[CANCER RESEARCH 49, 4636–4639, August 15, 1989]

Phase I Trial of *N*-(Phosphonacetyl)-L-aspartate, Methotrexate, and 5-Fluorouracil with Leucovorin Rescue in Patients with Advanced Cancer[1]

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ABSTRACT

Based on an animal model to improve the antitumor activity of 5-fluorouracil (FUra), a Phase I study of *N*-(phosphonacetyl)-L-aspartate, methotrexate, FUra, and leucovorin was conducted on 44 patients. Methotrexate was given in an intermediate dose (250 mg/m^2) to overcome potential drug resistance, and *N*-(phosphonacetyl)-L-aspartate was given at a low dose (250 mg/m^2) in order to allow escalation of FUra to toxicity. These two drugs were given 24 h before FUra to enhance maximal incorporation of FUra into RNA. Two schedules of administration were used; one every other week and one weekly for 2 weeks. The every other week schedule was well tolerated, with minimal gastrointestinal and hematological toxicity. However, the weekly for 2 weeks schedule was more toxic with increased mucositis, diarrhea requiring therapy, and decreased performance status of 20% in 4 of 6 patients. There were no responders in the every other week schedule. There was one partial response and three patients with stable disease in four evaluable patients on the weekly for 2 weeks schedule. At 24 h post-*N*-(phosphonacetyl)-L-aspartate-methotrexate treatment, PRPP levels were doubled in bone marrow biopsies, and increased 2.5- to 25-fold in tumor biopsies. We have currently added uridine rescue to this combination with the hope of further escalating the dose of FUra.

INTRODUCTION

5-Fluorouracil is the most active chemotherapeutic agent for the treatment of advanced colorectal cancer, although the response rate is only 15–20% (1). One strategy to improve its antitumor activity is to biochemically modulate FUra[3] to make its antitumor activity more selective. FUra exerts its cytotoxic effect by at least two mechanisms: *a*) incorporation of FUra as FUTP into RNA, and *b*) inhibition of thymidylate synthetase (2–3). It is not intuitively obvious whether the DNA- or RNA-dependent mechanism of FUra activity correlates with its cytotoxicity in various carcinomas cell lines. Evans *et al.* (4–5) have shown that low levels of FUra cause growth inhibition of Sarcoma 180 cells (a mouse sarcoma line) by inhibiting thymidylate synthetase, while at high levels of FUra the incorporation of RNA seems to be dose limiting. In contrast, the growth inhibition of human Hep-2 cells by FUra is due to its incorporation into RNA.

PALA is an inhibitor of aspartate transcarbamylase, one of the initial enzymes in the *de novo* pathway for the biosynthesis of pyrimidines. Thus, when PALA is administered prior to FUra, the consequent decrease in UTP allows greater utilization of FUTP by RNA polymerase, resulting in increased incorporation of FUra into tumor RNA, and enhanced antitumor activity in several animal tumor systems (6). Aspartate transcarbamylase, the target enzyme for PALA, is in lower concentration in human and murine tumor tissues than in normal tissues (7); thus, a low, nontherapeutic, but still biochemically active dose of PALA may selectively and safely modulate the anticancer activity of a maximally tolerated dose of FUra in patients, as it does in tumor-bearing mice (8–9). Casper *et al.* (10) have demonstrated in patients that excellent inhibition of whole body pyrimidine synthesis can be achieved with a low weekly dose (250 mg/m^2) of PALA, allowing the safe administration of FUra at its weekly maximally tolerated dose 24 h after PALA.

MTX is a folate antagonist that binds to and inhibits dihydrofolate reductase, resulting in depletion of tetrahydrofolates and the inhibition of both thymidylate production and *de novo* purine synthesis. The latter inhibition leads to an accumulation of PRPP, a substrate which can be rate limiting in the activation of FUra to its active nucleotides; thus, the MTX-induced increased PRPP levels result in greater intracellular conversion of FUra into its nucleotide and therefore greater antitumor activity (11). Synergism between these two drugs depends both on proper sequence; *i.e.*, MTX and then FUra, and the interval (18–24 h) between the two agents (12–13). Given the above information, a Phase I study was conducted with PALA (at low dose) and MTX (at an intermediate dose) 24 h prior to FUra (dose escalated), followed by leucovorin rescue to determine the maximum tolerated dose and toxicity of FUra.

MATERIALS AND METHODS

Patients

Forty-four patients with advanced cancer were entered in this protocol. Criteria for entry in this study included histologically confirmed cancer (42 patients with colorectal adenocarcinoma and 2 with epidermoid cancer of the head and neck), disease not curable by radiation therapy or surgery, Karnofsky performance status >50, life expectancy of 8 weeks, WBC >3,500 × 10^9/liter, platelet count >130,000 × 10^9/liter, bilirubin <1.5 mg/dl, and creatinine <1 mg/dl, or creatinine clearance >65 ml min. Previously treated patients were eligible if they had not received myelosuppressive chemotherapy within the previous 4 weeks (6 weeks for nitrosoureas and mitomycin C). Patients were not put on study if they had received radiation therapy to major bone marrow-containing areas within 4 weeks.

Of the 44 patients entered, 38 received every other week therapy, and 6 received weekly therapy for 2 weeks. Throughout this paper, they will be referred to as group 1 and group 2, respectively. Table 1 describes base-line characteristics of our patients.

Base-line laboratory studies included complete blood count, platelet count, differential, biochemical screening profile, chest radiograph, carcinoembryonic antigen, creatinine, and 24-h creatinine clearance. Radionuclear liver scan, bone scan, and abdominal computed tomography were performed if clinically indicated.

PRPP Determination

To ascertain the biochemical effects of PALA and methotrexate as modulators of PRPP levels, biopsies of tumor tissue were taken prior

Received 11/14/88; revised 4/5/89; accepted 5/10/89.

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[1] This work was supported in part by USPHS Grants N01-CM57732 and PO1-CA25842 awarded by the National Cancer Institute, Department of Health and Human Services, and in part by the Chemotherapy Foundation of New York.

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[3] The abbreviations used are: FUra, 5-fluorouracil; FUTP, 5-fluorouridine triphosphate; PALA, *N*-(phosphonacetyl)-L-aspartate; MTX, methotrexate; PRPP, 5-phosphoribosyl-1-pyrophosphate; HPLC, high pressure liquid chromatography; FUMP, fluorouridine monophosphate.

Table 1 *Base-line characteristics of Phase I patients*

	Group 1 (median)	Group 2 (median)
No. of patients	38	6
Age	58	61
Sex	25 M; 13 F	4 M; 2 F
WBC	7.2	6.6
Platelets	325	209
Albumin	4.15	4.25
Alkaline phosphatase	120	108
Carcinoembryonic antigen	25.1	117
Lactate dehydrogenase	249	256
Phosphatidylserine	80	80
Prior therapy with FUra	36	5
Site of measurable disease		
Liver	7	1
Lung	15	1
Abdomen	4	1
Pelvic mass	0	1
Chest wall mass	1	0
Median no. of previous treatments with FUra-containing regimen	1 (range, 0–5)	1 (range, 0–4)

to the administration of these two drugs, and again approximately 24 h later just prior to the administration of FUra. Tumors were pulverized under liquid nitrogen, suspended in four volumes of extraction media (0.2 M Tris-HCl buffer, pH 7.4, 33 mM sodium fluoride, 0.5 mM 2,3-diphosphoglyceric acid, and 5 mM EDTA; Sigma Chemical Co., St. Louis, MO) and filtered through Amicon CF25 filter cones. An aliquot of the homogenate was assayed for protein content. The filtrates were stored at −70°C or immediately assayed for PRPP content. The assay, as described by Houghton *et al.* (14), is based on the conversion of [^{14}C] orotic acid (New England Nuclear, Boston, MA) to uridine monophosphate with release of $^{14}CO_2$ by orotidine-5-phosphate pyrophosphorylase plus orotidine 5-decarboxylase (Sigma Chemical Co.). The 200-μl incubation mixture contained 1.0 mM dithiothreitol, 5.0 mM magnesium sulfate, 0.312 mg enzyme freshly dissolved in 0.01 ml Tris-HCl buffer, 0.43 μCi [^{14}C]orotic acid, 2.5 mM Tris-HCl buffer, pH 7.4 (Sigma Chemical Co.). The incubation was carried out in scintillation vials. Absorbent paper (Whatman No. 3 MM 2.3-cm diameter circle) saturated with NCS tissue solubilizer (Amersham) was placed in the cap before closing. After a 1 h incubation at 37°C in a shaking water bath, 50 μl 60% perchloric acid were added, and the incubation continued at 37°C for 40 min. At the end of the second incubation, the filters were placed in 8.5 ml Hydrofluor (National Diagnostics) plus 1.0 ml water and 0.5 ml NCS tissue solubilizer to remove the counts from the surface of the filter into the scintillation fluid and the ^{14}C was counted. The results are expressed as pmol PRPP/mg protein for the tumors and pmol PRPP/10^6 cells for the human bone marrows. All tumor biopsies had microscopic confirmation of colorectal cancer.

HPLC Measurement of RNA FUMP

Nucleated cells from bone marrow aspirates were separated by Ficoll-Hypaque centrifugation. The cells were resuspended in 0.01 M Tris-HCl, 0.15 M NaCl, 0.005 M EDTA buffer (pH 7.4), counted, and extracted twice with phenol:cresol saturated with the same buffer and once with chloroform:isoamyl alcohol (24:1). Nucleic acids were recovered by ethanol precipitation. The ethanol precipitate was dissolved in 0.4 N NaOH and incubated at 37°C for 3 h. DNA was removed by the addition of excess perchloric acid. The acidfied alkaline hydrolysate was then placed in a boiling water bath for 15 min in order to convert purine nucleotides to purine bases. After cooling, the hydrolysate was neutralized by extraction with 3 volumes of a 1:2 mixture of trioctylamine in Freon. HPLC analysis of 2′,3′-FUMP was done using a C_{18} column and a buffer of 10 mM KH_2PO_4 in 5 mM tetrabutylammonium hydrogen sulfate, pH 2.7.

Dosage Schedules

Group 1 (Every Other Week Schedule). PALA was given as a rapid i.v. infusion on days 1 and 15 at 250 mg/m^2 followed by i.v. methotrexate, 250 mg/m^2 over 2 h. FUra was given as an i.v. bolus injection on days 2 and 16, 24 h after methotrexate. The initial dose of FUra was 300 mg/m^2 and was escalated by 50 mg/m^2 to 900 mg/m^2. Leucovorin, 10 mg p.o., was given every 6 h for eight doses starting 24 h after the methotrexate was given.

Group 2 (Weekly for 2 Weeks Schedule). PALA and methotrexate were given in the same doses and schedule as above, except now on 2 consecutive weeks. FUra was given as above on days 2 and 9. The initial dose of FUra was 600 mg/m^2.

In both scedules, cycles were repeated every 28 days. Three patients were entered at each dose level. At a dose producing significant toxicity, 6 patients were entered to further document its extent.

Methotrexate levels were measured in posttreatment plasma at 24 h. If the level was greater than 5×10^{-7} mm/liter, the patient returned for a 48-h methotrexate level and continued leucovorin at the same dose for an additional 2 days.

Patients with measurable disease were assessed for therapeutic response by the following criteria. Partial remission was defined as a 50% decrease in the sum of the product of the diameters of all measured lesions. A minor response is an objective decrease of disease in excess of 25% but less than that required for a partial remission. Stable disease was defined as a <25% measurable change in tumor size over a period of 90 days. Progressive disease is an unequivocal 25% increase in the size of any measured lesion or significant new lesion.

A minimum of one complete cycle of chemotherapy in the absence of toxicity, or fewer doses in the presence of dose-limiting toxicity, was required for a patient to be considered adequate for the Phase I dosage objective. Therapy was withheld if severe toxicity developed.

RESULTS

Toxic Effects (Every Other Week Schedule). The dose of FUra was begun at 300 mg/m^2 and escalated to 900 mg/m^2. In the initial part of this study chemotherapy was given every other week in order to minimize toxicity. All patients were evaluable for toxicity. Table 2 shows the fraction of patients experiencing myelosuppression. Myelosuppression was minimal or nonexistent at all dose levels of FUra up to 900 mg/m^2, with the exception of 4+ toxicity in one patient at the 600-mg/m^2 dose level of FUra, who died from neutropenia and sepsis 8 days after starting therapy. At 900 mg/m^2, 2 of 3 patients had grade 3+ myelosuppression. At all dose levels, gastrointestinal toxicity was minimal and easily controlled (see Table 2). Only 2 patients had mucositis requiring a liquid diet, 1 at the 600-mg/m^2 level, and 1 at the 700-mg/m^2 dose level. At the 500-mg/m^2 dose level, 1 patient experienced a decrease in creatinine clearance to less than 60 ml/min. Two patients experienced headache at the 600-mg/m^2 dose level of FUra.

Toxic Effects (Weekly for 2 Weeks Schedule). In an effort to intensify the regimen, the schedule was changed to weekly for 2 weeks. At 600 mg/m^2 there was mild myelosuppression. Two

Table 2 *Toxic effects of PALA plus MTX plus FUra plus leucovorin regimens*

	WBC			Nausea/vomiting		Diarrhea	
Dose (mg/m^2)	100–1,000	1,000–2,500	Platelets >130,000	1+, 2+	3+, 4+	1+, 2+	3+, 4+
Group 1							
300	0/3	0/3	0/3	3/3	0/3	2/3	0/3
400	0/3	0/3	0/3	0/3	0/3	0/3	0/3
500	0/4	0/4	1/4	0/4	0/4	1/4	0/4
600	1/10	0/10	1/10	7/10	0/10	2/10	1/10
650	0/3	0/3	1/3	0/3	0/3	0/3	0/3
700	0/5	0/5	1/5	1/5	0/5	0/5	0/5
750	0/3	1/3	1/3	2/3	0/3	0/3	0/3
800	0/4	0/4	0/4	0/4	0/4	2/4	0/4
900	0/3	2/3	0/3	2/3	1/3	0/3	0/3
Group 2							
600	0/6	1/6	3/6	2/6	0/6	1/6	2/6
500	0/4	2/4	2/4	0/4	0/4	4/6	0/4

patients had diarrhea, causing dehydration and requiring therapy. Nausea and vomiting were minimal at this dose level, but 1 patient had mucositis, requiring a liquid diet. Although objective parameters of toxicity were not significant, symptomatic tolerance was severely impaired with 4 of 6 patients having a >20% decrease in performance status on treatment, with improvement of performance status when therapy was delayed or stopped. Four of the 6 patients continued therapy at 500 mg/m^2. This did not cause a decrease in performance status, but 2 of the 4 patients had grade 2+ hematological toxicity and 2 had grade 2+ diarrhea.

PRPP Levels. Bone marrow and cancer biopsies were analyzed for levels of PRPP in pretreatment and posttreatment (24 h after PALA and MTX) tissues from the same patient. PRPP levels were enhanced. Findings are in Tables 3 and 4, respectively.

RNA Containing Incorporated FUra Residues in Bone Marrow. Fig. 1 is a HPLC separation of an alkali digest of RNA prepared from nucleated bone marrow cells; Fig. 1*A* is bone marrow taken prior to FUra administration, and Fig. 1*B* is bone marrow 2 h after FUra administration (650 mg/m^2) in the same patient. Note the appearance of a small peak in Fig. 1*B* that is absent in Fig. 1*A*. The identity of the peak indicated as 2′,3′-FUMP was established by its absence in the chromatogram of pretreatment marrow, and by its cochromatographing with authentic 2′,3′-FUMP. In this particular sample, there was 446 pmol of FUra incorporated into RNA/10^6 cells.

Therapeutic Results. Although this study was a Phase I trial, we nevertheless evaluated 27 patients with measurable disease in the first group and 4 patients with measurable disease in the second group for antitumor response. There were no responses in the first group (every other week schedule), but 3 patients with previous progressive disease had stabilization of their disease for a median of 349 days (range, 95–586). In the second group (weekly for 2 weeks schedule), 1 of 4 evaluable patients had a partial response (>50% decrease in an abdominal wall mass), and 3 who had progressed on previous FUra-containing regimens had stablization of disesae for a median of 189 days (range, 92–236).

DISCUSSION

In an attempt to increase the efficiency of FUra, numerous trials have been designed to biochemically modulate FUra.

Table 3 *PRPP content in bone marrow before and 24 h after two biochemical modulators*[a]

Patient	Pretreatment (pmol/10^6 cells)	Posttreatment (pmol/10^6 cells)	Pre/post ratio
1	5.6	12.2	2.2
2	11.7	21.6	1.9
3	24.4	41.8	1.7
4	41.5	30.0	0.7
5	15.7	38.3	2.4

[a] PALA, 250 mg/m^2 plus MTX, 250 mg/m^2.

Table 4 *PRPP levels in human colorectal tumors before and 24 h after treatment with two biochemical modulators*[a]

Biopsy	Site	Speciman	PRPP levels (pmol PRPP/mg protein)	Fold increase (treatment/control)
Patient 1				
852804	Abdominal wall mass	Pretreatment	39.0	
8613296		Posttreatment	961.0	25.0 ×
Patient 2				
31:43270	Rectal mass	Pretreatment	124.0	
31:43291		Posttreatment	309.0	2.5 ×

[a] PALA, 250 mg/kg plus MTX, 250 mg/kg.

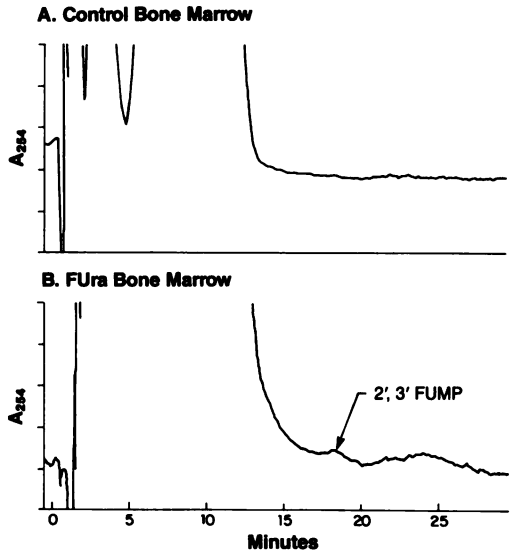

Fig. 1. Identification of FUMP in RNA from bone marrow of a FUra-treated patient. Bone marrow aspirates were obtained prior to and again 2 h after FUra (650 mg/m^2). Nucleic acids from WBC were prepared as detailed in "Materials and Methods." RNA was digested with alkali, after which purine nucleotides were converted to purine bases by heating the acidified digest. The resulting mixture of 2′,3′-pyrimidine nucleoside monophosphates and purine bases was separated by ion-pairing HPLC. *A*, chromatogram of pretreatment marrow; *B*, post-FUra sample. 2′,3′-FUMP (*B, arrow*) was identified by its cochromatographing with authentic 2′,3′-FUMP. In murine studies, this peak was further characterized by treatment with bacterial acid phosphatase with subsequent recovery of fluorouridine.

Ardalan *et al.* (8) have shown in *in vitro* studies that the synergistic effect of the combination of PALA and FUra on human mammary carcinoma cell lines correlates with an increased proportion of FUTP in the pyrimidine nucleotide pool, and, consequently, with an enhanced incorporation of FUra into RNA. Similar results, including enhanced antitumor activity, were also demonstrated *in vivo* preclinically with low doses of PALA prior to FUra (15).

PALA, inactive clinically as a single agent, has been used in a number of clinical studies as a biochemical modulator of FUra. However, the appropriate ratios of agents and the sequence and time interval between administration of agents are critical determinants of success, and these parameters were frequently not incorporated into the design of the clinical protocols (16). Buroker *et al.* (17), using PALA at 625 mg/m^2 followed in 4 h by FUra at 300 mg/m^2 for 5 consecutive days, obtained only an 11% response rate. Muggia *et al.* (18), using a high dose of PALA (1.5 g/m^2) combined with escalating doses of FUra to 800 mg/m^2 given every other week, obtained a partial response rate of 26%. It is possible that these high doses of PALA did not permit appropriate escalation of FUra or more frequent weekly administration of the PALA/FUra combination which may have led to a better response rate. In contrast, Ardalan *et al.* (19) used a weekly schedule with a low dose of PALA (250 mg/m^2), followed in 24 h with a continuous infusion of FUra at 2600 mg/m^2 over 24 h, and obtained a response rate of 48% in previously untreated patients.

The latter study (19) suggests that a low PALA dose is capable of selectively potentiating the effects of FUra in human tumor cells with relative sparing of host tissues, thus improving the therapeutic index. In mice, a low nontherapeutic dose of PALA can lower UTP pools in a spontaneous breast tumor model and can safely be administered 24 h prior to the maximum tolerated dose of FUra resulting in enhanced anticancer activity (9).

Cadman *et al.* (11) have shown that a MTX concentration of

10 μM was necessary to maximally increase intracellular PRPP. They administered MTX doses of 200 mg/m^2 in order to achieve the desired MTX concentration (20). We thus selected 250 mg/m^2 as our dose, since this dose was safely administered in most of the clinical investigations with sequential MTX and FUra (13), and would give us the desired micromolar concentration.

The PRPP data indicate that the levels of PRPP in human colorectal tumors (Table 4) can be markedly increased (25-fold in one patient) 24 h following PALA and MTX treatment, in contrast to a 5-fold increase in the CD8F1 tumor. The data in Table 3 suggest that PALA-MTX combination can have some modulating effect upon the content of PRPP in bone marrow cells. The magnitude of the change observed was 2-fold in our study, but has not been reproducibly measured in the murine model. It is our future hope to compare the amount of FUra incorporated into RNA after pretreatment with PALA/MTX relative to that incorporated into RNA after FUra alone in human tumors.

The recommended Phase II dose for the every other week schedule is FUra at 800 mg/m^2, and the dose for the weekly for 2 weeks schedule is FUra at 500 mg/m^2.

Although the actual dose given in the every other week schedule was greater than the weekly for 2 weeks schedule, slight activity was seen only with this latter regimen. Calculations of dose intensity gives equal importance to time delays and actual dose (21). It may be that giving the FUra 1 week earlier is more advantageous.

Our current interest is to decrease toxicity of FUra with the addition of uridine. In murine studies, the toxicity was reduced by the addition of uridine rescue permitting escalation of FUra and consequent marked antitumor effects (14, 22). New studies demonstrate that uridine rescue is effective in controlling FUra toxicity in patients (23–24) as well. A Phase I study of PALA, MTX, FUra, leucovorin, and uridine has been initiated.

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