR in mice

Mice received 5-AZA-CdR and/or zebularine by i.v. infusion for 4 h. Blood samples were obtained by cardiac puncture. A standard clonogenic assay as described above was then performed on L1210 cells with different concentrations of 5-AZA-CdR for a standard curve and with different dilutions of plasma from the drug-treated mice. The plasma concentration of 5-AZA-CdR in presence or absence of zebularine was estimated from the standard curve [19].

Statistical considerations

For statistical analysis of in vitro experiments, the one-way ANOVA was performed and the P value was evaluated accordingly to Tukey's method. The Kaplan–Meier method was used to analyze the survival curves using the GraphPad Prism software (San Diego, CA, USA). A value $P < 0.05$ was taken to indicate statistical significance. The data correspond to the mean values ± SD for $n \geq 3$.

Results

Analysis of extracts of V5 cells indicated that these transduced cells contained 25-fold greater CR deaminase activity than the wild type 3T3 cells (Table 1), as reported previously [12, 14]. Enzyme kinetic analysis on CR deaminase from V5 cell showed that zebularine was a competitive inhibitor of the deamination of 5-AZA-CdR (Fig. 1). The estimated apparent Km value for 5-AZA-CdR was 47.7 μM, whereas the estimated inhibitory constant (Ki) for zebularine was 0.95 μM.

Clonogenic assay showed that the V5 cells were less sensitive to the antineoplastic action of 5-AZA-CdR as compared to the 3T3 cells (Table 2). The antineoplastic activity of zebularine for both these cell lines was similar, but very weak. When zebularine was used in combination with 5-AZA-CdR, it produced a marked enhancement of the antineoplastic activity of this cytosine nucleoside analog on the V5 cells, but not the 3T3 cells (Fig. 2). A similar interaction between these two agents was observed for both the 24 and 48 h exposure times.

Zebularine also enhanced the survival time of mice with L1210 leukemia treated with 5-AZA-CdR (Fig. 3). About 27% of the mice treated with this drug combination had a survival time longer than the mice treated with only 5-AZA-CdR ($P < 0.05$). Mice treated with zebularine alone had a survival time that was not significantly different from the control mice [15]. In this mouse model, the leukemic cells were injected i.v. to give an anatomical distribution similar to the human disease. The mice received a 15 h i.v. infusion of 5-AZA-CdR (2 mg/kg) and/or zebularine (20 mg/kg). From the published pharmacokinetic data on zebularine [20], we estimated that the steady state plasma level of this agent was in the range of 6 μM (~1,400 ng/ml). In order to determine if zebularine increased the plasma level of 5-AZA-CdR by inhibition of CR deaminase, we determined the steady state plasma level of this cytosine nucleoside analog (Table 3). Co-infusion of zebularine increased the 5-AZA-CdR plasma level by 2-fold.

Table 1 CR deaminase activity in 3T3 and V5 cells

Cell line	CR deaminase activity (units/mg)
3T3 cells	14.7 ± 6.2[a]
V5 cells	338 ± 75

CR deaminase activity was obtained by CR deaminase assay using a radioactive substrate

[a] Mean value ± SD $n \geq 4$

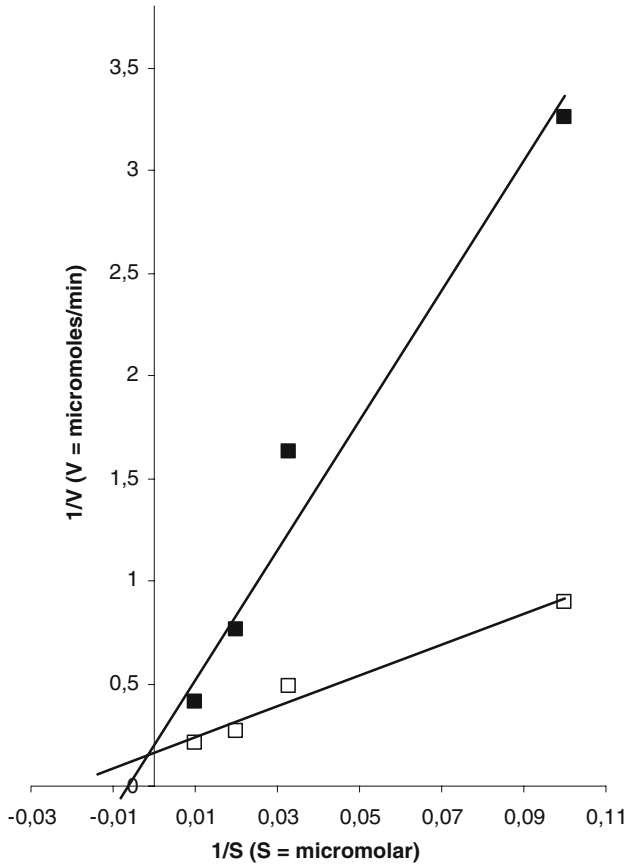

Fig. 1 Lineweaver–Burk plot of the effect of zebularine on the deamination of different concentrations of 5-AZA-CdR by CR deaminase. 5-AZA-CdR plus 500 ng/ml of zebularine (*black squares*); 5-AZA-CdR alone (*white squares*). *S* = μM 5-AZA-CdR; zebularine concentration 2.19 μM

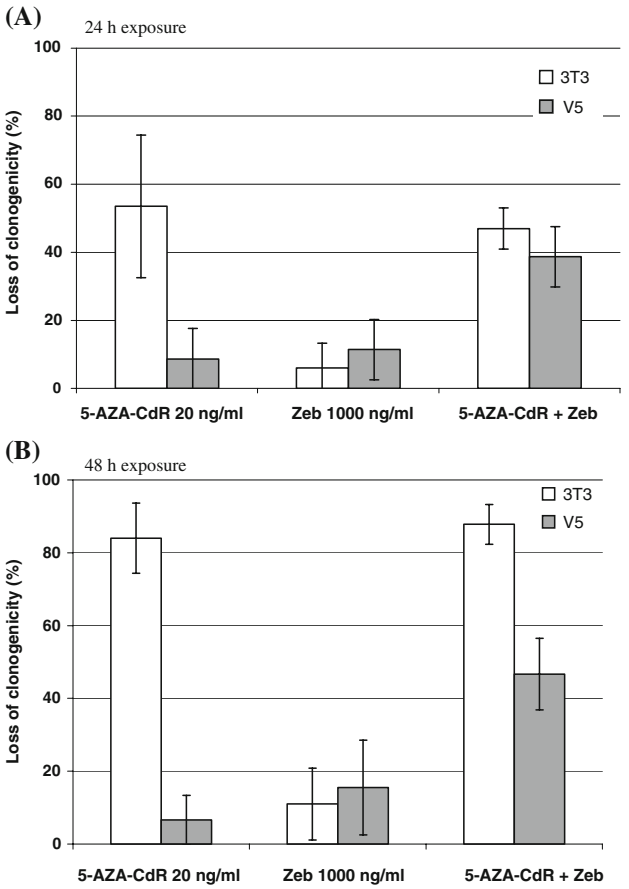

Fig. 2 Effect of zebularine on antineoplastic action of 5-AZA-CdR on 3T3 cells and V5 cells transduced with CR deaminase gene. The cells were exposed to 20 ng/ml 5-AZA-CdR and/or 1,000 ng/ml zebularine (*Zeb*) for either 24 h (**a**) or 48 h (**b**). Cell survival was determined by clonogenic assay. For V5 cells, the loss of clonogenicity of 5-AZA-CdR plus zebularine was greater than 5-AZA-CdR or zebularine alone ($P < 0.05$)

Table 2 Effect of different concentrations and exposure times of 5-AZA-CdR on loss of clonogenicity of 3T3 and V5 cells

Drug concentration (ng/ml)	Loss of clonogenicity (%)			
	3T3 exposure time (h)		V5 exposure time (h)	
	24	48	24	48
5-AZA-CdR				
1	22.6 ± 12.7[a]	19.0 ± 18.9[a]	6.1 ± 7.2[a]	9.4 ± 12.2[a]
10	28.6 ± 13.3	54.9 ± 20.1	6.8 ± 10.1	7.1 ± 7.5
20	58.6 ± 14.3	85.3 ± 9.4	8.6 ± 9.5	6.9 ± 7.6
100	91.3 ± 4.2	98.0 ± 2.5	23.1 ± 13.8	44.5 ± 25.3
Zebularine				
1	22.0 ± 17.4	14.0 ± 13.2	19.0 ± 18.0	15.2 ± 19.7
10	9.0 ± 13.1	6.0 ± 7.4	3.0 ± 4.7	9.4 ± 10.8
100	9.0 ± 12.7	4.0 ± 4.9	9.0 ± 11.6	9.0 ± 11.3
1,000	12.0 ± 11.6	11.0 ± 9.9	11.4 ± 8.9	15.5 ± 13.0

[a] Mean value ± SD $n \geq 4$

Discussion

Zebularine is an interesting antineoplastic agent [1]. Its capacity to inhibit DNA methylation can result in reactivation of silent tumor suppressor genes [2–5]. Zebularine shows inhibitory activity against both tumor and leukemic cells [3–5]. In mouse models, zebularine at high doses shows significant antitumor [3] and antileukemic activity [6]. Zebularine is also a potent inhibitor of CR deaminase [8, 16], which is of great importance since this enzyme inactivates cytosine nucleoside analogs [9, 13]. We reported previously that zebularine enhances the curative activity of the potent inhibitor of DNA methylation 5-AZA-CdR in mice with L1210 leukemia [15]. An important question is whether this enhancement of the epigenetic action of 5-AZA-CdR is due to the additional inhibition of DNA methylation or

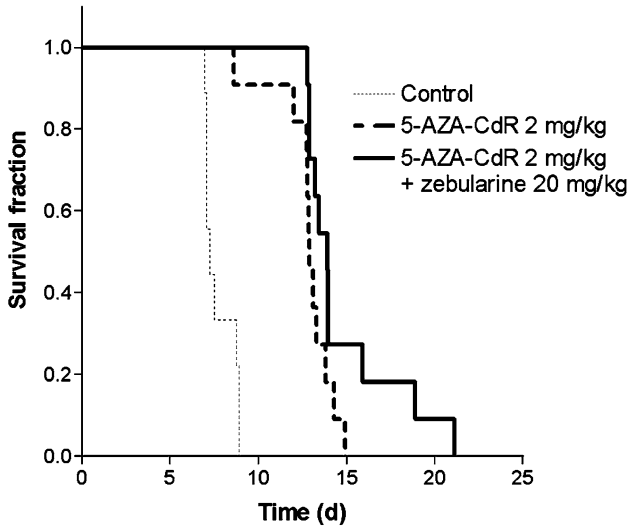

Fig. 3 Kaplan–Meier survival curve of mice with L1210 leukemia treated with zebularine and 5AZA-CdR. The mice received an i.v. injection of 10^4 L1210 leukemic cells day 1. On day 2 the mice were administered a 15 h i.v. infusion of 5-AZA-CdR (2 mg/kg) and/or zebularine (20 mg/kg). The control leukemic mice without treatment survived 7.3 days. Mice treated with zebularine alone had a survival time that was not significantly different from the control mice (data not shown) [15]. About 27% of the mice that received 5-AZA-CdR plus zebularine survived longer than those who were treated with 5-AZA-CdR alone ($P < 0.05$)

Table 3 Pharmacokinetic analysis of 5-AZA-CdR during i.v. infusion in mice

Drug	Plasma level	
	Rate drug infusion [mg/(kg h)]	5-AZA-CdR (ng/ml)
5-AZA-CdR	0.133	71.4 ± 3.3[a]
Zebularine	1.33	0
5-AZA-CdR + zebularine	0.133 + 1.33	149.2 ± 34.2

5-AZA-CdR and/or zebularine were administered as a 15 h i.v. infusion at total doses of 2 and 20 mg/kg, respectively. Blood samples were obtained after 4 h i.v. infusion and plasma concentration of 5-AZA-CdR was determined by bioassay on L1210 leukemic cells

[a] Mean value ± SD $n = 4$

reduction in its inactivation by zebularine. We showed previously that co-incubation of HL-60 human myeloid leukemic cells with 5-AZA-CdR and zebularine gives a greater re-expression of the tumor suppressor gene p57KIP2 compare to each agent alone, whereas zebularine by itself does not demethylate the gene [15]. Also, zebularine is reported to sustain the demethylation produced by 5-AZA-CdR on human bladder cells [4]. In this report, we focus on the ability of zebularine to inhibit the CR deaminase and to prevent the degradation of 5-AZA-CdR to explain the longer survival time of mice with L1210 leukemia treated with both compounds.

In order to clarify the interaction between 5-AZA-CdR and zebularine, we used a murine fibroblast cell line (V5) that was transduced with the CR deaminase gene and expresses very high levels of CR deaminase activity [12, 14; Table 1]. We observed that the transduced V5 cells showed significant drug resistance to 5-AZA-CdR as compared to the wild type cells (Table 2) and therefore represent a trustable model of resistance to cytosine nucleoside analogues. The addition of zebularine to 5-AZA-CdR enhances its antineoplastic activity against the V5 transduced cells, but not the wild type 3T3 cells (Fig. 2). Enzymatic analysis of CR deaminase purified from the V5 cells showed that zebularine was a competitive inhibitor of the deamination of 5-AZA-CdR (Fig. 1). In this cellular model, the enhancement of the antineoplastic activity of 5-AZA-CdR by zebularine was due primarily to its inhibition of CR deaminase. We based this conclusion on the fact that zebularine did not produce a significant enhancement of 5-AZA-CdR on the wild type cells.

In order to show that the enhancement of antineoplastic activity of 5-AZA-CdR was due to the inhibition of CR deaminase by zebularine, we administered a 15 h i.v. infusion of low dose 5-AZA-CdR with and without zebularine to mice with L1210 leukemia. The low dose 5-AZA-CdR was used to clarify the in vivo role of CR deaminase with respect to the metabolism of this analog. The addition of zebularine to 5-AZA-CdR increased significantly the survival time of the leukemic mice as compared to the mice administered 5-AZA-CdR alone (Fig. 3). Pharmacokinetic analysis showed that the addition of zebularine significantly increased the plasma level of 5-AZA-CdR in the mice (Table 3). These observations are supportive to our hypothesis that the enhancement of the antineoplastic action of 5-AZA-CdR by zebularine is due to reduction in the catabolism of this cytosine nucleoside analog. Our results may have clinical application for the therapy of leukemia in patients. Due to the very high levels of CR deaminase in human liver [21], this organ may act as a "biochemical sanctuary" for leukemic stem cells during therapy with 5-AZA-CdR [22]. The use of zebularine in combination with 5-AZA-CdR may be a way to overcome this problem and increase the clinical efficacy of this epigenetic agent in patients with hematological malignancies.

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