Potentiation of 5-Fluorouracil Efficacy by the Dihydrouracil Dehydrogenase Inhibitor, 5-Benzylxobenzyluracil1

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ABSTRACT

5-Benzylxobenzyluracil (BBU) is the most potent inhibitor (K1 ~30 nm) of dihydouracil dehydrogenase (EC 1.3.1.2), the first enzyme in the catabolic pathway of pyrimidine bases and their analogues, including 5-fluorouracil (FUra). The effect of BBU on modulating the chemotherapeutic efficacy and host toxicity of FUra was evaluated using human colon carcinoma DLD-1 grown in culture and as xenografts in anti-thymocyte serum (ATS)-immunosuppressed mice. The effect of BBU on FUra-induced host toxicity was also studied in nontumor-bearing-ATS-immunosuppressed and immunocompetent mice. At 0.2 μM, BBU potentiated growth inhibition by FUra of DLD-1 cells in culture (the concentration that produces 50% inhibition of cell growth was 0.48 μM at 3 h) by 1.3-fold (from 45 to 28% growth). BBU also enhanced the cytotoxic effect of FUra (0.48 μM, 3 h) against DLD-1 grown in soft agar by 3-fold (from 45 to 15% growth). In ATS-immunosuppressed mice bearing DLD-1 xenografts, coadministration of BBU with FUra enhanced not only the efficacy of FUra in killing the tumor but also protected the host from FUra-induced host toxicity. This was particularly evident at low doses of FUra. Coadministration of BBU (10 mg/kg/day × 2) with FUra at 30 mg/kg/day × 2 reduced tumor weight by 16-fold (from 799 to 49 mg) and increased host survival from 83 to 100%. The enhancement of tumor kill and protection from host toxicity induced by FUra was also evident at higher doses of FUra, albeit to a lesser degree. At 120 mg/kg/day × 2 FUra, coadministration of BBU (10 mg/kg/day × 2) reduced tumor weight from 44 to 10 mg and increased survival of the animals from 33 to 50%. Host protection from FUra-induced toxicity was corroborated further by the protective effect of BBU, inferred from the increase in the dose that produces 50% mortality in ATS-immunosuppressed (from 135 to 195 mg/kg/day × 2) and immunocompetent (from 250 to 300 mg/kg/day × 2) mice. Therefore, coadministration of BBU improved the therapeutic index of FUra by 5.5-fold (from 2.3 to 12.6) as a result of potentiating the antitumor efficacy of FUra and reducing its induced host toxicity. This protection by BBU sharply contrasts with the effect of most other dihydrouracil dehydrogenase inhibitors, which at therapeutic doses increase host toxicity. These findings may lead to a more successful use of FUra in cancer chemotherapy.

INTRODUCTION

For over three decades, FUra3 despite its clinical toxicity, has remained among the few drugs effective against solid tumors in humans. Although the anabolism of FUra and its nucleosides is the main factor accountable for its chemotherapeutic effects, catabolism plays a major role in therapy by this fluoropyrimidine. Indeed, over 80% of clinically administered FUra is rapidly degraded, via the catabolic route, with loss of efficacy (1–3). Increasing the administered dose of the drug is limited by host toxicity. DHUDase (EC 1.3.1.2) is the first and rate-limiting enzyme in the catabolic pathway of pyrimidine bases (except cytosine) and their analogues, including FUra (4, 5). FUra is a better substrate for DHUDase than the natural metabolites, uracil and thymine (5–9).

The importance of DHUDase as a target for chemotherapy has been established by several recent studies. For example, in patients receiving continuous infusion of FUra at a constant rate, it was found that the plasma concentration of FUra varied significantly during treatment (10, 11). This variation followed a circadian rhythm, which was the inverse of that observed for DHUDase activity (11); i.e., a high plasma concentration of FUra was associated with low DHUDase activity and vice versa. A significant correlation between the circadian rhythm of DHUDase activity and that of the anticancer efficacy of FUra has also been reported (12). Hence, a strong association exists between the level of DHUDase activity and the bioavailability and efficacy of FUra. Moreover, inhibitors of DHUDase administered by various routes (p.o., i.p., etc.), increased both the concentration and half-life of FUra in plasma (12–15) and strongly enhanced the chemotherapeutic efficacy of various 5-fluorinated pyrimidines (e.g., FUra, Florafor, etc.) in vitro and in vivo (12–24). Nonetheless, coadministration of DHUDase inhibitors with FUra has not been popular for two main reasons: (a) it was generally believed that tumors lack or possess very little DHUDase activity (25–27); hence, FUra by itself was considered selectively toxic to tumors; and (b) although the available inhibitors of DHUDase enhanced the efficacy of FUra, at curative doses most inhibitors increased host toxicity (12–14, 16, 17, 20–24).

We have identified BBU as the most potent inhibitor (K1 ~30 nm) of DHUDase (28) yet known and an effective inhibitor of the catabolism of FUra (29). We have also demonstrated that DHUDase activity is substantially present in a wide range of human tumors, most notably solid tumors of the colon, lung, and pancreas (5). In the present study, we examined the effects of BBU on the efficacy and toxicity of FUra against human tumors in vitro and in vivo. The results indicate that coadministration of BBU improves the antitumor efficacy of FUra. Most importantly, BBU does not increase the host toxicity of FUra but may indeed protect from FUra-induced host toxicity. These findings may lead to a more successful use of FUra in chemotherapy. A preliminary report has been presented (30).

MATERIALS AND METHODS

Chemicals. RPMI 1640, FBS, phosphate-buffered saline, Difco Noble agar, streptomyacin and penicillin, and trypsin-EDTA (0.5% trypsin-5.3 mM EDTA) were purchased from Gibco (Grand Island, NY); ATS was obtained from Accurent Chemical and Scientific Corp. (Westbury, NY); and BBU was synthesized and generously provided by Dr. Shih-Hsi Chu (Brown University, Providence, RI). All other chemicals (highest grade) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture Studies. The toxicity of BBU, alone and in combination with FUra, was determined using DLD-1 human cells in culture and on soft agar. DLD-1 is a carcinoma of the human sigmoid colon, morphologically heterogeneous, varying from moderately to poorly differentiated, and characterized

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3 The abbreviations used are: FUra, 5-fluorouracil; DHUDase, dihydrouracil dehydrogenase; BBU, 5-benzyloxybenzyluracil; FBS, fetal bovine serum; ATS, anti-thymocyte serum; IC50, the concentration that produces 50% inhibition of cell growth; DMSO, dimethyl sulfoxide; LD10, the dose that produces 50% mortality; T:C, the ratio of tumor weight in the treated group to that in the corresponding untreated control; ED10, the dose required to achieve a T:C value of 50%.
by a moderate DHUDe activity (5). The cells (passages 10—20) were routinely grown in RPMI 1640-modified growth medium [10% FBS, 100 μg/ml streptomycin and 100 units/ml penicillin in RPMI 1640 (pH 7.15)] in a humidified incubator maintained at 37°C and 95% air/5% CO2. Determination of Doubling Time. The doubling time was determined by daily counts of viable untreated cells using a hemacytometer (Bright-Line; Reichert Scientific Instruments) and a microscope (Nikon Labophot type 104), or a Coulter counter (Coulter Electronics, Inc.) connected to a Coulter channelyzer.

Determination of IC50 of BBU and FUra. BBU was dissolved in DMSO as a stock solution (13 mm). The solution was diluted with normal saline (0.9% NaCl) to give the appropriate concentration before its use. The final concentration of DMSO in the media ranged between 0.02—1.0%. FUra was dissolved in normal saline. Plastic plates (35 × 15 mm) were seeded with a 2-ml suspension of 2 × 10^6 cells/ml in modified RPMI 1640 growth medium and incubated at 37°C. After 24 h, 100 μl of BBU or FUra (to give a final concentration of 0.1, 1.0, 10, or 100 μM) were added to the cell cultures. Controls received normal saline only (containing appropriate concentrations of DMSO, when required). After 3 h, the growth medium was replaced with fresh medium without BBU or FUra. Twenty-four h thereafter, control and treated cells were counted daily for 3 consecutive days by the trypan blue exclusion method. All experiments were carried out in duplicate. IC50 was estimated from the plot of the number of cell doublings versus the logarithm of drug concentration.

Determination of the Effect of BBU on FUra-treated Cells by Trypan Blue Exclusion. Plastic tissue culture plates (35 × 15 mm) were seeded with a 2-ml suspension of 2×10^6 cells/ml in modified RPMI 1640 growth medium and incubated at 37°C. After 24 h, 100 μl of saline containing BBU, FUra, or their combinations were added to the cell cultures. The final concentration of BBU was 2 μM and FUra its IC50 (0.48 μM). When the combination was tested, the cells were preincubated with BBU for 30 min before addition of FUra. After 3 h, the medium was replaced with fresh medium without FUra or BBU. After an additional 24 h, control and drug-treated cells were counted daily for 3 consecutive days by the trypan blue exclusion method. All experiments were carried out in duplicate. The degree of survival was expressed as a percentage of the untreated controls.

Determination of the Effect of BBU on FUra-treated Cells by Soft Agar Cloning. Noble agar at 0.5 and 0.3% in RPMI 1640 (pH 7.15) containing 20% FBS was prepared immediately before its use. The medium of log phase cells (24 h), growing in plastic dishes (35 × 15 mm), was replaced by fresh medium. After approximately 24 h, the exponentially growing cells were treated for 3 h with BBU, FUra, or their combinations as described above. Afterwards, the cells were recovered by trypsinization (0.005% trypsin and 0.002% EDTA in Hanks’ balanced salt solution, free of calcium, magnesium, and bicarbonate) and diluted with 0.3% nutrient agar to yield 100 colonies/dish (approximately 200 cells/ml). A 1-ml suspension of this preparation was added to dishes containing 2 ml of the 0.5% nutrient agar precooled to 37°C. The cell suspension and agar were mixed gently but quickly and kept on ice until a gel began to form (approximately 15 min). The plates were left at room temperature for an additional 45 min and then incubated at 37°C for 2—3 weeks before counting with a dark-field colony counter (American Optical). The degree of survival was expressed as the fraction of colonies that survived (number of colonies/number of cells cultured) relative to untreated controls.

Animal Studies. Female CD-1 mice, approximately 22 g (Charles River Laboratories, Wilmington, MA), were maintained under controlled temperature (25°C) and light (12-h light/12-h-dark), with food and water ad libitum. When required, animals were immunosuppressed by i.p. injections (0.3 ml/mouse/day) of ATS (5 mg/kg/day) on days 0, 1, 3, 5, and 7 and twice weekly thereafter, of a 30-day experimental schedule. FUra and BBU were dissolved in normal saline and injected i.p. in amounts of 0.3 ml/mouse/day. Since BBU was dissolved in DMSO, it was diluted with normal saline to give the appropriate concentration without any sediments before it was injected into the mice. The final concentration of DMSO in the injection solution did not exceed 15%. The control group received only saline (with the appropriate concentration of DMSO, when required).

Evaluation of Toxicity in Mice and Determination of LD50. Toxicity was evaluated in nontumor-bearing immunocompetent and immunosuppressed mice (6—39 mice/group). On days 1 and 2, immunocompetent animals were injected i.p. for 2 consecutive days with BBU (0—20 mg/kg/day), FUra (0—300 mg/kg/day), or their combinations dissolved in normal saline. ATS-immunosuppressed mice received BBU (5 and 10 mg/kg/day), FUra (0—120 mg/kg/day), or their combinations. The control group received only saline with the appropriate concentration of DMSO when required. Animals were checked daily for body weight loss and mortality. The LD50 was expressed as the dose required to achieve a ratio of 0.5 average mortality between the treated group and the corresponding untreated control, as estimated by the end point method (31).

Chemotherapy. The effectiveness of BBU, FUra, or their combinations in vivo was determined in ATS-immunosuppressed mice bearing DLD-1 human tumor xenografts. Immunosuppressed mice were injected on day 0 with DLD-1 cells. Cultured cells were washed with RPMI 1640, resuspended in RPMI 1640, and injected s.c. (10—15 × 10^6 cells) into the left inguinal area. On days 1 and 2, the drug-treated groups were injected i.p. (0.3 ml/mouse) for 2 consecutive days with BBU (5 and 10 mg/kg/day), FUra (0—120 mg/kg/day), or their combinations dissolved in normal saline. The control group received saline only (with the appropriate concentration of DMSO). On day 1, the mixture also included ATS. Tumor growth was monitored by the measuring, with calipers, of two perpendicular diameters of the implant. Tumor volume (mm3) was expressed as the square root of the product, long diameter (mm) × short diameter (mm). After 30 days, the tumor was excised and weighed. T/C and ED50 were estimated. LD50 was estimated by end point analysis (31), and therapeutic index was calculated as LD50/ED50.

Statistical Analysis. A paired t test was used to determine the degree of significance of the difference in survival between treated and untreated cells, seeded from the same culture flask, and the difference in animal survival, body weight, and tumor weight between treated and untreated animals.

RESULTS

IC50 of FUra. To evaluate the effect of BBU on the efficacy of FUra on DLD-1 grown in vitro, it was necessary to determine the IC50 of FUra in this cell line. The IC50 of FUra was subsequently used in combination with BBU to study the modulation of FUra cytotoxicity by BBU. The IC50 of FUra was estimated from the plot of the percentage growth inhibition versus log concentrations of FUra to be 0.48 ± 0.2 μM.

Effects of BBU on Cytotoxicity of FUra in Vitro. At concentrations higher than 1 μM, BBU showed slight toxicity toward cells in culture (data not shown). This toxicity, however, was caused mainly by the DMSO in which BBU was dissolved. Hence, BBU at 0.2 μM (0.02% DMSO), a concentration yielding less than 5% growth inhibition of DLD-1 cells in culture, was chosen for the in vitro experiments. Table 1 shows that BBU (K1 ~30 nm) at 0.2 μM, while not toxic to the DLD-1 cell line, enhanced cell kill by FUra (at its IC50) by 1.6-fold. These experiments were repeated using the soft agar cloning technique. Table 2 shows that BBU (0.2 μM) enhanced the efficacy of FUra against DLD-1 by 3-fold, confirming the results obtained by trypan blue exclusion presented in Table 1.

Effects of BBU on the Toxicity and LD50 of FUra in Immunocompetent Mice. The effect of BBU on the toxicity of different doses of FUra was studied in nontumor-bearing immunocompetent mice.
The data in Table 3 shows that BBU caused no mortality in these mice up to the highest dose tested (20 mg/kg/day × 2). Furthermore, there was no weight loss as a result of BBU administration (data not shown). The LD₅₀ of BBU was not determined because higher concentrations of BBU could not be used due to the limited solubility of the compound. Table 3 also shows that coadministration of BBU at 10 mg/kg/day × 2 with FUra at all doses tested (100–300 mg/kg/day × 2) did not increase toxicity induced by FUra. In fact, BBU appears to protect the mice from this toxicity, because there was always better survival when BBU was coadministered with FUra than when FUra was used alone. This protection was also evident from the estimates of the LD₅₀ of FUra in the absence and presence of BBU. In the 2-day schedule used, the LD₅₀ of FUra alone was 250 mg/kg/day. Co-administration of BBU (10 mg/kg/day × 2) increased the LD₅₀ of FUra from 250 to 300 mg/kg/day × 2.

Effects of BBU on the Toxicity and LD₅₀ of FUra in ATS-immunosuppressed Mice. Since it was necessary to immunosuppress the mice to insure the growth of DLD-1 human tumor xenografts, we evaluated the toxicity of different doses of FUra on ATS-immunosuppressed mice and studied the effect of BBU on modulating this toxicity. Table 4 shows the results of this experiment. From these data, the LD₅₀ of FUra alone was estimated to be 135 mg/kg/day × 2. Thus, it appears that ATS renders FUra more toxic to the animals, since the LD₅₀ of FUra alone decreased from 250 (Table 2) to 135 mg/kg/day × 2 (Table 4) as a result of immunosuppression with ATS. Nevertheless, even under these conditions, coadministration of BBU (10 mg/kg/day × 2) increased the LD₅₀ of FUra from 135 to 195 mg/kg/day × 2, confirming the results in Table 3 in which BBU protected the animals from FUra toxicity.

Effects of BBU on Chemotherapy by FUra in Vivo. Table 5 shows that, while BBU has no host toxicity on its own in tumor-bearing ATS-immunosuppressed mice, it improved the efficacy of FUra against the human colon carcinoma DLD-1 grown as xenografts in these mice. This enhancement of the chemotherapeutic efficacy of FUra was particularly evident at the lower doses of FUra. FUra alone at 30 mg/kg/day × 2 had no significant effect on reducing tumor weight but induced some host toxicity (17% mortality). In contrast, coadministration of BBU with this dose of FUra caused no host mortality yet significantly decreased the tumor weight by approximately 16-fold (from 799 to 49 mg). Furthermore, coadministration of BBU with FUra at 30 mg/kg/day × 2 reduced the %T:C of tumor weight by 18-fold (from 70 with FUra alone to 4 by the combination of BBU plus FUra). Coadministration of BBU also rendered the antitumor efficacy (%T:C = 4) of FUra at 30 mg/kg/day × 2 as effective as that obtained by a 4-fold higher concentration of FUra alone (120 mg/kg/day × 2). It should also be noted that the combination of BBU plus FUra (30 mg/kg/day × 2) did not produce the significant host toxicity (67% mortality) observed with FUra alone at 120 mg/kg/day × 2. In fact, it improved the survival of tumor-bearing mice treated with 120 mg/kg/day × 2 FUra from 33 to 50%. It is also important to mention that coadministration of BBU with the higher doses of FUra (100 and 120 mg/kg/day) resulted in an almost complete cure of the tumors from some mice without increasing FUra-induced host toxicity. Table 6 shows that the potentiation of the antitumor efficacy of FUra, and the reduction of FUra-induced host toxicity by BBU, resulted in an improvement of the therapeutic index of FUra by 5.5-fold, from 2.3 to 12.6.

**DISCUSSION**

The present results clearly demonstrate that BBU enhanced cell kill by FUra at its IC₅₀ in the human colon tumor DLD-1 grown in vitro. The present results also show that coadministration of BBU with FUra to mice bearing human colon carcinoma DLD-1 resulted not only in enhancement of the efficacy of FUra in killing the tumor but also protected the host from FUra-induced host toxicity. This was particularly evident at low doses of FUra. At 30 mg/kg/day FUra, BBU caused a 16-fold enhancement of FUra antitumor activity and increased host survival from 83 to 100%. The enhancement of tumor kill...
and protection from FUra-induced host toxicity was also evident, albeit to a lesser degree, at higher doses of FUra. At 120 mg/kg/day × 2 FUra, coadministration of BBU reduced tumor weight from 44 to 10 mg and increased survival of the animals from 33 to 50%.

It is noteworthy that the LD₅₀ of FUra decreased from 250 to 135 mg/kg/day × 2 as a result of immunosuppressing the mice. This is the first report of increased toxicity arising from coadministration of ATS with FUra. In retrospect, this result is not surprising since in murine animals as well as primates and humans, FUra is known to be a strong myelosuppressive agent (32).

The present results could have far reaching implications in improving chemotherapy with FUra. FUra is the most commonly used anticancer drug for the treatment of solid tumors of the breast, colon, head and neck, and stomach (33). Its advantage in chemotherapy stems from the fact that, in the course of combination therapy, it shows synergism with most other anticancer agents (34). Nevertheless, clinical use of FUra is limited by its rapid degradation by DHUΔase (1–3). Both the liver and peripheral blood mononuclear cells contain substantial amount of this activity (5), and FUra is a better substrate for DHUΔase than the natural substrates, uracil and thymine (5–9). Consequently, FUra, which is usually administered i.v. as a bolus or continuous infusion, is subjected to massive catabolism before reaching its target tissue(s). Therefore, it is conceivable that coadministration of BBU with FUra would enhance the chemotherapeutic activity of FUra in cancer patients. Another difficulty with treatment by FUra stems from circadian variations in DHUΔase activity (11, 35), which is reflected in variations in plasma levels of FUra during continuous drug infusions (10, 11). The direct effect of this phenomenon would be loss of efficacy if the timing of administering FUra coincided with the time of DHUΔase peak activity. Therefore, coadministration of BBU with FUra is likely to overcome circadian variations of DHUΔase activity and, as such, prevent unnecessary loss of efficacy or/and increased host toxicity. Finally, recent studies have implicated α-fluoro-β-alanine, a catalobate of FUra, in host neurotoxicity, hepatobiliary toxicity, and cardiotoxicity associated with therapy by FUra (36–38). BBU, by blocking the conversion of FUra to α-fluoro-β-alanine, should prevent such toxicities.

In conclusion, BBU, the most potent inhibitor of DHUΔase known to date (28), is unique among available inhibitors of DHUΔase in that it can improve the therapeutic index of FUra by enhancing its efficacy as well as reduce its host toxicity. These findings may have great significance in improving treatment of cancer by FUra.

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Potentiation of 5-Fluorouracil Efficacy by the Dihydouracil Dehydrogenase Inhibitor, 5-Benzyloxybenzyluracil

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