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# Biochemical modulation of 5-fluorouracil with or without leucovorin by a low dose of brequinar in MGH-U1 cells*

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**Summary.** Combination of low doses of de novo pyrimidine biosynthesis inhibitors with 5-fluorouracil (FU) has been proposed to increase the antitumor activity of FU. Brequinar is such an inhibitor that has little clinical antitumor effect when used alone. We determined the clonogenic survival of MGH-U1 cells treated with FU ± leucovorin (LV) ± brequinar and examined the effects of these treatments on thymidylate synthase (TS). After 24 h exposure, the concentrations resulting in 50% inhibition of cell growth ($IC_{50}$) for brequinar, FU, and FU + LV (100 μM) were 0.4, 20, and 10 μM, respectively. Both 24 h pretreatment and 48 h continuous treatment with the $IC_{10}$ (0.1 μM) of brequinar increased the cytotoxicity of FU but did not enhance that of FU + LV. Simultaneous 24 h exposure to 0.1 μM brequinar and FU ± LV did not increase the cytotoxicity of FU ± LV. Intracellular cytidine triphosphate (CTP) and uridine triphosphate (UTP) pools, free TS binding sites, and levels of free fluorodeoxyuridine monophosphate (FdUMP) and deoxyuridine monophosphate (dUMP) were measured in cells pretreated with 0.1 μM brequinar for 24 h alone or followed by a 2-h exposure to FU (25 μM) ± LV (100 μM). In brequinar-treated cells, CTP and UTP pools amounted to 68% and 46% of control values, respectively. The free TS binding sites remaining amounted to 70% of control values in cells treated with FU and 9% of control levels in those treated with FU + brequinar. Free FdUMP levels increased 5-fold in cells pretreated with brequinar as compared with those treated with FU alone. The increased formation of FdUMP was inhibited by simultaneous exposure to 100 μM hypoxanthine and 25 μM FU. Intracellular dUMP levels were not affected by brequinar. We conclude that a low dose of brequinar increases the cytotoxicity of FU but does not enhance that of FU + LV when exposure to brequinar precedes FU treatment. This potentiation appears to be mediated by the increased formation of FdUMP as a consequence of an increase in the cosubstrate phosphoribosyl pyrophosphate (PRPP).

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## Introduction

5-Fluorouracil (FU) has shown limited clinical efficacy in the treatment of patients with various malignancies [13, 26, 27]. Attempts to increase its antitumor activity through biochemical modulation have been reported. The combination of leucovorin (LV) and FU is an example of this modulation and has led to an increased response rate in patients with colorectal cancer [7, 10, 13, 25, 32, 40].

To exert its cytotoxicity, FU must be intracellularly activated to fraudulent pyrimidine nucleotides [9, 15, 29, 37, 44] (Fig. 1). These fraudulent pyrimidine nucleotides compete with their normal counterparts in pyrimidine metabolism [20]. The inhibition of TS by fluorodeoxyuridine monophosphate (FdUMP), an active FU metabolite, is one of the main mechanisms underlying the action of FU [37]. Inadequate FdUMP formation in patients following FU treatment is a prominent mechanism of tumor resistance to TS inhibition [43]. Inhibition of de novo pyrimidine biosynthesis may increase the activation of FU and favor utilization of these fraudulent pyrimidine nucleotides by selectively decreasing pools of the competing normal pyrimidine nucleotides [15, 20, 23].

De novo pyrimidine biosynthesis inhibitors are capable of blocking specific steps in the formation of pyrimidine nucleotides. Whereas *N*-(phosphonacetyl)-L-aspartate (PALA) has been evaluated extensively [14], brequinar is a novel agent that inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, the fourth enzyme in the de novo pyrimidine biosynthesis pathway [6] (Fig. 2). Although brequinar has demonstrated a broad spectrum of antitumor activity against various tumor cell lines [4, 8, 17, 34, 42], clinical trials of this compound alone have revealed little antitumor efficacy [2, 3, 30].

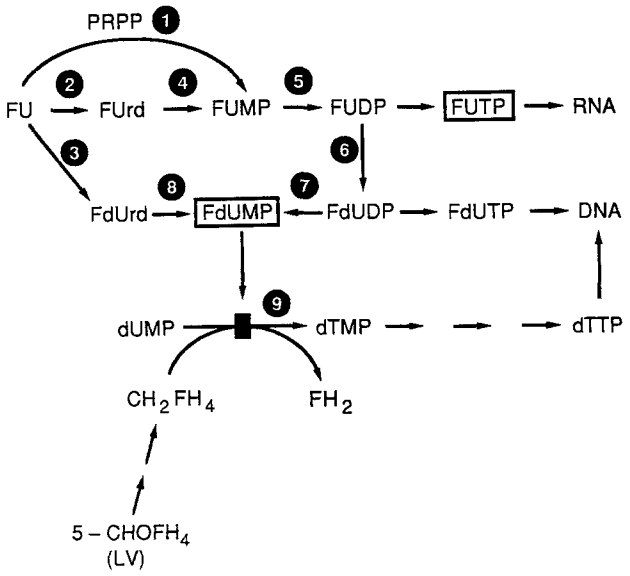


**Fig. 1.** Anabolism and sites of action of FU in the presence of the following enzymes: *1*, phosphoribosyl transferase; *2*, uridine phosphorylase; *3*, thymidine phosphorylase; *4*, uridine kinase; *5*, uridine monophosphate kinase; *6*, ribonucleotide reductase; *7*, phosphatase; *8*, thymidine kinase; and *9*, thymidylate synthase. The activation of FU can be bidirectional. *Arrows* are drawn in one direction only for clarity. *PRPP*, Phosphoribosyl pyrophosphate; *FUrd*, fluorouridine; *FUMP*, fluorouridine monophosphate; *FUDP*, fluorouridine diphosphate; *FUTP*, fluorouridine triphosphate; *FdUrd*, fluorodeoxyuridine; *FdUMP*, fluorodeoxyuridine monophosphate; *FdUDP*, fluorodeoxyuridine diphosphate; *FdUTP*, fluorodeoxyuridine triphosphate; *dTMP*, deoxythymidine monophosphate, *dTTP*, deoxythymidine triphosphate

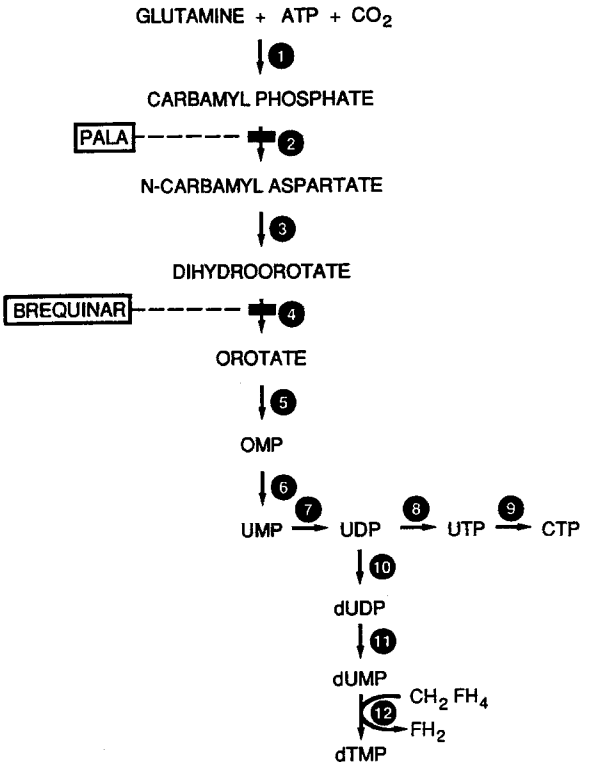


**Fig. 2.** De novo pyrimidine biosynthetic pathway and sites of action of PALA and brequinar in the presence of the following enzymes: *1*, carbamyl phosphate synthase; *2*, aspartate transcarbamylase; *3*, dihydroorotase; *4*, dihydroorotate dehydrogenase; *5*, orotate phosphoribosyltransferase; *6*, orotidylic acid decarboxylase; *7*, uridine monophosphate kinase; *8*, uridine diphosphate kinase; *9*, cytidine triphosphate synthase; *10*, ribonucleotide reductase; *11*, deoxyuridine diphosphatase; and *12*, thymidylate synthase. *ATP*, Adenosine triphosphate; *UMP*, uridine monophosphate; *UTP* uridine triphosphate; *CTP*, cytidine triphosphate; *dUDP*, deoxyuridine diphosphate; *dUMP*, deoxyuridine monophosphate; *dTMP*, deoxythymidine monophosphate

Recently, the concomitant administration of a low dose of PALA has been proposed to modulate FU toxicity [1, 5, 22, 31]. Pizzorno and colleagues [38] have reported that brequinar produces a synergistic effect when it is given in combination with FU to mice bearing colon tumor 38. Studies on the biochemical effects of a low dose of brequinar together with FU ± LV on TS inhibition have not yet been performed. The purpose of the present study was to determine whether a low dose of brequinar could alter the formation of cytidine triphosphate (CTP), uridine triphosphate (UTP), FdUMP, and deoxyuridine monophosphate (dUMP) and to investigate its effects on the TS binding and cytotoxicity of FU ± LV.

## Materials and methods

*Drugs and chemicals.* FU and LV (Sigma Chemical Co., St. Louis, Mo.) were dissolved in distilled water at a final concentration of 10 mM. FU solutions were made every 2 weeks and kept at –20° C; LV was made fresh daily and protected from light. Brequinar was obtained from the National Cancer Institute, National Institutes of Health (Bethesda, Md.); it was dissolved in distilled water and kept at –20° C for up to 6 months. Hypoxanthine (Sigma) was dissolved in 0.1 *N* sodium hydroxide at a final concentration of 10 mM. [6-$^3$H]-FdUMP (18–20 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, Calif.), and [5-$^3$H]-dUMP (18–20 Ci/mmol) was obtained from Amersham Co. (Oakville, Ontario, Canada); both were stored at –20° C in a 1:1 (v/v) mixture of ethanol: water. TRIS was obtained from International Biotechnologies Inc. (New Haven, Conn.). L. Casei TS (53.4 μmol $h^{-1}$ $ml^{-1}$, 38.3 mg/ml) was purchased from Biopure Co. (Boston, Mass.) and stored at –20° C. Methanol and acetonitrile were obtained from BDH Inc. (Toronto, Canada). Aqueous counting scintillant (ACS) was purchased from Amersham. All other chemicals were purchased from Sigma. Cell-culture medium and trypsin were obtained from Gibco Laboratories, Life Technologies, Inc. (Grand Island, N. Y.). Fetal calf serum (FCS) was purchased from Bockneck Laboratory Inc. and was dialyzed at the Ontario Cancer Institute (Toronto, Canada). Nunclon 80-$cm^2$ flasks were supplied by Gibco. Falcon 60-$mm^2$ dishes were obtained from Becton Dickinson Canada Inc. (Mississauga, Canada).

*Working buffers and solutions.* Buffers were prepared as follows: buffer A consisted of 31 mg dithiothreitol, 31 mg magnesium chloride, 121 mg TRIS, and 200 mg bovine serum albumin (BSA) in a final volume of 100 ml (pH 7.4); buffer B comprised 200 mM TRIS-HCl buffer (pH 7.4). L. Casei TS was diluted in buffer A. Acid-washed activated-charcoal stock was made as a 10% (w/v) suspension supplemented with 2.5% (w/v) BSA and 0.25% (w/v) dextran. The cofactor solution consisted of 10 mg 5,10-tetrahydrofolate, 10 μl formaldehyde (37% aqueous solution by weight), and 100 μl 2-mercaptoethanol (1 ml = 1.1168 g) in 10 ml buffer B.

*MGH-U1 cells.* MGH-U1, a human transitional bladder-carcinoma cell line, was originally obtained from Dr. G. Prout (Boston, Mass.). The cells were maintained as a monolayer as described elsewhere [11]. For clonogenic and biochemical experiments, cells were grown in α-mini-

mum essential medium (α-MEM) containing no nucleosides and supplemented with 10% dialyzed FCS. The concentration of brequinar producing 10% inhibition of cell growth ($IC_{10}$, 0.1 μM) was used in all combination treatments. FU (25 μM), LV (100 μM), and hypoxanthine (100 μM) were used in the biochemical studies.

*Clonogenic assay.* Logarithmically growing cells were harvested, and a million cells were seeded in each flask. The dose-response relationship for brequinar was tested over a concentration range of 0.01 – 100 μM for a 24-h exposure period. Drugs were added on day 2 (24 h after seeding) and incubated for 24 h. In combination treatments, cells were treated with the $IC_{10}$ of brequinar (0.1 μM) on day 1 (immediately after seeding, pretreatment), on day 2 (24 h after seeding, simultaneous treatment), or on day 1 for 48 h continuous treatment followed by FU (0–100 μM) ± LV (100 μM) during the last 24 h, i.e., on day 2. On day 3 (48 h after seeding), cells in each flask were trypsinized, counted, diluted, and plated in 60-mm² Falcon dishes at varying concentrations in replicates of three. At 14 days after plating, the colonies were fixed and stained with 2% methylene blue in a 1 : 1 (v/v) mixture of methanol and water and then counted by eye. The surviving fraction was expressed as a fraction of the plating efficiency of control cells. All experiments were repeated to ensure reproducibility.

*CTP and UTP measurement by high-performance liquid chromatography.* The CTP and UTP levels in control cells and brequinar-treated cells were measured by a modified ion-pair reverse-phase high-performance liquid chromatographic (HPLC) method [36] using a Waters HPLC system (Waters Associates, Milford, Mass.) consisting of an M6000 solvent-delivery pump, a model 440 UV absorbance detector (254 nm), a model U6K injector, and a Radial-Pak cartridge. The chromatogram was recorded using a model CR 501 Shimadzu Chromatopac integrator (Shimadzu Corp., Analytical Instruments Division, Kyoto, Japan). The mobile phase comprised 0.005 M tetrabutylammonium phosphate in 0.01 M ammonium dihydrogen phosphate buffer (pH 7.0) supplemented with 15% (v/v) acetonitrile. The separation was achieved on an 8- × 100-mm Nova-Pak $C_{18}$ column (Waters) at a flow rate of 2 ml/min using isocratic elution. The retention times of CTP and UTP were 15 and 20 min, respectively. The nucleotide levels in cell extracts were calculated from a standard curve generated for the AUC versus the concentration of CTP or UTP in nanomoles. The cell extracts were prepared as described below for the free FdUMP assay.

*[³H]-FdUMP binding assay.* A modification of the [³H]-FdUMP binding assay of Moran [28] was used to measure cytosolic free TS binding sites as well as to measure free FdUMP levels using L. Casei TS as the enzyme source. For measurements of TS binding sites, logarithmically growing cells were trypsinized, counted, and centrifuged at 1,000 *g* for 5 min. The supernatant was discarded and the pellets were resuspended with buffer A to give a final concentration of $5 \times 10^7$ cells/ml. The suspension was freeze-thawed three times, sonicated for 1 min with a model VC600 sonicator (power setting of 5) using a 2-inch cup horn (Sonics & Material, Danburg, Conn.), and centrifuged at 16,000 *g* for 15 min. To 100 μl of this extract were added 50 μl [³H]-FdUMP (5 pmol) in distilled water and 50 μl cofactor solution (final folate concentration, 400 μM). The mixture was incubated at 30° C for 15 min, and 0.5 ml ice-cold charcoal suspension was added to bind free nucleotides. The mixture was centrifuged at 16,000 *g* for 10 min to separate protein-bound radioactivity from free nucleotides, and 0.4 ml supernatant was added to 10 ml ACS for counting. The background counts determined using buffer A, [³H]-FdUMP, and cofactor solution averaged 500 cpm. Results were expressed as a percentage of the TS binding sites in controls.

For the free FdUMP assay, cell extracts were prepared as previously described [33, 41]. Levels of free FdUMP were determined using the [³H]-FdUMP binding assay described above, except that 500 pmol L. Casei TS was used instead of the cytosolic enzyme. A standard curve was constructed each time, and the amount of FdUMP in unknown samples was determined by reference to these standard curves. The amount of [³H]-FdUMP bound was inversely proportional to the amount of cold FdUMP in the reaction mixture. In the presence of excess cofactor, coincubation of dUMP with FdUMP at a ratio of 1,000 : 1 (dUMP : FdUMP) did not inhibit the binding of [³H]-FdUMP to L. Casei TS.

*Free dUMP assay.* This assay was a modification of the tritium-release assay of Roberts [39]. The cell extracts were prepared in the same manner as for the free FdUMP assay. To 100 μl cell extract were added 50 μl [³H]-dUMP (1 pmol) in water, 25 μl L. casei TS (5 pmol), and 25 μl cofactor solution. The tubes were incubated at 37° C for 30 min. The reaction was stopped by the addition of 100 μl cold 20% trichloroacetic acid (TCA) solution and 0.5 ml charcoal suspension to bind free nucleotides. The mixture was vortexed and centrifuged at 16,000 *g* for 10 min, and the supernatant (0.5 ml) was removed for counting. The background counts obtained using buffer A and [³H]-dUMP averaged <200 cpm. A standard curve was constructed as described above for the FdUMP assay.

*Data analysis.* Multiregression analysis and one-way analysis of variance (ANOVA) were used to analyze cytotoxicity data and biochemical data (CTP and UTP pools), respectively [24]. These data analyses were performed on a VAX system (Digital Equipment Corporation, USA) using MINITAB analysis software (State College, Pa.). *P* values of <0.02 and <0.05 were considered to be significant for the cytotoxicity and biochemical data, respectively.

## Results

### *Dose-response curve for brequinar in MGH-U1 cells*

The dose-response curve generated for MGH-U1 cells treated with brequinar for 24 h is shown in Fig. 3. The $IC_{10}$ and $IC_{50}$ values were 0.1 and 0.4 μM, respectively. The cytotoxicity was dose-dependent over the concentration range of 0.01 – 10 μM.

### *Intracellular CTP and UTP pools in cell extracts*

After a 24-h exposure to 0.1 μM brequinar, intracellular CTP and UTP pools amounted to 68% and 46% of control values, respectively (Fig. 4). The difference in UTP pools noted between brequinar-treated cells and controls was statistically significant ($P < 0.05$), but no difference was found in CTP pools.

### *Cytotoxicity of FU ± LV ± brequinar*

The clonogenic survival of MGH-U1 cells treated with FU ± LV alone following 24 h preincubation and 48 h continuous incubation with the $IC_{10}$ of brequinar is shown in Fig. 5. The cytotoxicity of FU ± LV was dose-dependent over the FU concentration range of 0–100 μM. The $IC_{50}$ value for FU was 20 μM, and that for FU + LV was 10 μM at an LV concentration of 100 μM. The potentiation of FU cytotoxicity by LV was statistically significant ($P < 0.00001$). The cytotoxicity of FU was increased by 24 h pretreatment with or 48 h continuous exposure to brequinar + FU during the last 24 h ($P = 0.002$ and $P = 0.009$, respectively). The cytotoxicity of FU + LV was not increased by 24 h pretreatment with or 48 h continuous exposure to brequinar. Simultaneous 24 h exposure to brequinar along with FU ± LV did not affect the cytotoxicity of FU ± LV (data not shown).

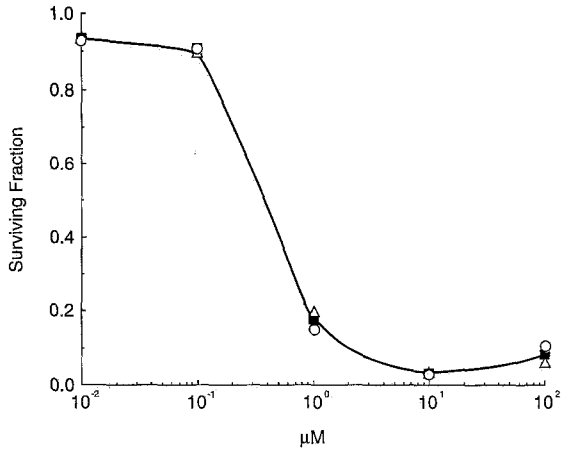


**Fig. 3.** Dose-response curve generated for brequinar after 24 h drug exposure. Each *open symbol* represents 2 separate experiments; each *solid square* represents the mean value for the 2 experiments. The plating efficiency of controls was 85%. The variation between these values was <2 orders of magnitude

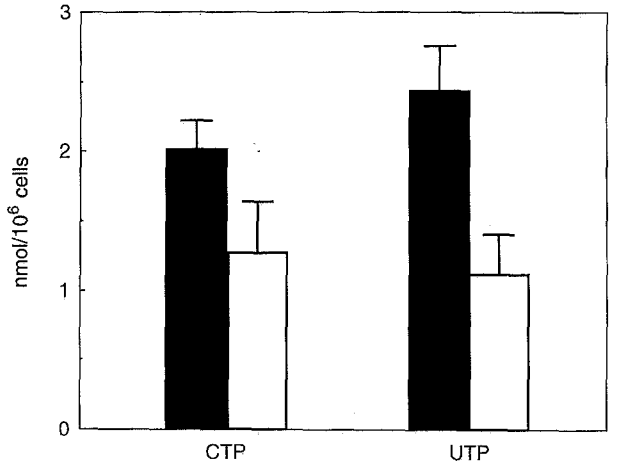


**Fig. 4.** CTP and UTP pools in control cells, and cells treated with the $IC_{10}$ of brequinar for 24 h. ■, controls; □, brequinar-treated cells. Data represent mean values ± SD for 3 separate experiments

### *Effect of brequinar and LV on TS binding sites in FU-treated cells*

The free TS binding sites remaining in the cells after various treatments are shown in Fig. 6. Neither LV nor brequinar alone affected the level of TS binding sites. The free TS binding sites amounted to 70% of the control value following 2 h exposure of cells to FU alone and 9% of the control level for cells pretreated with brequinar and subsequently exposed to FU. The addition of LV did not further change the quantity of TS binding sites remaining.

### *Effect of brequinar and hypoxanthine on FdUMP formation*

Free FdUMP levels remaining in the cells after various treatments are shown in Table 1. Pretreatment of cells with the $IC_{10}$ of brequinar for 24 h followed by 2 h exposure to 25 μM FU resulted in a 5-fold increase in free FdUMP levels as compared with those resulting from exposure to FU alone. The accumulation of FdUMP in cells treated with FU alone or with FU + brequinar was inhibited by simultaneous exposure to 100 μM hypoxanthine.

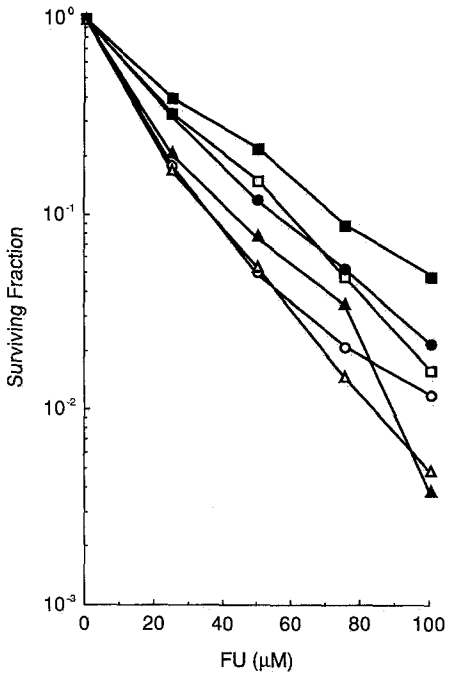


**Fig. 5.** Survival curves generated for MGH-U1 cells treated with FU ± LV ± brequinar. The brequinar concentration was maintained constant at the $IC_{10}$. The concentration of LV was kept constant at 100 μM. The exposure period for FU ± LV was 24 h. ■, FU; □, 24 h pretreatment with brequinar + FU; ●, 48 h continuous treatment with brequinar + FU; ○, FU + LV; ▲, 24 h pretreatment with brequinar + FU + LV; △, 48 h continuous treatment with brequinar + FU + LV. Each *point* represents 2 separate experiments. The plating efficiency of controls was 85%. The variation between these values was <2 orders of magnitude

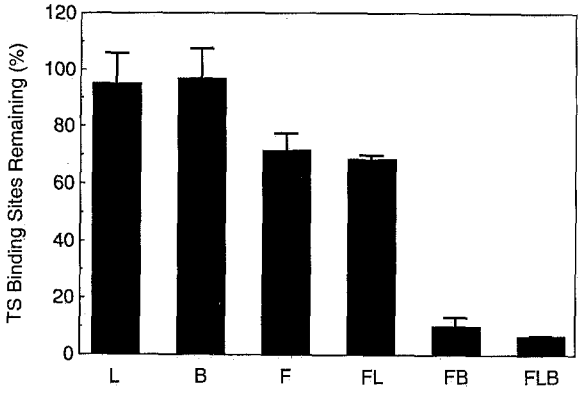


**Fig. 6.** TS binding sites remaining in cells after various treatments as described in Materials and methods. *L*, LV; *B*, brequinar; *F*, FU; *FL*, FU + LV; *FB*, FU + brequinar; *FLB*, FU + LV + brequinar. Total TS binding sites amounted to 1.1 pmol/$10^7$ cells in untreated controls. Data represent mean values ± SD for 3 separate experiments

**Table 1.** Intracellular levels of free FdUMP in MGH-U1 cells

| Drugs | FdUMP (fmol/$10^6$ cells) | Drugs | FdUMP (fmol/$10^6$ cells) |
|---|---|---|---|
| F | 147 ± 15.3 | FH | ND |
| FB | 767 ± 224.8 | FBH | ND |

MGH-U1 cells were treated with 25 μM FU ± 100 μM hypoxanthine for 2 h or pretreated with the $IC_{10}$ of brequinar for 24 h followed by FU ± hypoxanthine. Free FdUMP levels were measured by a modified [$^3$H]-FdUMP assay using L. Casei TS as the enzyme source (see Materials and methods for details). Data represent mean values ± SD for 3 separate experiments. F, FU; H, hypoxanthine; B, brequinar; ND, not detectable

*Effect of brequinar on dUMP levels*

Mean intracellular levels of free dUMP were 25.8 ± 1.7 and 24.3 ± 1.7 pmol/$10^6$ cells ($n$ = 3 separate experiments) in controls and in cells treated for 24 h with the $IC_{10}$ of brequinar, respectively.

## Discussion

Modulation of FU cytotoxicity by de novo pyrimidine biosynthesis inhibitors is an attractive approach to increasing the effect of FU. The aim of such modulation is to achieve an ultimate improvement of the therapeutic index, i.e., to increase the selectivity of an anticancer agent for tumor versus normal cells [16, 18, 21]. To this end, the dose of the modulating agent used is critical. Martin et al. [23] have argued that the dose of a modulator should be biochemically active but not necessarily biologically effective.

The biochemical rationale for combining brequinar with FU is that inhibition of de novo pyrimidine biosynthesis may increase levels of phosphoribosyl pyrophosphate (PRPP), a cosubstrate involved in the anabolism of FU, and/or may deplete competing intracellular nucleotides such as UTP and dUMP. On this basis, we determined the cytotoxicity and the biochemical effects on TS of a low dose of brequinar used alone or in combination with FU ± LV in MGH-U1 cells. MGH-U1 cells were selected because they have been reported to be sensitive to *N*-(phosphonacetyl)-L-aspartate (PALA) [12], an inhibitor of de novo pyrimidine biosynthesis, and because LV potentiates the cytotoxicity of FU in these cells [11]. Brequinar is a novel de novo pyrimidine biosynthesis inhibitor that acts at a site different from that used by PALA (Fig. 2).

Figure 3 illustrates the dose-response relationship of brequinar in MGH-U1 cells after a 24-h exposure period. The cytotoxic potency of brequinar was greater than that of PALA in these cells (unpublished data). The measurement of intracellular CTP and UTP pools after treatment with 0.1 μM brequinar demonstrated a significant decrease in UTP and a smaller decrease in CTP. Thus, the $IC_{10}$ of brequinar was selected for use in the combination studies as it was relatively nontoxic yet retained some biochemical effects. Furthermore, the degree to which UTP levels were decreased in these cells was similar to that previously observed by Martin et al. [22] in CD8F1 mice bearing breast tumors after treatment with a therapeutically inactive dose of PALA. This approach is also consistent with the rationale proposed by Martin et al. [23].

The cell-survival studies confirmed that LV can potentiate the cytotoxicity of FU in these cells. The experiments using brequinar + FU revealed that brequinar could potentiate the effects of FU alone when 24 h exposure to brequinar preceded the FU treatment. A similar cytotoxic effect was seen when cells were exposed to brequinar for 48 h, with FU being added during the last 24 h; however, simultaneous exposure of cells to brequinar and FU did not potentiate the cytotoxicity of FU. This finding suggests that prolonged exposure to brequinar is not necessary for the achievement of such potentiation but that pretreatment is a prerequisite.

Interestingly, we did not see any increase in cytotoxicity when brequinar was added to cells undergoing treatment with FU + LV on any of the three exposure schedules used. The reason for this lack of potentiation is not evident from our results. However, two possible explanations for this observation can be put forth. In the presence of an excess of reduced folate cofactor, the binding of FdUMP to TS may be sufficiently tight that the contribution of additional FdUMP would be slight. Alternatively, additional effects of brequinar that limit the cytotoxicity of FU may be operative, and only the total impact would be reflected in the cytotoxicity studies. Nevertheless, our results suggest that the addition of brequinar to the combination of FU + LV is of limited value. This observation requires further evaluation in other cell lines.

The apparent lack of effect of LV on the remaining TS binding sites determined using the FdUMP binding assay may have been secondary to the presence of endogenous free FdUMP in the cell extracts. Increased binding of endogenous free FdUMP following LV treatment would lead to an underestimation of the true quantity of TS binding sites remaining in the cells. The studies of TS binding indicated that brequinar could decrease the levels of TS binding sites. At the same time, free FdUMP levels were increased. Taken together with the cytotoxicity data, these results indicate that the mechanism underlying the increase in cytotoxicity obtained using brequinar pretreatment is partly attributable to an enhanced synthesis of FdUMP, thus overcoming one limitation of ternary complex formation [43]. A similar finding has been reported for the combination of PALA and FU in sarcoma 180 cells [19]. In contrast to our studies, in which a low concentration of brequinar was used, Liang et al. [19] used a very high concentration of PALA.

The complete abrogation of free FdUMP accumulation observed in the presence of 100 μM hypoxanthine in the present study strongly suggests that the primary role for brequinar in this interaction is to increase the accumulation of PRPP [15]. Due to its decreased utilization in the conversion of orotate to OMP, the accumulated PPPP would thus be available for anabolism of FU. This observation is consistent with the finding of Peters et al. [35] that activation of FU proceeds preferentially through its one-step conversion to FUMP by phosphoribosyl transferase in tumor cells. Our failure to observe a decrease in dUMP

levels when cells were treated with brequinar alone implies that a depletion of this natural competing substrate did not contribute to the recorded decrease in free TS binding sites. The decrease in UTP and increase in PRPP resulting from brequinar treatment may increase the incorporation of FU into RNA as well. This second potential effect of brequinar on the mechanism of FU cytotoxicity further strengthens the rationale for combining brequinar with FU.

In conclusion, we found (a) that brequinar can increase the cytotoxicity of FU when it is applied prior to FU treatment, (b) that brequinar increases the formation of FdUMP, and (c) that the enhanced formation of FdUMP is primarily mediated by the increased anabolism of FU by phosphoribosyl transferase.

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