Effect of Plasma Concentrations of Uridine on Pyrimidine Biosynthesis in Cultured L1210 Cells*

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The concentration of uridine in the media of cultured L1210 cells was maintained within the concentration range found in plasma (1 to 10 μM) to determine if such concentrations are sufficient to satisfy the pyrimidine requirements of a population of dividing cells and to determine if cells utilize de novo and/or salvage pathways when exposed to plasma concentrations of uridine. When cells were incubated in the presence of N-(phosphonacetyl)-L-aspartate to block de novo biosynthesis, plasma concentrations of uridine maintained normal cell growth. De novo pyrimidine biosynthesis, as determined by [14C]sodium bicarbonate incorporation into uracil nucleotides, was affected by the low concentrations of uridine found in the plasma. Below 1 μM uridine, de novo biosynthesis was not affected; between 3 and 5 μM uridine, de novo biosynthesis was inhibited by approximately 50%; and above 12 μM uridine, de novo biosynthesis was inhibited by >95%. Inhibition of de novo biosynthesis correlated with an increase in the uracil nucleotide pool. The de novo pathway was much more sensitive to the uracil nucleotide pool size than was the salvage pathway, which was much more sensitive to the uracil nucleotide pool size than was the salvage pathway. Likewise, Hoogenraad and Lee (9) reported a 70% decrease in de novo pyrimidine biosynthesis when cultured rat hepatoma cells were incubated with 500 μM uridine; a uridine concentration that is 50- to 500-fold higher than plasma concentrations. It is not clear from these studies whether the high concentration of uridine was required to offset the high rate of uridine utilization and/or catabolism or to an inability of lower concentrations to satisfy cellular pyrimidine requirements.

In the present study, cultured L1210 cells were exposed to fixed concentrations of uridine by either adjusting the cell number for minimal uridine removal or by infusion into the culture at a rate to offset uridine removal. The data demonstrate that plasma concentrations of uridine can satisfy the cell requirements for pyrimidines in cells where de novo biosynthesis is blocked; that cells exposed to plasma concentrations of uridine utilize their salvage pathways and turn off de novo biosynthesis; and that the de novo pyrimidine synthetic pathway is more sensitive to cellular uracil nucleotide concentrations than is the salvage pathway. These data indicate that circulating uridine is a factor in antipyrimidine cancer chemotherapy and possibly a determinant of the marginal clinical effectiveness so far achieved with inhibitors of de novo pyrimidine biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—Nucleosides, enzymes, and tri-n-octylamine were purchased from Sigma. PALA (NSC 224131) was supplied by the Drug Synthesis Branch, National Cancer Institute. All media components and fetal calf serum were purchased from Grand Island Biological
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Co. (GIBCO). Sodium [14C]bicarbonate (7.8 mCi/mmol) and [U-14C] uridine (522 mCi/mmol) were obtained from New England Nuclear.

L1210 cells, originating from the cultures of Moore et al. (10), were maintained at 37 °C in RPMI 1630 medium supplemented with penicillin, streptomycin, and 10% fetal calf serum. The stocks were diluted and given fresh media at least 3 times a week in order to maintain the cells in logarithmic growth. All experiments were performed using 20% fetal calf serum unless otherwise stated.

Quantitation of Media Uridine—To 0.5 to 2.0 ml of media, 10 nmol of the internal standard 5-methylcytidine was added and the sample volume was brought up to 2 to 3 ml with water. The samples were then centrifuged through Amicon Centricon CF25 membrane cones (Amicon Corp., Cambridge, MA) and the filtrate containing the nucleosides was lyophilized. Radioactive media were treated with barium hydroxide prior to centrifugation to precipitate the excess [14C]bicarbonate as barium carbonate.

The lyophilized samples were re-dissolved in 200 μl of water and 5 μl of xanthine oxidase (Grade III) were added to oxidize xanthine and hypoxanthine. A 100-μl aliquot was analyzed on an Altex Model 312 high pressure liquid chromatograph equipped with a Whatman Partisil PXS 5/25 ODS-3 column. The samples were eluted with an acetate buffer (0.01 M sodium acetate plus 0.01 M acetic acid, pH 4.0) at 1.2 ml/min. Between each run, the column was washed with 95% methanol and 5% buffer. Uridine was detected at both 254 and 280 nm and peak heights were used for uridine quantitation (4).

Reversal of PALA Growth Inhibition by Uridine—L1210 cells in conditioned media were distributed in 25-cm2 culture flasks (1000 cells/ml, 5 ml/flask) and were incubated overnight in order to deplete the uridine present in the serum. The following morning, solutions of uridine and PALA were sonicated for 5 min in warm water to prevent air bubbles from forming in the infusion tubing. All remaining procedures were performed in a 37 °C water bath. Twenty-four disposable 1-ml syringes were filled through a 23 gauge, 1-inch needle. With a 20-gauge needle, a hole was made in 24 tissue culture flask caps of the soft plastic variety. Using tweezers, a 30-cm piece of PE50 tubing was threaded through the cap hole leaving 10 cm on the back side of the cap. The tubing was then attached to the syringe needle and the syringes were set into a set of 23 gauge, 1-inch needle. With a 20-gauge needle, a hole was made in 24 tissue culture flask caps of the soft plastic variety. Using tweezers, a 30-cm piece of PE50 tubing was threaded through the cap hole leaving 10 cm on the back side of the cap. The tubing was then attached to the syringe needle and the syringes were set into a modified infusion pump rack capable of holding multiple syringes. The infusion pump plate was pushed up behind the syringes filling the PE50 tubing with uridine solution, and the pump motor was turned on. PALA (80 μl of a 5 mM solution, in H2O neutralized with NaOH) and uridine (to give an initial concentration of 2.5 to 10 μM) were added to 12 flasks and uridine alone was added to another 12 flasks. These 24 flasks were attached to the infusion pump ensuring that the end of the tubing was resting in the media. A set of noninfused, PALA-treated (100 μM) flasks was also prepared. All uridine solutions were in H2O and the infusion pump (Harvard Apparatus Compact Infusion Pump Model 975, Harvard Apparatus Company, Inc., South Natick, MA) was run on the slowest speed noninfused, PALA-treated (100 μM) flasks was also prepared. All uridine solutions were in H2O and the infusion pump (Harvard Apparatus Compact Infusion Pump Model 975, Harvard Apparatus Company, Inc., South Natick, MA) was run on the slowest speed.

Measurement of Uridine Salvage—L1210 cells at 6 × 10⁵ cells/ml were incubated overnight at 37 °C in 25-cm² culture flasks. The following day, 3 flasks were made 100 μM in PALA and 3 flasks each were made 100 μM in PALA (0.5 μM), 100 μM in PALA (10 μM), and 100 μM in uridine. Cell growth was measured at 72 h. At 72 h, control flasks contained 2.32 × 10⁵ cells/ml ± 1.6 (S.E., n = 3).

| Initial PALA concentration | Per cent growth of untreated control |
|---------------------------|-------------------------------------|
| 10 μM                     | 38 ± 2.5*                           |
| 10 μM                     | 24 ± 0.7                            |
| 1 μM                      | 15 ± 2.1                            |
| 0 μM                      | 17 ± 2.6                            |

*Mean ± S.E. of 6 samples.

FIG. 1. Uridine reversal of PALA toxicity in L1210 cells at low cell density. The 48-h cell growth was measured in cell cultures treated initially with uridine (0 to 10 μM) or with uridine plus 2 mM PALA. Dyeing of L1210 cultures with uridine containing dialyzed serum, conditioned media, prepared as described under "Experimental Procedures," was used in this experiment.
the shaking water bath for an additional 30 min with [U-14C]uridine
(2.5 μM, 0.25 μCi). The cells were then centrifuged, the pellet was
resuspended in 2 ml of H2O, the sample was filtered through DE81
filter disks, and the disks were counted. An aliquot of cells taken just
prior to the final incubation was analyzed to determine the size of
the uracil nucleotide pool.

RESULTS

Effect of Uridine on the Inhibition by PALA of L1210 Cell
Growth—The data in Table I show that plasma concentra-
tions of uridine (1 to 10 μM) are ineffective in rescuing
cultured L1210 cells from growth inhibition by PALA. When

![Graphs](attachment:image.png)

**Fig. 2.** Uridine reversal of the growth-inhibitory effects of PALA on L1210 cells at high cell density.
The cell cultures were infused with uridine in order to maintain a relatively constant level of media uridine. Details
of the procedure used are given under “Experimental Procedures.” Each composite graph depicts the media uridine
concentration for the uridine-infused flasks and the cell counts at 0, 4, 24, 48, and 72 h for an individual experiment
performed in triplicate. Bars, S.E. The S.E. of the cell counts was less than ±5%.
PALA-treated L1210 cells were incubated with uridine for 72 h. 1 μM uridine caused only minimal reversal of growth inhibition and even 100 μM uridine did not completely reverse the toxic effects of PALA (Table I). These data are similar to the observations of Swyryd et al. (7) and Tauboi et al. (8) who found that 100 μM uridine was required to reverse the growth-inhibitory effects of PALA in cultured SV40-transformed baby hamster kidney cells and in cultured HT-29 human colonic epithelial cancer cells, respectively.

In each of the above studies, the uridine concentration required to maintain normal growth is at least 10-fold higher than plasma concentrations. However, depletion of uridine from the culture media may explain the high concentration of uridine required in the experiment detailed in Table I. When L1210 cell cultures at 1 to 3 x 10^5 cells/ml were made 100 μM in uridine, uridine disappeared from the media at a rate of 0.3 to 1.0 nmol/h/10^6 cells during the first 24 h of growth. By 48 h, media uridine concentrations fell below 0.2 μM. Addition of 100 μM PALA did not significantly affect the rate of depletion of uridine during the first 24 h when the initial media uridine concentration was 100 μM. Uridine was not detectable in the media (0.2 μM) after 24 h of incubation when the initial concentration of media uridine was 10 μM or less. Less than a 5% loss of uridine was detected when 20 μM uridine was incubated at 37 °C in RPMI 1640 media. Fetal calf serum was found to be approximately 1 μM in uridine and the presence of 20% fetal calf serum did not alter the loss of media uridine.

The addition of tracer amounts of [14C]uridine to the media of L1210 cells demonstrated 67 to 85% of the radiolabel was retained in the media after 24 h. By collecting and counting the uridine peak from the high performance liquid chromatography analyses of the media, it was determined that the specific activity of the media uridine remained unchanged after 24 h of incubation. Since the specific activity of uridine remains unchanged and the amount of media uridine at 24 h is approximately 25% of its original level, the majority of the radiolabel retained in the media after 24 h is no longer in the form of [14C]uridine. No significant amounts of radiolabeled uracil or dihydrouracil were found in the media.

Since L1210 cells at high density (1 x 10^6 cells/ml) rapidly depleted the media uridine, uridine concentrations were measured at 48 h. Media uridine concentration measurements at 48 h revealed that cultures initially 10 μM in uridine were 6.2 ± 0.6 μM (S.D.) and those initially 5 μM were 1.7 ± 0.2 μM (S.D.) in the absence of PALA and were 5.7 ± 0.4 μM (S.D.) and 1.5 ± 0.2 (S.D.), respectively, in the presence of PALA. No uridine (<0.2 μM) was detected at 48 h in the cultures in which the media uridine was originally 1 μM or less. The results in Fig. 1 demonstrate that even at the lowest concentrations of uridine, the 48-h growth of PALA-treated L1210 cells was almost completely rescued at 48 h. Thus, at low cell density, plasma concentrations of uridine will maintain normal growth in cultures when de novo synthesis is blocked.

In order to examine the effect of constant levels of uridine on the growth inhibition caused by PALA of L1210 cells at high cell density, it was necessary to infuse uridine into the cultures to offset the removal of media uridine. An infusion system was developed to supply uridine to the cultured cells at a rate that approximated the rate of uridine depletion by the cells. Although it was not possible to maintain constant uridine concentrations, the infusion system maintained uridine concentrations close to those found in plasma (Fig. 2). The rate of consumption of infused uridine generally correlated with the rate of cell division between each time point. In the uridine infusions experiments, it was found through experimental trial to be necessary to infuse uridine into the PALA-treated cultures than into the untreated cultures in order to maintain the media uridine concentration in the PALA-treated cultures close to the level of the untreated cultures. The results in Fig. 2 show that infusion of L1210 cultures with plasma concentrations of uridine (1 to 10 μM) reverse the growth-inhibitory effects of PALA.

Cadmans (11) determined the minimum cellular requirement of exogenous uridine necessary to maintain normal cell growth in cells where de novo pyrimidine biosynthesis had been inhibited by pyrazofurin and reported that the amount of uridine required for cell division for L1210 cells under these conditions is 53.3 fmol/cell. The experiments in Fig. 2 were averaged yielding a rate of media uridine consumption for the L1210 cells for the first 48 h of 83 ± 24 (S.D.) fmol/cell division for the uridine-infused cells in the absence of PALA 160 ± 48 (S.D.) fmol/cell division for the uridine-infused cells in the presence of PALA. These values are similar to the rate of uridine consumption of 185 fmol/cell division observed for the first 24 h for noninfused cultures which were initially 100 μM uridine.

**Effect of Exogenous Uridine on de Novo Pyrimidine Biosynthesis**—The effect of exogenous uridine on de novo pyrimidine biosynthesis in L1210 cells was examined by measuring the incorporation of [14C]bicarbonate into uracil nucleotides at different media uridine concentrations. These experiments were carried out using 7 x 10^4 to 1 x 10^5 cells/ml. At 1 x 10^6 cells/ml, uridine depletion from the media was found to be 12 to 17.5 nmol/ml/h at an initial uridine concentration of 20 to 50 μM. In order to maintain relatively constant media uridine levels, cell cultures were infused with uridine as described under *Experimental Procedures*.

Fig. 3 plots the per cent inhibition of the incorporation of [14C]bicarbonate into the intracellular uracil nucleotide pool versus the media uridine concentration at the beginning and end of a 1-h incubation with [14C]bicarbonate. The results were calculated by comparing the amount of carbon 14 incorporated into the uracil nucleotides of the uridine-infused cultures to the carbon 14 incorporation into the uracil nucleotide pool of control cultures infused with PBS. The data in Fig. 3 show that media uridine concentrations below 0.5 μM.
have no effect on de novo pyrimidine biosynthesis in L1210 cells, while media uridine concentrations in the range of 3 to 5 \( \mu M \) generally produce approximately a 50% inhibition of de novo pyrimidine biosynthesis and media uridine concentrations above 12 \( \mu M \) cause a >95% inhibition of de novo pyrimidine biosynthesis. Thus, in the range of plasma uridine concentrations in man, mice, and rats are in the range of 1 to 10 \( \mu M \) (4). Uridine concentrations remain within this range throughout the day and are unaffected by a 24-h fast. Previous studies have shown that plasma uridine concentrations in man, mice, and rats are in the range of 1 to 10 \( \mu M \) (4). Uridine concentrations remain within this range throughout the day and are unaffected by a 24-h fast. Previous studies have shown that >100 \( \mu M \) uridine is necessary to rescue cultured cells from growth inhibition by PALA (7, 8). Accordingly, Table I shows that 100 \( \mu M \) uridine partially reverses PALA inhibition of L1210 cell growth. However, PALA concentrations of uridine are required because there is rapid removal of uridine from the media. When the cell number was adjusted to minimize uridine
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deployment or when cell cultures were infused with uridine to maintain a constant concentration, it was found that plasma concentrations of uridine can reverse growth inhibition by PALA (Figs. 1 and 2). Thus, the concentration of uridine in plasma is sufficient to maintain uridine nucleotide pools when cells are forced to use their salvage pathway, as in the presence of PALA.

The data in Fig. 3 demonstrate that L1210 cells incubated in the presence of physiologic concentrations of uridine, in the absence of PALA, utilize their salvage pathway in preference to their de novo pathway. The decrease in de novo synthesis in the absence of plasma concentrations of uridine is directly related to the size of the uracil nucleotide pool (Fig. 4). UTP is known to be an allosteric inhibitor of both carbamyl phosphate synthetase II (the first enzyme of the de novo pathway) and uridine/cytidine kinase (12-16). UTP inhibition of carbamyl phosphate synthetase II and uridine/cytidine kinase has never been compared in the same system, so the relative affinity of UTP for the 2 pathways cannot be assessed from values reported in the literature. Anderson and Brockman (16) reported 84% inhibition of uridine/cytidine kinase from P815 mouse tumor cells when both uridine and UTP were at a 10 mM concentration. Carbamyl phosphate synthetase II, isolated from rat liver (15), rat hepatomas (12, 15), and mouse spleen (13, 14) is inhibited by 70 to 90% when the ATP to UTP ratio (each at millimolar concentrations) ranged from 4:1 to 1:1. Levine et al. (13) reported a K of 0.11 mM for UTP for mouse spleen carbamyl phosphate synthetase II when ATP varied between 1 and 25 mM and UTP ranged from 0 to 4 mM. Our data demonstrate that in intact L1210 cells the de novo pathway is considerably more sensitive to intracellular uracil nucleotides than is the salvage pathway. When the uracil nucleotide pool is doubled (Fig. 4) and de novo synthesis is nearly completely inhibited (Fig. 3), the uracil nucleotide pool continues to expand in the presence of exogenous uridine and the salvage of [14C]uridine is inhibited by less than 50% (Table II).

Since the concentration of uridine in the plasma is adequate to satisfy the requirements of L1210 cells for uracil nucleotides and since L1210 cells preferentially utilize their salvage pathway over their de novo pathway when exposed to plasma concentrations of uridine, plasma uridine may be an important factor in antipyrimidine chemotherapy. It would appear that the highly vascularized areas of a tumor are exposed to a concentration of uridine sufficient to circumvent growth inhibition by inhibitors of de novo biosynthesis. These data support the development of inhibitors of uridine/cytidine kinase as agents for use in combination with inhibitors of de novo pyrimidine biosynthesis in the treatment of cancer.

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