Plasma Uridine Changes in Cancer Patients Treated with the Combination of Dipyridamole and N-Phosphonacetyl-L-aspartate

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ABSTRACT

Dipyridamole (DP) and N-phosphonacetyl-L-aspartate (PALA) act synergistically in vitro against many cell lines and in vivo against human ovarian carcinoma xenografts. We have conducted a phase I clinical trial of DP p.o. (50 mg/m², every 6 h) in combination with PALA (starting at 500 mg/m² i.v. with 300-mg dose escalations). Sixty-five patients were entered into this study, and we have established the maximum tolerated dose of PALA to be 4.5 g/m² when combined with DP, which is approximately 80% of the previously reported maximum tolerated dose for PALA alone. The observed toxicities of DP plus PALA were mild and were similar to those reported for PALA alone. Bone marrow toxicities were not evident at any PALA dose. Ten patients with a mean pretreatment plasma uridine concentration of 3.49 ± 1.28 (SD) μM had their plasma uridine reduced to 2.29 ± 0.70 μM 9 h after DP p.o. A peak plasma DP concentration of 1.86 ± 0.59 μM was achieved approximately 2 h after p.o. dosing. Nine patients who had a reduced plasma uridine concentration of 2.46 ± 0.61 μM after 1 week of DP had their plasma uridine further reduced by PALA to 0.87 ± 0.23 μM 7 h post-PALA. Daily plasma uridine measurements in two patients during their DP treatment confirmed the previously described pattern for Day 1, but the data suggest a slight recovery (15%) in plasma uridine by Day 2. Daily sampling in two other patients after a single PALA dose of 4.2 g/m² showed that their plasma uridine declined 5 h after the PALA dose and remained depressed for 6 days in one patient and 11 days in the other. These results suggest that DP worked in synergy with PALA to reduce circulating uridine in cancer patients. The mechanism for the ability of DP to reduce plasma uridine is not known, but there is evidence that DP can inhibit cellular efflux of uridine as well as its uptake. DP may reduce plasma nucleoside pools in addition to blocking nucleoside salvage and therefore have general applicability in other chemotherapy regimens.

INTRODUCTION

PALA is a potent inhibitor of ATCase, the enzyme responsible for the biosynthesis of carbamyl-L-aspartate in the pyrimidine metabolic pathway. Pyrimidine biosynthesis in mammalian cells was virtually completely blocked by PALA (3). In experiments with mice, PALA was found to markedly inhibit liver ATCase activity within 15 min of administration, and its effects persisted for at least 72 h after a single dose (4). Preclinical studies found PALA to have a unique antitumor spectrum with the rapidly proliferating murine leukemias being resistant and the more slowly growing Lewis lung carcinoma and B16 melanoma being sensitive to the effects of the drug (5, 6). In phase I pharmacological studies, PALA was found to have an initial phase t₀ of 1 h and a terminal phase tₘₖ of 5 h (7, 8). The drug is excreted unchanged and its renal clearance appears to be linearly related to creatinine clearance. Excretion is nearly complete at 24 h (7, 8) with over 85% of the administered dose being found in the urine of patients (8). The major side effects of PALA have included skin rash, diarrhea, stomatitis, and in several patients seizures (9–21). Myelosuppression has not been a problem in any of the clinical trials which made the drug an attractive chemotherapeutic agent.

Although PALA showed promising antitumor activity in preclinical studies, several phase I (9–14) and phase II (15–21) clinical trials have failed to reveal significant activity of PALA against any of the tumors evaluated. The reasons for the lack of activity of PALA in patients were investigated in our laboratory. One of our working hypotheses was that even with near complete or complete inhibition of ATCase activity by PALA, pyrimidine synthesis can proceed normally through the salvage pathway by reutilization of circulating uridine. It has been established in experimental systems that uridine can reverse both the toxic and antitumor effects of PALA (3, 22). Studies on mice seem to suggest that 95% of the circulating uridine is metabolized by the liver in a single pass, and new uridine is synthesized and released by this same organ (23). DP, a drug used clinically as a vasodilator and antiplatelet agent, is a potent inhibitor of membrane uridine transport (24, 25) and therefore was thought useful in preventing salvage activity in tumor cells. DP is a particularly attractive agent for clinical use; previous experience with this drug in thousands of patients has established its effective dose and demonstrated its lack of serious toxicity in humans. Previous results from this laboratory have shown that dipyridamole can augment the activity of PALA (26) against tumor cells and that the mechanism of synergy relates to inhibition of uridine salvage by DP (27). We have therefore initiated a phase I clinical trial of DP p.o. in combination with PALA in cancer patients. In conjunction with this trial, we have also studied the effects of PALA and DP on plasma uridine levels in these patients to examine the effects of DP and PALA on uridine metabolism in humans.

MATERIALS AND METHODS

Patients. A total of 65 patients were admitted into this phase I trial. To be eligible for entrance, the patient had to have a histologically confirmed malignancy and failure or refusal of conventional therapies of proven merit or be previously untreated and have a malignancy where there is no established form of treatment. There were 44 male and 21 female patients; the mean age for the male patients was 55 years and for the female patients it was 58 years. Table 1 categorizes the patients into subgroups according to diagnosis. All patients had an Eastern Cooperative Oncology Group performance status of 3 or less, and informed consent was obtained before the initiation of chemotherapy.

Drugs and Chemicals. Dipyridamole (Persantine, 75 mg) was supplied by Boehringer Ingelheim, Ltd. (Ridgefield, CT) and PALA disodium was provided by the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). All chemicals used in this study were purchased from Fisher Scientific (Fairlawn, NJ) except for the nucleoside standards, which were purchased from Sigma Chemical Co. (St. Louis, MO).
analyses and radiograms were performed on Day 1. The patients were then given dipyridamole p.o. at a dose of 50 mg/m² (most patients received 75 mg) and were instructed to repeat every 6 h for as long as they remained on the study. On Day 7, the patients were brought back into the clinic for their PALA dose (starting at 500 mg/m² in the first few patients with 300-mg dose escalations as i.v. infusion over 20 mm) and 1 ml of plasma was removed and frozen at —20°C for DP measurement of plasma uridine and to serve as a control for multiple blood sampling, detailed time courses of plasma uridine levels were studied during their first dipyridamole doses. Anti-coagulated (EDTA) blood samples were obtained 2 and 1 h prior to DP dosing and at hourly intervals after the DP dose via an indwelling venous catheter. The samples were centrifuged (500 × g) immediately and 1 ml of plasma was removed and frozen at —20°C for DP measurements while the remaining plasma was aliquoted into ultrafiltration membrane cones (Amicon CF-25) and centrifuged at 1000 × g for 30 min. The ultrafiltrates were either stored frozen or analyzed immediately for uridine as described below. In 9 patients, the same blood sampling regimen was followed after their initial PALA dose (2.0 g/m²) to study the acute effects of PALA on plasma uridine concentration.

In two patients, we have extended the monitoring of plasma uridine after their initial DP dose to beyond 15 h on Day 1 and subsequent blood sampling at 10 a.m. daily (to minimize diurnal variations) for 1 week. The same procedure was followed in two other patients after they had received their PALA dose (4.5 g/m²), except blood samples were drawn daily for 3 weeks.

Plasma Uridine Measurements. The HPLC system used in uridine measurements consisted of the following Waters Associates (Milford, MA) equipment: Model 6000A pump; Model 440 UV absorbance detector; Model U6K injector; Z-module radial compression unit; and Model 730 data module. The column was a Waters Associates C18 Bondapak radial compression cartridge with a proximal guard column of the same material. The isocratic buffer used was 50 mM KH₂PO₄, pH 3.75, maintained at a flow rate of 2.0 ml/min. Uridine identification was confirmed by 254 nm/280 nm absorbance ratios and coelution with purified standards. The typical retention time for uridine in this HPLC system was 10 mm and a representative chromatogram of a plasma ultrafiltrate is shown in Fig. 1. In separate experiments, we have established that uridine was completely recovered from the ultrafiltration process, and the nucleoside is stable at —20°C for 6 months if stored as plasma or ultrafiltrate. The detection limit of this assay is equivalent to 0.1 μM uridine in plasma.

Plasma DP Measurement. The HPLC method used in DP measurements was a modification of that reported by Wolfram and Bjornsson (28). Briefly, 1.0 ml of plasma was mixed with 20 μl of the internal standard solution (quinidine·HCl, 64 μg/ml) and 1.0 ml of 1 M sodium hydroxide. The sample was then extracted with 5.0 ml of diethyl ether by vortexing the mixture vigorously for 20 s. The organic phase was removed and evaporated to dryness under nitrogen. The samples were reconstituted in 100 μl of the mobile phase and a carefully measured volume was injected into the HPLC. The HPLC system consisted of the following Waters Associates equipment: Model 6000A pump; Model U6K injector; Z-module fitted with a C₁₈-Bondapak cartridge and a proximal guard column of the same material; and a Model 420 fluorescence detector with an excitation wavelength set at 285 nm and an emission cutoff filter of 470 nm. DP and the internal standard were eluted with a mobile phase of methanol-water (60/40) containing 1-heptanesulfonic acid, sodium salt (0.005 M) and acetic acid (0.1%), delivered at 2.5 ml/min through the column. Typical retention time for quinidine was 3.8 min, and that for DP was 5.8 min. The extraction procedure routinely recovers over 98% of quinidine and 95% of DP from the aqueous phase.

RESULTS

Toxicities of DP and PALA. DP p.o. at 50 mg/m² (every 6 h) was well tolerated by the patients in this study, with mild headache being the most common complaint. Five patients reported severe headaches with four of them requiring a dose reduction to 50 mg every 6 h. Two other patients reported nausea and upper abdominal pain. One patient withdrew from this study because of headache and twelve patients withdrew before receiving PALA due to disease progression. A total of 128 courses of PALA in combination with DP was administered to 52 patients. The toxicity of PALA plus DP was mild below the 3000-mg/m² dose level. The commonly observed toxicities above this dose were mucositis, rash, diarrhea, and abdominal cramps. We have established the maximum tolerated dose of PALA in combination with DP to be 4500 mg/m². The commonly observed toxicities above this dose were mucositis, rash, diarrhea, and abdominal cramps. We have established the maximum tolerated dose of PALA in combination with DP to be 4500 mg/m², with the dose limiting toxicities being severe diarrhea and abdominal pain (1). There was no evidence of bone marrow, renal, hepatic, or neurotoxicities. Nausea was mild and rarely observed, and several patients noticed tingling sensations in their hands and lips during PALA infusion which persisted for 10–30 min.

Effects of DP Treatment on Plasma Uridine. The acute effects of DP treatment on plasma uridine concentration were examined in a group of ten patients. Fig. 2 shows the averaged plasma uridine concentrations in these patients as a function of time before and during their DP treatment. Their mean pretreat-

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**Table 1** Classification of patients according to diagnosis

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>17</td>
</tr>
<tr>
<td>Colorectal</td>
<td>14</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>10</td>
</tr>
<tr>
<td>Adenocarcinoma of unknown primary</td>
<td>5</td>
</tr>
<tr>
<td>Pancreas</td>
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</tr>
<tr>
<td>Ovary</td>
<td>3</td>
</tr>
<tr>
<td>Melanoma</td>
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</tr>
<tr>
<td>Head and neck</td>
<td>2</td>
</tr>
<tr>
<td>Breast</td>
<td>1</td>
</tr>
<tr>
<td>Endometriium</td>
<td>1</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>1</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>1</td>
</tr>
<tr>
<td>Gastric</td>
<td>1</td>
</tr>
<tr>
<td>Small bowel</td>
<td>1</td>
</tr>
<tr>
<td>Prostate</td>
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</tr>
<tr>
<td>Glioblastoma</td>
<td>1</td>
</tr>
<tr>
<td>Renal</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>66*</td>
</tr>
</tbody>
</table>

*One patient had primary breast tumor and sarcoma.*
Effects of DP plus PALA Treatment on Plasma Uridine. In a group of nine patients who had a mean pretreatment plasma uridine concentration of 3.67 ± 1.22 μM which dropped to 2.46 ± 0.61 μM (P < 0.05, paired t test) after 1 week of DP, the acute effects of the further addition of PALA treatment on plasma uridine were studied. Their plasma uridine concentrations started falling 2 h after receiving PALA to a steady level of 0.87 ± 0.23 μM 7 h post-PALA. Fig. 3 shows the averaged plasma uridine concentration in these patients as a function of time. Plasma uridine was reduced markedly by the PALA treatment. In another two patients, the long term effects of a single PALA dose on plasma uridine concentration were studied. The mean plasma uridine concentration was 3.49 ± 1.28 (SD) μM, which began to drop 5 h after the first p.o. DP dose to a nadir of 2.29 ± 0.70 μM (P < 0.05) 9 h after the initiation of DP p.o. The peak plasma DP concentrations (mean) achieved in these patients was 1.86 ± 0.99 μM approximately 2 h after the first p.o. dose. In another two patients, we studied the long term effects of continuous DP treatment on plasma uridine concentrations. Blood sampling was extended to 15 h on Day 1 and then the patients were asked to come back daily for blood sampling for 1 week (before they received their PALA). The data confirmed the effects of DP in lowering plasma uridine concentration on Day 1 as reported in Fig. 2. By Day 2, there was a slight recovery of the plasma uridine concentration (approximately 15% higher than Day 1) but the concentration remained below the pretreatment level throughout the week such that after 7 days, their mean plasma uridine concentration was 2.51 μM (the mean pre-DP value was 3.51 μM).

DISCUSSION

We have demonstrated previously that of all the commonly encountered nucleosides, uridine was the only one capable of antagonizing the cytotoxic effects of DP and PALA in vitro. The mechanism of synergy between these two drugs seems to reside in the inhibition of de novo pyrimidine biosynthesis by PALA and the concurrent blockade of uridine salvage by DP (27). In addition to the usual objectives of a phase I clinical trial, we are also interested in the effects of DP and PALA on the circulating uridine levels in cancer patients. While PALA alone specifically decreases the level of pyrimidines in sensitive tumor cells compared to normal tissues (27, 29), the reported decrease in circulating uridine levels after PALA treatment in vivo was not dramatic. In mice, PALA treatment either as a single dose or as four daily doses reduced serum uridine level by 55% (30), while in the Sprague-Dawley rat uridine depression was found to be less than 40% 24 h after treatment (31). Similarly, in a phase I trial in patients receiving PALA (1000–
2000 mg/m²/day), serum uridine levels were noted to be maximally depressed by 37–85% (32). It is possible, in fact, that the observation of decreased circulating levels represents not only a decline secondary to the inhibition of pyrimidine de novo synthesis by PALA but also an increased uptake of uridine by the cells to be used in the salvage pathway. Several lines of evidence support this hypothesis: (a) enzyme activity of the salvage pathway has been demonstrated to be increased in tumors made resistant to PALA (33); and (b) while leukocyte ATCase activity is rapidly and markedly inhibited by PALA with 280 h being required for 50% recovery of ATCase activity (34), there was no evidence of myelotoxicity under these conditions suggesting the efficient use of salvage pathways by the bone marrow.

Our current findings provide some interesting information on the effects of DP and PALA in cancer patients. The observation that DP alone reduced the circulating level of uridine in our patients was unexpected. If this drug works via the blockade of cellular uptake of uridine, we might expect the plasma uridine to increase after treatment due to impaired hepatic extraction and blocked cellular uptake of uridine from the plasma. Such an increase has been observed in the case of plasma adenosine concentrations in patients and several species of laboratory animals being treated with DP (35). The mechanism by which DP decreases plasma uridine may be related to its ability to inhibit nucleoside efflux in addition to blocking influx, as has been shown recently in several mammalian cell lines (36–38).

If the liver is indeed the major site of uridine biosynthesis as proposed by Gasser et al. (23), DP may inhibit the release of this nucleoside from the liver into the systemic circulation resulting in a drop in circulating uridine after DP treatment. More detailed pharmacokinetic studies on uridine metabolism in vivo are required before we can be certain of an explanation for this observation.

In combination with DP, PALA reduced the plasma uridine in these patients consistently to approximately 20% of the pretreatment level. The reduction is more reproducible and larger in magnitude than the previously reported 45% reduction in patients treated with PALA alone (32). We have also demonstrated that a single dose of PALA resulted in a prolonged reduction of plasma uridine which did not recover to pre-PALA levels until 9–13 days posttreatment. This finding is very valuable for the design of further trials in terms of the optimum frequency and dosage of chemotherapy to be used, if we can assume that plasma uridine is a biological indicator of the overall biochemical effects of the PALA-DP combination. Other nucleosides measured concurrently did not show any remarkable trend during DP and PALA treatment (data not shown).

Although the overall response rate in this phase I trial was not striking, we are particularly encouraged by the responses of the patients with soft tissue sarcomas to the DP-PALA regimen. We are unaware of any effective second line chemotherapeutic regimen to date for the treatment of this type of advanced malignancy. We plan to investigate further the efficacy of DP and PALA in the treatment of soft tissue sarcoma in a phase II trial. We do not know the mechanism by which DP lowers the plasma uridine concentration at the present time. The ability of this drug to effectively reduce the body’s plasma uridine pool (and possibly other metabolites) has important clinical implications. It is possible that DP can limit the availability of salvage metabolites in the body in addition to blocking their uptake.

Other workers have already reported synergy between DP and antimitabolites such as methotrexate (39) and acivicin (40, 41) against tumor cells in vitro. Reports on the results from clinical trials using DP in combination with other anticancer drugs will undoubtedly be forthcoming.

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