Tissue-specific Enhancement of Uridine Utilization and 5-Fluorouracil Therapy in Mice by Benzylacyclouridine

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ABSTRACT

Benzylacyclouridine (BAU), a potent inhibitor of uridine phosphorylase, delays the disappearance of uridine from plasma, affects the utilization of uridine by selected tissues, and enhances the therapeutic effects of 5-fluorouracil (FUra) in female C57BL/6 mice. A single 30-mg/kg i.v. injection of BAU lengthens the plasma half-life of both a tracer dose of \(^{3}H\)-uridine (3 µg/kg) and a pharmacological dose of uridine (250 mg/kg) by 250 and 83%, respectively. This dose of BAU also increases the normal plasma concentration of uridine about 4-fold to 9 µM and sustains these levels for 4 h. Four injections of BAU at 30 mg/kg over 6 h or a single injection at 240 mg/kg increases the plasma concentration of uridine over 10-fold to approximately 50 µM. In addition to affecting the pharmacokinetics of uridine, a 30-mg/kg dose of BAU selectively increases up to 4-fold the ability of normal host tissues to salvage a tracer dose of \(^{3}H\)-uridine for nucleic acid biosynthesis, the uracil nucleotide pool size, and the incorporation of uridine into nucleic acids. However, uridine salvage from plasma by colon tumor 38 is increased only slightly by BAU, while the uracil nucleotide pool size and uridine incorporation into tumor nucleic acids are actually decreased by 15 and 37%, respectively. The selective effect of BAU on uridine utilization is reflected in the ability of BAU to modify FUra-induced host toxicity. The dose of FUra required to kill 50% of the treated normal mice (350 mg/kg) is modestly increased by “rescue” regimens consisting of the subsequent administration of repeated injections of either BAU alone (30 mg/kg/injection) or uridine alone (250 mg/kg/injection). However, an increase of 54% is achieved when repeated injections of the combination of BAU and uridine are administered. In C57BL/6 mice bearing advanced transplants of colon tumor 38, the period of tumor growth inhibition resulting from multiple courses of FUra-containing drug regimens can be increased by the delayed administration of BAU alone or BAU combined with uridine.

INTRODUCTION

The pyrimidine analogue FUra has remained an important antineoplastic agent for the treatment of certain solid carcinomas for more than 25 yr (1). Its use is limited, however, by its low therapeutic index. This has led to studies aimed at increasing its antitumor activity and decreasing its toxic effects on host tissue by combining FUra with agents such as methotrexate (2, 3), N-(phosphonacetyl)-L-aspartate (4), and allopurinol (5, 6). Susceptible normal tissues can also be selectively “rescued” by the subsequent administration of large doses of uridine (7–9). This treatment presumably increases the availability of uridine for salvage by host tissues and has been postulated to increase tissue pools of uracil nucleotides. The increased cellular concentration of uridine and its nucleotides apparently competes with FUra nucleotides and accelerates the clearance of FUra from host tissue RNA (7). Certain tumors are less efficient at salvaging uridine under these conditions, and this may be the basis for the increased therapeutic effectiveness of FUra when rescued with uridine. While this approach has shown promise in murine systems, its clinical implementation has been hampered by toxicity associated with the administration of high doses of uridine, such as phlebitis and pyrogenic reactions (10). These heroic doses of uridine are required, however, because of the remarkable efficiency with which the liver and other tissues catabolize uridine, presumably by uridine phosphorylase (11–13).

Chu and co-workers (14, 15) have synthesized a series of pyrimidine acyclo-nucleosides that are capable of the potent and specific inhibition of this enzyme. These authors have also shown that BAU and benzylxobenzylacyclouridine inhibit nucleoside transport at high concentrations (\(K_{i} = 250 \mu M\)) (16) and can enhance the growth-inhibitory potential of fluorodeoxyuridine (17, 18). In addition, Monks et al. (19) have demonstrated that, in a perfused rat liver, BAU was capable of inhibiting the clearance of uridine from the perfusate. The above results suggest the use of BAU as an inhibitor of uridine catabolism in uridine rescue regimens, thus increasing the therapeutic effectiveness of FUra with lower doses of uridine.

In the present study, we have investigated the ability of BAU in vivo to increase the plasma concentration of uridine and inhibit its turnover. We also present evidence that suggests that BAU has a selective effect on the salvage and utilization of uridine for nucleic acid biosynthesis in different tissues. Finally, we demonstrate that, administered alone or combined with uridine, BAU can enhance the therapeutic effectiveness of FUra without increasing its toxicity. Preliminary aspects of these findings have been communicated (20, 21), and the results are discussed in light of their obvious clinical implications.

MATERIALS AND METHODS

Animals. All experiments utilized 3-mo-old female C57BL/6 mice obtained from the NIH. The therapeutic effectiveness of various drug combinations was studied in C57BL/6 mice bearing advanced (<300 mg) s.c. transplants of colon tumor 38 (22, 23).

Drugs. FUra was purchased from Adria (Columbus, OH) as a solution of 50 mg of FUra per ml. BAU was the generous gift of Dr. S. Chu of Brown University. Uridine was purchased from Sigma (St. Louis, MO). \([5,6-^{3}H]\)-Uridine (40 Ci/mmol) was obtained from New England Nuclear (Boston, MA). For injection purposes, FUra was diluted with, and uridine dissolved in, saline. BAU was dissolved in ethanol and then diluted 1:5.
in saline. Unless otherwise noted, all injections were i.v. (tail) with 0.1 ml injected per 10 g of body weight.

The effect of BAU on the pharmacokinetics of uridine was studied in C57BL/6 mice that received either a single injection of BAU at 30 or 240 mg/kg (due to its limited solubility, the maximum i.v. dose which could be administered) or four 30-mg/kg injections, one every 2 h, over a 6-h period. Control animals received an equivalent volume of saline. Thirty min after the 30-mg/kg BAU injection, animals received either a tracer dose of [3H]uridine (3 μg/kg, 10 μCi/mouse) or a pharmacological dose of uridine (250 mg/kg). Whole blood (200 μl) was then collected in a heparinized Natelson pipet, and the plasma was added to 2 volumes of 15% TCA at 0°C. The acid-soluble material was extracted with 1 volume of 1 N triacylamine in 1,1,2-trichlorotrifluoroethane (Freon) and stored at −20°C until analysis. Uridine was assayed by a modification of HPLC methods previously reported (4), utilizing an Altex Model 100 pump and Altex Model 153 UV detector (254 nm) connected to a Kipp-Zonen Model BD-41 chart recorder. The column was a Rainin Microsorb C-18 (25 cm x 4.6 mm) maintained at 30°C with a mobile phase of 10 mM H3PO4 containing 30 μM heptane sulfonic acid and adjusted to pH 3.1 with NaOH. Plasma extracts (200 μl) were injected with a Waters Model WISP 710A automated sample processor, and the column was eluted at 1 ml/min; the retention time of uridine was 24.5 min.

To monitor the turnover rate of [3H]uridine in the plasma after the tracer dose, the uridine peak from HPLC fractions was collected on a LKB Model 7000 fraction collector, and 1-ml fractions were added to 5 ml of Liquisicint (National Diagnostics, Somerville, NJ) and assayed for [3H]uridine in Beckman Model LS7000 liquid scintillation counter. In addition, the total radioactivity of each plasma sample was determined by adding 10 μl of the appropriate sample to 4 ml of Liquisicint and assaying as above.

The total uracil nucleotide content of tissues and the utilization of [3H]uridine for uracil nucleotide and nucleic acid biosynthesis was analyzed by a modification of previously described methods (24-27). C57BL/6 mice bearing colon tumor 38 received a 30-mg/kg injection of BAU. Thirty min later, they received either a tracer (3 μg/kg, 10 μCi/mouse) or pharmacological dose (250 mg/kg, 10 μCi/mouse) of [3H]uridine. After 2 h, the mice were killed by cervical dislocation, and liver, tumor, spleen, and kidneys were quickly removed, weighed, and homogenized in 2 volumes of ice-cold 15% TCA. Ten cm of intestine starting at the pyloric sphincter were flushed with ice-cold saline and processed as the other tissues. Acid-soluble fractions were extracted with the triacylamine/freon procedure described above, and the acid-insoluble pellet was washed twice with 3 ml of 15% TCA at 0°C and dissolved in 2 volumes of 1 N NaOH by incubation overnight at 37°C. The solubilized tissue (0.2 ml), representing the total nucleic acid fraction, was added to glacial acetic acid (0.12 ml), 1.6 ml of H2O, and 20 ml of Liquisicint for the assay of total radioactivity. Uracil nucleotides in the acid-soluble extract were converted to UM P by boiling for 15 min before neutralization and extraction. HPLC analysis was as described above but with a Whatman Partisil PXS10/SA25 (25 cm x 4.6 mm) column maintained at 20°C with a mobile phase of 35 mM sodium formate and 0.7 mM sodium phosphate (pH 4.0) and eluted at 0.7 ml/min. Under these conditions, the retention time for UMP was 15.0 min. The salvage of [3H]uridine for uracil nucleotide synthesis was determined by assaying the specific activity of UMP by collecting the peak described above for uridine.

The LD50 of FUra was determined in experiments consisting of 5 groups of mice with each group containing 5 to 10 animals. After receiving a challenge dose of FUra (150 to 700 mg/kg), BAU (30 mg/kg/injection) or uridine (250 mg/kg/injection) was administered as 4 i.p. injections, over a 6-h period, on each of 2 days following FUra administration, was also tested. The dose of FUra administered in each group was chosen to be approximately the LD50 with mice given injections of FUra alone or FUra+BAU receiving a FUra dose of 300 mg/kg. Mice rescued by uridine or FUra+uridine received FUra doses of 400 and 500 mg/kg, respectively. This injection regimen was repeated at 16-day intervals for a total of 2 or 3 courses of therapy. Mice in each group were weighed, and their tumors were measured twice weekly. Tumor growth in all groups was compared to a no-treatment control group, a group that received only FUra, and a group that received only multiple injections of BAU. Differences in tumor size among groups were considered significant if their P values, as determined by the Student t test, were ≤ 0.05.

RESULTS

The effect of BAU on the turnover of the plasma pool of uridine is presented in Chart 1 and indicates that BAU reduced the total radioactivity, representing intact [3H]uridine and its breakdown products, in plasma 10 min after [3H]uridine injection by more than 50% but increased the half-life of intact [3H]uridine in plasma by more than 2-fold from a value of 2 min in control mice to 5 min in animals that had received BAU. These findings are consistent with the in vitro results of Monks et al. (19) and suggested that BAU could also inhibit the clearance of larger pharmacological doses of uridine. Indeed, a similar effect was seen when mice received a single injection of uridine at a dose of 250 mg/kg (Chart 2). Under these conditions, the plasma half-life of uridine was increased from 12 to 22 min.

A single 30-mg/kg injection of BAU also increased the plasma concentration of uridine over 3-fold to approximately 9 μM (Chart 3). These elevated plasma uridine concentrations were sustained...
In all normal tissues, BAU increased up to 3-fold the utilization of uridine for nucleic acid biosynthesis as reflected by the increase in the specific activity of tissue uracil nucleotides in all normal tissues. The slight increase in the kidney, as reflected by the increase in the specific activity of tissue uracil nucleotides, suggests a high degree of uridine utilization in this tissue. However, in colon tumor 38, incorporation was actually decreased by over 37%. Administering BAU in combination with a pharmacological dose of \([^3H]uridine\) decreased the incorporation of the label into uracil nucleotides and the nucleic acid fraction of all tissues when compared to that seen with a tracer dose of \([^3H]uridine\), as expected by dilution of the labeled precursor, and did not have a significant effect on uracil nucleotide concentrations in tissues. Administering BAU in combination with this large dose of uridine did not cause the marked tumor-specific decrease in uridine salvage and utilization that was observed when BAU was given alone. However, under these conditions, spleen and gut did not efficiently salvage plasma uridine. Analysis of \([^3H]uridine\) incorporation into the nucleic acid fraction of these tissues indicated that BAU caused a modest increase in the degree of incorporation compared to this dose of uridine administered alone except in gut, where the incorporation was decreased by 12%.

Since others have shown improvement in the therapeutic index of FUra when the concentration of uridine in plasma was increased by the administration of pharmacological doses of uridine (7–9), it was of interest to determine if BAU could also modify FUra toxicity. The \(LD_{50}\) of FUra was determined, therefore, in mice that subsequently received 8 injections, beginning 18 h after FUra, of either BAU, uridine, or the BAU+uridine combination. These studies (Table 1) indicated that treatment with either uridine alone or BAU alone slightly increased the \(LD_{50}\), but the most dramatic increase was achieved when the combination of BAU+uridine was administered. The \(LD_{50}\) of FUra in this case was increased more than 50%.

To determine if this decrease in the toxicity of FUra was associated with enhanced therapeutic effectiveness, experiments were performed in mice bearing transplants of colon tumor 38. All FUra treatments significantly inhibited tumor growth after the first course of therapy (Chart 5A), with a 5 to 10% drug-related mortality in all groups. In this system, the subsequent administration of uridine did not improve the effectiveness of FUra even though its administration allowed the injection of more FUra. In contrast, both BAU-containing regimens slightly enhanced FUra effectiveness and also extended the period of tumor growth inhibition. This was particularly evident with 2 courses of FUra therapy. In these preliminary experiments, however, the 2 courses of FUra therapy resulted in drug-related mortality of between 8 and 19% in all groups with a maximum weight loss of 15% experienced by the group rescued by BAU alone. The maximum weight loss in the other groups ranged from 7 to 11%.

Since our studies had indicated that a single 240-mg/kg dose of BAU also caused a pronounced increase in plasma uridine concentrations without evidence of toxicity, single injections of this dose were given for 2 days after FUra (Chart 5B). This regimen also enhanced FUra effectiveness and extended the period of tumor growth inhibition. More importantly, however, these large single doses of BAU significantly decreased FUra-related toxicity and allowed the administration of 3 courses of therapy. The enhanced therapeutic effect was associated with less weight loss and only 12% FUra-related mortality on Day 48, compared to 18% with the same dose of FUra alone.

**DISCUSSION**

The administration of BAU, presumably by its potent and specific inhibition of uridine phosphorylase, elevates uridine concent-

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5366

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**Legend:**

1. **Chart 2.** Effect of a single injection of BAU (30 mg/kg) on the plasma clearance of a pharmacological dose of uridine (250 mg/kg) in C57BL/6 mice (O). Control animals (C) received no BAU. Points, mean of 3 animals; bars, SE.

2. **Chart 3.** Effect of a single injection of BAU at a dose of either 30 mg/kg (O), or 240 mg/kg (△), or 4 injections of BAU (30 mg/kg/injection) over 6 h (△△) on the plasma concentration of uridine in C57BL/6 mice. Control animals (C) received no BAU. Points, mean of 6 animals except the multiple- and high-dose injection group where each point represents the average of 2 animals; bars, SE.

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**Note:**

For 4 h with peak levels at 2 h. A single injection of BAU at 240 mg/kg or injecting BAU (30 mg/kg) at 2-h intervals for a total of 4 injections increased the plasma uridine concentration more than 10-fold to approximately 50 \(\mu\)M with the uridine concentration remaining elevated for over 7 h.

In addition to increasing the concentration of uridine in plasma, the administration of BAU also caused a tissue-specific increase of up to 4-fold in the salvage of uridine from plasma (Chart 4A) as reflected by the increase in the specific activity of tissue uracil nucleotides in all normal tissues. The slight increase in the kidney, when compared to its very high control value, suggests a high degree of uridine utilization in this tissue. However, BAU only modestly increased the uracil nucleotide pool size by up to 45% in normal tissues. In colon tumor 38, BAU administration actually decreased the uracil nucleotide pool size by approximately 15%, an effect that is partially responsible for the increase in the specific activity of the uracil nucleotide pool in this tissue.

In all normal tissues, BAU increased up to 3-fold the utilization of salvaged uridine for nucleic acid biosynthesis as reflected by the incorporation of the label into the nucleic acid fraction of these tissues. However, in colon tumor 38, incorporation was actually decreased by over 37%. Administering BAU in combination with a pharmacological dose of \([^3H]uridine\) decreased the incorporation of the label into uracil nucleotides and the nucleic acid fraction of all tissues when compared to that seen with a tracer dose of \([^3H]uridine\), as expected by dilution of the labeled precursor, and did not have a significant effect on uracil nucleotide concentrations in tissues. Administering BAU in combination with this large dose of uridine did not cause the marked tumor-specific decrease in uridine salvage and utilization that was observed when BAU was given alone. However, under these conditions, spleen and gut did not efficiently salvage plasma uridine. Analysis of \([^3H]uridine\) incorporation into the nucleic acid fraction of these tissues indicated that BAU caused a modest increase in the degree of incorporation compared to this dose of uridine administered alone except in gut, where the incorporation was decreased by 12%.
centrations in plasma and also causes a significant decrease in the concentration of uridine breakdown products. BAU also accentuates the marked differences in the potential of various tissues to salvage plasma uridine for uracil nucleotide biosynthesis. The finding that tumor tissue was not as efficient at utilizing plasma uridine after exposure to BAU suggested the possibility that uridine salvage in colon tumor 38 tissue was impaired under these conditions.

That combining BAU with a pharmacological dose of uridine did not accentuate the poor salvage capacity of tumor tissue and, in fact, masked this effect was unexpected. It was also remarkable that diluting [³H]uridine in plasma more than 1000 times with a pharmacological dose of uridine only decreased the specific activity of the uracil nucleotide pool and nucleic acid fraction by a factor of 4. Furthermore, contrary to earlier postulates (7), the large dose of uridine administered in these experiments did not significantly increase the tissue concentration of uracil nucleotides. These paradoxical findings may be explained by our recent observation that this dose of uridine only increases the total body pool of uridine 3- to 4-fold.⁴

That multiple injections of BAU did not affect the LD₉₀ of FUra appears to contradict the biochemical data and suggests that the enhanced salvage and utilization of uridine by host tissues under these conditions were not sufficient to reduce FUra cytotoxicity. A significant increase in the LD₉₀ of FUra was observed, however, when BAU was combined with uridine. Thus, the modest increase, under these conditions, in both uracil nucleotide levels as well as the utilization of these nucleotides for nucleic acid biosynthesis may be sufficient to increase the clearance of FUra nucleotides out of RNA. Of potential clinical relevance is the fact that a 50% increase in the LD₉₀ of FUra was obtained with a total uridine dose of 2000 mg/kg when combined with BAU. This increase may be compared to that reported by Martin et al. (7) to occur when a total uridine dose of 7000 mg/kg is used in uridine rescue regimens. Clearly, BAU has the potential to augment uridine rescue regimens and may thus limit some of the clinical toxicities associated with the use of large doses of uridine in rescue trials (10).

The evidence for the improved therapeutic effectiveness of FUra regimens that include BAU against colon tumor 38 was consistent with the biochemical data and suggested that, while BAU did not significantly decrease the toxicity of FUra to the host, it did increase the sensitivity of tumor tissue to FUra. The inhibition of tumor growth by repeated courses of drug therapy in this advanced tumor model was intended to mimic multiple courses of drug therapy in humans and suggests that high single daily doses of BAU after FUra may be most effective. Further preclinical toxicity and pharmacokinetic studies appear to be warranted along with an exploration of this effect in a wider range of tumor models.

*J. W. Damowsk and R. E. Handschumacher, unpublished observations.
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