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Cytidine 5′-Triphosphate Synthetase as a Target for Inhibition by the Antitumor Agent 3-Deazauridine[1]

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SUMMARY

The activity of a highly purified cytidine triphosphate synthetase from calf liver was shown to be inhibited by 3-deazauridine triphosphate, a major metabolite of 3-deazuridine (deaza-UR) in tumor cells. The inhibition is competitive with respect to uridine triphosphate, and the average K_i value, determined by Dixon and Lineweaver-Burk plots, is 5.3×10^{-6} M. Neither deaza-UR nor deazauridine monophosphate inhibited the enzyme to a significant extent. The amination of uridine triphosphate to cytidine triphosphate in extracts of leukemia L1210 cells was also inhibited by deazauridine triphosphate. Coupled with the finding that the inhibition of the *in vitro* growth of leukemia L1210 cells by deaza-UR is reversed by cytidine and to a lesser extent by uridine and 2′-deoxycytidine, but not by thymidine or 2′-deoxyuridine, this observation suggests that deaza-UR exerts its growth-inhibitory activity by interfering with the activity of the cytidine triphosphate synthetase.

INTRODUCTION

deaza-UR,[3] a structural analog of uridine synthesized by Robins and Currie (12), inhibits the growth of *Escherichia coli*, EAC, and leukemia L1210 cells *in vitro* (13). deaza-UR is also active against leukemia L1210 *in vivo*, increasing the life-span of tumor-bearing male DBA2/Ha mice by approximately 65% (2). Wang and Bloch (19) found that in cell-free extracts of EAC and in intact EAC and leukemia L1210 cells, deaza-UR was converted to deaza-UTP. Furthermore, the cell-free extracts of EAC did not cleave deaza-UR to deazauracil, under conditions where uridine was readily converted to uracil, and deaza-UR was not incorporated into the RNA or DNA of the tumor cells.

Because deaza-UR is metabolized in the cells to deaza-UTP, it was considered possible that the analog may act, at this metabolic stage, as an inhibitor of CTP synthetase (EC 6.3.4.2) (5, 9, 14), particularly since Brockman *et al.* (3) had shown that, in addition to uridine and cytidine, 2′-deoxycytidine but not 2′-deoxyuridine or thymidine alleviated the inhibition of the growth of Ca 755 cells caused by deaza-UR. The availability of a highly purified calf liver CTP synthetase and of deaza-UTP in our laboratories permitted exploration of this possibility. Inhibition of CTP synthetase in extracts of L1210 cells was also examined.

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[3] The abbreviations used are: deaza-UR, 3-deazauridine; EAC, Ehrlich ascites carcinoma.

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MATERIALS AND METHODS

Compounds

deaza-UR was obtained from ICN Corp., Irvine, Calif. deaza-UMP and deaza-UTP were synthesized according to the methods of Yoshikawa *et al.* (21) and Sowa *et al.* (17), respectively.

Nonlabeled nucleotides were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. UTP-2-^{14}C in 50% aqueous ethanol was purchased from Schwarz/Mann, Orangeburg, N. Y. This solution was adjusted to pH 7.4 and evaporated to dryness in a vacuum. deaza-UR-^{3}H was prepared according to the Wilzbach technique by New England Nuclear, Boston, Mass., and was purified as described previously (19). All concentrations of nucleosides and nucleotides were determined in a Zeiss PMQ II spectrophotometer, using published constants.

Enzyme Assay and Inhibition Studies

Purified Calf Liver CTP Synthetase. A 350-fold-purified calf liver CTP synthetase (designated Fraction V) (14), purified an additional 3-to-5-fold by differential ammonium sulfate precipitation (6) and centrifugation through sucrose density gradients (10), was used for the assays. The specific activity of the enzyme in several preparations was 0.35 to 0.90 unit/mg protein. The protein concentration was determined by the method of Warburg and Christian (20), utilizing the absorbance ratio 280/260 nm. One unit of enzyme is defined as that amount catalyzing the formation of 1 μmole of CTP per hr at 37°.

The standard assay mixture (14) contained, in μmoles/ml: ATP, 8; UTP-2-^{14}C, 0.2 (specific activity, 1.0×10^{6} dpm/μmole); GTP, 0.2; L-glutamine, 55; 2-mercaptoethanol, 50; $MgCl_2$, 18; Tris-HCl, 35; and the enzyme at the concentration indicated in the tables and charts. GTP is an allosteric activator of the enzyme (5) and, in its absence, the activity of the enzyme is reduced by about 85% (14). The final pH was 7.2. The time of incubation was 1 hr at 37°.

The amount of CTP-^{14}C formed was determined (5, 14) by acid hydrolytic conversion of the nucleoside triphosphate to CMP-^{14}C, which was separated from UMP-^{14}C by chromatography on Dowex 50-H$^+$, followed by scintillation spectrometry (14, 18). The cpm were converted to dpm by the instrumental standard curve.

The inhibitor studies were carried out by determining the initial velocity of the enzyme reaction as a function of the UTP concentration in the presence and absence of a constant concentration of deaza-UTP, and a Lineweaver-Burk plot was constructed from the resulting data. The reaction mixture was as described above, except that UTP-^{14}C was varied from 0.039 to 0.2 μmole/ml. The K_i was determined by a Dixon plot, the concentration of deaza-UTP being varied from 8.90×10^{-3} to 5.23×10^{-2} μmole/ml in the presence of the standard assay concentration of UTP or one-half the standard UTP concentration.

Leukemia L1210 Cell Extracts. L1210 cells (3×10^9 cells) were harvested by centrifugation (1640 × *g*, 10 min, 4°) after 40 hr of growth in Roswell Park Memorial Institute 1630 medium containing 10% calf serum, penicillin, streptomycin, and 2 mM *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.4 (11). The cells were washed once with 0.154 M NaCl/0.01 M sodium phosphate buffer, pH 7.4. Approximately 3 ml of packed cells were obtained, to which 9 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.25 M sucrose and 0.05 M 2-mercaptoethanol, were added. The cells were disrupted, at ice-bath temperature, in a glass homogenizer with a motor-driven Teflon pestle. Succeeding operations were carried out at 4°. The homogenate was centrifuged at 15,000 × *g* for 10 min and the supernatant solution was drawn off with a Pasteur pipet and centrifuged for 1 hr at 105,000 × *g*. The supernatant cytosol (8.6 ml) was removed with a Pasteur pipet; contact with the sediment was avoided. A 6-ml portion of the supernatant cytosol was brought to 35% saturation with ammonium sulfate at pH 7.2 and mixed well. After 30 min, the precipitate was collected by centrifugation at 15,000 × *g* for 10 min. The precipitate was dissolved in 1 ml of 0.1 M glutamine/0.035 M Tris buffer, pH 7.4, containing 0.05 M 2-mercaptoethanol to give Fraction II [so designated in analogy (14) with the purification step of the liver enzyme]. The protein concentration of this fraction was 15.9 mg/ml. A portion of Fraction II was dialyzed, until sulfate free (Ba^{+2}), against 1 mM L-glutamine in 0.035 M Tris buffer, pH 7.4, containing 0.05 M 2-mercaptoethanol. L-Glutamine was included in the buffer to increase the stability of the enzyme, as has been shown for the liver enzyme (14).

The cytosol and both the undialyzed and dialyzed portions of Fraction II were assayed for CTP synthetase activity as described above.

Conversion of deaza-UR to deaza-UTP in Intact Leukemia L1210 Cells

L1210 cells were harvested by centrifugation from 3 liters of the medium described above, washed once with 40 ml of cold 0.154 M NaCl, and centrifuged at approximately 1640 × *g* for 5 min. They were then resuspended in 150 ml of the medium (free of serum and antibiotics) in a 500-ml Erlenmeyer flask (1.8×10^7 cells/ml), and 0.4 ml of a solution of deaza-UR-^{3}H (42 mM; specific activity, 2.1×10^5 dpm/μmole) was added. The mixture was incubated at 37° in a water-bath shaker for 60 min, the flask was quickly removed and plunged into ice water, and 100 ml of 0.154 M NaCl at 0° were added to hasten cooling. The cells were then collected by centrifugation, washed with 400 ml of 0.9% NaCl solution, and extracted with perchloric acid as described previously (19). The extract was evaporated to dryness in a flash evaporator at 30°, and 1 ml of H_2O was added to the dried residue. The resulting mixture was clarified by centrifugation. The tritiated nucleotides in the supernatant solution were separated by ion-exchange chromatography (8) and their radioactivity was determined by scintillation spectrometry.

Inhibition Analysis *in Vitro*

The inhibition analysis (16) was carried out by a microtechnique (2). Aliquots (0.25 ml) of the culture medium (11) containing deaza-UR at 4 times the desired final concentration were introduced into 16- x 125-mm screw-cap culture tubes, followed by 0.25-ml aliquots of medium containing 1 of the natural pyrimidine nucleosides listed in Chart 3 at 4 times the final concentration. To this mixture, 0.5-ml aliquots of the medium containing 3×10^5 leukemia L1210 cells were added, to a final volume of 1.0 ml. The cultures were incubated at 37° for 40 hr, during which time the cell number increased approximately 8- to 9-fold in the absence of deaza-UR, with an average cell viability of 99% as determined by trypan blue exclusion. The inhibitor was used at concentrations ranging from 5×10^{-6} to 1×10^{-4} M, and the natural pyrimidine nucleosides were used at concentrations ranging from 1×10^{-6} to 1×10^{-4} M. Thymidine was used maximally at 1×10^{-5} M because higher concentrations were toxic.

RESULTS

Table 1 shows the extent of inhibition of the activity of a highly purified CTP synthetase from calf liver in the presence of deaza-UR, deaza-UMP, or deaza-UTP. Only deaza-UTP

Table 1

Effect of deaza-UR and its 5'-mono- and triphosphate derivatives on the activity of a purified CTP synthetase from calf liver

The standard reaction mixture was incubated for 1 hr at 37° in the absence or presence of deaza-UR or its 5'-mono- or triphosphate derivatives at the concentrations specified. Two separate enzyme preparations (0.082 and 0.136 mg protein; specific activity, 0.35 and 0.90 unit/mg) were used for Experiments 1 and 2.

Compound	Concentration (mM)	% inhibition of CTP formation from UTP	
		Experiment 1	Experiment 2
deaza-UR	0.198	7.0	2.7
	0.396	6.7	3.5
deaza-UMP	0.193	11.4	1.7
	0.385	13.1	11.0
deaza-UTP	0.0178	48.4	45.0

inhibited 2 different preparations of the enzyme to a significant extent, and the mode of this inhibition was studied in detail. Chart 1 presents a Lineweaver-Burk plot relating the velocity of the reaction to the substrate concentration in the presence and absence of inhibitor, obtained with one of the enzyme preparations. A similar result with a 2nd preparation was obtained when the concentration of deaza-UTP was 1.78×10^{-5} M. These results make it apparent that deaza-UTP acts as a competitive inhibitor of the substrate UTP for the CTP synthetase. It should be noted that the classical Michaelis-Menten behavior observed with this liver enzyme (Chart 1) has also been seen with other liver enzyme preparations (14).

The K_i values obtained from the Lineweaver-Burk plots were calculated to be 7.6×10^{-6} M (Chart 1) and 5.9×10^{-6} M, respectively. The K_i for the inhibitor was also determined by plotting the initial velocity of the reaction as a function of the concentration of the inhibitor in the presence of 2 different concentrations of UTP. The K_i value obtained from the Dixon plot shown in Chart 2 was 3.0×10^{-6} M obtained with one enzyme preparation; with another preparation of enzyme it was 4.7×10^{-6} M. The average K_i for all the determinations was 5.3×10^{-6} M.

Since deaza-UR is an effective inhibitor of the growth of leukemia L1210 cells *in vivo* and *in vitro* (2, 13) it was of interest to determine whether deaza-UTP inhibits the amination of UTP to CTP in extracts of these cells. As is shown in

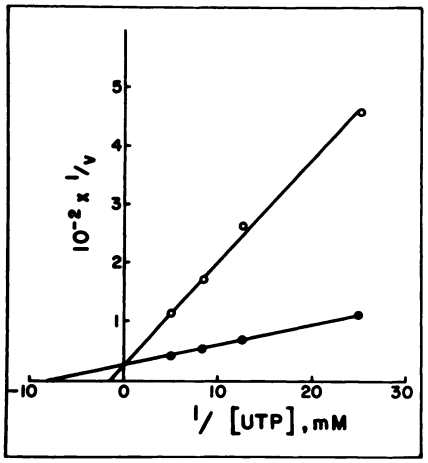

Chart 1. Effect of deaza-UTP on liver CTP synthetase activity. Double recriprocal plot of the velocity of the enzymatic reaction as a function of UTP concentration using 3.50×10^{-5} M deaza-UTP. The amount of protein used per assay mixture was 0.098 mg; •, no deaza-UTP; ○, deaza-UTP present.

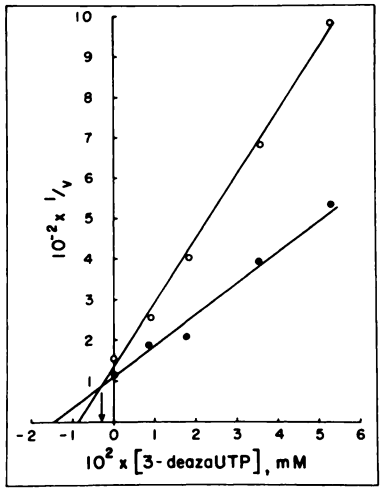

Chart 2. Dixon plot of the velocity of the CTP synthetase reaction as a function of the deaza-UTP concentration. The standard reaction mixture contained 0.2 mM (•) or 0.1 mM (○) UTP, and 0.049 mg of enzyme protein. *Arrow*, K_i value.

Table 2

Inhibition by deaza-UTP of the formation of CTP from UTP in cell-free preparations from leukemia L1210 cells

Enzyme source	Amount of protein added to reaction mixture (mg)	Reaction mixture used	CTP formed (μmoles $\times 10^3$)	Decrease in amount of CTP formed (%)
Cytosol	1.85	Complete	18.6	0
Cytosol	1.85	Without ATP	0.38	98
Cytosol	1.85	Without GTP	8.8	53
None	0	Complete	0.44	98
Fraction II, dialyzed	0.25	Complete	11.9	0
Fraction II, dialyzed	0.25	Without glutamine[a]	2.3	81
None	0	Complete	0.68	94
Fraction II	0.40	Complete	10.8	0
Fraction II	0.40	Without GTP	5.9	46
		With deaza UTP		
Fraction II	0.40	0.036 mM	4.5	59
Fraction II	0.40	0.072 mM	2.7	75
None	0	Complete	0.44	96

[a] The concentration of L-glutamine in this case was 0.05 mM and represents the carryover from the dialysis procedure (see "Materials and Methods"). The concentration is 55 mM in all the other experiments.

Table 2, this is indeed the case, CTP formation being inhibited 59% at 0.036 mM deaza-UTP. The fact that the amount of CTP formed in these extracts was dependent upon the presence of ATP and L-glutamine, and that the reaction was stimulated 2-fold when GTP was added to the mixture, constitutes strong evidence that the amination is catalyzed by a CTP synthetase (5, 14). If an effective cellular locus for the action of exogenous deaza-UR in these cells were the CTP synthetase, it would be expected that a significant amount of intracellular deaza-UR should be present as the triphosphate. Indeed, 79% of the total radioactivity found in the acid-soluble fraction of a suspension of washed leukemia L1210 cells incubated for 60 min with deaza-UR-^{3}H was present as deaza-UTP. This corresponds to an approximate intracellular concentration of 1.3×10^{-4} M.

Similarly, if the CTP synthetase is a major target for inhibition by deaza-UR, it would be expected that the inhibition of cell growth could be relieved by exogenous sources of the end product, CTP, or by metabolites that compete with the phosphorylation of deaza-UR. Accordingly, an inhibition analysis was carried out with L1210 cells. The results are shown in Chart 3. Among the pyrimidine nucleosides added to the growth medium containing the drug, cytidine was most effective in preventing the inhibition. Uridine and 2′-deoxycytidine were somewhat less effective, whereas 2′-deoxyuridine and thymidine did not prevent the inhibition at all. A similar reversal pattern was obtained by Brockman *et al.* with Ca 755 cells (3).

DISCUSSION

That the inhibition of CTP synthesis from UTP in leukemia L1210 cells may be a major site of action of deaza-UR following its conversion to deaza-UTP is indicated by the inhibition analysis. Cytidine was most effective in relieving the inhibition of L1210 cell growth caused by deaza-UR. The phosphorylation of cytidine gives rise to the ribonucleotide derivatives of cytosine and, following reduction at the ribonucleoside diphosphate level (7) to cytosine 2′-deoxyribonucleotides. Thus, cytidine can satisfy the cellular requirements for cytosine derivatives, whose concentration would be expect to be low if CTP synthetase activity were impaired. Indeed, Brockman *et al.* (4) have reported that, in deaza-UR-treated L1210 cells, the pools of CMP, CDP, CTP, and dCTP were markedly decreased. In addition to supplying the product of the inhibited reaction, cytidine may also compete with deaza-UR (a uridine analog) for phosphorylation (1), thereby decreasing the inhibitory activity of the drug even more effectively.

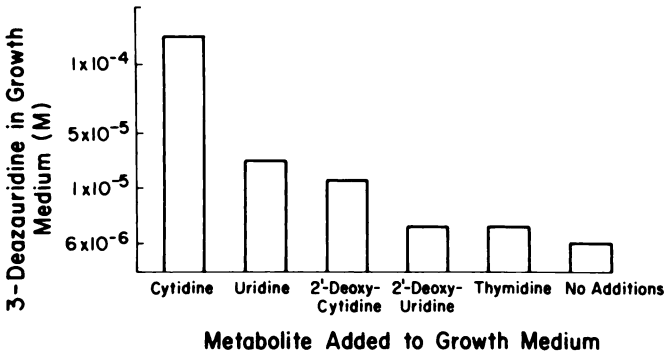

Chart 3. Ability of pyrimidine nucleosides to partially prevent the inhibition of the *in vitro* growth of leukemia L1210 cells, exerted by 3-deaza-UR. *Bars,* concentration of inhibitor at which 50% growth occurs in the presence of 1×10^{-4} M metabolite. Thymidine was used at 1×10^{-5} M, because higher concentrations are toxic.

2′-Deoxycytidine is expected to be a less effective reversing agent than cytidine, since it can supply only the requirement for the deoxyribonucleotides. In addition, after phosphorylation to dCMP, it becomes subject to deamination by dCMP deaminase (15), which also reduces its effectiveness. The fact that 2′-deoxyuridine and thymidine lack the ability to alleviate the inhibition provides further support for the assumption that depletion of cytosine nucleotides is an essential component of the mechanism leading to the observed inhibition of L1210 cell growth by deaza-UR.

The ability of uridine to partially prevent the inhibition may be ascribed not only to its ability to compete with deaza-UR for phosphorylation to the triphosphate stage, but also to the ability of UTP, derived from uridine, to competitively relieve the inhibition of CTP synthetase exerted by deaza-UTP.

To our knowledge, deaza-UTP is the first reported competitive inhibitor of a mammalian CTP synthetase. In the case of the *E. coli* enzyme, CTP was reported to compete with UTP for the enzyme with a K_i of 1.1×10^{-4} M (9).

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