Biochemical Pharmacology, Vol. 33, No. 23, pp. 3739–3742, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

REGULATION OF PYRIMIDINE BIOSYNTHESIS IN CULTURED L1210 CELLS BY 3-DEAZAURIDINE

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(*Received* 3 *October* 1983; *accepted* 28 *February* 1984)

Abstract—The effect of 3-deazauridine on the synthesis of uracil nucleotides by *de novo* and salvage pathways was investigated in intact cultured L1210 cells. *De novo* pyrimidine biosynthesis, as measured by sodium [^{14}C]bicarbonate incorporation into uracil nucleotides, was inhibited 40–85% at intracellular 3-deazauracil nucleotide concentrations of 1–6 nmoles/10^6 cells. The inhibition was not due to an increase in the size of the uracil nucleotide pool since this pool was only 97–66% of control level at 3-deazauracil nucleotide concentrations of 1–6 nmoles/10^6 cells. Furthermore, intracellular 3-deazauracil nucleotide concentrations of 0.5 to 5.2 nmoles/10^6 cells inhibited the salvage of [^{14}C]uridine by 25–75%. The data indicate that 3-deazauridine may potentiate its own inhibition of CTP synthetase by reducing the concentration of competing uracil nucleotides by inhibiting *de novo* pyrimidine biosynthesis and pyrimidine salvage. It is postulated that the biochemical mechanism by which 3-deazauridine inhibits uracil nucleotide synthesis is by acting as a fraudulent allosteric regulator of carbamyl phosphate synthetase II and uridine/cytidine kinase.

DeazaUR† is a uridine analogue synthesized by Robins and Currie [1]. It has antitumor activity against several murine tumors [2, 3] and has undergone Phase I and Phase II clinical testing [4–7]. Wang and Bloch [8] found that deazaUR is successively phosphorylated to the triphosphate by intact L1210 cells and by free extracts of Ehrlich ascites and L1210 cells. In its triphosphate form, deazaUR is a potent inhibitor of CTP synthetase and is competitive with respect to UTP with a K_i of 5.3 μM for calf liver enzyme [9]. Brockman *et al.* [3] also found deazaUTP† to inhibit the synthesis of CTP from UTP in enzyme preparations from L1210 cells. The triphosphate is not incorporated into RNA or DNA *in vitro* [8] or in L1210 cells *in vivo* [10].

UTP acts as an allosteric inhibitor of both carbamyl phosphate synthetase II [11–14], the first enzyme of mammalian *de novo* pyrimidine biosynthesis, and uridine/cytidine kinase [15], a salvage enzyme. Recent studies in our laboratory [16] have shown that an increase in the uracil nucleotide pool of intact L1210 cells is associated with inhibition of both *de novo* pyrimidine biosynthesis and uridine phosphorylation. Since deazaUTP is an analogue of UTP, it was anticipated that deazaUTP might reduce *de novo* and salvage biosynthesis of uracil nucleotides either indirectly, by causing an elevation of UTP through inhibition of CTP synthetase, or directly, by acting as a fraudulent allosteric inhibitor of carbamyl phosphate synthetase II and uridine/cytidine kinase. We examined these possible effects of deazaUR on pyrimidine biosynthesis in cultured L1210 cells. The results are contained in this report.

MATERIALS AND METHODS

Materials. Nucleosides, enzymes, and tri-*n*-octylamine were purchased from the Sigma Chemical Co. (St. Louis, MO). 3-Deazauridine (NSC 126849) was supplied by the Drug Synthesis Branch, National Cancer Institute. All media components and fetal calf serum were purchased from HEM Research, Inc. (Rockville, MD). Sodium [^{14}C]bicarbonate (7.8 mCi/mmole) and [^{14}C(U)]uridine (522 mCi/mmole) were obtained from the New England Nuclear Corp. (Boston, MA).

L1210 cells, originating from the cultures of Moore *et al.* [17], were maintained at 37° in RPMI 1630 medium supplemented with penicillin, streptomycin, and 20% fetal calf serum. The stocks were diluted to 10^4 cells/ml with fresh media at least three times a week in order to maintain cells in exponential growth (12 hr doubling time).

Quantitation of uracil and 3-deazauracil nucleotide pools by high pressure liquid chromatography. The L1210 cell pellet was first vortexed with 1 ml of H_2O to disrupt the cell membranes. Then 0.8 ml of a cold 10% trichloroacetic acid solution was added with vortexing. After adding 10 nmoles of 5-methylcytidine (internal standard), the samples were centrifuged at 800 *g* for 15 min, and the supernatant fraction was collected. The supernatant fraction was neutralized by vortexing with 2 ml of a trichlorotrifluoroethane–tri-*n*-octylamine solution (3:1). The aqueous layer was transferred to a vial and incubated overnight at 37° with phosphodiesterase I (Type VI, 1 mg) and alkaline phosphatase (Type I, 5 units) to

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† Abbreviations: deazaUR, 3-deazauridine; and deazaUTP, 3-deazauridine triphosphate.

convert the nucleotides of uracil and 3-deazauracil to uridine and deazaUR, respectively.

The lyophilized samples were redissolved in 200 μl of water, and 5 μl of xanthine oxidase (Grade III, 0.1 unit) were added to oxidize xanthine and hypoxanthine which may interfere with uridine in the chromatogram [18]. A 100-μl aliquot was analyzed on an Altex model 312 high pressure liquid chromatograph equipped with a Waters Radial Compression Module containing a 5 mm × 10 cm C18 Radial-PAK cartridge with a 5 μm partical size. The samples were eluted at 2.0 ml/min with an acetate buffer (0.01 M acetic acid, 0.01 M sodium acetate, 0.05% triethylamine, and 5% methanol). Comparison of the 280 nm/254 nm ratio of peak heights for the uridine, 3-deazauridine, and 5-methylcytidine peaks obtained from the samples to the ratio obtained from standards demonstrated the purity of these peaks [19]. A typical chromatogram is shown in Fig. 1.

Measurement of de novo *pyrimidine biosynthesis.* L1210 cells were distributed into 15-ml culture tubes (8×10^5 to 1×10^6 cells/ml, 5 ml/tube) and incubated for 2 hr in a shaking 37° water bath with deazaUR at concentrations ranging from 1 μM to 1 mM. Sodium [^{14}C]bicarbonate (25 μCi) was added, and the incubation was continued for 1 hr. The cells were pelleted at 200 *g* for 10 min and analyzed as described in the previous section. The amount of radioactivity incorporated into the uracil nucleotides was determined by counting the uridine peak collected from each chromatographic run and normalizing the cpm obtained with the amount of internal standard in the chromatographic run.

Measurement of uridine salvage. L1210 cells at 5–8 × 10^5 cells/ml were placed into 15-ml culture tubes (5 ml/tube) and incubated in a shaking 37° water bath with 0 or 5 μM to 1 mM deazaUR. After 2 hr the cells were washed twice with medium and incubated in the shaking water bath for an additional 1 hr with fresh medium containing [^{14}C(U)]uridine (10 μM, 0.2 μCi). The cells were then centrifuged, the pellet was resuspended in 2 ml H_2O, the sample was filtered through DE81 filter disks [10], and the disks were washed with 30 ml of H_2O. The radioactive nucleotides and macromolecules are adsorbed onto the disks and counted. An aliquot of the resuspended cell pellet was used to determine the intracellular 3-deazauracil nucleotide concentration.

RESULTS

Effect of deazaUR on uracil nucleotide pools. Since deazaUTP inhibits the conversion of UTP to CTP, incubation of cultured L1210 cells with deazaUR may result in an increase in the intracellular uracil nucleotide pool. However, as shown in Fig. 2, as 3-deazauracil nucleotides accumulated within the cell, the uracil nucleotide pool did not increase, but rather decreased by 20–30%. These results are in agreement with Brockman *et al.* [3] who found that the UTP pool of cultured L1210 cells declined 30% after a 4-hr exposure to 4 μM deazaUR.

Effect of deazaUR on de novo *pyrimidine biosynthesis. De novo* biosynthesis of uracil nucleotides in intact L1210 cells was monitored by quantitating the incorporation of sodium [^{14}C]bicarbonate into uracil nucleotides. DeazaUR inhibited the formation of

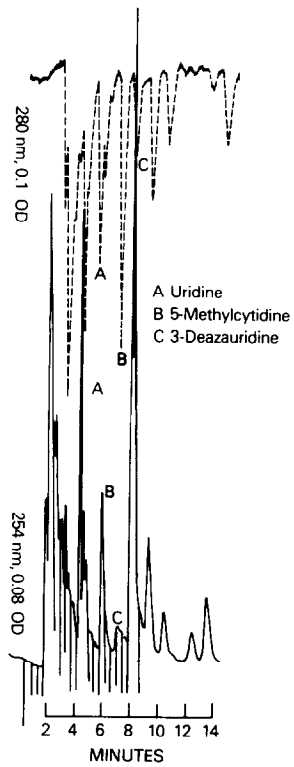

Fig. 1. High pressure liquid chromatogram of uridine and deazaUR obtained from L1210 cells that had been exposed to 50 μM deazaUR for 3 hr. All nucleotides were enzymatically hydrolyzed to nucleosides as described in the text. The 5-methylcytidine was added during sample preparation and served as an internal standard.

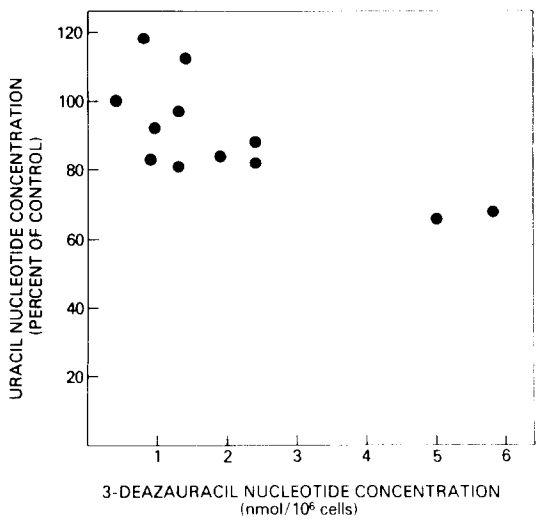

Fig. 2. Intracellular uracil nucleotide concentration of L1210 cells plotted against the intracellular 3-deazauracil nucleotide concentration at the end of a 3-hr incubation with 5 μM to 1 mM deazaUR. All concentrations were measured by high pressure liquid chromatography as described in the text. S.D., <5%.

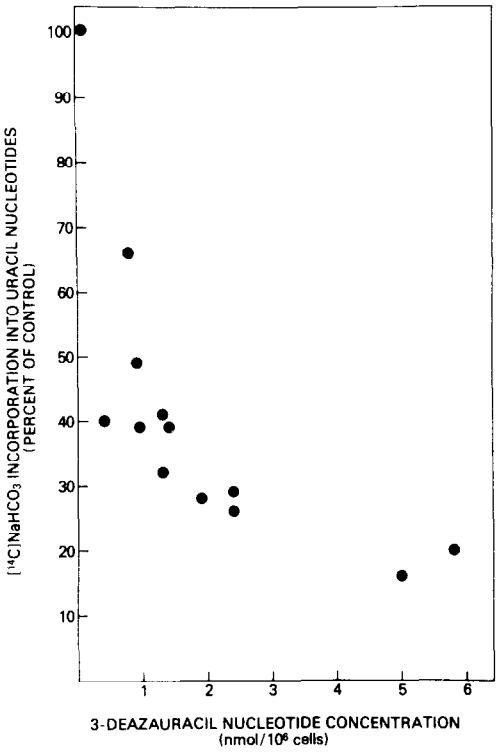

Fig. 3. Incorporation of sodium [^{14}C]bicarbonate into uracil nucleotides as a function of the intracellular 3-deazauracil nucleotide concentration at the end of a 3-hr incubation with 1 μM to 1 mM deazaUR. The L1210 cells were preincubated for 2 hr with deazaUR followed by a 1-hr incubation with both deazaUR and sodium [^{14}C]bicarbonate as described in the text. The level of radioactivity found in the uracil nucleotide pool of untreated cells was 6540 cpm/10^6 cells ± 370 (S.E., N = 12).

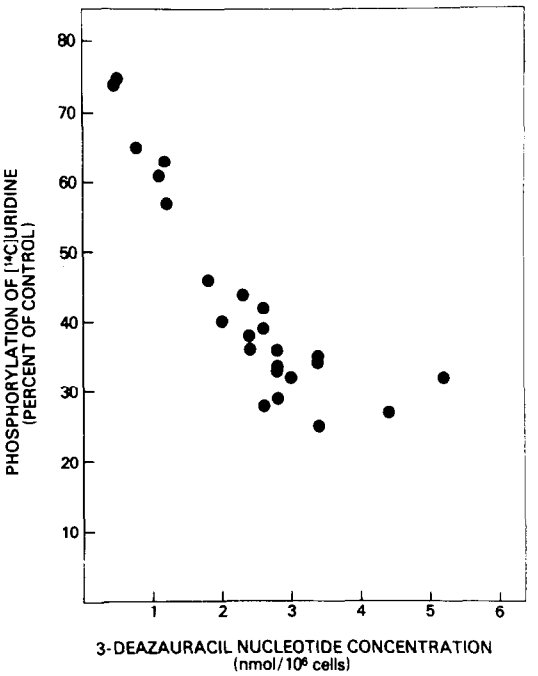

Fig. 4. Effect of deazaUR on [^{14}C]uridine phosphorylation by L1210 cells. The cells were preincubated with various concentrations of deazaUR for 2 hr, washed, and incubated with 10 μM [^{14}C]uridine for 1 hr. All intracellular phosphorylated products were counted, and the intracellular 3-deazauracil nucleotide concentration was measured. The control level of phosphorylated [^{14}C]uridine was 13,110 cpm/10^6 cells ± 290 (S.E., N = 3).

labeled uracil nucleotides and reduced the intracellular uracil nucleotide pool up to 30% in 3 hr. In Fig. 3, the incorporation of sodium [^{14}C]bicarbonate into uracil nucleotides in L1210 cells treated with various concentrations of deazaUR is plotted against the intracellular 3-deazauracil nucleotide concentration at the end of a 3-hr exposure. At intracellular 3-deazauracil nucleotide concentrations of 1–6 nmoles/10^6 cells, *de novo* biosynthesis of uracil nucleotides was inhibited by 40–85%. Since, as shown in Fig. 2, the uracil nucleotide pool was not expanded, the inhibition of *de novo* pyrimidine biosynthesis appears to be due to a direct effect of deazaUR or an anabolite such as 3-deazaUTP.

Effect of deazaUR on uridine salvage. The rate of phosphorylation of [^{14}C]uridine by cultured L1210 cells that were preincubated with deazaUR was compared to the rate of phosphorylation of [^{14}C]uridine in control L1210 cells. To eliminate any competition for membrane transport when high deazaUR concentrations were used, the deazaUR-treated cells were washed free of drug prior to exposure to [^{14}C]-uridine. A medium concentration of 10 μM [^{14}C]uridine was chosen to be within the range of human uridine plasma concentrations [19]. Cellular 3-deazauracil nucleotide concentrations of 0.5 to 5.2 nmoles/10^6 cells caused a 25–75% inhibition of [^{14}C]uridine phosphorylation (Fig. 4).

DISCUSSION

DeazaUR is successively phosphorylated to deazaUTP which potently inhibits CTP synthetase [3, 9]. Inhibition of UTP to CTP conversion should result in an increase in the uracil nucleotide pool. However, as shown in Fig. 2, the uracil nucleotide pool did not increase when cultured L1210 cells were exposed to deazaUR. At intracellular concentrations of 3-deazauracil nucleotides ≥ 2 nmoles/10^6 cells, a 20–30% decline in the uracil nucleotide pool was observed. Brockman *et al.* [3] reported a similar decrease in the UTP pool of cultured L1210 cells exposed to deazaUR. Thus, the observed inhibition by deazaUR of uracil nucleotide synthesis by both *de novo* and salvage pathways (Figs. 3 and 4) was not due to feedback inhibition by an expanded UTP pool. Our data suggest that the reduction in uracil nucleotide synthesis was accomplished by deazaUTP acting as a fraudulent feedback inhibitor of carbamyl phosphate synthetase II, the first enzyme of *de novo* pyrimidine biosynthesis, and uridine/cytidine kinase, the salvage enzyme. The reduction in synthesis of uracil nucleotides may be an important aspect of the mechanism of chemotherapeutic action of deazaUR.

The effect of intracellular 3-deazauracil nucleotides on pyrimidine biosynthesis in intact L1210 cells

differs quantitatively from the effect of intracellular uracil nucleotides. A 2-fold increase in intracellular uracil nucleotides, which corresponds to approximately 4 nmoles/10^6 cells, inhibits *de novo* biosynthesis by >95% and inhibits uridine phosphorylation by 40% [16]. By comparison, an intracellular 3-deazauracil nucleotide concentration of 4 nmoles/10^6 cells inhibited uridine phosphorylation by 75% (Fig. 4), which is nearly twice the inhibition found for uracil nucleotides at this concentration. In contrast, uracil nucleotides appeared to be more potent inhibitors of *de novo* biosynthesis than 3-deazauracil nucleotides. Above uracil nucleotide concentrations of 4 nmoles/10^6 cells, *de novo* biosynthesis was nearly completely inhibited, whereas 15–40% of the activity remained when the sum of the uracil nucleotides and 3-deazauracil nucleotides was 4 nmoles/10^6 cells. When the 3-deazauracil nucleotide concentration itself was 4 nmoles/10^6 cells, 15–20% of the activity was still observed (Fig. 3).

The concentration of 3-deazauracil nucleotides found in this study to alter pyrimidine biosynthesis is achievable *in vivo*. Following the administration of deazaUR (300 mg/kg, ip, CDF_1 mice), the concentration of 3-deazauracil nucleotides in *i.p.* and *s.c.* implanted L1210 cells is in the range of 0.4 to 1.0 nmoles/10^6 cells in the first 2 hr following deazaUR injection [10]. Time points beyond 2 hr were not reported. These values are in the range of concentrations capable of inhibiting *de novo* biosynthesis of uracil nucleotides by up to 60% and salvage of uridine by up to 40% in cultured L1210 cells. After an equivalent dose of deazaUR, the mouse tissue levels of 3-deazauracil nucleotides in normal tissues (i.e. lung, heart, liver, spleen, and small intestine) were much lower (generally < 0.2 nmole/10^6 cells). Uracil nucleotide synthesis in these tissues should not be affected.

The ability of deazaUR to inhibit biosynthesis of uracil nucleotides should be considered when deazaUR is combined with other antitumor agents in the treatment of human malignancies. Since L1210 tumors accumulate 3-deazauracil nucleotides many times more than normal tissues, uracil nucleotide synthesis in tumor cells may be decreased in comparison to normal cells. Thus, the combination of deazaUR with other pyrimidine antimetabolites may result in an enhanced selective toxicity of tumor cells.

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