Brequinar Potentiates 5-Fluorouracil Antitumor Activity in a Murine Model Colon 38 Tumor by Tissue-specific Modulation of Uridine Nucleotide Pools

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ABSTRACT

Modulation of pyrimidine metabolism or the metabolic fate of 5-fluorouracil by a number of different agents has permitted a significant increase in the response rate to this agent, particularly for colorectal cancers. Brequinar, a noncompetitive inhibitor of mitochondrial dehydroorotate dehydrogenase has been shown to achieve a tumor-specific modulation of the therapeutic effect of 5-fluorouracil.

A selective decrease of uridine nucleotide pools in Colon tumor 38 compared to normal tissues of C57/BL6 mice was observed after Brequinar administration. This effect was achieved with very low nontherapeutic doses of Brequinar (8 to 27% of the maximum tolerated dose in this model). Pretreatment with Brequinar and 24 h prior to administration of [3H]fluorouracil significantly increased incorporation of the fluoropyrimidine into Colon 38 tumor RNA, while minimal effects were seen in normal tissues of C57/BL6 mice.

Brequinar (15, 30, and 50 mg/kg) was administered 4 h prior to fluorouracil (85 mg/kg) on a weekly basis in Colon 38-bearing mice. All combinations potentiated 5-fluorouracil antitumor activity and the lowest dose of Brequinar (15 mg/kg) showed a reduced toxicity (weight loss) compared to the same dose of 5-fluorouracil as a single agent. When Brequinar preceded fluorouracil by 24 h, greater toxicity and less antitumor activity were observed.

A comparison of the optimal Brequinar-fluorouracil regimen with a previously optimized N-(phosphonoacetyl)-L-aspartic acid-fluorouracil combination in Colon 38 tumor indicated that Brequinar-fluorouracil was more effective and less toxic.

INTRODUCTION

FUra has been an important agent in the treatment of gastrointestinal malignancies but yields a limited response rate of 10–20% in colorectal cancer with an average duration of 6–8 months. In recent years, progress in understanding the biochemical pharmacology of FUra has led to a more rational approach in designing new drug combinations involving this fluoropyrimidine (1, 2). Several of these combinations, or biochemical modulations, have been shown to be effective not only in preclinical studies but also in the clinical arena. Leucovorin (3, 4), methotrexate (5–8), levamisole (9, 10), and α-interferon (11) with appropriate scheduling have improved the therapeutic efficacy of FUra in human disease.

Recently Ardan et al. (12) and O’Dwyer et al. (13) have reported 43–45% response rate when PALA, an inhibitor of aspartate transcarbamylase (14), was used as a modulator of FUra in colorectal cancer based on preliminary studies by Martin et al. (15). PALA was shown to deplete the intracellular pyrimidine nucleotide pools and modulate the incorporation of FUra into RNA with some indication of selectivity for tumors at low concentrations of PALA.

Brequinar, a fluorinated carboxyquinoline derivative, inhibits de novo pyrimidine synthesis by noncompetitive inhibition of mitochondrial dihydroorotate dehydrogenase (16–23) and has shown excellent antineoplastic activity in a number of murine tumors and human xenografts (16, 24, 25). Phase II trials as a single agent conducted in the United States and Europe in a variety of solid tumors, however, have yielded very modest antitumor activity in breast and colon cancers (26–31). Since Brequinar acts on the de novo pathway for pyrimidine but at a different target than PALA, it was evaluated as a modulator of FUra therapy in a murine model Colon 38 tumor.

We report here the effect of Brequinar on uridine and uridine nucleotide pools in plasma, normal tissues, and neoplastic (Colon 38) tissues, and the antitumor activity of its combination with FUra as well as a comparison with a PALA-FUra regimen.

MATERIALS AND METHODS

Drugs and Chemicals. 5-Fluorouracil was purchased from Sigma (St. Louis, MO). Brequinar sodium (DuP-785) was obtained from Du Pont Pharmaceuticals (Wilmington, DE). PALA was a generous gift from Dr. David A. Cooney, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). All the drugs were reconstituted in 0.85% saline and administered i.p. (0.1 ml of injected solution for 10 g of body weight). [6-3H]FUra was purchased from Moravek Biochemicals (Brea, CA).

All other chemicals were purchased from Sigma or Schwarz-Mann (Cleveland, OH).

Animal Experiments. Two- to 3-month-old female C57/BL6 mice were purchased from National Cancer Institute (Bethesda, MD), and were inoculated s.c. in each flank with 1-mm³ fragments of Colon 38 tumor. Treatment was started when the tumors were 50–250 mg, determined by caliper measurement according to the formula (15):

\[
\text{Tumor wt (mg)} = \text{length (mm)} \times \text{width}^2 (\text{mm}^2) + 2
\]

Groups of six mice, each containing a similar tumor size distribution, were treated on a weekly basis and tumor size was determined once or twice a week (32). Both Brequinar and 5-fluorouracil were dissolved in 0.85% NaCl solution and administered i.p. in a volume of 0.1 ml/10 g of body weight on a weekly basis. 5-Fluorouracil was administered at 85 mg/kg, the MTD in C57/BL6 mice for a weekly schedule. Brequinar was administered at 15, 30, and 50 mg/kg 4 or 24 h prior to 5-fluorouracil injections.

Differences in tumor size and weight among groups were evaluated by analysis of variance, followed by Bonferroni t-tests.

Toxicity was evaluated by changes in body weight and was expressed as a percentage of the initial body weight of the animal.

Tissue Uridine and Nucleotide Determination. Normal tissues and tumors were removed and frozen in liquid nitrogen with less than a 5-s delay after removal from the vascular bed. Tissue samples were homogenized in 2 volumes of ice-cold 15% TCA. One-half of the acid-soluble fraction was neutralized by trioctylamine-Freon extraction and stored at −20°C until analyzed for uridine by high-pressure liquid chromatography (33).

To determine total uridine nucleotides, the acid-soluble fraction was heated for 15 min at 100°C to convert them to UMP prior to Trioclylamin-Freon extraction.

Uridine was analyzed on a Rainin Microsorb C18 column (25 cm x 4.6 mm) eluted at 1 ml/min with 10 mM H₃PO₄ containing 30 μM...
heptane sulfonic acid, pH 3.1, at 8°C.

UMP was determined on an Altex Partisil SAX_10 column (25 cm x 4.6 mm) at room temperature with a mobile phase of 10 mM sodium phosphate, pH 3.3, at 1.5 ml/min. The recovery was 75 and 85% for uridine and UXP, respectively.

[¹³C]Fluorouracil Incorporation into RNA. Two h after FUra administration the animals were sacrificed by cervical dislocation. Tissues of mice given injections of [¹³C]FUra were immediately removed and frozen. Frozen samples were weighed and homogenized in 2 volumes of ice-cold 15% TCA. The pellet was washed twice with TCA at 0°C and dissolved in 2 volumes of 1 N NaOH by incubating overnight at 37°C (32). The solubilized tissues were neutralized with HCl before radioactivity determination.

Thymidylate Synthase Determination. Tumor and normal tissues were removed and frozen in liquid nitrogen. Thymidylate synthase inhibition was determined in tissue extracts by using the method of Fernandes and Cranford for the analysis of free and FdUMP-bound enzyme (34).

RESULTS

Appropriate doses and scheduling of Brequinar therapy were selected by evaluation of changes in de novo pyrimidine biosynthesis as reflected in the concentration of uridine and uracil nucleotides in tissues.

Treatment of C57/BL6 mice bearing Colon 38 tumor with a single i.p. administration of Brequinar at 50 mg/kg caused a rapid decrease in uridine concentrations in plasma, as well as in neoplastic and normal tissues (Fig. 1A). In plasma, liver, kidney, and spleen the uridine concentration returned to normal levels within 24 h. Gut and Colon 38 uridine pools, however, were still depleted after 48 h.

Similar decreases in total uridine nucleotide concentrations were observed after 2 h in both neoplastic and normal tissues (Fig. 1B). UXP pools returned to control levels within 4 to 6 h in all the normal tissues but in the Colon 38, the UXP pools were still reduced by 80% after 24 h. A smaller dose of Brequinar (15 mg/kg) caused a similar selective depletion of UXP pools in Colon 38 (Fig. 2) that returned to normal concentrations within 24 h.

This selective effect of Brequinar on the concentration of UXP in tumors compared to normal tissues prompted a study of the therapeutic efficacy of weekly schedules of Brequinar and FUra in mice bearing advanced Colon 38 tumor.

FUra was administered weekly at 85 mg/kg (a dose equivalent to its MTD in this model), 4 h after Brequinar, 15, 30, and 50 mg/kg (8, 16, and 27% of the MTD of this drug).
At these doses Brequinar had no antitumor activity and FUra as a single agent was only able to limit the tumor growth for the first 3 weeks. Brequinar (30 mg/kg) in combination with FUra completely inhibited tumor growth for up to 6 weeks (P = 0.015 at day 40 versus FUra alone) without additional toxicity compared to the same dose of FUra alone (85 mg/kg) (Fig. 3). Similar results were obtained with Brequinar at 15 and 50 mg/kg (data not shown). Higher doses of Brequinar (80, 110, and 150 mg/kg) in combination with FUra produced toxic deaths within 3 weeks without a significant improvement in the antitumor effect of Brequinar (data not shown).

The interval between Brequinar and FUra administration affects the antitumor activity and toxicity of the combinations. Mice bearing smaller Colon 38 tumors (50 mg) were treated with Brequinar at 15 or 50 mg/kg, 4 and 24 h prior to FUra. Brequinar alone had no antitumor effect. Fig. 4 shows that Brequinar at 15 mg/kg, 4 h prior to FUra, potentiated the tumor inhibition of FUra (P = 0.001). A similar effect was seen with the other doses and schedules. However, the time interval between Brequinar and FUra administration had a major impact on the toxicity (Table 1). Brequinar (15 mg/kg) administered 4 h prior to FUra was no more toxic than FUra alone and cured 83% of tumors (no evidence of tumor 60 days after the end of the therapy). The same dose of Brequinar given 24 h prior to FUra was more toxic (P < 0.05 compared to Brequinar, 15 mg/kg 4 h prior to 5-FUra) and was less effective; only 8% of the tumors completely regressed. A higher dose of Brequinar (50 mg/kg) was as effective as the low dose regimen when administered 4 h prior to FUra, but was much more toxic if administered 24 h prior to FUra.

Because of the proven efficacy (preclinical and clinical) of PALA plus FUra (12, 13, 15), we compared the antitumor
Fig. 4. Antitumor activity of the combination Brequinar-5-fluorouracil on Colon 38 tumors in C57/BL6 mice. C57/BL6 mice bearing Colon 38 tumors averaging 75 mg were randomized into groups of 6 mice each. Brequinar was administered on a weekly basis at 15 mg/kg, 4 or 24 h prior to FUra (85 mg/kg). The tumor size and mouse weights were measured twice a week as described in “Materials and Methods.” Arrows indicate time of administration of chemotherapy.

Table 1  Effect of Brequinar administered 4 and 24 h prior to 5-fluorouracil (85 mg/kg) on the toxicity and antitumor activity of 5-fluorouracil in mice bearing Colon 38 tumor

<table>
<thead>
<tr>
<th>Experimental groups (6 mice each)</th>
<th>Initial wt (g ± SD)</th>
<th>% of wt loss *</th>
<th>% of toxic deaths</th>
<th>% tumor free</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FUra, 85 mg/kg</td>
<td>17.5 ± 1.1</td>
<td>12 ± 6</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Brequinar, 15 mg/kg 4 h prior to 5-FUra, 85 mg/kg</td>
<td>18.0 ± 0.8</td>
<td>6 ± 5</td>
<td>0</td>
<td>83</td>
</tr>
<tr>
<td>Brequinar, 15 mg/kg 24 h prior to 5-FUra, 85 mg/kg</td>
<td>18.1 ± 1.6</td>
<td>18 ± 3 *</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Brequinar, 50 mg/kg 4 h prior to 5-FUra, 85 mg/kg</td>
<td>18.0 ± 0.5</td>
<td>15 ± 4 *</td>
<td>0</td>
<td>92</td>
</tr>
<tr>
<td>Brequinar, 50 mg/kg 24 h prior to 5-FUra, 85 mg/kg</td>
<td>17.7 ± 1.2</td>
<td>22 ± 9 *</td>
<td>100</td>
<td>92</td>
</tr>
</tbody>
</table>

* Compared to initial weight.
* P < 0.05 compared to Brequinar, 15 mg/kg 4 h prior to 5-FUra, 85 mg/kg.

These results indicate that Brequinar can improve the therapeutic index of FUra in the therapy of Colon 38 tumor by augmentation of its antitumor activity and can actually reduce its host toxicity.

DISCUSSION

These results indicate that Brequinar can improve the therapeutic index of FUra in the therapy of Colon 38 tumor by augmentation of its antitumor activity and can actually reduce its host toxicity.

Since Brequinar is apparently equally distributed in tumor and normal tissues and its elimination rate from all tissues was approximately equal (35), it is unlikely that these factors could account for its specific effect at the tumor level. The presence of greater uridine concentrations in normal tissues compared to Colon 38 tumor and possibly a different enzymatic capability to utilize uridine through the salvage pathway, may be responsible. The Na⁺-dependent active transport system for uridine is minimal for these tumors that have also limited uridine kinase activities (36).

Since thymidylate synthase is the other primary target for FUra, we also analyzed the effect of Brequinar plus FUra on this enzyme. No significant difference was seen in the extent of thymidylate synthase inhibition by FUra or the combination in Colon 38 tumor for periods up to 72 h after FUra administration (data not shown).
BREQUINAR POTENTIATION OF FUra THERAPY

Fig. 5. Comparison of Brequinar-FUra versus PALA-FUra in mice bearing Colon 38 tumors. C57/BL6 mice bearing advanced Colon 38 tumors averaging 150 mg were randomized into groups of 5 mice each. Brequinar was administered on a weekly basis at 15 mg/kg, 4 h prior to FUra (85 mg/kg). Treatment with PALA (100 mg/kg) preceded FUra (85 mg/kg) by 24 h. The tumor size and animal weights were measured twice a week as described in "Materials and Methods." Arrows indicate time of administration of chemotherapy.

Colon 38 tumor used in this study.

The observation that Brequinar had its best effect on FUra therapy when given at 4 h rather than at 24 h is difficult to explain. The nadir for UXP concentration in the tumor was reached 24 h after injection of 50 mg/kg of Brequinar. Combining fluorouracil after this interval of time did not result in an improved therapeutic effect and in fact resulted in increased toxicity. Thus, UXP depletion alone cannot be the explanation for this potentiation. We assumed that a depletion of the uridine diphosphate pools would result in a diminished formation of dUMP and thus less competition with FdUMP at the level of thymidylate synthase. Our results show that the degree or period of inhibition of thymidylate synthase in Colon 38 tumor was not affected by the Brequinar modulation. However, we cannot rule out the possibility that higher concentrations of Brequinar may enhance or prolong thymidylate synthase inhibition by FUra in normal tissues and result in increased toxicity.

The improved results seen with the Brequinar-FUra combination compared to PALA-FUra may reflect the relatively low activity of dihydroorotate dehydrogenase compared to aspartate transcarbamylase activity (1:100), the enzyme target for PALA in human lymphocytes (38, 39). Previous clinical experiences with PALA have emphasized the importance of the dosage ratio between modulator and effector agent and timing, and the current results emphasize the importance of appropriate scheduling. In summary, low, nontherapeutic doses of Brequinar can potentiate the antitumor activity of a given dose of fluorouracil and at the same time reduce its host toxicity if a correct schedule of administration is followed.
REFERENCES


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