Selective Inhibition of Pyrimidine Synthesis and Depletion of Nucleotide Pools by N-(Phosphonacetyl)-L-aspartate¹

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INTRODUCTION

PALA³ is a rationally designed transition state analog inhibitor of aspartate transcarbamylase, the enzyme catalyzing the second step of pyrimidine synthesis de novo (6, 21, 27). PALA has shown activity against a selected group of transplantable solid tumors including Lewis lung carcinoma, B16 melanoma, and colon carcinoma 26 (10, 11, 23). It is curative of Lewis lung carcinoma at doses well below toxic levels (11) but is ineffective against the common murine leukemia models.

In cell culture, PALA is capable of producing a nearly complete inhibition of pyrimidine synthesis and reduces intracellular UTP levels (11, 12). Attempts to measure inhibition of pyrimidine synthesis in animals by use of radioactive sodium bicarbonate and aspartate have been unsuccessful because incorporation of these compounds into pyrimidines is inefficient (27). Evidence concerning the degree of inhibition of aspartate transcarbamylase produced in animals by PALA treatment was obtained by Yoshida et al. (27), who found that the specific activity of aspartate transcarbamylase in extracts of spleens from animals treated with PALA was only 20% of that found in control mouse spleen. The residual activity was considered an artifact. Since there are many difficulties in extrapolating in vitro enzyme assays to the in vivo situation, we have taken some alternative approaches to measure the effectiveness of PALA inhibition in vivo.

Inhibition of orotidylic decarboxylase by the antibiotic pyrazofurin leads to accumulation of the uridine precursors, orotate and orotidine, and excretion of these compounds in the urine of patients treated with this antibiotic (3, 17). We have developed methods for the rapid quantitation of these compounds in biological samples. Since these compounds are known to arise only via de novo synthesis, they can serve as a measure of this process in the animal. Evidence for limited inhibition at therapeutically effective doses in normal tissues but a marked effect on a sensitive tumor, Lewis lung carcinoma, has been obtained. We have also measured the effects of PALA on the pyrimidine nucleotide pools of tissues. The expected consequence of inhibition of synthesis would be depletion of these pools in vivo as had been demonstrated in vitro. Here again, the normal tissues showed minimal depletion while the Lewis lung tumor had significantly reduced pools of these nucleotides. This combination of techniques affords a means for the analysis of PALA actions in vivo. Portions of this work have been reported previously (16).

MATERIALS AND METHODS

Drugs and Chemicals. Nucleosides, nucleotides, and orotic acid were purchased from Sigma Chemical Co. (St. Louis, Mo.) or P-L Biochemicals (Milwaukee, Wis.). Pyrazofurin was kindly supplied by Eli Lilly and Co. (Indianapolis, Ind.). PALA [NSC 224131; Lots He21-84-1 and MK2-73-1] was supplied by Dr. Harry Wood of the National Cancer Institute and prepared daily in sterile 0.9% NaCl solution. All drugs were sterilized by filtration through 0.22-μm Millipore filter.

Animals, Tumors, and Cell Cultures. Female BALB/c X DBA/2 F₁ (hereafter called CD2F₁) mice were purchased from Charles River Laboratories (Cambridge, Mass.). C57BL/6 and C57BL X DBA/2 F₁ (hereafter called BD2F₁) mice were from The Jackson Laboratory (Bar Harbor, Maine). The mice were fed Purina laboratory chow ad libitum. The Lewis lung tumor was passaged by trocar in C57BL mice as a s.c. implant. L1210 cells were grown in Fischer’s medium with 10% horse serum (Grand Island Biological Co., Grand Island, N. Y.) as a suspension at 37°C in an incubator containing 5% CO₂. Lewis lung cells were grown in 25-sq cm plastic flasks (Costar,
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Cambridge, Mass.) in a modified minimal essential medium (Eagle's) (24 containing 10% fetal bovine serum (Grand Island Biological Co.). Serum contained <4 μM uridine which gave <0.4 μM uridine on dilution in medium.4

Determination of Urinary Excretion. Urinary excretion of pyrimidine precursors was determined by housing animals in individual metabolic cages with access to 2% sucrose in water. The collected urine and cage washes were filtered through a 0.22-μm filter and used directly for chromatography in System 1 as described below.

Tissue Levels of Orotate, Orotidine, and Total Acid-soluble Uracil and Cytosine Nucleotides. Tissues for analysis were placed in liquid N2 immediately after cervical dislocation and dissection. The frozen tissues were homogenized at 4°C in 5 to 10 volumes of 1.0 M perchloric acid using a glass-Teflon homogenizer. The acid-insoluble material was removed by centrifugation, and a 1.0-ml aliquot of the acid-soluble extract was placed in a water bath at 100°C for 14 min. Under these conditions, the pyrimidine nucleoside polyphosphates and coenzymes are converted to UMP and CMP, and the purine nucleotides are deproteinized to yield adenine and guanine which chromatograph near the void volume in the chromatographic system described below. Orotate is stable to this treatment, but orotidine is about 60% hydrolyzed to orotidine. After hydrolysis, the solution is adjusted to pH 3 to 4 with KOH; the potassium perchlorate is removed, and the sample was filtered before chromatography in System 3 described below. The Lewis lung cells were grown in culture were centrifuged in tabletop centrifuge at 5°C for 7 min. The medium was removed, and the cell pellet was extracted with 1 ml of 0.5 M HC104. The suspension was centrifuged at 5°C to remove the protein precipitate, then neutralized with 10 N KOH, and stored frozen. The KClO4 precipitate was removed, and the sample was filtered before chromatography in System 2 described below. The Lewis lung cells were grown in 150-sq cm flasks. The medium was decanted, and 10 ml of ice-cold 0.5 M HC104 were added. The flask was kept on ice with occasional gentle rocking for 10 min before the acid solution was removed. This extraction was then repeated, the two 10-ml portions were combined, and the protein was removed by centrifugation. The supernatant was neutralized with KOH, the KClO4 was removed, and the sample was then lyophilized. The sample was reconstituted to 1.8 ml with System 2 buffer, and the insoluble material was removed and chromatographed in System 2 described below.

High-performance Liquid Chromatograph. Chromatography was performed with a system consisting of an Altex Model 100 pump, a Partisil PXS 10/25 SAX anion exchange column (Whatman, Inc., Clifton, N. J.) equipped with a guard column, 2 Altex Model 153 UV spectrophotometers monitoring 254 and 280 nm, and a linear Instrument Corporation Model 485 dual channel recorder. In some experiments, a WISP F10 automatic sample injector (Waters Associates) was used to permit continuous overnight operation. Peaks were identified by retention times and absorbance factors were determined daily. All buffers were filtered through a 0.2-μm filter before use. The following buffer systems were used. System 1 consisted of 0.05 M sodium formate, pH 4.1, 1.0 ml/min. Under these conditions, free purines elute in <5 min as do uric acid, pyrazofurin, and uridine. The retention times of the compounds of interest were: orotidine, 9.8 min; orotate, 11.4 min; CMP, 19.7 min; and UMP, 26 min. An example of a separation of orotate and orotidine in a urine sample is given in Chart 1A. System 2 consisted of 0.4 M sodium phosphate (Baker), pH 3.3, 1.5 ml/min. In this system, the following retention times were observed: CTP, 13.5 min; UTP, 16.5 min; ATP, 19.5 min; and GTP, 37.5 min. System 3 consisted of 0.05 M formate and 0.001 M sodium phosphate, pH 4.1, 1.0 ml/min. In this system, CMP and UMP had retention times of 13.2 and 18.3 min, respectively; this system could be used for more rapid quantitation than System 1 when orotidine and orotidine were not present. An example of the separation which can be obtained using biological samples is given in Chart 1B.

RESULTS

Orotate and orotidine can be easily and rapidly quantitated by the procedure described in "Materials and Methods." The urinary excretion of orotic acid and orotidine by mice treated with pyrazofurin is dose dependent and reaches a maximum at pyrazofurin doses of >10 mg/kg (Chart 2). This excretion decreases with time but is still above normal 6 days after treatment as shown in Chart 3. Since excretion of orotic acid and orotidine by mice treated with 0.9% NaCl solution is below the level of detection (<0.08 μmol/day), a partial inhibition of orotidylic decarboxylase is indicated even 6 days after a single dose of pyrazofurin (10 mg/kg).
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mylase is rapid in onset and long lasting. Nonetheless, we performed urinary excretion studies on mice pretreated with PALA for 1 hr before pyrazofurin administration, and we also made 6-hr urine collections (data not shown). These experiments revealed a similar degree of inhibition of orotate excretion as was observed in the 24-hr collections in which the 2 drugs were given simultaneously. In BD2F1 mice commonly used for antitumor studies with PALA, pyrazofurin induced excretion of orotate, and orotidine was reduced 50% after treatment with PALA (50 mg/kg), a result similar to that seen with CD2F1 mice.

The duration of inhibition by PALA was examined by treating mice with PALA (500 mg/kg) and then with pyrazofurin at various times thereafter (Chart 5). The inhibition produced by PALA was still significant on Day 3, but orotidine and orotate excretion induced by pyrazofurin returned to that seen in

Administration of uridine to replete pools of pyrimidine nucleotides reduces but does not eliminate excretion of orotic acid and orotidine. Mice treated with pyrazofurin (10 mg/kg) excrete a total of 23 μmol of orotate and orotidine in the 24 hr after treatment. This is reduced to 13 μmol by a single i.p. injection of 2.4 g of uridine per kg and to 8.7 μmol by a series of 4 s.c. injections of 3 g of uridine per kg. Using the techniques described in "Materials and Methods," we found that 3 hr after a s.c. injection of 3 g of uridine per kg the uracil nucleotide content of liver increased from 1.3 ± 0.1 (S.D.) in controls to 2.9 ± 1 μmol/g in the treated animals.

The excretion of orotate and orotidine after pyrazofurin treatment also affords a convenient measure of inhibition of prior steps in pyrimidine synthesis de novo by such compounds as PALA. Mice treated with pyrazofurin (10 mg/kg) and simultaneously given PALA (50 mg/kg) show a 55% decrease in orotic acid excretion relative to pyrazofurin-treated controls. Even 10 times this dosage (500 mg/kg) inhibited orotate and orotidine excretion only 80% (Chart 4). Studies by Yoshida et al. (27) suggest that PALA inhibition of aspartate transcarboxylase

Chart 2. Orotate and orotidine excretion after pyrazofurin treatment. CD2F1 mice were treated with pyrazofurin by i.p. injection, and urine was collected over the subsequent 24 hr. Orotate (□) and orotidine (△) were determined as described in "Materials and Methods"; the sum is also shown (○). Each point is the average of determinations on 4 to 8 mice.

Chart 3. Time course of orotate and orotidine excretion after pyrazofurin treatment. CD2F1 mice were treated with pyrazofurin (10 mg/kg i.p.) on Day 1. Twenty-four-hr urine collections were made for 5 days, and orotate and orotidine were determined as described in "Materials and Methods." Excretion in mice treated only with 0.9% NaCl solution was <0.08 μmol/day. Each value is the average of 4 mice.

Chart 4. Effect of PALA treatment on pyrazofurin-induced orotate and orotidine excretion. CD2F1 mice received pyrazofurin (10 mg/kg) and the indicated dose of PALA simultaneously by i.p. injection. Urinary excretion of orotic acid and orotidine over the subsequent 24 hr was quantitated as described in "Materials and Methods." Each point is an average of the sum of orotate and orotidine in 8 to 14 mice. Bars, S.E.

Chart 5. Time course of PALA inhibition of orotate and orotidine excretion induced by pyrazofurin. Ten CD2F1 mice received PALA (500 mg/kg i.p.) on Day 1. On this day and on each day of the next 4 days, 2 mice were given pyrazofurin (10 mg/kg) after which 24-hr urinary excretion of orotate and orotidine was determined as in "Materials and Methods." The results from 2 experiments were combined; each point represents data from 4 mice. In these experiments, control mice treated with pyrazofurin but no PALA excreted 24 μmol of orotate and orotidine in the subsequent 24-hr period.
pyrazofurin-treated controls by the fifth day after PALA treatment.

Since tissues such as spleen and solid tumors are very poorly permeable to orotate and orotidine, it seemed possible that high concentrations of these compounds would be found after pyrazofurin treatment. Chart 6 shows this occurred in both spleen and Lewis lung tumor 3 hr after treatment with pyrazofurin. Neither tissue normally contains these compounds in measurable amounts (<0.03 μmol/g). PALA treatment as either a single dose or a 3-day regimen is only partially effective in preventing orotate and orotidine accumulation in spleen. In contrast, PALA was highly effective in preventing accumulation of orotate and orotidine in the Lewis lung tumor.

PALA inhibits the growth of both L1210 and Lewis lung cells in culture, but higher concentrations are required to inhibit the leukemia as had been previously reported (Chart 7; Ref. 11). PALA (1 mM or 0.3 mM) reduced the UTP and CTP pools of L1210 cells by >50% in 3 hr (data not shown). Lower doses (0.1 mM) produced <20% depletion of pools at 6 hr but depleted pools by 60% at 10 hr of treatment. Examination of nucleotide pools after 24 hr of exposure to PALA showed that in both L1210 and Lewis lung cells UTP and to a lesser degree CTP pools were depleted (Chart 8). Quantitatively, however, Lewis lung cells showed reduced pools even at 5 μM PALA, whereas L1210 pools were only slightly perturbed by 20 μM PALA. In both cell lines, complete growth inhibition required depletion of pools by more than 50%. Comparison of Chart 7 with Chart 8 reveals that a 50 to 70% reduction of pools in L1210 cells gave only a moderate decrease in growth, and growth at 50% of control rate was observed in Lewis lung cells with pyrimidine pools depleted by 60 to 80%.

PALA produced a striking depletion of total acid-soluble pyrimidine nucleotides in 24 hr in Lewis lung tumors in vivo even at doses as low as 6.25 mg/kg (Chart 9). A maximal depletion of 75% of normal uracil nucleotides was seen over the range of 25 to 500 mg/kg; decreases in cytosine nucleotides showed a similar dose dependence. Within 3 hr of PALA treatment (500 mg/kg), a 66% depletion of uracil nucleotide pools was observed. In contrast to the dramatic decreases seen in Lewis lung tumor, host spleen and liver experienced no depletion even at doses of 500 mg/kg (Chart 9). Similar experiments were also performed in non-tumor-bearing CDF mice, and PALA (500 mg/kg) produced only a minimal (<20%) reduction of either cytosine or uracil nucleotides in liver, spleen, or lung at 3 or 24 hr after PALA treatment.

DISCUSSION

The extensive and prolonged excretion of orotate and orotidine after administration of pyrazofurin has been demonstrated in the mouse (Charts 2 and 3) and reported elsewhere in patients undergoing therapy (3, 17). Since excretion in untreated mice is negligible and these compounds arise only via the pyrimidine-biosynthetic pathway, this excretion serves as a minimal estimate of pyrimidine production in the mouse. It is minimal because some conversion to UMP doubtless does
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Chart 9. Effect of PALA on total acid-soluble pyrimidine nucleotides in Lewis lung tumor, spleen, and liver of C57BL mice. Mice bearing tumors implanted days earlier (~0.5 to 1 g) were treated with the indicated doses of PALA by i.p. injection. Control mice received 0.9% NaCl solution. At 24 hr, mice were sacrificed, acid-soluble extracts were prepared, and LiMP (•) and CMP (○) were analyzed in chromatographic System 3 as described in “Materials and Methods.” Data from 2 experiments are combined, and each point is the mean ± S.E. of 4 to 10 animals.

Relations to decrease biosynthesis reflect the extensive breakdown of uridine to uracil by the liver (4, 22, 26).

Relatively large doses of PALA are necessary to achieve extensive inhibition of overall pyrimidine synthesis in the mouse (Chart 4). Even a 500-mg dose of PALA per kg allows synthesis to continue at 20% of the control rate. Doses of 50 and 20 mg/kg, reported curative in chemotherapy of Lewis lung carcinoma (11), produced only 35 and 10% inhibition of synthesis, respectively; thus, therapeutic effectiveness can be obtained without extensive inhibition of pyrimidine biosynthesis in host tissues. Most other responsive tumors require doses of 200 to 500 mg/kg (11) which produce more extensive inhibition in normal tissues and, therefore, a poorer therapeutic index. The inhibition by PALA persists 4 days after a single injection as measured by this method. Other methods have also shown a long duration of action (27).

The effectiveness of PALA against Lewis lung tumor at doses which only slightly depressed total synthesis in mice suggested a selective effect. Evidence for this selectivity is seen in Chart 6. Although PALA (500 mg/kg) produced only ~50% inhibition of orotidine accumulation in the spleen, it was highly effective in the tumor. We have not been able to extend this technique to the liver probably because a highly effective biliary excretion mechanism exists that rapidly eliminates orotate via the bile (7). In other studies, we have found that PALA inhibits orotate production in the liver of pyrazofurin-treated rats by 75 to 87% at a dose of 100 mg/kg.5 The data comparing spleen and tumor show that a selective inhibition can be obtained although the mechanism remains to be explained.

PALA has been shown to deplete UTP pools of cultured hepatoma cells (12) and transformed hamster cells (11). In a preliminary communication, Brockman et al. (1) report depletions of UTP and CTP pools in P388/ara-C-resistant cells and colon carcinoma 26 after PALA treatment in vivo. The studies here extend the results in culture and present new data concerning the effects on pools of normal and neoplastic tissues in vivo as related to growth inhibition.

PALA is more potent as an inhibitor of growth of Lewis lung cells than of L1210 (Chart 7), as has been reported elsewhere (11). This is also reflected in the different concentrations required to deplete pools in the 2 cell lines (Chart 8). Both cell lines show similar changes at high concentrations of PALA, i.e., marked decreases in UTP and CTP pools with increases in the pools of ATP and GTP. Similar increases in ATP levels were seen in L5178Y cells when pyrimidine synthesis was inhibited by 6-azauridine (9). Conversely, inhibition of purine biosynthesis by 6-mercaptopurine riboside led to decreases in ATP and GTP and increased concentrations of UTP and CTP (25). Comparison of the effects on growth with effects on pools shows that decreases of 50% in UTP pools appeared to result in only minor decreases in growth rate in these cells (Charts 7 and 8). It is not surprising that a substantial decrease must be produced in order to slow growth since the Kₗ of RNA poly-erase appears to be 10-fold lower than the concentrations normally found in tissues (5). Experiments to quantitate the effect of PALA on deoxynucleotide pools are in progress.

The effect of PALA on pyrimidine nucleotide concentrations of spleen, liver, and lung is minimal at 3 and 24 hr even after doses of 500 mg/kg. In contrast, Lewis lung carcinoma cells

5 J. D. Moyer, J. T. Oliver, and A. E. Handschumacher, unpublished data.

occur although the extent of this conversion is not known. The excretion of orotate and orotidine has been used as a measure of inhibition of pyrimidine synthesis by PALA and uridine. Uridine can reduce urinary excretion of orotate and orotidine of spleen, liver, and lung is minimal at 3 and 24 hr even after doses of 500 mg/kg. In contrast, Lewis lung carcinoma cells...
experience a decrease of >70% at doses as low as 12.5 mg/kg at 24 hr. Both uracil and cytosine nucleotides are depleted in this tumor. This selective effect could represent a slower rate of depletion in the slowly growing tissues as well as a difference in the degree of inhibition achieved as shown in Chart 6. The depletion of tumor pools after doses of 12.5 mg PALA per kg is particularly notable since the total capacity of the animal to produce pyrimidines is unaffected (Chart 4).

Brockman et al. (1) have reported in a preliminary communication that PALA (400 mg/kg) can deplete UTP and CTP pools by 80% in P388 tumors in vivo and that similar depletions occur in colon carcinoma 26. Earlier studies by this group showed that pyrazofurin produced a maximum 80% depletion of uracil nucleotides in colon carcinoma 38 (2). These studies along with ours suggest that a reduction of the concentration of pyrimidine nucleotides below 20% of normal is very difficult to achieve in vivo. Microscopic examination of the tumor samples shows a high degree of cellularity with minimal lymphatic tissue or connective tissue, but the residual pools of pyrimidine nucleotides may be associated with nontumorous tissue.

Another factor complicating consideration of pool depletion after inhibition of synthesis de novo is salvage of circulating pyrimidines. It has been suggested that circulating pools of pyrimidines exist (8, 18, 20) and that the liver may serve as a source of circulating pyrimidines (14); evidence to the contrary has also been presented (19). Competition for this circulating nucleoside may be a factor in maintenance of pools in some tissues. We have found that the plasma concentration of uridine in mouse and rat is quite low (<2 μM), and investigation of the possible physiological role of this material is underway in our laboratory.

The current study has shown that PALA can inhibit synthesis and deplete pools in Lewis lung tumor at doses that are virtually without effect on total synthesis in the animal and have no effect on pyrimidine pools in liver, spleen, or lung. The mechanism of this selectivity is not known, but it has been suggested by Johnson et al. (11) that the Lewis lung tumor is sensitive by reason of relatively low levels of aspartate transcarbamylase. It remains to be shown whether RNA synthesis, DNA synthesis, or reactions involving pyrimidine nucleotide coenzymes become the limiting process for growth when nucleotide concentrations fall to the levels indicated here.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Frank Schabel of the Southern Research Institute for his gift of the Lewis lung cells and Dr. R. W. Brockman for sending a copy of his paper before publication. We thank Robert Dreyer for assistance in the development of the chromatographic systems described here and Connie Lehman for assistance with tissue culture. The efforts of Paula Sullivan and Arlene Cashmore in preparation of the manuscript are greatly appreciated.

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