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Inhibition of cytidine deaminase by 2-oxopyrimidine riboside and related compounds

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Cytidine deaminase catalyzes the hydrolytic deamination of several cytosine nucleosides including cytosine arabinoside, a drug used extensively in the treatment of leukemic patients [1]. We have been engaged in studies of the design and synthesis of new cytidine deaminase inhibitors that may be more potent and more selective than the standard inhibitor of this enzyme, 3,4,5,6-tetrahydrouridine (I; [2,3]). In the course of these studies, we found some interesting structure–activity relationships among inhibitors of cytidine deaminase, and these relationships are the subject of this communication.

All nucleoside derivatives tested were synthesized in these laboratories by condensing silylated heterocyclic bases with a suitably protected sugar derivative, employing the approach described by Niedballa and Vorbruggen [4], involving the use of stannic chloride as catalyst. Tetrahydrouridine was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. All compounds synthesized were characterized by appropriate spectroscopic methods (u.v., n.m.r. g.c.-m.s.), as well as by elemental analysis. The detailed description of the synthetic procedures of these and related compounds will be published elsewhere. Mouse kidney cytidine deaminase was isolated and partially purified from mouse kidney acetone powder (prepared with acetone only), obtained from the Sigma Chemical Co., St. Louis, MO. The powder (3 g) was extracted at 55–60° for 5 min with pH 8.0 phosphate buffer (0.05 M) and the extract was filtered through a Nalgene filter unit (0.45 μm grid membrane) to yield a clear yellow filtrate. The filtrate was fractionated with ammonium sulfate, essentially as described by Wentworth and Wolfenden [3], and the active ammonium sulfate fraction was dissolved in 2.0 ml of phosphate buffer (0.05 M; pH 7.0). The K_m for deamination of cytidine using this preparation was found to be 5×10^{-5} M, in good agreement with the value of 7×10^{-5} M reported previously by Tomchick *et al.* [5] for mouse kidney cytidine deaminase. A commercially available yeast enzyme concentrate (Sigma Chemical Co.) was used as the source of yeast cytidine deaminase; extraction of 100 mg of the yeast enzyme concentrate with phosphate buffer gave a preparation suitable for spectroscopic studies, but the preparation was also fractionated with ammonium sulfate, as described for the mouse kidney enzyme. The K_m value that we determined for cytidine, in the yeast system, was 2.3×10^{-4} M, a value very close to that (2.5×10^{-4} M) reported in the literature [6].

Table 1. Inhibition of mouse kidney cytidine deaminase activity by 2-oxopyrimidine riboside (II)

Conc of inhibitor (M)	Per cent control activity*
0	100
2×10^{-6}	78
5×10^{-6}	63
2×10^{-5}	42
5×10^{-5}	30
1×10^{-4}	10

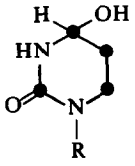
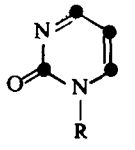
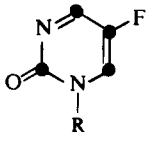
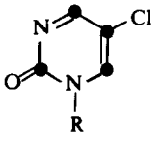
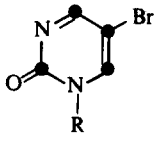
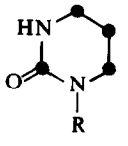
* Activity in the absence of inhibitor was 0.015 absorbance units over 10 min; the substrate (cytidine) was present at a concentration of 1×10^{-4} M.

Rhesus monkey liver was obtained at autopsy, through the cooperation of Dr. Richard Adamson (Laboratory of Chemical Pharmacology, National Cancer Institute), and cytidine deaminase was immediately isolated and purified in the manner described for the human liver enzyme [3]. We measured cytidine deaminase activity by following the decrease in absorbance at 282 nm (290 nm for crude preparations) that characterizes the conversion of cytidine to uridine [7]; all assays were performed at pH 7.0 (phosphate buffer) and 37° with substrate (cytidine) at 1×10^{-4} M. Spectroscopic determinations were carried out with a Beckman model 34 kinetic spectrophotometeric system with the recorder set for full-scale deflection in the range 0.0 to 0.1 absorbance units. Candidate inhibitors were incubated with the enzyme for 2 min prior to initiation of the enzymatic reaction by addition of cytidine.

2-Oxopyridimidine riboside (II). may be considered to be the 'deamino' analog of the natural nucleoside, cytidine (2-oxo-4-amino pyrimidine riboside). When II was present in the cytidine deaminase reaction mixture, at a concentration comparable to that of the substrate, enzyme activity was inhibited strongly (Table 1); as summarized in the table, a reasonable dose–response relation was observed for this compound as an inhibitor of cytidine deaminase. Inhibition of enzymatic activity was not increased, for a given concentration of inhibitor, by prolonging the preincubation period from 2 min to 10 min. Kinetic studies showed inhibition to be formally of the competitive type; the apparent K_i value estimated [8,9] for compound II, as an inhibitor of mouse cytidine deaminase, was 2×10^{-6} M. For comparative purposes we determined the K_i for tetrahydrouridine (I; Table 2) with the mouse kidney enzyme and obtained a value of 2×10^{-7} M; it should be pointed out that this value for the K_i of tetrahydrouridine is somewhat higher than that reported by Wentworth and Wolfenden for the human liver enzyme (4×10^{-8} M; [3]) but quite close to values reported by others for cytidine deaminase from human liver and monkey serum [10,11]. The ability of the 4-deamino analog of cytidine (II) to inhibit cytidine deaminase is paralleled by the ability of the '6-deamino' analog of adenosine, nebularine, to act as a moderately potent inhibitor of adenosine deaminase; for example, nebularine has an affinity ($K_i = 1 \times 10^{-5}$ M) for calf intestine adenosine deaminase that is slightly higher than that of the natural substrate, adenosine ($K_m = 3 \times 10^{-5}$ M; [12]). Considerable structural specificity characterizes the interaction of II with cytidine deaminase. Removal of the ribose function on N-1 or its replacement by a benzyl group results in a pronounced decrease of inhibitory activity, since neither the heterocyclic base itself nor the 1-benzyl derivative produces significant inhibition when evaluated at 1×10^{-4} M. The 2-oxopyridine analog of II was approximately one order of magnitude less active than the analogous pyrimidine derivative, an observation which suggests that the 3-nitrogen of compound II plays a significant role in the reaction involved in binding to the enzyme.

In order to evaluate the relative importance of electronic and/or steric influences on the activities of pyrimidine

Table 2. Inhibition of cytidine deaminase by 2-oxopyrimidine riboside analog*

Compound No.	Compound structure†	K_i (mouse enzyme)	K_i (yeast enzyme)
I		2.2×10^{-7} m	5×10^{-8} M
II		2×10^{-6} M	3×10^{-6} M
III		2×10^{-7} M	1×10^{-7} M
IV		3×10^{-7} M	2×10^{-5} M
V		7×10^{-7} M	$>5 \times 10^{-5}$ M
VI		4×10^{-6} M	4.5×10^{-5} M

* Methods are as described in the text.
† R = 1-β-D-ribofuranosyl.

nucleosides as inhibitors of cytidine deaminase, we initiated investigations of 5-halogen substituted derivatives of II. Introduction of a fluorine substituent into position 5 was associated with a 10-fold increase in inhibitory potency; indeed, the 5-fluoro derivative (III) inhibited mouse cytidine deaminase as well as does tetrahydrouridine, the most potent cytidine deaminase inhibitor yet discovered. The increased activity of the fluorinated pyrimidine nucleoside as an inhibitor of cytidine deaminase is of considerable interest in view of the observations by Kreis *et al.* [13] that introduction of a 5-fluoro function increased the deamination velocity of several cytidine derivatives. 5-Chloro-2-oxopyrimidine riboside (IV) was only slightly less potent than the 5-fluoro derivative, and introduction of a bromo substituent into position 5 resulted in a compound (V) that was intermediate in potency ($K_i = 7 \times 10^{-7}$ M) between the 'parent' nucleoside (II) and its 5-fluoro analog.

Studies with yeast cytidine deaminase were carried out in order to compare the interactions of the above nucleoside derivatives and this fungal enzyme with those observed with the mammalian enzyme. Compound II inhibited yeast cytidine deaminase as effectively ($K_i = 3 \times 10^{-6}$ M) as it inhibited the mouse kidney enzyme; on the other hand, the 5-chloro analog of II was less effective ($K_i = 2 \times 10^{-5}$ M) than the parent compound as an inhibitor of the yeast enzyme. Monkey liver cytidine deaminase resembles the mouse kidney enzyme in that the 5-chloro compound inhibited more strongly than does II. The lower activity of the 5-chloro compound as an inhibitor of yeast cytidine deaminase may reflect more stringent steric requirements for binding of the 2-oxopyrimidine derivatives to this enzyme, relative to those for the mammalian enzyme. Such an interpretation is consistent with observations [14] that 5-methyl cytidine is deaminated effectively by mammalian cytidine deaminase, but not by the yeast enzyme, and with our observation that 5-fluoro-2-oxopyrimidine riboside (III), with a 5-substituent considerably smaller than the chloro substituent of (IV), was a potent inhibitor ($K_i = 1 \times 10^{-7}$ M) of the yeast enzyme. It should be mentioned that steric considerations may also account for the lower rate of deamination of 5-methyl cytidine derivatives, compared with cytidine derivatives, by mammalian enzymes [5, 13].

Compound II was reduced catalytically to 3,4,5,6-tetrahydro-2-oxopyrimidine riboside (VI) which retained a considerable degree of activity ($K_i = 4 \times 10^{-6}$ M) as an inhibitor of cytidine deaminase; the activity of this reduced uridine derivative (VI) is perhaps not suprising in view of its structural resemblance to tetrahydrouridine.

Compound II and its halogenated derivatives were tested as inhibitors of the growth of leukemia cells (murine L1210; RPMI medium; assay at 48 hr) in culture. Compound II inhibited growth of these cells by 50 per cent at 1×10^{-5} M and its 5-fluoro analog was somewhat less potent, producing equivalent inhibition of growth at a concentration of 3×10^{-5} M. Tetrahydrouridine, a more potent inhibitor of cytidine deaminase than II, did not inhibit cell growth at concentrations as high as 1×10^{-4} M. The 5-chloro and 5-bromo analogs of II also did not produce significant suppression of cell growth when present in the growth medium in the concentration range of 1×10^{-7}–1×10^{-4} M. Growth suppression by II and its 5-fluoro analog may involve a variety of mechanisms, but it is pertinent to stress that a possible mechanism of action involves intracellular anabolism to a deoxyribotide derivative which may then inhibit a suitable target enzyme, such as thymidylate synthetase, in a manner analogous to that described earlier in a bacterial system [15]. Compound II exhibited modest antineoplastic activity *in vivo*, producing a 50 per cent increase in life span of leukemic mice (P388) when administered daily (days 1–9) at a high dose (400 mg/kg). More extensive studies of the biological actions of compound II and related compounds are in progess.

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