Pharmacological and Biochemical Interactions of N-(Phosphonacetyl)-L-aspartate and 5-Fluorouracil in Beagles

Antonis A. Miller, E. Colleen Moore, Robert B. Hurlbert, John A. Benvenuto, and Ti Li Loo

Departments of Developmental Therapeutics [A. A. M., J. A. B., T. L. L.] and Tumor Biochemistry [E. C. M., R. B. H.], The University of Texas System Cancer Center M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77030

ABSTRACT

N-(Phosphonacetyl)-L-aspartate (PALA) and 5-fluorouracil (FUra) are both antimetabolites that affect the biosynthetic pathways of pyrimidines. To determine whether these two drugs exhibit synergistic pharmacological or biochemical interactions, we determined the pharmacological and biochemical parameters of PALA and [14C]FUra in 14 beagle dogs which received i.v. bolus administrations of either the single agents or the drug combination. The pharmacokinetic parameters of PALA (four dogs, 20 mg/kg) in plasma, cerebrospinal fluid, and urine were not changed by FUra (10 mg/kg, 30 min after PALA). The pharmacokinetics of [2-14C]FUra (six dogs, 10 mg/kg, 20 μCi/kg) was characterized by higher FUra plasma concentrations after pretreatment with PALA (20 mg/kg, 30 min before FUra); this led to a significantly larger area under the drug concentration-time curve, a decreased volume of distribution, and a reduced clearance rate and was associated with higher cerebrospinal fluid concentrations of FUra. The FUra plasma and cerebrospinal fluid half-lives, however, were not significantly altered by PALA. The biochemical determinants of PALA and FUra activity were studied in intestinal mucosa, liver, thymus, spleen, and bone marrow of four dogs. Although the activity of the target enzyme of PALA, L-aspartate carbamoyltransferase, in tissue extracts was decreased at least 50% at 18 to 24 hr after PALA administration (50 mg/kg), the uridine nucleotide pools remained remarkably stable. Intracellular FUra concentrations were not influenced by PALA. The incorporation of 5-fluorouridine triphosphate into RNA was enhanced in intestinal mucosa and liver. In other tissues, however, fluorouridine nucleotide concentrations were not affected by PALA. Free 5-fluorodeoxyuridine monophosphate had the highest concentration in liver and was detectable in all tissues, but it was not altered by PALA treatment.

Our results show that the pharmacological and biochemical events after FUra exposure are marginally modulated by PALA in normal dogs. If sensitive tumors with a higher degree of toxicity to normal tissues can be expected.

INTRODUCTION

Since their introduction by Heidelberger in 1957 (14), the fluoropyrimidines have been widely used for the treatment of various solid tumors (13). Some possible mechanisms of action of these fluoropyrimidines have been explored (1), including RNA- and DNA-directed actions of the FUra4 nucleotides FUTP and FdUMP (9, 22) and cell surface alterations after exposure to FUra (18); however, the exact mode of their antitumor action remains unknown. Recently, interest has grown in improving the response to FUra by combining it with other chemotherapeutic agents that may alter its metabolism or mechanism of action. Based on biochemical considerations, combinations of FUra with PALA (20), thymidine (28), methotrexate (4), or lactic acid (10) were evaluated, and enhanced activity of FUra seemed to be apparent.

We were interested in the pharmacological and biochemical interactions of FUra with PALA, a transition-state analogue that inhibits an early step in the de novo biosynthesis of pyrimidines (8), that had shown remarkable activity against a spectrum of murine tumors (16). The inhibition of ACTase by PALA causes diminished intracellular concentrations of pyrimidine nucleotides (Chart 1). As the concentration of UTP decreases, the incorporation of its analogue FUTP into RNA may become increased (20). Furthermore, lowering the concentration of dUMP may potentiate the DNA-directed activity of FUra, since dUMP competes with FdUMP for the binding site on thymidylate synthetase (26). The early (PALA) and late (FdUMP) block of the pyrimidine biosynthesis (Chart 1) interferes with the formation of RNA and DNA and comprises the rationale for the combination of PALA and FUra. We decided to test the hypothesis that these 2 drugs are biologically interactive and administered them as single agents or in combination to 14 beagles. Pharmacological and biochemical parameters were evaluated in biological fluids, expired air, and tissue specimens.

MATERIALS AND METHODS

Chemicals. PALA, FUra, and FdUrd were supplied by the Drug Development Branch of the National Cancer Institute, NIH (Bethesda, Md.). FUra was obtained from Roche Laboratories (Nutley, N. J.), and [2-14C]FUra (100 μCi/mg) was from Stanford Research Institute (Menlo Park, Calif.). FdUMP and all naturally occurring nucleobases, nucleosides, and nucleotides used as standards were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-[U-14C]Aspartic acid (specific activity, 192 mCi/mmOL) was supplied by New England Nuclear. Glass-distilled solvents for HPLC were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). Other reagents and chemicals were purchased from regular commercial suppliers and were reagent grade or higher.

Dog Studies. Beagles were lightly anesthetized with sodium pentobarbital. An endotracheal tube was inserted, and the bladder was catheterized. Throughout the experiment, a slowly dripping i.v. infusion of

---

1 Supported by Contract NO1-CM-87185 from the National Cancer Institute, NIH, USPHS.
2 Recipient of a grant from Deutsche Krebsfalle, Federal Republic of Germany.
3 To whom requests for reprints should be addressed.

Received November 30, 1981; accepted March 1, 1983.
5% dextrose in water was maintained. All groups of dogs were comparable in weight and sex distribution.

**Pharmacokinetics.** To study the effect of FURA on the pharmacokinetics of PALA, 2 dogs were given this agent (20 mg/kg, 400 mg/sq m, i.v.) alone. For comparison, another 2 dogs received the combinations of PALA (20 mg/kg) and FURA (10 mg/kg, 200 mg/sq m) simultaneously as necessitated by the short plasma half-life of FURA. Similarly, [2-¹⁴C]FURA (10 mg/kg, 20 µCi/kg) was administered to 3 dogs as a single agent and to 3 other dogs 30 min after PALA (20 mg/kg) to simulate as closely as possible the initial clinical protocol of the PALA-FURA combination at our institution. Blood samples were drawn from a contralateral extremity at various times, collected in glass tubes containing heparin as the anticoagulant, and centrifuged at 2000 x g for 10 min at 25°C. The plasma was separated from the cells and frozen until analysis. CSF samples were obtained by cisternal puncture and frozen immediately. Urine was collected by an indwelling Foley catheter, and portions were kept frozen until analysis. Labeled [¹³CO₂] was trapped by passing the expired air through a solution of phenethylamine and methanol (1:1, v/v) which was changed hourly. Samples were collected for 6 hr after drug administration. The dogs were killed by an injection of T-61 euthanasia solution (National Laboratories Corp., Somerville, N.J.).

**Disposition.** For tissue studies, dogs were divided into 2 groups. [2-¹⁴C]FURA (10 mg/kg, about 87 µCi/kg) was administered to 2 dogs as a single agent. Two dogs received the combination of PALA (50 mg/kg, 1000 mg/sq m) and [2-¹⁴C]FURA (10 mg/kg, about 74 µCi/kg) with an interval of 18 and 24 hr between i.v. bolus injections. One hr after FURA, portions of liver, spleen, and thymus were taken from the anesthetized animal and placed immediately into liquid nitrogen to prevent possible changes in nucleotide concentrations caused by tissue autolysis. A segment of the small intestine was flushed in situ with ice-cold normal 0.9% NaCl solution; the mucosa thereof was scraped off onto an ice-cold glass plate and similarly immersed in liquid nitrogen. Bone marrow speedily aspirated from the iliac crest of the dog was likewise frozen in liquid nitrogen. The collection of all of these specimens required about 7 min. The frozen samples were removed from the liquid nitrogen and stored at −80°C until analysis.

**PALA Assay.** PALA in biological fluids and tissue specimens was quantified by its inhibition of partially purified ACTase from rat liver (11). Carbamoylphosphate and radioactive aspartate were used as substrates, and the product radioactive carbamoyl aspartate was separated by passage through a cation exchange resin and determined radiochemically.

**ACTase Assay.** ACTase activities in tissue specimens were determined as reported previously (21). A Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) was used to measure the protein concentrations of the samples. The results are expressed in nmol of aspartate converted per mg of protein in 30 min.

**Sample Preparation for HPLC.** Plasma was deproteinized by ultrafiltration using Amicon CF25 membrane cones (Amicon Corp., Lexington, Mass.). CSF samples were not pretreated.

Unine samples (15 ml) were lyophilized, reconstituted with water (1.5 ml), adjusted to pH 10 with KOH, and chromatographed on Bio-Rad AG 1-X8 anion exchange resin (200 to 400 mesh, formate form, 10-ml disposable column). The urea and fluoropyrimidines were eluted off with 20 ml water (adjusted to pH 11 with KOH) and 40 ml of 2.5 M formic acid, respectively (6). [¹⁴C]Urea was measured radiochemically. The eluates containing the [¹⁴C]-labeled fluoropyrimidines were lyophilized and subjected to HPLC after reconstitution. The recovery of total radioactivity (about 75%) was determined for each sample, and corrections were made accordingly.

While still frozen, tissue samples were weighed (0.5 to 1.0 g) and immediately homogenized with 3 volumes (v/v) of 0.5 M HOAc in an ice bath. The homogenates were centrifuged for 15 min at 1000 x g in the cold. The acid-soluble supernatant was neutralized by extraction with an equal volume of 0.4 M Alamine in Freon (19) and washed with equal volumes of 0.04 M Alamine in Freon and pure Freon. This method of neutralization was chosen because nucleobases, nucleosides, and nucleotides were not extracted into the organic layer; no volume change of the aqueous phase occurred; and it was compatible with subsequent HPLC analyses. The perchloric acid precipitate was analyzed for RNA, DNA, and protein-bound FdUMP as described below.

**Chromatography.** HPLC analyses were performed with a Waters Associates (Milford, Mass.) liquid chromatograph equipped with an M6000A and an M45 solvent delivery system, an M660 solvent programmer, and a U6K universal injector. A Varian-chrom variable wavelength UV detector set at 280 nm and a Model 9176 recorder from Varian Associates (Palo Alto, Calif.) were used. Peak areas were determined by a Varian CDS 111 integrator. All solvents were freshly prepared and vacuum degassed before use, and all samples were centrifuged before injection.

**Reverse-Phase HPLC.** Samples of ultrafiltered plasma, CSF, and pretreated urine were injected onto a Spherisorb octadeclsilyle column (4.6-mm inside diameter x 250 mm, 5-µm particle size) from Custom LC (Houston, Texas) and eluted isocratically with 0.1 M acetate buffer (pH 4.5) at a flow rate of 2 ml/min. Fractions of the eluate were collected every 30 sec for radiochemical measurements of FURA, FdUMP, and FdUrd.

Neutralized acid-soluble extracts of tissues were injected onto the same column and eluted with 0.05 M (NH₄)₂HPO₄ adjusted to pH 3.5 with phosphoric acid at a flow rate of 1.5 ml/min (24). Fractions of the eluate were collected every min for radiochemical determinations of FURA, FdUMP, and FdUrd.

**Anion Exchange HPLC.** Nucleotides in tissue extracts were separated on a Whatman (Clifton, N.J.) Partisil PSX 10/25 strong anion exchange column (4.6 x 250 mm, 10-µm particle size). A 1-hr gradient from 0.005 M (NH₄)₂HPO₄, 0.0025 M sodium acetate buffer, pH 5.0, to 0.5 M (NH₄)₂HPO₄, 0.25 M sodium acetate buffer, pH 5.0, was generated by the solvent programmer (Curve 7) at a flow rate of 1.5 ml/min. The fractions of the eluate containing FUMP, FdUMP, FdUDP, and FdUMP were collected for radiochemical determinations. UMP, UDP, and UTP were separated from all other naturally occurring nucleotides and were determined by their UV absorbance.

**Separation of Ribonucleotides.** Since FUMP and FdUMP coeluted under the conditions of the anion exchange HPLC, they were separated by the following procedure. The fractions containing the FUMP-FdUMP peak from the HPLC column were combined, lyophilized, reconstituted with 4 ml of a solution containing 0.05 M potassium acetate...
and 0.05 mM magnesium acetate, and adjusted to pH 8.5 with KOH. Affi- 
Gel 601 (Bio-Rad), a cross-linked, beaded polystyrylamine gel with phenyl 
boronate functional groups covalently attached to the matrix, was pre-
pared (1.5 ml) in Bio-Rad glass barrel Econocolumns by washing it with 
the above potassium acetate:magnesium acetate solution (pH 8.5). The 
flow rate was restricted to 0.5 ml/min by tightly fitting discs of filter paper 
over the frit. The samples (4 ml) were applied onto the boronate gel 
followed by 6 ml of the 0.05 M potassium acetate:0.05 M magnesium 
acetate solution (pH 8.5). The deoxyribonucleotides were in the 10-ml 
effluent. The ribonucleotides were eluted from the column with 5 ml of 
0.25 M ammonium acetate, pH 5.0, and the elution was repeated. All 
euates were collected in counting vials, lyophilized, reconstituted with 2 
ml of water, and analyzed radiochemically. The recovery of this procedure 
was 89% for ribo- and deoxyribonucleotides.

The columns were regenerated with 10 ml of 0.1 M Na2SO4 stirring 
up the boronate gel to allow swelling. The gel was then washed with 
water and stored in 50 mM sodium azide.

RNA and DNA Analyses. The acid precipitates of the tissue homog-
enates were washed twice with 10 ml of 0.4 M HClO4 and once with 10 
ml of ethanolic, rinsing the entire inner surface of the tubes and breaking 
up the precipitates. After centrifugation for 10 min at 1000 × g in the 
cold, the supernatants were discarded, and the precipitates were ex-
tracted with hot potassium acetate as described previously (27). RNA 
and DNA in these extracts were precipitated with 2 volumes (v/v) of 
ethanol in the freezer and then separated by incubation with 0.2 M NaOH 
and precipitation with 4 M HClO4 (27). Portions of the resulting samples, 
RNA and DNA concentrations were determined according to published 
procedures (3, 15). The rest of these samples were analyzed radiochemically.

Protein-bound FUrA Metabolites. Following the hot potassium ace-
etrate extraction, tissue precipitates were washed with 2 ml of 0.4 M HClO4 
(100°, 10 min) to remove traces of RNA and ONA. After centrifugation, 
cotton-tipped applicators were inserted into the precipitates which were 
then frozen in dry ice. The gel was then washed with water and stored in 50 mM sodium azide.

Radiochemical Technique. Radioactivity was determined with a 
Packard Tri-Carb Model 2650 liquid scintillation spectrometer; quenching 
was corrected by the external standard channel ratio method, and all 
results were expressed in dpm. PCS scintillant (Amersham Corp., Arling-
ton Heights, Ill.) was used as the counting fluid unless otherwise stated. 
In addition to plasma, CSF, urine samples derived from the above 
procedures, and 14CO2 trapped in phenethylamine:methanol solution 
were likewise counted for total radioactivity.

Protein Binding. [2-14C]FUrA was added to freshly obtained plasma 
of 4 different dogs in concentrations encountered in the pharmacokinetic 
studies. The plasma was ultrafiltered through Amicon CF25 cones (mo-
olecular weight cutoff, 25,000), and the filtrates were analyzed for radio-
activity. Drugs diluted in 0.9% NaCl solution were processed identically 
as a control. FurA concentrations in this study were corrected to repres-
ten total plasma concentrations.

RESULTS

Pharmacokinetics of PALA. The pharmacokinetic parameters (12, 23) of PALA administered as a single agent or in combination 
with FurA are shown in Table 1. The means of the terminal 
plasma half-life (1.5 hr), the apparent volume of distribution (0.4 
liter/kg), and the total clearance rate (2.8 ml/kg/min) were identi-

cal in both groups, and other values for the plasma kinetics and 
urinary excretion were similar. The peak PALA concentration in 
CSF was reached between 2 and 3 hr but did not exceed 10% 
of the concurrent plasma concentration. The CSF half-life of 
PALA was about 3 times longer than the terminal plasma half-
life.

Pharmacokinetics of FurA. The pharmacokinetic parameters (12, 23) of FurA (detection limit, 1 nmol/ml) are shown in Table 2. 
PALA did not cause a significant change of the terminal plasma half-life of FurA. When compared with the control group, PALA 
pretreatment increased the area under the concentration-time

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Pharmacokinetic parameters of PALA in beagles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma PALA</td>
<td>f1/2 (T1/2)</td>
</tr>
<tr>
<td>(C₀)ₘₐₜ (nmol/ml)</td>
<td>Initial AUC (hr/ml)</td>
</tr>
<tr>
<td>PALA (n = 2)</td>
<td>595.4</td>
</tr>
<tr>
<td>PALA:FurA (n = 2)</td>
<td>623.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Pharmacokinetic parameters of FurA in beagles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma FurA</td>
<td>f1/2</td>
</tr>
<tr>
<td>(C₀)ₘₐₜ (nmol/ml/min)</td>
<td>Initial AUC (min)</td>
</tr>
<tr>
<td>FurA (n = 3)</td>
<td></td>
</tr>
<tr>
<td>FurA after PALA (n = 3)</td>
<td>329.2 ± 36.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> (C₀)ₘₜ, initial plasma concentration; AUC, area under curve; Vₐ, volume of distribution (12, 23).

<sup>b</sup> Mean ± S.D.

<sup>c</sup> With an interval of 30 min.

<sup>d</sup> Significantly different at p = 0.05.
curve, decreased the apparent volume of distribution, and reduced the total clearance rate (Chart 2; Table 2). The urinary excretion of FUra and total radioactivity were increased when the combination was given. These changes were statistically significant at the 0.05 level. The catabolism of FUra to urea and radioactive CO₂ remained unchanged. The peak CSF concentration of FUra was reached 1 hr after its administration and was comparable to that in plasma (Chart 3). The CSF half-life was longer than the terminal plasma half-life and not influenced by PALA (Chart 3).

Protein Binding. PALA had no significant influence on the plasma protein binding of FUra. Whether PALA was present or not, FUra was 21% protein bound (data not shown). PALA itself was insignificantly bound to plasma protein as reported previously (21).

Disposition. Table 3 summarizes the parameters measured in tissue samples. The highest FUra concentrations were observed in the liver followed by the intestinal mucosa, thymus, spleen, and bone marrow. No differences were observed whether the samples were obtained 18 or 20 hr after PALA administration. Highest ACTase activities were found in extracts from the thymus and intestinal mucosa. When compared with FUra alone, decreases in ACTase activity were seen in all tissue extracts from the dog treated with PALA:FUra. The greatest reduction was noted in the liver (93%) followed by intestinal mucosa (63%), thymus (56%), bone marrow (55%), and spleen (50%). The naturally occurring nucleotides UDP and UTP were quantified; UMP was not detectable (<10 nmol/g), suggesting that the degradation of nucleotides had been largely prevented by rapid sample freezing. The concentrations of UDP and UTP did not show consistent changes attributable to the activity of PALA.

The disposition of FUra and its anabolites is shown in Table 3. The highest free FUra concentrations were found in bone marrow aspirates, and the liver contained the lowest amounts. FUra concentrations ranged from 1.5 to 5.8 nmol/g tissue, and PALA exerted no significant influence on them. The nucleosides FUrd and FdUrd were not detectable (<20 pmol/g) in biological fluids or tissue specimens. Acid-soluble, free FdUMP was identified in all tissue samples, with the highest concentrations in the liver and a narrow range of 50 to 60 pmol/g in other tissues. The residual radioactivity in the protein fraction after washing with hot perchloric acid was taken as "bound FdUMP" (see below) (26, 29). The tissue concentrations of bound FdUMP decreased in the order: liver; intestinal mucosa; thymus; spleen; and bone.

![Chart 2. Plasma concentration of FUra administered as a single agent (●) or 30 min after PALA (△) in 3 beagle dogs. Bars, S.D.](image)

![Chart 3. CSF concentration of FUra administered as a single agent (●) or 30 min after PALA (△) in 3 beagle dogs. Bars, S.D.](image)

| Distribution of PALA, ACTase, and labeled FUra and its metabolites in beagles |
|-----------------|----------|----------|----------|----------|----------|----------|----------|
|                  | Tissue   | PALA(g)  | ACTase   | UDP      | UTP      | FUra     | FdUMP    | FUTP     |
|                  |          |          |          |          |          |          |          |          |
| FUra only (n = 2)| Intestinal mucosa | 24.6     | 155      | 253      | 4.8      | 0.06     | 2.3      | 1.4      | 2.4      | 0.6      |
| Liver            | 2.8      | 252      | 188      | 1.5      | 0.39     | 5.9      | 0.6      | 1.0      | 0.3      |
| Thymus           | 59.2     | 125      | 271      | 4.5      | 0.05     | 0.6      | 0.4      | 1.2      | 0.5      |
| Spleen           | 2.6      | 86       | 105      | 2.6      | 0.05     | 0.2      | 0.3      | 0.7      | 0.6      |
| Bone marrow aspirate | 2.9   | 33       | 26       | 5.5      | 0.06     | 0.1      | 0.1      | 0.2      | 0.9      |

PALA:FUra(g) (n = 2)

|                  | Intestinal mucosa | 3.9      | 9.2      | 127      | 249      | 4.9      | 0.07     | 3.3      | 1.2      | 3.5      | 1.2      |
| Liver            | 12.3     | 0.2      | 241      | 220      | 1.6      | 0.37     | 8.0      | 0.9      | 1.8      | 0.6      |
| Thymus           | 2.0      | 26.3     | 115      | 244      | 4.4      | 0.05     | 0.5      | 0.3      | 1.0      | 0.7      |
| Spleen           | 1.4      | 1.3      | 84       | 104      | 2.6      | 0.06     | 0.2      | 0.2      | 0.9      | 0.7      |
| Bone marrow aspirate | 0.6   | 0.6      | 31       | 17       | 5.8      | 0.07     | 0.1      | 0.3      | 0.2      | 0.9      |

All parameters expressed in nmol per g of wet tissue except for ACTase activity (nmol of substrate converted in 30 min per mg protein) and FUTP incorporation into RNA (nmol per mg RNA).

6 FUra given 18 to 20 hr after PALA.
marrow. The liver contained more than 50-fold-higher amounts than bone marrow aspirates. FU TP represented the FUra nucleotide with the highest concentration in each sample. FUMP, in concentrations from 158 to 190 pmol/g, was measurable in the liver but not (below 25 pmol/g) in other tissues. PALA had a limited effect on FUra nucleotide concentrations in 2 tissues; the sum of FUDP and FU TP was increased in intestinal mucosa (from 3.8 to 4.7 nmol/g) and liver (from 1.6 to 2.7 nmol/g). The FU TP incorporation into RNA, as measured by the amount of radioactivity present in the RNA preparation, was most prominent in bone marrow aspirates when FUra was given alone. After the administration of the drug combination, higher levels of incorporation were observed in intestinal mucosa and liver. No significant changes of the FUra ribonucleotide pools and FU TP incorporation into RNA were noted in other tissues after PALA pretreatment.

DISCUSSION

Combination chemotherapy with FUra has been used extensively (1). Of special biochemical interest are those combinations that lead to a sequential inhibition of the pyrimidine-biosynthetic pathway. Among the agents that modulate the effects of FUra are PALA, an inhibitor of ACTase, hydroxyurea, an inhibitor of ribonucleotide reductase, and pyrazofurin and 6-azauridine, inhibitors of orotidylate decarboxylase. Of these, only PALA inhibits an early step of the pyrimidine pathway (8) and does not interfere with the metabolic activation of FUra (8). FUra must be activated to nucleotides. FU TP is incorporated into RNA, thereby affecting RNA processing and maturation, while FdUMP is a potent inhibitor of thymidylate synthetase (26).

For the measurements of PALA and ACTase, sensitive assays were developed in this laboratory (11, 21). As expected, the pharmacokinetic parameters of PALA (Table 1) showed no significant differences whether it was administered as a single agent or together with FUra. PALA reached all investigated tissues with highest concentrations in the liver and lowest in bone marrow aspirates, in agreement with other reports that PALA lacks myelosuppressive toxicity in animals and humans (8, 17) and that toxic effects in mice were evident in the liver as revealed by histological evaluation (17). ACTase, the target enzyme of PALA, was apparently inhibited in all tissue extracts (Table 3). Higher intracellular amounts of PALA in liver and intestinal mucosa were associated with greater degrees of ACTase inhibition. However, the observed enzyme inhibition did not translate into diminished nucleotide pools. The intracellular concentrations of UDP and UTP (Table 3) remained remarkably stable in spite of the presence of PALA. The following factors might account for this observation. Since the in vivo concentration of the competing substrate carbamoyl phosphate at the enzyme complex was unknown, and since the PALA in the tissue was diluted 35-fold in the assay mixture, the actual extent of in vivo ACTase inhibition, though obscure, may have been even greater. Because ACTase is present in great excess over carbamoyl phosphate synthetase, the limiting enzyme in the pathway (8), a small fraction of residual ACTase activity may be sufficient to maintain a significant rate of pyrimidine synthesis. Furthermore, a “salvage pathway” exists in mammalian cells which can alternatively provide necessary pyrimidines. Therefore, it seems that tissues can effectively maintain constant concentrations of pyrimidine nucleotides by the residual activity of the de novo route and the salvage pathway. Other investigators (25) reported minimal depletion of nucleotides in normal mouse tissues at therapeutically effective doses of PALA; a sensitive tumor, Lewis lung carcinoma, however, showed markedly reduced pools of pyrimidine nucleotides.

After PALA pretreatment, plasma FUra concentrations were elevated as reflected in the larger area under the drug concentration-versus-time curve as compared with that when FUra was administered alone (Chart 2). Also, the apparent volume of distribution and the total clearance of FUra were noticeably decreased, whereas the catabolism of FUra to urea and carbon dioxide remained unchanged. However, the diminished total clearance was accompanied by an increase rather than a decrease in the 6-hr cumulative urinary excretion of the unchanged drug (Table 2). This apparent paradox could be resolved if the effects of PALA on all routes of elimination of FUra from the plasma were separately considered; these routes obviously included renal, biliary, catabolic, anabolic, and binding to macromolecules. The biliary excretion of FUra has been investigated by Douglass and Mittelman (7) who administered a tracer dose of [2-14C]FUra i.v. to 2 patients. From their results, we estimated that the total excretion of FUra in the bile was less than 0.08% of the administered dose. As alluded to before, PALA exerted no influence on FUra catabolism and binding to plasma protein. Possibly, therefore, as a compensatory mechanism to meet cellular needs for pyrimidines, the alternative salvage pathway of pyrimidine biosynthesis may have become stimulated in response to the PALA inhibition of the de novo pathway. The heightened demand by cells for preformed pyrimidines, such as uracil, could thus interfere with the uptake, anabolic utilization of FUra, as well as with its binding to macromolecules other than plasma protein. This is consistent with our findings that as a result of PALA pretreatment, the total clearance of FUra was reduced, because the anabolism and binding of FUra were inhibited even though the urinary excretion of the drug was increased; after all, renal clearance contributed less than 6% to the total clearance (Table 2). The expected reductions in the biochemical effects were, however, not apparent from our distribution studies (Table 3), probably because our analytical methodologies were not sufficiently sensitive to allow their detection.

Our observations that FUra was present in lowest concentrations in the liver (Table 3) and that more than 60% of the administered radioactivity appeared in the expired air as 14CO2 within 6 hr (Table 2) are in agreement with the fact that the catabolic enzyme dihydrouracil dehydrogenase (EC 1.3.1.1) is predominantly located in the liver where FUra is rapidly degraded (1). The nucleosides FUrd and FdUrld were not detectable.

FUra was converted to ribonucleotides in all tissues, but FUDP and FU TP comprised only a small portion of the nucleotide pool in comparison with the naturally occurring UDP and UTP (Table 3). After PALA administration, the sum of the FUDP and FU TP concentrations was elevated in intestinal mucosa and liver, and this was correlated with a higher incorporation into RNA. In other tissues, PALA did not influence the ribonucleotide pools of FUra or the incorporation into RNA. Less than 1% of RNA uracil was replaced by FUra, which is probably a consequence of the similar proportion of FUTP to UTP (9). The DNA fraction contained only small quantities of radioactivity that was not further characterized; it could represent RNA contamination. Neither the degree of apparent incorporation of FdUTP nor the total amount of DNA was changed by PALA pretreatment. Although FdUTP is formed in mammalian cells and is a substrate for DNA polymerase, it is
rapidly hydrolyzed by the enzyme deoxyuridine triphosphatase or removed from DNA by uracil-DNA glycosylase (5); the incorporation of larger quantities of FdUTP into DNA is thereby prevented.

The presence of free FdUMP in all tissues suggests some inhibition of thymidylate synthetase (6). Since FdUMP has been shown to be protein bound to a certain extent (26, 29), measurement of free FdUMP alone does not adequately assess DNA-directed activity of FUra. The $^{14}$C from FUra in the insoluble protein residue was assumed to be enzyme-bound FdUMP, which is known not to dissociate after acid precipitation of the protein (26, 29). According to Washtien and Santi (29), essentially all of the acid-insoluble label in their cultured L1210 cells could be accounted for by either RNA (84%) or enzyme-bound FdUMP (18%). However, it seems unlikely that the thymidylate synthetase activity or the concentration of bound FdUMP in the liver could be higher than that in the more actively growing tissues (Table 3). We were unable to demonstrate release of FdUMP from liver or mucosal specimens were homogenized in buffer and warmed to 65° (29), although the procedure was not definitive because of the action of degradative enzymes, as shown by an increase in free FUra and a decrease in RNA and ribonucleotides.

All tissues contained more protein-bound label than free FdUMP (Table 3), and a considerable amount of FUra was incorporated in this fraction. Increases of protein-bound label were observed in intestinal mucosa and liver after PALA pretreatment, whereas unbound FdUMP concentrations showed no change. Apparently, the bound FdUMP included unidentified FUra metabolites in addition to FdUMP bound to enzyme.

The relative importance of RNA-directed effects of FUra versus the inhibition of thymidylate synthetase is still controversial (26). We were able to demonstrate that considerable differences of FUra activation existed among the tissues investigated (Table 3). The liver contained the highest concentrations of bound and free FdUMP, yet the amount of FUTP incorporated into RNA was among the lowest. These suggest a greater DNA- and RNA-directed activity of FUra in the liver. In bone marrow aspirates, concentrations of bound FdUMP were the lowest, while FUTP incorporation into RNA was relatively high. This may result in more RNA-directed toxicity. Bound FdUMP, free FUTP, and FUTP incorporated into RNA were all present in relatively large quantities in the intestinal mucosa; this combination of both RNA- and DNA-directed effects may account for the predominant gastrointestinal toxicity observed in other studies (2).

Our results demonstrate that the intracellular biochemical events after FUra exposure were minimally modulated by PALA. Although FUra and PALA interact only marginally in normal tissues, they have shown synergistic effects in some tumor systems (20). A recent clinical trial in patients with colon cancer revealed that the PALA:FUra combination was not more effective than FUra alone (2). Our pharmacological studies in patients tend to suggest that PALA does not significantly alter the pharmacokinetic parameters of FUra.

REFERENCES

Pharmacological and Biochemical Interactions of \( N \)-(Phosphonacetyl)-l-aspartate and 5-Fluorouracil in Beagles

Antonius A. Miller, E. Colleen Moore, Robert B. Hurlbert, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/6/2565

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.