Effect of Inhibitors of the de Novo Pyrimidine Biosynthetic Pathway on Serum Uridine Levels in Mice

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ABSTRACT

Since C57BL × DBA F1 (hereafter called BDF,) mice possess a relatively constant concentration of serum uridine [9.7 ± 1.3 (S.D.) nmol/ml], circulating uridine is available to cells with an intact pyrimidine salvage pathway and thus could influence the effectiveness of certain antitumor agents which inhibit de novo pyrimidine biosynthesis and whose cytotoxic properties are reversed by uridine. Three inhibitors of the de novo pyrimidine biosynthetic pathway were studied to determine their effects on circulating uridine concentrations in BDF, mice. Pyrazofurin and 6-azauridine were found to have no significant effect on serum uridine levels when administered as a single dose or on 4 consecutive days. In contrast, N-(phosphonacetyl)-L-aspartate reduced serum uridine levels by 55% when administered either as a single dose or on 4 consecutive days. This reduction could contribute to the antitumor effectiveness of N-(phosphonacetyl)-L-aspartate by limiting the rescue of cells possessing a salvage pathway.

D-Galactosamine, a stimulator of the de novo pyrimidine pathway, was also studied and found to increase total liver uridine (uridine plus uracil nucleotides and uridine diphosphate esters) by 4-fold at 8 hr, returning to normal by 24 to 48 hr. However, these large effects were not reflected in the serum.

INTRODUCTION

PALA2 is a new antitumor agent that is a potent inhibitor of aspartate transcarbamylase (12, 29), an early step in the de novo pyrimidine biosynthetic pathway. The toxic effects of PALA (13, 29) and certain other inhibitors of the de novo pyrimidine biosynthetic pathway (1, 5, 10) can be reversed by uridine, a nucleoside that can be utilized via the salvage pathway to maintain the intracellular pool of pyrimidine nucleotides. Recently, we developed methodology for quantitating uridine concentrations in serum and plasma (16) and found that the circulating level of uridine in humans, rats, and mice is relatively constant for each species. The serum is an available source of endogenous uridine that has the potential of reversing the toxic effects of inhibitors of the de novo pyrimidine pathway in cells that have an intact salvage pathway.

In a recent study involving patients from a Phase I trial of PALA, we found that PALA had a limited reductive effect on serum levels of uridine (17). PALA has been found to be clinically ineffective against most tumors (2, 6, 7, 11, 22, 31), even though marked inhibition of tumor aspartate transcarbamylase is observed (18, 26, 31). These clinical findings of limited reduction of circulating uridine, marked inhibition of tumor aspartate transcarbamylase, and clinical ineffectiveness could be explained by a lack of effect of PALA on the de novo pyrimidine biosynthetic pathway in donor organ(s) resulting in a continuous supply of circulating preformed uridine that could rescue the tumor cells.

In contrast to its clinical ineffectiveness, PALA is curative in C57BL × DBA F1 (hereafter called BDF,) mice bearing Lewis lung carcinoma, a slow-growing solid tumor that is resistant to many other antitumor agents (15). To gain a better understanding of the biological effects of PALA in vivo, we initiated a study to determine the effects of PALA on circulating levels of uridine. We also included, for comparison, 2 other inhibitors of the de novo pyrimidine biosynthetic pathway (6-azauridine and pyrazofurin) and δ-galactosamine, a stimulator of this pathway. The results of the study are contained in this report.

MATERIALS AND METHODS

Drugs and Chemicals. Nucleosides, nucleotides, enzymes, tri-n-octylamine (about 95%), and δ(+)-galactosamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ammonium acetate (Certified A.C.S.), sodium acetate (Certified A.C.S.), and 1,1,2-trichlorotrifluoroethane (reagent grade) were obtained from Fisher Scientific Co. (Fair Lawn, N. J.) PALA (NSC 224131), 6-azauridine (NSC 32074), and pyrazofurin (NSC 143095) were supplied by the Drug Synthesis Branch, National Cancer Institute. Amicon Centriflo CF25 membrane cones were purchased from Amicon Corp. (Lexington, Mass.), and Affi-Gel 601 boronate gel was obtained from Bio-Rad Laboratories (Richmond, Calif.).

Drug Studies. Male BDF, mice (20 to 27 g; NIH breeding colonies) were maintained on Purina mouse chowRalston Purina Co., St. Louis, Mo.) and water. In the 5-day studies, mice were given injections of a single dose or daily doses for 4 days of PALA (200 mg/kg in water, neutralized with 10 N NaOH), pyrazofurin (10 mg/kg in water), or 6-azauridine (1000 mg/kg in water). The dose of each drug was chosen to be at a level which is therapeutic towards mouse tumors (9, 14, 15, 28). Blood samples were taken at 1, 2, 4, 8, 24, 48, 72, and 96 hr. In the multiple-dose experiments, blood samples were collected just prior to the next injection. δ(+)-Galactosamine HCl was given at a dose of 2000 mg/kg in water, and blood and liver samples were taken at 1, 2, 4, 8, 16, 24, and 48 hr. All drugs were administered i.p. in a volume of 0.01 mg/g body weight.

The mice were anesthetized with 0.3 ml of 5% chloral hydrate, i.p., and bled from the brachial artery. Immediately after bleeding, the livers were removed and frozen in liquid N2. Livers from 10 individual mice were combined prior to analysis. In each experiment, at each time point, the blood from 5 mice was combined, and in the galactosamine studies, the livers from the 5 mice were combined prior to sample preparation and analysis.

Sample Preparation. The blood samples were allowed to clot at room temperature and then were centrifuged. The serum was collected and frozen at −20° until used. The nucleosides were isolated from serum by one of 2 methods. Method A is a modification of the method described by Kratulovic et al. (23) and consists of adding 10 nmol of the internal standard 5-methylcytidine to 500 μl of serum. While vortexing the sample, 1 ml of a cold 6% trichloroacetic acid solution was added dropwise, and the resulting suspension was centrifuged at
The supernatant was transferred to another tube and vortexed with an equivalent volume of a 2:1 solution of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine. The tubes were centrifuged at 1000 × g for 5 min, and the top layer containing the nucleosides was collected. In Method B, after the addition of the internal standard, the samples were diluted with 2 ml of H2O and centrifuged at 1000 × g for 20 min through an Amicon cone. The filtrate containing the nucleosides was collected.

To further purify the serum nucleosides, a 10-× 30-mm column of polyacrylamide-boronate gel, Affi-Gel 601, was equilibrated with 10 ml of 0.25 M ammonium acetate solution (pH 8.8). The nucleoside fraction obtained from Method A or B was made about 0.25 M in ammonium acetate, placed on the column, and washed with 10 ml of 0.25 M ammonium acetate solution. The ribonucleosides were eluted with 15 ml of 0.1 M formic acid, collected, and lyophilized. Under these conditions, the relative quantities of uridine and 5-methylcytidine were unchanged after chromatography. The lyophilized serum samples were redisolved in 100 μl of H2O, and 5 μl of xanthine oxidase were added to oxidize any remaining xanthine or hypoxanthine which would otherwise interfere with the uridine peak (23). The boronate column was regenerated by washing with 20 ml of 0.1 M formic acid and stored in the refrigerator between uses. The boronate column procedure is a modification of the affinity column procedure described by Davis et al. (3).

The frozen livers were weighed, a measured amount of 5-methylcytidine (about 500 nmol for every 0.5 g of liver) was added, and the livers were homogenized in a cold 6% trichloroacetic acid solution. A sample of homogenized liver, about 100 mg, was purified by Method A. The resulting nucleotide fraction was treated with xanthine oxidase (Grade III) to remove the hypoxanthine and xanthine and incubated at 37° overnight with alkaline phosphatase (type I) and phosphodiesterase I (type VI) to convert all uracil nucleotides and uridine diphosphate esters to uridine.

High-Pressure Liquid Chromatography. A 50- to 100-μl aliquot of serum or liver sample was analyzed on an Altex Model 312 high-pressure liquid chromatograph equipped with a Partisil PXS 5/25 ODS column (Whatman, Inc., Clifton, N. J.). The samples were eluted with acetate buffer (0.01 M sodium acetate plus 0.01 M acetic acid, pH 4.5) at 1.5 ml/min. Between each run, the column was washed for 15 min with 95% methanol and 5% acetate buffer. Peak heights were determined at 254 nm using an Altex Model 153 UV detector. To ensure purity of the uridine and 5-methylcytidine peaks, an Altex Model 153-30 variable wavelength UV detector set at 280 nm was used in series. The variation of individually analyzed replicate samples was less than 10%. Additional details of the purification and chromatography are published elsewhere (16). Typical chromatograms for mouse serum and liver are shown in Chart 1.

RESULTS AND DISCUSSION

The effects of PALA (200 mg/kg, i.p.), pyrazofurin (10 mg/kg, i.p.), and 6-azauridine (1000 mg/kg, i.p.) on serum uridine levels in the BDF, mouse are illustrated in Chart 2. Each graph shows serum uridine levels for daily studies in which the mice received daily doses for 4 days and for the single-dose studies. The shaded section of each graph represents the serum uridine level for 10 untreated mice [9.7 ± 1.3 (S.D.) nmol/ml] (16).

In the PALA-treated mice (Chart 2C), serum uridine levels remained near untreated levels for the first 4 to 8 hr but declined to 4.3 ± 0.5 (S.E.) nmol/ml by 24 hr and remained close to this level for at least 96 hr in both the daily- and single-dose studies. Thus, PALA at a dose level curative of Lewis lung carcinoma in BDF1 mice (15) effectively lowered mouse serum uridine levels by 55%. This result is similar to the effect of PALA on serum uridine in patients receiving PALA (17). A reduction from predose serum uridine levels was observed in these patients 24 to 96 hr after receiving their first dose of PALA, the largest drop for each patient being 37 to 85% below predose levels. Thus, since PALA does not reduce circulating uridine in the mouse to a much greater extent than in humans, the lack of therapeutic effect exerted by PALA in humans (2, 6, 7, 11, 22, 31) versus the curative capability of PALA on certain mouse tumors (15) is not readily explained by the effect of PALA on circulating uridine which is available for use via the salvage pathway. Other factors such as age of tumor, tumor vascularity which determines the capability of circulating uridine to reach the tumor, and tumor blood flow which determines the amount of circulating uridine to reach the tumor may also be playing an important role. It may be significant to note that, although patient serum uridine levels did decrease from pre-dose levels, they generally remained within the range of normal human serum uridine concentrations whereas BDF1 mouse serum uridine consistently decreased to a level about one-half of the normal range. The results portrayed in Chart 2C also demonstrate (a) that the effect of PALA on serum uridine was not immediate, requiring over 4 hr to lower serum levels significantly and (b) that a single dose of PALA produced nearly the same effect as multiple doses. Since no further decrease of serum uridine levels was observed in the mice given multiple doses, a single dose of 200 mg/kg was sufficient to produce maximal effect, and the effect is long-lasting. The long-lasting effect of a single PALA dose is consistent with other investigations (27, 32) which have shown that the inhibition of de novo pyrimidine
biosynthesis by PALA can persist as long as 4 days after a single injection.

Unlike PALA, pyrazofurin (Chart 2A) and 6-azauridine (Chart 2B), also inhibitors of the de novo pyrimidine biosynthetic pathway (9, 28), had little effect on serum uridine levels either as a single dose or multiple dose. Pyrazofurin and 6-azauridine, therefore, do not decrease the supply of circulating uridine. Although the lowered circulating uridine levels caused by PALA may be beneficial toward the antitumor effectiveness of PALA, the antitumor effectiveness of pyrazofurin and 6-azauridine must not be dependent upon their decreasing serum levels of preformed uridine available for rescue via the salvage pathway.

For drugs that require uridine-cytidine kinase for activation, such as 6-azauridine (28) or 5-azacytidine (24), combination in vivo with PALA may prove to be therapeutically beneficial. The effect of PALA could be 2-fold: (a) in lowering serum uridine levels which would reduce the amount of endogenous uridine available for competing with 6-azauridine or 5-azacytidine as substrates for uridine-cytidine kinase; and (b) in reducing intracellular UTP and CTP (15, 19, 27, 32) which by feedback regulation would augment uridine-cytidine kinase activity (24). In support of the latter, Grant et al. (8) recently reported an enhancement of intracellular accumulation of phosphorylated 5-azacytidine following PALA pretreatment of P388 and L1210 cells in culture. Preceding with PALA by 24 hr may be sufficient to increase utilization and effectiveness of these drugs.

Galactosamine, a stimulator of the de novo pyrimidine biosynthetic pathway, was given to BDF1 mice to determine what effect stimulation of pyrimidine biosynthesis would have on circulating uridine levels. Galactosamine was expected to cause an increase in de novo pyrimidine biosynthesis by depleting the end product UPT (4), an inhibitor of the de novo pyrimidine biosynthetic pathway (25, 30). Total liver uridine (uridine plus uracil nucleotides and uridine diphosphate esters) was measured to show that galactosamine affects total liver uridine levels in the mouse in a manner similar to the effects of galactosamine on rat livers, which have been studied extensively (4, 20, 21). A single 2000-mg/kg dose of galactosamine was injected i.p., and the results are shown in Chart 3. Total liver uridine in 10 untreated mice ranged from 1440 to 1910 nmol/g (1730 ± 180 (S.D.)). In the galactosamine-treated mice, de novo pyrimidine biosynthesis in the liver greatly increased such that total liver uridine levels increased 4-fold at 8 hr, returning close to normal by 24 to 48 hr. These mouse liver data correspond well to earlier studies of the effects of galactosamine on Wistar rat livers (4, 20, 21). Rat liver UTP was found to be at its lowest level at 3 hr (only 8% of control), returning to normal by 20 hr. At 400 mg/kg, the ΣUMP plus sugars reached 3 times normal within 8 hr, returning to normal at 25 to 40 hr. The increased ΣUMP did not result from increased breakdown of RNA.

In contrast to the changes in total liver uridine, serum uridine showed only small deviations from untreated levels following galactosamine treatment. Neither the depletion of intracellular UDP used for elimination of the galactosamine nor the stimulation of de novo pyrimidine biosynthesis significantly affected serum uridine levels.

In conclusion, PALA, pyrazofurin, and 6-azauridine lack large effects on mouse serum uridine even though, at the dose...
studied, all 3 drugs are therapeutic (9, 14, 15, 28). The antitumor effectiveness of pyrazofurin and 6-azauridine must not be dependent upon their decreasing circulating levels of preformed uridine available for rescue via the salvage pathway. PALA does significantly reduce mouse serum uridine levels, and this effect may be beneficial towards the antitumor effectiveness of PALA.

REFERENCES


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