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Potent inhibitors for the deamination of cytosine arabinoside and 5-aza-2′-deoxycytidine by human cytidine deaminase*

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Summary. Deamination of the nucleoside analogues ARA-C and 5-AZA-CdR by CR deaminase results in a loss of antileukemic activity. To prevent the inactivation of these analogues, inhibitors of CR deaminase may prove to be useful agents. In the present study we investigated the effects of the deaminase inhibitors Zebularine, 5-F-Zebularine, and diazepinone riboside on the deamination of CR, ARA-C, and 5-AZA-CdR using highly purified human CR deaminase (EC 3.5.4.5). These inhibitors produced a competitive type of inhibition with each substrate, the potency of which followed the patterns diazepinone riboside >5-F-Zebularine and THU > Zebularine. 5-AZA-CdR was more sensitive than ARA-C to the inhibition produced by these deaminase inhibitors. The inhibition constants for diazepinone riboside lay in the range of 5–15 nM, suggesting that this inhibitor could be an excellent candidate for use in combination chemotherapy with either ARA-C or 5-AZA-CdR in patients with leukemia.

Introduction

Certain analogues of CdR have been demonstrated to be active antileukemic agents. ARA-C is one of the most effective drugs for the treatment of acute myeloid leukemia [11]. Another analogue, 5-AZA-CdR, is an experimental agent that shows stronger antileukemic activity in animal models than does ARA-C [21, 25]. In a phase I study, 5-AZA-CdR has been shown to be an active antileukemic agent in man as well [26]. It is currently under clinical investigation in phase II studies in patients with acute leukemia.

Analogues of CdR must undergo phosphorylation to become active inhibitors. This phosphorylation is catalyzed by CdR kinase (EC 2.7.1.14) [18]. ARA-C and 5-AZA-CdR are also subject to degradation by CR deaminase (EC 3.5.4.5) [2, 4]. The deamination of ARA-C produces ARA-U, which is about 100 times less inhibitory than the parent analogue [23]. 5-AZA-UdR, the product of deamination of 5-AZA-CdR, is completely inactive (R. L. Momparler, unpublished data). The major sites of deamination in man are the liver and spleen [9]; the high level of CR deaminase in these tissues is responsible for the short half-life of these analogues (15–20 min) [9, 26].

It has been reported that in some patients with acute leukemia who were treated with ARA-C or 5-AZA-CdR at the time of relapse, the leukemic cells showed an increase in CR deaminase activity [24, 27]. However, Tattersall et al. [28] observed only a slight increase in CR deaminase activity in ARA-C-resistant leukemic cells from patients, but the difference was not statistically significant as compared with sensitive cells. In a previous study in HL-60 myeloid leukemic cells, we also observed that 5-AZA-CdR produced a significant increase in CR deaminase activity [19]. To avoid this type of resistance to ARA-C or 5-AZA-CdR, inhibitors of CR deaminase could be useful adjuvants in combination chemotherapy. THU was the first strong inhibitor of CR deaminase to be identified [2]. The inhibition by THU was studied using CR deaminase purified from human granulocytes and was found to be competitive with the natural substrate CR [3]. The clinical importance of THU was demonstrated by Kreis et al. [13], who observed that this combination significantly increased the plasma level of ARA-C in patients with solid tumors.

In the search for more potent inhibitors of CR deaminase, new compounds were synthesized [12, 15–17]. The chemical structures of these inhibitors are shown in Fig. 1. In preliminary studies using a crude deaminase and CR as the substrate, some of these inhibitors showed stronger

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Abbreviations: CdR, deoxycytidine; CR, cytidine; ARA-C, cytosine arabinoside; ARA-U, uridine arabinoside; 5-AZA-CdR, 5-aza-2′-deoxycytidine; 5-AZA-UdR, 5-aza-2′-deoxyuridine; THU, tetrahydrouridine

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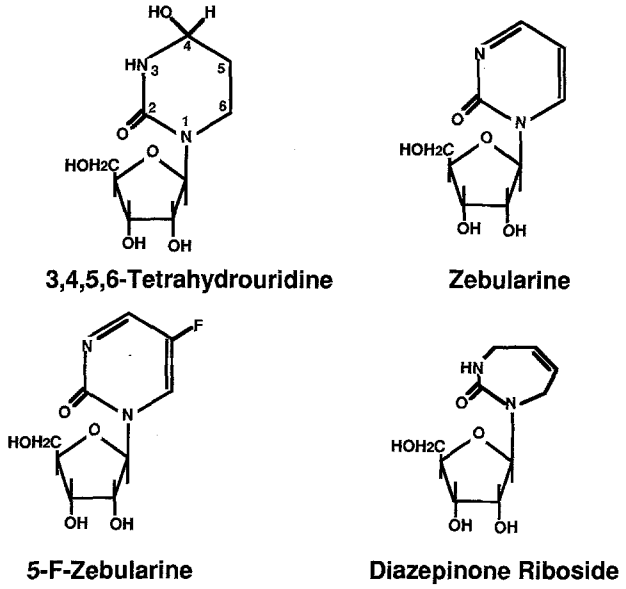

Fig. 1. Chemical structures of CR deaminase inhibitors

activity than THU [16, 17]. Since to our knowledge there has been no report on the influence of these inhibitors on the deamination of CdR analogues, we performed an in-depth kinetic analysis of their effect on the deamination of ARA-C and 5-AZA-CdR using highly purified human CR deaminase.

Materials and methods

Materials. CR, ARA-C, and 5-AZA-CdR were obtained from Boehringer Mannheim Laval (Quebec), Upjohn Canada (Montreal), and the Mack Co. (FRG), respectively. THU was obtained from Calbiochem (La Jolla, Calif.). Zebularine, 5-F-Zebularine, and diazepinone riboside were obtained by chemical synthesis as described elsewhere [15, 17].

Enzyme purification. CR deaminase from human placenta was purified using the method of Chabner et al. [3], with the following modifications. Nucleic acids in the cell extract were removed by precipitation with streptomycin sulfate. The precipitation of CR deaminase by ammonium sulfate occurred at between 40% and 55% of saturation. After a heat treatment, the ammonium sulfate fraction was purified by gel-filtration chromatography (Sephacryl S-200, Pharmacia) and ion-exchange chromatography (Mono-Q, Pharmacia) using the fast protein liquid chromatography (FLPC) system of Pharmacia to yield a highly purified enzyme (approximately 5,000-fold purification, resulting in a major band on polyacrylamide gel). The specific activity of the purified CR deaminase was 11,000 units/mg protein; 1 unit of enzymatic activity was defined as the amount of enzyme that catalyzes the deamination of 1 nmol cytidine/min at 37° C. The protein concentration was determined by the dye-binding procedure of Bradford [1] using the microassay method and bovine serum albumin as the standard (Bio-Rad Laboratories, Mississauga, Ontario).

Enzyme assay. CR deaminase activity was determined by spectrophotometric assay. The reaction mixture (0.2 ml) contained 20 mM $KHPO_4$ (pH 7.5), 100 mM KCl, the indicated concentration of substrate, and about 0.15 μg (1.5 unit) purified CR deaminase from human placenta. The mixture was incubated at 37° C in a 10-mm light-path cuvette, and the rate of deamination was determined at 286 (CR and ARA-C) or 245 nm (5-AZA-CdR) using a Gilford 260 spectrophotometer. For the conversion of CR to UR and of ARA-C to ARA-U, a ΔE_M value of 3,000 at 286 nm was used to calculate the amount of substrate deaminated. For the conversion of 5-AZA-CdR to 5-AZA-UdR, the ΔE_M value at 245 nm was estimated to be 4,500 [22]. In this assay, the daily deviation was less than 5%.

Kinetic analysis. The Michaelis-Menten constant (K_m) and the maximal velocity (V_{max}) were determined from double-reciprocal plots of enzyme activity as a function of changing substrate concentration according to the method of Lineweaver-Burk. The inhibition constant (K_i) was determined by a Dixon plot [6] of the reciprocal velocity of the enzyme reaction as a function of changing inhibitor concentration at a constant substrate concentration.

Table 1. K_m and V_{max} values for different substrates of CR deaminase

Substrate	K_m (μM)	V_{max} (nmol/min)
CR	15.2± 3.7	0.93±0.05
ARA-C	139.8±19.2	0.92±0.08
5-AZA-CdR	320.8± 8.7	0.40±0.01

Data represent mean values ± SE ($n = 3$)

Table 2. Inhibition of deamination of substrates for CR deaminase by different inhibitors

Inhibitor	Concentration (μM)	Inhibition of deamination (%)	
		ARA-C (100 μM)	5-AZA-CdR (100 μM)
THU	0.01	4.9±2.6	27.0±2.5
	0.02	14.7±1.7	39.0±3.4
	0.04	22.1±3.2	78.0±1.2
5-F-Zebularine	0.01	5.1±1.0	14.4±4.6
	0.02	11.1±1.0	28.4±1.1
	0.04	18.2±1.7	66.3±1.0
Diazepinone riboside	0.01	15.2±1.9	34.8±3.5
	0.02	19.1±2.5	60.1±3.6
	0.04	40.0±2.9	87.2±2.1

Data represent mean values ± SE ($n = 3$)

Results

The K_m and V_{max} values for the different substrates of CR deaminase are summarized in Table 1. The natural substrate CR had the lowest K_m value, indicating that the enzyme exhibited the highest affinity for this nucleoside. The analogues of CdR showed less affinity. The K_m values for ARA-C and 5-AZA-CdR were about 9- and 21-fold that for CR, respectively. The V_{max} values for CR and ARA-C were similar, whereas that for 5-AZA-CdR was lower.

The effect of the CR deaminase inhibitors THU, 5-F-Zebularine, and diazepinone riboside on the deamination of the analogues is shown in Table 2. At identical substrate concentrations (100 μM), 5-AZA-CdR was more sensitive than ARA-C to the inhibition produced by these substances. At a concentration of 0.04 μM, diazepinone riboside inhibited the deamination of ARA-C and 5-AZA-CdR by 40% and 87%, respectively.

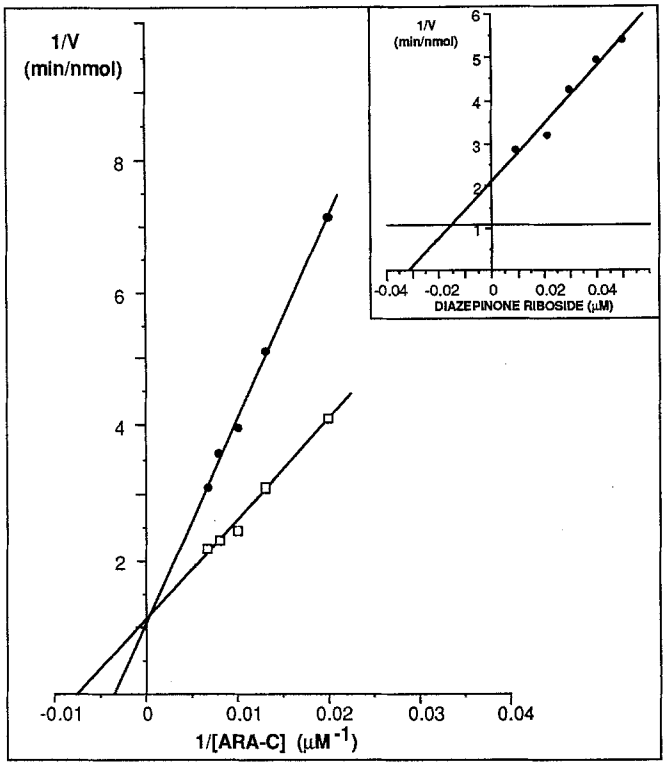

Fig. 2. Lineweaver-Burk plot for the substrate ARA-C in the absence of inhibitor (□——□) and in the presence of 0.02 μM diazepinone riboside (●——●); 1.5 unit enzyme was used in each assay. *Inset:* Dixon plot of 1/*v* for substrate ARA-C (100 μM) vs different concentrations of diazepinone riboside (●——●); SE, <5%. *Horizontal line*, $1/V_{max}$ at ARA-C concentration → ∞ in the absence of inhibitor

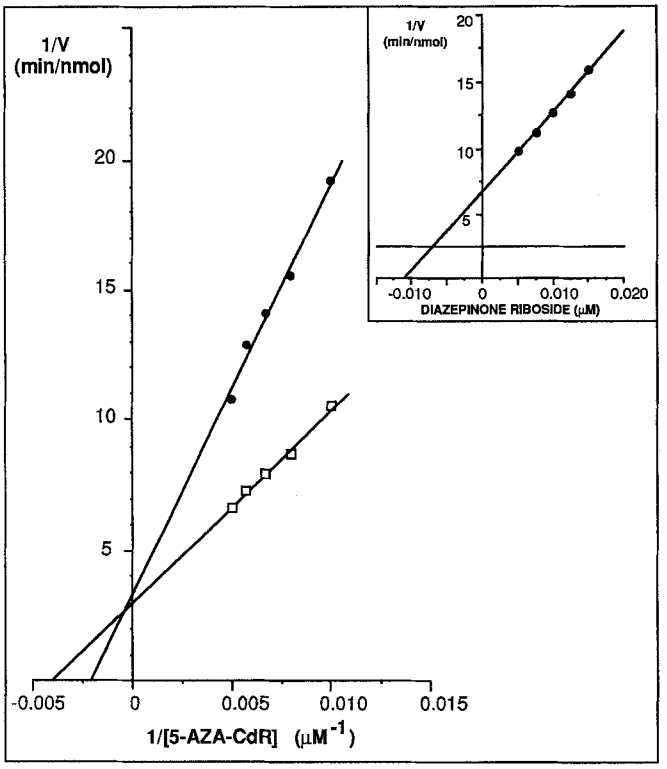

Fig. 3. Lineweaver-Burk plot for the substrate 5-AZA-CdR in the absence of inhibitor (□——□) and in the presence of 0.01 μM diazepinone riboside (●——●); 1.5 unit enzyme was used in each assay. *Inset:* Dixon plot of 1/*v* for substrate 5-AZA-CdR (150 μM) vs different concentrations of diazepinone riboside (●——●); SE, <5%. *Horizontal line*, $1/V_{max}$ at 5-AZA-CdR concentration → ε in the absence of inhibitor

Table 3. Determination of K_i values of different CR deaminase inhibitors

Substrate	Type of inhibition	K_i values (nM)			
		THU	Zebularine	5-F-Zebularine	Diazepinone riboside
CR	Competitive	51.3±16.4	816.3± 7.7	40.4±2.7	17.5±2.3
ARA-C	Competitive	26.3± 4.8	275.0±13.5	34.7±1.9	15.0±0.5
5-AZA-CdR	Competitive	9.8± 0.7	54.2± 6.2	10.1±0.3	5.4±0.6

Data represent mean values ± SE (n = 3)

In Fig. 2, the effect of 0.02 μM diazepinone riboside on the deamination of different concentrations of ARA-C is shown in a Lineweaver-Burk plot. The plot indicates that the inhibition produced by diazepinone riboside was competitive with respect to ARA-C. To evaluate the K_i value, the Dixon plot was used (Fig. 2, inset); the K_i value for diazepinone riboside was estimated to be 15 nM.

Figure 3 shows the effect of 0.01 μM diazepinone riboside on the deamination of different concentrations of 5-AZA-CdR. This Lineweaver-Burk plot demonstrates that the inhibition produced by diazepinone riboside was also competitive with 5-AZA-CdR. The inset illustrates the Dixon plot for this inhibitor vs 5-AZA-CdR; the estimated K_i value was 5.4 nM. Lineweaver-Burk and Dixon plots were also used to estimate the K_i values for the other inhibitors (data not shown).

The type of inhibition produced by each of these inhibitors is shown in Table 3 along with the corresponding K_i values. With each substrate, all inhibitors of CR deaminase displayed competitive inhibition. The K_i values obtained demonstrate that the CR deaminase inhibitors prevent the deamination of CdR analogues more effectively than that of CR itself. These results are in accord with the data shown in Table 2. For all inhibitors, 5-AZA-CdR was most sensitive to the inhibition. The strongest inhibitor was diazepinone riboside as shown by its K_i value, which was the lowest determined for each substrate.

Discussion

One of the reasons for the failure of chemotherapy of acute leukemia is the development of drug resistance. For CdR

analogues such as ARA-C or 5-AZA-CdR, the biochemical mechanism of drug resistance can be due to a deficiency of CdR kinase, an increase in the pool of dCTP, or an increase in CR deaminase [20]. In cases of elevated CR deaminase activity in leukemic cells at the time of relapse [24, 27], the use of CR deaminase inhibitors in combination with CdR analogues may be one way to avoid this type of drug resistance. Another possible benefit of using deaminase inhibitors is to prevent leukemic stem cells from escaping from the cytotoxic effects of CdR analogues due to their presence in tissues that contain very high levels of CR deaminase, such as the liver, the spleen, and the intestinal mucosa. Ho [9] has reported that in patients, the liver can deaminate about 2 g ARA-C per hour.

To study the enzyme kinetics of CR deaminase inhibitors, we extensively purified CR deaminase from human placenta. Using the highly purified enzyme, we investigated the affinity of this enzyme for its natural substrate and related analogues. Table 1 shows the K_m and V_{max} values determined for CR, ARA-C, and 5-AZA-CdR. The kinetic values we obtained for the substrates are similar to previously reported data, with slight variations [3, 4]. The affinity of our enzyme preparation for the substrates was slightly decreased as illustrated by moderately higher K_m values as compared with published data. Chabner et al. [3] and Chabot et al. [4] reported a K_m value of about 88 μM for ARA-C, whereas we observed a value of 140 μM. Chabot et al. [4] obtained a K_m value of 250 μM for 5-AZA-CdR as compared with our finding of 320 μM. The differences in the purity of the enzyme preparation or the reaction buffer may be the explanation for the discrepancies between these kinetic values. Due to the chemical instability of 5-AZA-CdR [14], we replaced TRIS buffer with phosphate.

To illustrate the interaction of inhibitors with analogues of CdR, we compared the inhibition of deamination produced by different concentrations of inhibitors. At a substrate concentration of 100 μM, which corresponds to the plasma level of high-dose ARA-C or 5-AZA-CdR, we observed a strong inhibition of the deamination of these two analogues by the different inhibitors (Table 2).

The inhibition produced by diazepinone riboside is illustrated in Figs. 2 and 3. This inhibitor did not alter the maximal velocity of the human placenta enzyme with either ARA-C or 5-AZA-CdR, but changed the affinity (K_m) of the enzyme. These properties are in accord with a competitive type of inhibition. The method used to determine the different dissociation constants for the enzyme-inhibitor complex (K_i) was very reproducible using the Dixon plot. The finding that all of the inhibitors produce a competitive inhibition enabled the calculation of the K_i values by Dixon plots.

In Table 3, the K_i values for the different inhibitors and substrates studied are summarized. The K_i value obtained in the present study for THU (51 nM) using CR as the substrate is similar to those reported by other investigators (29–54 nM) [3, 29]. However, in another study [16], THU showed less inhibitory activity (K_i, 100 nM). The difference between these values may be due to the origin and purity of the enzyme preparation. The results obtained using the inhibitors Zebularine, 5-F-Zebularine, and diazepinone riboside and the substrate CR showed some variation with the published data [7, 15]. Liu et al. [15] reported that diazepinone riboside (molecule 17 in their report) was the strongest inhibitor of human liver CR deaminase, exhibiting a K_i value of 25 nM. With our enzyme preparation, this inhibitor displayed similar potency (K_i, 17 nM). Using CR deaminase purified from mutants of *Escherichia coli*, Frick et al. [7] found apparent K_i values of 360 and 35 nM for Zebularine and 5-F-Zebularine, respectively (molecules IV and V in their report). In the present study, the inhibitory strength of Zebularine against CR (816 nM) was lower than the value reported by Frick et al. [7], whereas that obtained for 5-F-Zebularine was about the same as the previous finding. All of the inhibitors tested were more effective against CdR analogues than against CR itself. THU and 5-F-Zebularine exhibited comparable potency against each analogue. The most potent inhibitor appeared to be diazepinone riboside, as has previously been reported by Liu et al. [15].

In summary, CR deaminase inhibitors are excellent candidates for use in combination therapy with either ARA-C or 5-AZA-CdR. In support of this proposal is the in vitro study of Grant et al. [8], who have reported that THU can increase ARA-CTP formation in the human KG-1 leukemic cell line, which contains a high level of CR deaminase. This in vitro effect has also been demonstrated in leukemic cells from patients by Chou et al. [5] and Ho et al. [10]. In addition, THU has been shown to increase significantly the plasma concentration of ARA-C in patients [13]. The use of potent CR deaminase inhibitors merits further clinical investigation in leukemic patients undergoing treatment with CdR analogues in an attempt to increase the effectiveness of chemotherapy.

References

1. Bradford MM (1972) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
2. Camiener GW (1968) Studies of the enzymatic deamination of aracytidine: V. Inhibition in vitro and in vivo by tetrahydrouridine and other related pyrimidine nucleosides. Biochem Pharmacol 17: 1981–1991
3. Chabner BA, Johns DG, Coleman CN, Drake JC, Evans WH (1974) Purification and properties of cytidine deaminase from normal and leukemic granulocytes. J Clin Invest 53: 922–931
4. Chabot GG, Bouchard J, Momparler RL (1983) Kinetics of deamination of 5-aza-2′-deoxycytidine and cytosine arabinoside by human liver cytidine deaminase and its inhibition by 3-deazauridine, thymidine or uracil arabinoside. Biochem Pharmacol 32: 1327–1328
5. Chou T-C, Arlin Z, Clarkson BD, Philips FS (1977) Metabolism of 1-β-D-arabinofuranosylcytosine in human leukemic cells. Cancer Res 37: 3561–3570
6. Dixon M, Webb EC (1964) Enzymes. Academic Press, New York
7. Frick L, Yang C, Marquez VE, Wolfenden R (1989) Binding of pyrimidin-2-one ribonucleoside by cytidine deaminase as the transition-state analogue 3,4-dihydrouridine and the contribution of the 4-hydroxyl group to its binding affinity. Biochemistry 28: 9423–9430
8. Grant S, Bhalla K, McCrady C (1991) Effect of tetrahydrouridine and deoxytetrahydrouridine on the interaction between 2′-deoxycytidine and 1-β-D-arabinofuranosylcytosine in human leukemia cells. Leuk Res 15: 205–213

9. Ho DHW (1973) Distribution of kinase and deaminase of 1-β-D-arabinofuranosylcytosine in tissues of man and mouse. Cancer Res 33: 2816–2820
10. Ho DHW, Carter CJ, Brown NS, Hester J, McCredie K, Benjamin RS, Freireich EJ, Bodey GP (1980) Effects of tetrahydrouridine on the uptake and metabolism of 1-β-D-arabinofuranosylcytosine in human normal and leukemic cells. Cancer Res 40: 2441–2446
11. Keating MJ, McCredie KB, Bodey GP, Smith TL, Gehan E, Freireich EJ (1982) Improved prospects for long-term survival in adults with acute myelogenous leukemia. JAMA 248: 2481–2486
12. Kim C-H, Marquez VE, Mao DT, Haines DR, McCormack JJ (1986) Synthesis of pyrimidin-2-one nucleosides as acid-stable inhibitors of cytidine deaminase. J Med Chem 29: 1374–1380
13. Kreis W, Chan K, Budman DR, Schulman P, Allen S, Weiselberg L, Litchman S, Henderson V, Freeman J, Deere M, Andreeff M, Vinciguerra V (1988) Effect of tetrahydrouridine on the clinical pharmacology of 1-β-D-arabinofuranosylcytosine when both drugs are coinfused over three hours. Cancer Res 48: 1337–1342
14. Lin KT, Momparler RL, Rivard GE (1981) High performance liquid chromatographic analysis of chemical stability of 5-aza-2′-deoxycytidine. J Pharm Sci 70: 1228–1232
15. Liu PS, Marquez VE, Driscoll JS, Fuller RW, McCormack JJ (1981) Cyclic urea nucleosides. Cytidine deaminase activity as a function of aglycon ring size. J Med Chem 24: 662–666
16. Marquez VE, Liu PS, Kelley JA, Driscoll JS, McCormack JJ (1980) Synthesis of 1,3-diazepin-2-one nucleosides as transition-state inhibitors of cytidine deaminase. J Med Chem 23: 713–715
17. McCormack JJ, Marquez VE, Liu PS, Vistica DT, Driscoll JS (1980) Inhibition of cytidine deaminase by 2-oxopyrimidine riboside and related compounds. Biochem Pharmacol 29: 830–832
18. Momparler RL, Fisher GA (1968) Mammalian deoxyribonucleoside kinase: I. Deoxycytidine kinase: purification, properties, and kinetic studies with cytosine arabinoside. J Biol Chem 243: 4298–4304
19. Momparler RL, Laliberté J (1990) Induction of cytidine deaminase in HL-60 myeloid leukemic cells by 5-aza-2′-deoxycytidine. Leuk Res 14: 751–754
20. Momparler RL, Onetto N (1988) Drug resistance to cytosine arabinoside. In: Kessel D (ed) Resistance to antineoplastic drugs. CRC Press, Boca Raton, pp 353–367
21. Momparler RL, Momparler LF, Samson J (1984) Comparison of the antileukemic activity of 5-aza-2′-deoxycytidine, 1-β-D-arabinofuranosylcytosine and 5-azacytidine against L1210 leukemia. Leuk Res 8: 1043–1049
22. Momparler RL, Rossi M, Bouchard J, Vaccaro C, Momparler LF, Bartolucci S (1984) Kinetic interaction of 5-aza-2′-deoxycytidine-5′-monophosphate and its 5′-triphosphate with deoxycytidylate deaminase. Mol Pharmacol 25: 436–440
23. Müller WEG, Zahn RK (1979) Metabolism of 1-β-D-arabinofuranosyluracil in mouse L5178Y cells. Cancer Res 39: 1102–1107
24. Onetto N, Momparler RL, Momparler LF, Gyger M (1987) In vitro biochemical tests to evaluate the response to therapy of acute leukemia with cytosine arabinoside or 5-aza-2′-deoxycytidine. Semin Oncol 14 [Suppl 1]: 231–237
25. Richel DJ, Colly LP, Lurvink E, Willemze R (1988) Comparison of the antileukaemic activity of 5-aza-2′-deoxycytidine and 1-β-D-arabinofuranosylcytosine in rats with Ara-C resistant and sensitive myelocytic leukaemia. Br J Cancer 58: 730–733
26. Rivard GE, Momparler RL, Demers J, Benoit P, Raymond R, Lin KT, Momparler LF (1981) Phase I study on 5-aza-2′-deoxycytidine in children with acute leukemia. Leuk Res 5: 453–462
27. Steuart CD, Burke PJ (1971) Cytidine deaminase and the development of resistance to cytosine arabinoside. Nature New Biol 233: 109–110
28. Tattersall MHN, Ganeshaguru K, Hoffbrand AV (1973) Mechanisms of resistance of human acute leukaemia cells to cytosine arabinoside. Br J Haematol 27: 39–46
29. Wentworth DF, Wolfenden R (1975) On the interaction of 3,4,5,6-tetrahydrouridine with human liver cytidine deaminase. Biochemistry 14: 5099–5105