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Biochemical modulation of 5-fluorouracil with brequinar: results of a phase I study

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Abstract Biochemical modulation can increase the efficacy of 5-fluorouracil (5-FU). Pizzorno et al. have previously shown that brequinar, a de novo pyrimidine synthesis inhibitor, enhances the antitumor effect of 5-FU in vivo [Cancer Res 52: 1660–1665, 1992]. On the basis of their data, we conducted a phase I study of brequinar in combination with 5-FU in patients with refractory solid tumors. The initial dose (100 mg/m^2) of brequinar was raised in 100-mg/m^2 increments in cohorts of three assessable patients. The initial dose of 5-FU was 500 mg/m^2, but escalation was allowed in patients who showed no significant toxic reaction. Brequinar was administered over 1 h and 5-FU over 2 h starting 18–20 h after the initiation of infusion of brequinar. Treatments were repeated weekly. Responses were evaluated after 4 weeks (one course) and then every 8 weeks thereafter. Pharmacokinetics of brequinar and determination of plasma uridine levels were performed in at least three patients at each dose level. Of the 25 patients registered in the study, 21 were assessable for toxicity studies. The dose of brequinar was escalated up to 600 mg/m^2. In addition, the dose of 5-FU was increased to 600 mg/m^2 as a result of a lack of a significant toxic reaction in the first nine patients. No objective responses were observed. One patient developed grade 3 stomatitis, and one developed grade 3 esophagitis at the 400 and 600 mg/m^2 dose of brequinar, respectively. Brequinar produced a dose-dependent decrease in plasma uridine levels at doses up to 500 mg/m^2. No additional decrease in plasma uridine occurred with higher doses of brequinar, thus suggesting a plateau effect. This observation prompted us to terminate the study before reaching the maximum tolerated dose of brequinar. Our data indicate that brequinar in doses $\geq$ 400 mg/m^2 results in significant biochemical modulation. The lack of toxicity seen at these doses of brequinar suggests that the initial dose of the effector agent 5-FU should be increased in future studies.

Key words Brequinar · 5-Fluorouracil · Biochemical modulation

Abbreviations *5-FU* 5-Fluorouracil · *PALA N*-(phosphonacetyl)-L-aspartic acid · *UXP* total uridine nucleotide · *AGC* absolute granulocyte count · *MTD* maximum tolerated dose · *ECOG* Eastern Cooperative Oncology Group

Introduction

Inhibition of de novo pyrimidine biosynthesis increases the activation and utilization of 5-FU by selectively decreasing pools of the competing normal pyrimidine nucleotides [1–3]. Figure 1 shows the site of action of the best-characterized inhibitors of de novo pyrimidine biosynthesis. Of these, PALA, an inhibitor of aspartate transcarbamylase, has been the most extensively evaluated in both preclinical and clinical studies. Preliminary results of clinical studies using low doses of PALA in combination with full doses of 5-FU in colorectal cancer have been encouraging, with response rates of the order of 40% [4, 5].

Brequinar is a fluorinated derivative of carboxyquinoline that inhibits the mitochondrial enzyme

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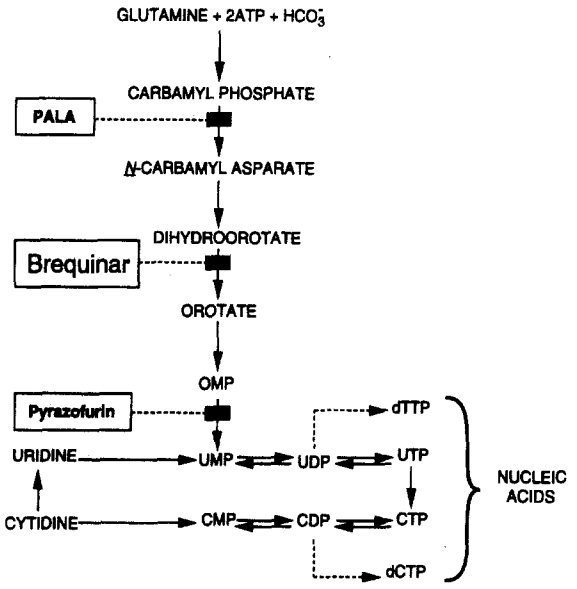

Fig. 1 De novo pyrimidine synthesis pathway showing sites of action of PALA, brequinar, and Pyrazofurin (Lilly)

dihydroorotate dehydrogenase, the fourth step in the de novo pyrimidine biosynthesis pathway (Fig. 1) [6–9]. Brequinar, as a single agent, shows significant antitumor activity in a number of murine and human xenografts [10, 11]. Phase II clinical studies, however, have shown no significant antitumor activity in colon cancer and other malignancies [12]. Since brequinar also inhibits de novo pyrimidine biosynthesis but acts on a different target from PALA, Pizzorno et al. [13] evaluated the effects of brequinar as a modulator of 5-FU cytotoxicity in C57/BL6 mice bearing the Colon 38 tumor. They observed that low, nontherapeutic doses of brequinar, in the range 8–27% of the MTD caused a rapid decrease in the concentration of uridine in plasma, and in tumor and normal tissues. In the plasma, liver, kidney, and spleen, the uridine concentration returned to normal levels within 24 h. However, the uridine pools in gut and Colon 38 remained depleted after 48 h. Similar decreases in UXP pools were observed after 2 h in both tumor and normal tissues. UXP pools returned to control levels within 4 to 6 h in all the normal tissues. However, in the Colon 38, the UXP pools remained reduced by 80% after 24 h.

Reasoning that selective depletion of uridine and UXP pools in tumor tissue might sensitize cells to 5-FU, Pizzorno et al. [13] studied the combination of brequinar plus 5-FU in this model. They demonstrated that brequinar has a synergistic effect when combined with 5-FU and that pretreatment with brequinar significantly increases the incorporation of 5-FU into Colon 38 tumor RNA, whereas minimal effects are seen in normal tissues [13].

On the basis of these preclinical data, we designed a phase I clinical study to determine the MTD of brequinar in combination with 5-FU and to evaluate the effects of brequinar on plasma uridine in patients with refractory solid tumors.

Materials and methods

Patient population

Patients with solid tumors who had not responded to standard therapy or who were untreated but had solid tumors for which there was no effective therapy were eligible for the study. A signed, informed consent form was required prior to entry. Eligibility criteria included the presence of assessable or measurable disease, an expected survival of more than 8 weeks, an ECOG performance status of 0, 1 or 2, a WBC count $\geq 3{,}000/mm^3$, an AGC $\geq 1{,}500/mm^3$, a platelet count $\geq 100{,}000/mm^3$, a total bilirubin level ≤ 1.5 mg/dl, and a serum creatinine ≤ 2.0 mg/dl. Patients had to have recovered from any significant toxic reaction associated with previous chemotherapy (6 weeks for nitrosoureas and mitomycin C and 3 weeks for other cytotoxic agents), surgery, radiotherapy, and/or immunotherapy. Patients with symptomatic brain metastases were excluded.

Treatment plan

This study was approved by the Human Investigation Committee of the Yale University School of Medicine. Each patient received a single i.v. infusion of brequinar and a fixed dose of 5-FU starting 18–20 h from the initiation of the infusion of brequinar. The 18–20 h interval was chosen because, in murine studies, this is the time when uridine and UXP pools reach the lowest concentration in both plasma and tumor tissues compared with normal tissue in which the levels have already returned to normal. Treatments were repeated weekly for a minimum of 4 weeks (defined as one course) unless there was progression of disease.

Brequinar was generously supplied by DuPont Pharmaceuticals (Wilmington, Del.) as a lyophilized powder (100 or 500 mg/vial). The initial dose of brequinar was 100 mg/m^2 and corresponded to approximately 5–10% of the phase II recommended dose [14]. At each dose level, brequinar was mixed in 250 ml normal saline and administered i.v. over 1 h. 5-FU at 500 mg/m^2 was mixed in 500 ml normal saline and administered i.v. over 2 h starting 18–20 h after the initiation of the infusion of brequinar. Because there were no significant toxic reactions at this dose, the protocol was subsequently amended increasing the initial dose of 5-FU to 600 mg/m^2. The dose of brequinar was escalated by 100-mg/m^2 increments in cohorts of three assessable patients. At least 1 week had to elapse between the enrolment of the first and the next two patients within each dose level. At least 4 weeks had to elapse after the last patient was entered at the previous dose level before new patients began treatment at the next higher dose level. If two or more patients experienced a toxic reaction equal to or greater than grade 3 at a particular brequinar dose level, an additional three or four patients were treated at that level in order to carefully characterize the toxic reaction. Dose escalation of brequinar was not permitted in individual patients. Only the first course of therapy (first 4 weeks) was used for the determination of the MTD which was defined as the dose level at which at least half of the patients experienced a toxic reaction grade 3 or more. Patients were regarded as assessable for MTD and response if they received at least one full course of therapy. Toxic reactions were graded according to the National Cancer Institute Common Toxicity Criteria.

Treatment was administered weekly if all the following conditions were met: WBC $\geq 3{,}500/mm^3$, AGC $\geq 1{,}500/mm^3$, platelet count $\geq 100{,}000/mm^3$ and non-hematologic drug-related toxicity $\leq$ grade 1. If any of these parameters were not satisfied, treatment

was delayed for at least 1 week until recovery. In patients eligible for retreatment, the dose of 5-FU was adjusted, depending on the nadir of the blood counts and the nonhematologic toxicity of the previous course. The dose of 5-FU was increased by 20% if the previous nadir of WBC was $> 3{,}000/mm^3$, AGC $> 1{,}500/mm^3$, platelet count $> 100{,}000/mm^3$, and no evidence of nonhematologic toxicity (excluding nausea, vomiting, and alopecia). The dose of 5-FU was decreased by 20% if the previous nadir of WBC was $< 2{,}000/mm^3$, AGC $< 500/mm^3$, platelet count $< 75{,}000/mm^3$ or nonhematologic toxicity $\geq$ grade 2 or 3. Patients who experienced grade 4 nonhematologic toxicity were removed from the study.

Prior to the initiation of therapy, all patients had complete blood counts with differential and platelet counts, a chemistry profile, a urinalysis, an electrocardiogram, and computerized tomograms of the chest, abdomen, and/or pelvis. Blood counts and chemistry profiles were repeated weekly and every 4 weeks, respectively. Computerized tomograms were repeated after one course (4 weeks) of therapy for initial assessment of response (as well as to exclude patients with rapidly progressive disease) and then every two courses thereafter. Patients whose disease progressed on therapy were removed from the study. Response to treatment was evaluated according to the ECOG standard criteria of response [15].

Pharmacokinetic studies

The first treatment of each patient was administered in the Clinical Research Center of the Yale New Haven Hospital as an overnight hospital stay to allow blood collection for the determination of uridine and brequinar levels in plasma. A 5-ml sample of blood was collected into heparinized tubes at the following time points: immediately before and at the end of the infusion of brequinar; 1, 2, 4, 6 and 12 h after infusion of brequinar; and immediately prior to and at the end of the infusion of 5-FU. The plasma was separated immediately after centrifugation of the blood at 2000 *g* for 10 min in a refrigerated bench centrifuge and then stored at $-20°C$. Brequinar was analyzed by HPLC as previously reported [16]. Briefly, an internal standard (Du Pont S-6056) was added to each sample prior to the extraction procedure. Plasma was extracted with methylene chloride after the addition of tetrabutylammonium hydroxide. The organic solvent was evaporated under nitrogen and the samples reconstituted with mobile phase $CH_3CN/H_2O/H_3PO_4$ (55:25:20) before HPLC analysis. Separation was achieved using a Biophase Octyl column (4.6 mm × 25 cm) and the absorbance was monitored at 254 nm using an Altex 153 UV detector. The detection limit was 0.5 μM with a linear range from 0.5 to 500 μM. The intraday coefficient of variation was 2.7% to 11% over the linear range and the interday variation ranged from 3% to 10.5%.

The plasma samples for uridine determination were mixed with two volumes of 15% TCA and the supernatant neutralized by trioctylamine/freon (45:55) extraction. The extraction yield for uridine was 78%. The detection limit was 0.25 μM with a linear range from 0.25 to 100 μM. Uridine concentrations were measured after separation on a C18 Microsorb column (4.6 mm × 25 cm) eluted at 1 ml/min with 10 mM H_3PO_4 containing 30 μM heptane sulfonic acid, pH 3.1, at 8°C, and monitored at 254 nm [17]. Pharmacokinetic parameters of brequinar were determined by a noncompartmental model using the software PCNONLIN, version 4.2 (ClinTrial Inc., Lexington, Ky). Comparison among all groups was done using repeated measures analysis of variance and the *P*-values were obtained from the *F*-test.

Results

Patient characteristics

The characteristics of the 25 patients registered into the study are shown in Table 1. Four patients were not assessable for evaluation of MTD for the following reasons: two patients received an incorrect dose of brequinar (lower than stated in the protocol), one patient discontinued therapy prior to completing one full course of therapy because of social problems, and one patient was ineligible because he had received lomustine less than 6 weeks prior to registration. Of the 21 assessable patients, only 3 (1 with prostate cancer and 2 with non-small-cell lung cancer) had not received prior cytotoxic chemotherapy. Of the 21 assessable patients, 14 (67%) had not responded to a fluoropyrimidine-based regimen, usually of 5-FU and leucovorin.

Table 1 Patients' characteristics

Number of patients entered	25
Number of patients assessable for toxicity	21
Male/female	16/9
Median age (range) (years)	61 (29–81)
Performance status	
ECOG 0	8
ECOG 1	15
ECOG 2	2
Primary site	
Lung	8
Colorectal	8
Prostate	2
Soft tissue sarcoma	2
Stomach	1
Esophagus	1
Breast	1
Melanoma	1
Unknown primary carcinoma	1
Site of metastases	
Lung	15
Liver	12
Node	12
Bone	7
Pleura	6
Soft tissue	2
Adrenal	2
Bladder	1
Rectum	1
Brain	1
Prior therapy	
Surgery	17
Radiotherapy	11
Chemotherapy	21
Fluoropyrimidine-based	14
Nonfluoropyrimidine-based	7

Pharmacokinetic results

Figure 2 and Table 2 show a proportional increase in the area under the curve of brequinar in plasma with an increase in the dose of brequinar. The terminal half-life of brequinar averaged 7.8 ± 1.8 h. The total plasma clearance was similar at all dose levels, with a mean of

1463 ± 156 ml/h per m^2. The apparent volume of distribution was 15.0 ± 2.4 l/m^2. Figure 3 shows that brequinar produced a dose-dependent decrease in plasma uridine concentrations at doses up to 500 mg/m^2. The difference between all groups was statistically significant ($P = 0.04$). No additional decrease in plasma uridine occurred with doses of brequinar of 500 mg/m^2 or higher, thus suggesting a plateau effect in terms of biochemical modulation.

Dose escalation and toxicity

The number of patients entered and the maximum toxic reaction observed at each dose level are shown in Table 3. Because of the lack of significant toxic reactions observed in the first nine patients treated, the protocol was amended increasing the initial dose of 5-FU from 500 to 600 mg/m^2. Following the first full course of therapy, the dose of 5-FU was modified in 14 patients, as described in the Methods. Specifically, 5-FU was increased from 500 to 600 mg/m^2 in four patients, from 600 to 720 mg/m^2 in eight patients, and from 720 to 860 mg/m^2 in one patient. Of these patients 5-FU dose reduction was required in only one patient, who had received extensive prior radiotherapy to the lumbar spine.

Clinically relevant grade 3 toxicity (esophagitis) during the first course of therapy was observed in one patient treated with 600 mg/m^2 brequinar. One additional patient treated with 400 mg/m^2 brequinar developed a grade 3 toxicity (stomatitis) during her second course of therapy. According to the study design, if one of the three patients experienced grade 3 toxicity at the 600-mg/m^2 level, additional patients were to be enrolled at this dose level in order to determine the MTD. However, because our analysis of the effects of brequinar on plasma uridine suggested that

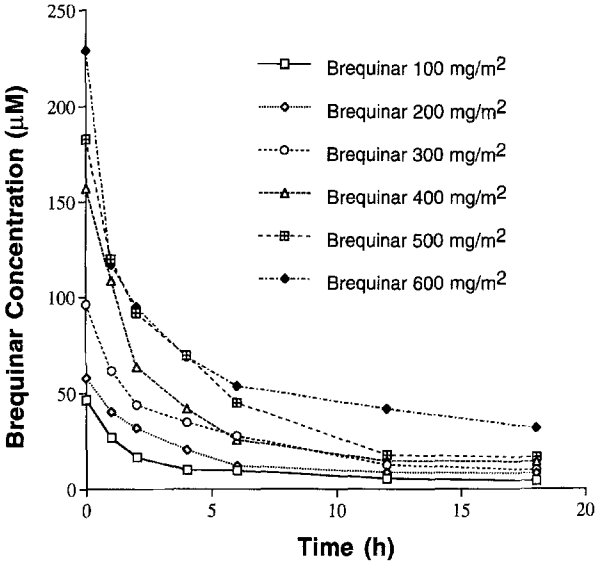

Fig. 2 Pharmacokinetics of brequinar at each dose level. Three patients were studied at each dose level except for the 200 mg/m^2 dose level at which four patients were evaluated

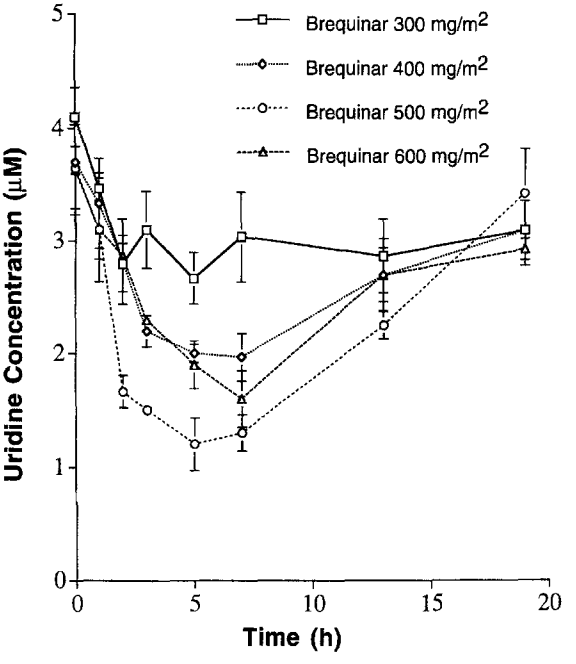

Fig. 3 Pharmacokinetics of plasma uridine after brequinar doses ranging from 300 to 600 mg/m^2. Four patients were studied at each dose level except for the 600 mg/m^2 dose level at which three patients were evaluated. *Error bars* indicate the standard error of the mean

Table 2 Pharmacokinetics of brequinar at the various dose levels studied (*AUC* area under the curve)

Dose (mg/m^2)	No. of patients	$AUC_0 \rightarrow \infty$ (μg·h/ml)	Terminal half-life (h)	Terminal plasma clearance (ml/h/m^2)	Volume of distribution (l/m^2)
100	3	80.7 ± 12.1	7.9 ± 0.8	1239 ± 146	15.5 ± 2.1
200	4	124.0 ± 15.2	8.1 ± 1.2	1613 ± 158	18.8 ± 2.0
300	3	214.3 ± 16.6	6.9 ± 1.0	1401 ± 109	13.2 ± 0.9
400	3	267.1 ± 27.8	6.8 ± 1.1	1498 ± 134	14.7 ± 1.7
500	3	364.2 ± 30.2	6.0 ± 0.9	1373 ± 165	11.9 ± 0.9
600	3	616.4 ± 89.2	11.1 ± 2.7	1655 ± 141	15.7 ± 1.5

Table 3 Maximum toxic reaction for all courses in relation to the dose of brequinar

Brequinar dose (mg/m^2)	No. of patients entered	No. of patients assessable	No. of courses	Maximum toxic reaction (no. of patients)
100	4	4	8	Grade 2 anemia (3)
200	5	3	9	Grade 2 anemia (3); grade 2 nausea (1); grade 2 fatigue (1)
300	5	4	9	Grade 2 anemia (3); grade 2 leukopenia (1); grade 4 lymphocytopenia (1); grade 2 vomiting (1); grade 2 fatigue (1)
400	4	3	6	Grade 2 anemia (1); grade 2 thrombocytopenia (1); grade 2 leukopenia (2); grade 2 neutropenia (1); grade 3 stomatitis (1); grade 2 hemorrhage (1)
500	3	3	4	Grade 2 anemia (1)
600	4	4	8	Grade 2 anemia (1); grade 2 thrombocytopenia (1); grade 2 diarrhea (1); grade 3 esophagitis (1)

a further increase in the dose of brequinar would not result in additional uridine depletion and would possibly increase the toxic reaction of the combination, we elected not to pursue the determination of the MTD.

Antitumor activity

No objective responses were observed in any of the 21 patients assessable for response.

Discussion

More than 30 years after its introduction to the clinic, 5-FU remains the most important drug in the treatment of gastrointestinal malignancies. A better understanding of its mechanism of action has allowed biochemical approaches to the modulation of the action of 5-FU at various intracellular loci. Inhibition of pyrimidine synthesis is one of the mechanisms by which the cytotoxicity of 5-FU can be enhanced. PALA is the pyrimidine synthesis inhibitor that has been most extensively studied, both in preclinical and clinical settings [18]. PALA, like brequinar, is inactive as a single agent in human malignancy. The initial clinical trials of PALA in combination with 5-FU were disappointing. These trials, however, used high doses of PALA that mandated a lower, less effective, dose of 5-FU [18]. Subsequent studies have shown that low, noncytotoxic doses of PALA could significantly affect nucleotide pools [2, 19]. The measurement of inhibition of pyrazofurin-induced orotic aciduria/orotidinuria by PALA in phase I studies have shown that higher doses are not more effective than lower doses in decreasing flux through the de novo pathway of pyrimidine synthesis [2, 19]. More recently, Ardalan et al. and O'Dwyer et al. have reported encouraging preliminary results of phase I trials that combined the lowest biochemically active dose of PALA with 5-FU in gastrointestinal malignancies [4, 5].

The results of our current study demonstrate that brequinar is capable of decreasing the concentration of plasma uridine at nontoxic doses when given with 5-FU. In addition, we showed that the maximum effect was achieved at a dose of 500 mg/m^2. The effects of brequinar on the concentration of plasma uridine in humans was first reported by Peters et al. [20]. In this series, brequinar in doses less than 600 mg/m^2 resulted in a greater than 50% decrease in plasma uridine levels in only 2 of 14 patients, whereas a greater than 50% decrease in plasma uridine levels was observed in 9 patients who received brequinar at doses between 600 and 2,250 mg/m^2. The nadir of plasma uridine occurred between 8 h and 3 days. In addition, a rebound of uridine plasma levels was noted 2–7 days later in 8 of 10 courses. In contrast, we observed a decline in uridine plasma levels at doses of 300 mg/m^2 or higher with a nadir at 5 h and recovery approaching baseline levels at 20 h following infusion. Uridine plasma levels decreased by 50% or more from baseline after doses of 400 mg/m^2or higher. In addition, there were no significant differences in the decreases in plasma uridine levels at doses of 400, 500 or 600 mg/m^2, thus indicating a plateau effect with doses of 400 mg/m^2 or higher.

Our phase I study was based on the premise that the optimal dose of the biochemical modulator should be the lowest biochemically active dose, whereas the effector agent should be used at full-dose to achieve maximal therapeutic benefit. Accordingly, we stopped the dose escalation of brequinar before reaching the MTD because no further decreases in uridine plasma concentrations were observed after the dose of 500 mg/m^2. The lack of significant toxic reaction observed at the highest brequinar dose levels indicates that in future studies the initial dose of 5-FU should be higher. Whether the 24-h weekly infusion of 5-FU used by

Ardalan et al. and O'Dwyer et al. is more active than 5-FU administered by i.v. push remains to be demonstrated [4, 5].

Previous animal studies have shown that the schedule of brequinar is more important than the dose [13]. The schedule used in our clinical study was based on extrapolation from animal data. In order to better define the optimal schedule of brequinar when combined with 5-FU, we are presently engaged in an additional phase I study specifically designed to study the effects of brequinar on tissue UXP pools. Patients with colorectal cancer undergoing resection of either their primary tumor or liver metastases are eligible for this study. Patients will be randomized to receive a single dose of brequinar either 4 h or 18–20 h prior to their surgery. The major objective of the study is to determine the effect of brequinar on UXP pools in both tumor and normal adjacent tissue. In order to preserve the biochemical integrity of the tissues, cryosurgery is used to freeze a portion of the tumor and of the normal adjacent tissue that is going to be resected. Both the tumor and the adjacent normal tissue are frozen before the vascular pedicle of the tumor is clamped. This prevents any period of ischemia and preserves uridine pools from degradation by uridine phosphorylase. By using two different schedules, this protocol should allow us to better determine the lowest dose of brequinar that will produce biochemical modulation in humans at the tissue level.

Our phase I study of brequinar in combination with 5-FU suggests that brequinar in doses ranging from 400 to 600 mg/m^2 (which correspond to approximately one-third of the recommended phase II dose of brequinar alone) can produce a significant decrease in plasma uridine levels. The lack of significant toxic reactions at biochemically active doses of brequinar indicates that the initial dose of the effector agent 5-FU should be higher in future studies. Further studies are needed to better define the optimal schedule between brequinar and 5-FU administration.

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