ABSTRACT

Doses of N-(phosphonacetyl)-L-aspartic acid (PALA) lower than those required for therapeutic activity against the spontaneous murine breast tumor (BALB/c x DBA/8 F1) were found to produce significant depression in uridine triphosphate pools in the tumor. Using such low, nontherapeutic, but biochemically active doses of PALA in combination with 5-fluorouracil (FUra), it was possible to maintain the dose of FUra at its full maximum tolerated single agent dose. In comparison with the maximum tolerated dose of FUra alone, the combination of FUra plus low-dose PALA produced a significant increase in the level of tumor FUra-containing RNA, only a slight increase in intestinal FUra-containing RNA, and no increase in bone marrow FUra-containing RNA. These biochemical results correlated with the therapeutic findings of significantly increased antitumor activity without an increase in host toxicity. A review of currently reported PALA plus FUra clinical protocols reveals that the experimental parameters which have produced successful therapeutic results in the laboratory (i.e., a low-PALA:high-FUra dosage ratio) have not yet been translated into clinical trial. Final judgment on the clinical efficacy of PALA:FUra combinations must await the results of proposed low-PALA:high-FUra clinical trials.

INTRODUCTION

PALA inhibits ACTase, the second enzyme in the de novo pathway for the biosynthesis of pyrimidines, by competing with its natural substrate, carbamyl phosphate (10, 29, 30, 51). Thus, when PALA is administered prior to FUra, the resulting decrease in UTP pools (6, 18, 26, 44, 45, 51, 52) allows greater utilization of 5-fluorouracil 5'-triphosphate by RNA polymerase, resulting in increased incorporation of FUra into tumor RNA (3, 32, 33, 36, 39-41) and enhanced antitumor activity in several animal tumor model systems (3, 23, 32, 33, 36, 39-41). In marked contrast to the potentiating effect of PALA on the antitumor activity of FUra in preclinical studies (24, 25, 39-41), the results of pilot clinical trials against advanced cancer with PALA:FUra combinations thus far have been disappointing as compared to historical controls receiving FUra alone (5, 14, 42, 46).

However, in all of these clinical studies, the dose of FUra administered to patients receiving the combination of PALA plus FUra was significantly lower than the dose of FUra administered as a single agent in historical studies used as reference controls. The fundamental scientific principle underlying comparative evaluation of alternative treatments in 2 groups of patients is that the groups must be alike in all important respects and differ only in the treatment that each group receives. Thus, in order to properly evaluate whether the combination (i.e., PALA plus FUra) is more effective than a maximally tolerated course of FUra as a single agent (as recorded in historical controls), the dose of FUra administered in the combination treatment should be at, or close as possible to, the dose used in the historical studies. This has not yet been accomplished.

In historical studies, the maximally tolerated dose of FUra when used as a single agent was 15 to 20 mg/kg (600 to 800 mg/sq m) i.v. once weekly (4, 21) or 12 mg/kg (480 mg sq/m) i.v. daily for 5 dosages followed by 6 mg/kg on alternate days until toxicity, followed by weekly doses of 15 mg/kg (1). However, in published clinical studies of PALA:FUra combinations (5, 14, 42, 46), relatively high doses of PALA were used, and, under this condition of PALA administration, patients only tolerated much less than the optimal historical doses of FUra. For example, in a PALA:FUra trial which initiated 2 FUra dose schedules, the initial doses of FUra (480 mg/sq m weekly and 400 mg/sq m/day daily for 5 dosages) were reduced by one half in 80% of patients because of intolerable toxicity (5). It was concluded in another study that the maximum tolerated dose of the PALA:FUra combination was approximately two-thirds of the maximum tolerated dose of each agent when used alone (42), and a more recent report (14) recommended only 200 mg FUra per sq m per day in combination and for only 4 days every 3 weeks.

Low doses of PALA may permit safe combination with high doses of FUra. If so, the low level of PALA's target enzyme, ACTase, in human tumors (35, 43, 53) and the fact that low levels are an important determinant of biochemical sensitivity to PALA (22, 28, 30, 34) suggest that low doses of PALA might inhibit this enzyme, thereby lowering the pyrimidine pools, and selectively augment the antitumor activity of maximally tolerated doses of a pyrimidine antagonist such as FUra. It therefore seems reasonable that low doses of PALA might permit therapeutically effective combination with high doses of FUra (i.e., maximally tolerated doses of FUra alone), but such dosage ratios have not yet been explored in the clinic in FUra-naive cancer patients.

In the laboratory, low-PALA:high-FUra dosage ratios were found equally active as high-PALA:low-FUra dosage combinations. However, these low doses of PALA were still capable of exerting anticancer activity when used alone against experimen-
The difference between the total and alkali-stable radioactivity was determined to determine alkali-stable, trichloroacetic acid-precipitable radioactivity. Other samples were first treated with alkali (0.4 M NaOH for 90 min at 37°), and extracted with phenol:resol (7:1, v/v). Samples of this material were resuspended in 0.01 M Tris-HCl (pH 7.6):0.15 M NaCl:0.001 M EDTA containing 1% Triton-X 100. The homogenate was dissolved in 0.85% NaCl solution immediately before use. PALA (mg/kg) was dissolved in 0.85% NaCl solution; the pH was adjusted to 7.2 to 7.5 with 1 N NaOH before adjustment to final volume. Both drugs were administered i.p. in a volume of 0.1 ml/10 g of mouse weight. [3H]FUra was obtained from Moravek Biochemicals.

**MATERIALS AND METHODS**

**Murine Tumor System.** Two- to 3-month-old male or female BALB/c x DBA/8 F1 (hereafter called CD8F1) mice bearing advanced (approximately 100 mg) first generation transplants of syngeneic, spontaneous breast tumors were used (37, 38). For each experiment, available tumors from a single transplant were measured, and the mice were distributed among the experimental groups of 10 mice each so that animals carrying approximately equal sized tumors were represented in each group. For the spontaneous tumor-bearing experiments (Table 1), the methodology was identical except that the CD8F1 spontaneous tumors initially averaged 200 to 300 mg, and approximately 14 mice were distributed into each experimental group. These female CD8F1 mice averaged 9 months in age.

**Drugs and Chemicals.** FUra and PALA were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. FUra was dissolved in 0.85% NaCl solution immediately before use. PALA was dissolved in 0.85% NaCl solution; the pH was adjusted to 7.2 to 7.5 with 1 N NaOH before adjustment to final volume. Both drugs were administered i.p. in a volume of 0.1 ml/10 g of mouse weight. [3H]FUra was obtained from Moravek Biochemicals.

**Tumor Measurements.** Two axes of the tumor (the longest axis, L, and the shortest axis, W) were measured with the aid of a vernier caliper 3 to 4 days after each course of treatment. Tumor weight was estimated according to the formula

\[ \text{Tumor wt (mg)} = \frac{L \times W}{2} \]

**Statistical Evaluation.** The Student t test was used for statistical evaluation of differences in mean tumor size between groups of treated mice. Differences between groups with a statistical probability of 0.05 or less were considered significant.

**Toxicity Measurements.** Animal body weights were recorded immediately before and biweekly after the initiation of treatment. Weight change was calculated as a percentage of the animal's initial body weight.

**Incorporation of [3H]FUra into RNA.** Tumor-bearing CD8F1 mice received the indicated dose of PALA (i.p.) 24 hr prior to [3H]FUra (50 mg/kg, 80 mCi/kg). After a 1- or 2-hr labeling period, the animals were sacrificed by cervical dislocation. Bone marrow was collected by flushing the femur with ice-cold 0.85% NaCl solution. Intestinal mucosa was collected by flushing an 8- to 10-cm section of small intestine with ice-cold 0.85% NaCl solution, slitting it open, and scraping off the lining cells with a glass microscope slide.

**Tumor Tissues.** Tumor tissues were homogenized in 0.01 M Tris-HCl (pH 7.6):0.15 M NaCl:0.001 M EDTA containing 1% Triton-X 100. The homogenate was treated with sodium dodecyl sulfate, sonicated, digested with Pronase for 60 min at 37° (0.2 mg/ml, predigested for 2 hr at 37°), and extracted with chloroform:isoamyl alcohol (24:1, v/v). Bone marrow and intestinal mucosa were processed the same way except processing began with suspending the pellets in 0.01 M Tris-HCl (pH 7.6):0.15 M NaCl:0.001 M EDTA followed by sonication. In some experiments, samples were also extracted with phenol:creosol (7:1, v/v). Samples of this material were precipitated with trichloroacetic acid to determine total radioactivity. Other samples were first treated with alkali (0.4 M NaOH for 90 min at 37°) to determine alkali-stable, trichloroacetic acid-precipitable radioactivity. DNA content was measured by the diphenylamine color reaction. The difference between the total and alkali-stable radioactivity was assumed to represent radioactivity in RNA.

**Tumor UTP Measurements.** Tumor-bearing CD8F1 mice received PALA i.p. 24 hr prior to sacrifice. Animals were anesthetized with sodium pentobarbital. As soon as the animal lost consciousness, the tumor was removed and immediately homogenized in 2 ml of ice-cold 1.2 n perchloric acid. After centrifugation of the homogenate, the supernatant containing the acid-soluble molecules was treated with 1.5 ml K2HPO4 to remove perchlorate and then filtered through a 0.22-μm Milipore filter prior to high-pressure liquid chromatography analysis. The precipitate pellet was washed with cold 0.6 n perchlorate and dissolved in 1.0 ml NaOH, and protein content was determined by the Lowry procedure. High-pressure liquid chromatographic analysis of nucleotides was done with a Du Pont Model 850 system using a Spectra-Physics calculating integrator. Nucleotides were separated isocratically using a Du Pont C8 column and a buffer consisting of 0.1 M KH2PO4 in 40 mw tetrabutylammonium hydrogensulfate (Aldrich Chemicals), 2 ml/min at 45°.

**RESULTS**

**Selection of a Therapeutically Synergistic Low PALA:High FUra Dosage Ratio.** The activity of PALA as a modulator was evaluated by examining the effect of PALA treatment of various doses upon the UTP pools of the CD8F1 breast tumor (Chart 1). In these experiments, UTP was measured 24 hr after PALA administration. Although the marginally effective therapeutic dose of PALA is 200 mg/kg in the CD8F1 breast tumor, nontherapeutic doses lower than 200 mg/kg were still able to decrease tumor UTP pools. For example, PALA at 100 mg/kg causes a 40% depletion of UTP pools (Chart 1). This low dose of PALA as a single agent lacks therapeutic activity against the advanced CD8F1 murine mammary tumor (Chart 2).

In the clinic, PALA is therapeutically ineffective. We therefore examined in advanced CD8F1 tumors whether a biochemically active, but therapeutically ineffective dose of PALA (100 mg/kg) could be administered safely in combination with FUra at its maximum tolerated dose alone (100 mg/kg). FUra alone at 100 mg/kg has therapeutic activity against these tumors, and the low-PALA (100 mg/kg):high-FUra (100 mg/kg) dosage combination produced a synergistic antitumor effect (p = <0.05) against this FUra-sensitive mammary tumor with acceptable toxicity (≠10% lethal dose) in the 179 mice summarized in Chart 2.

Since the incorporation of FUra (2) into RNA has been causally
associated with cytotoxicity in this tumor system (52, 53), we examined the effect of various doses of PALA administered 24 hr before FUra on the incorporation of FUra into RNA in the breast tumor, intestinal epithelium, and bone marrow. Results (Chart 3) indicated that in bone marrow, PALA did not affect the level of (FUra)RNA regardless of the PALA dosage administered. This negative result is consistent with the lack of PALA hematoxinity in mice reported by Harrison et al. (19). In contrast, the latter investigators reported a PALA-induced gastrointestinal toxicity, and in agreement with this finding, Chart 3 records that PALA augmented gut levels of (FUra)RNA. It is pertinent that substantial elevations of (FUra)RNA in intestine were seen only with PALA dosages of 200 mg/kg or higher, whereas PALA at 100 mg/kg caused only a slight elevation in the gut (FUra)RNA.

In contrast, the latter dose of PALA produced appreciable elevation of (FUra)RNA in the tumor.

In order to confirm antitumor results from the CD8F, first-generation tumor transplant experiments, which indicated that low-dose PALA could be therapeutically synergistic when combined with FUra at its maximum tolerated dose, we examined this combination in CD8F, mice bearing spontaneous, autochthonous breast tumors. Pooled results from 4 identical experiments are presented in Table 1. (Note that FUra was administered at a dose of 88 mg/kg in these experiments, which is the maximum tolerated dose in the spontaneous tumor-bearing CD8F, mice. This dose is slightly lower than the 100-mg/kg dose which is the maximum tolerated dose in the younger mice used in the CD8F, first-generation tumor transplant experiments.)

As indicated in Table 1, FUra alone (Group 2) was effective against the spontaneous tumors, and after 3 courses of weekly treatment, tumors had grown from an initial mean size of 270 mg to a mean size of only 579 mg, compared to 2548 mg in the 0.85% NaCl solution-treated controls (p = <0.001). FUra also produced a small, 7 of 55 (13%), but significant (p = <0.05) number of partial tumor regressions, and mortality was only 2 of 55 (4%) after 3 courses of treatment. In Group 3, the administrations of low-dose PALA:high-dose FUra combination against spontaneous, autochthonous CD8F, breast tumors

55 (4%) after 3 courses of treatment. In Group 3, the administration of low-dose PALA the day before each course of FUra at its maximum tolerated dose resulted in a striking increase in the therapeutic index. Mean tumor weight was reduced from a pretreatment level of 270 mg to 195 mg in mice treated with PALA plus FUra, which is to be compared with 579 mg in mice treated with FUra alone (p = 0.001). Pertinently, in terms of clinical application of this drug combination, the number of partial tumor regressions was increased from 7 of 55 with FUra alone to 31 of 55 with the PALA plus FUra combination (p = 0.001). Moreover, this was accomplished without any increase in mortality (2 deaths of 55 mice treated with FUra alone versus no deaths of 55 mice treated with PALA plus FUra).

**DISCUSSION**

The data presented here reveal that a biochemically selected, nontoxic, nontherapeutic dose of PALA, chosen on the basis of demonstrated biochemical effects at that dose in the tumor cells, can favorably ‘‘modulate’’ activity of FUra as an effective antitumor agent.
Based on the concepts emerging from these murine studies, we believe that clinical trials with the PALA:FUra combination should be conducted using the lowest dose of PALA which effectively interferes with pyrimidine biosynthesis along with the highest tolerable dose of FUra. A means of estimating this PALA dose in vivo was developed by Moyer and Handschumacher (44) in mice. The method is based on the fact that normally very small amounts of orotic acid and orotidine are excreted in the urine. Administration of PZF, an inhibitor of a late step in pyrimidine biosynthesis, results in a marked increase in the urinary excretion of these compounds (47). This observation can be used to assess the dose of PALA necessary to block the pathway of biosynthesis of pyrimidines. A dose which effectively blocks the PZF-induced excretion of orotic acid and orotidine presumably is adequate to inhibit whole-body pyrimidine biosynthesis. The minimum dose which achieves this inhibition is the dose of PALA desirable for use with FUra in patients with cancer. Although this test identifies doses of PALA that inhibit whole-body pyrimidine biosynthesis, since all PALA-sensitive laboratory tumors have possessed low ACTase activity, the lowest PALA dose identified by this test in patients similarly should be capable of inhibiting tumor ACTase in human tumors.

Several doses of PALA were evaluated clinically using this approach (9). Although the maximum tolerated dose of PALA when used as a single agent is approximately 4500 mg/sq m i.v. weekly (15), a dose of PALA (250 mg/sq m) adequately suppressed PZF-induced orotic aciduria and orotidineuria (9). This dose is far lower than the dose of PALA used in any of the above-mentioned clinical studies of PALA:FUra combinations. Furthermore, the inhibition with this low dose lasts for at least several days. As opposed to those trials where higher doses of PALA were administered, we have been able to give full doses of FUra (750 mg/sq m) weekly, 24 hr after PALA (250 mg/sq m), with acceptable toxicity (9). This low-dose PALA:full-dose FUra regimen now awaits clinical evaluation to assess its antitumor activity in comparison with historical results in FUra alone at its maximally tolerated dose.

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Therapeutic Utility of Utilizing Low Doses of N-(Phosphonacetyl)-l-aspartic Acid in Combination with 5-Fluorouracil: A Murine Study with Clinical Relevance


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