Allopurinol Modification of the Toxicity and Antitumor Activity of 5-Fluorouracil

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ABSTRACT

Allopurinol (HPP) reduced the mortality of 5-fluorouracil (FUra) to mice and rats by almost 2-fold. Toxic manifestations of FUra treatment, including weight loss, histological changes in the gastrointestinal mucosa, and reduction of bone marrow cell survival, were lessened by prior and concomitant administration of HPP. In mice given a 7-day schedule of FUra (30 mg/kg/day), marrow cellularity was reduced and only 1% of the stem cells per femur survived compared to controls; this same schedule of FUra given with HPP (50 mg/kg/day), starting 1 day prior to and thereafter 1 hr before each FUra dose, caused less of a reduction in marrow cellularity, and 17% of the stem cells in the femur survived compared to controls.

The toxicity of FUra on mouse bone marrow cells was also demonstrated in vitro as a reduction of the incorporation of [6-3H]deoxyuridine into the acid-insoluble fraction. Oxypurinol, the xanthine oxidase catabolite of HPP, but not HPP was capable of antagonizing this effect of FUra, suggesting that bone marrow cells are spared from FUra toxicity in vivo, possibly because HPP is rapidly metabolized by other tissues and distributed as oxypurinol.

A reduction in the lethal toxicity of high doses of FUra by HPP was also observed in mice bearing ascites P388 tumor or solid Colon Tumor 38. Increasing the dose schedule of FUra in combination with HPP did not increase the life span of P388-bearing mice beyond that seen with optimal schedules of FUra alone. In Colon Tumor 38-bearing mice, however, schedules of FUra given with HPP resulted in 100% greater delay in tumor growth than optimal schedules of FUra alone. The increase in the therapeutic index of FUra with HPP seen in this tumor is being examined in the treatment of human disease.

INTRODUCTION

Treatment of cancers of the gastrointestinal tract and breast with FUra is commonly limited by myelosuppression and occasionally by gastrointestinal mucositis (4, 8, 13). The onset and the degree of severity is dependent on the schedule and route of administration. Although such toxicity may become life threatening, it has been reported that optimal antitumor effects are achieved with doses of FUra that cause some degree of toxicity to host tissues (1). Numerous attempts to increase the therapeutic index of FUra with HPP seen in this tumor is being examined in the treatment of human disease.

The current study presents evidence that toxicity to host tissues caused by FUra can be reduced selectively by combination therapy with HPP. In cell cultures, we have seen that HPP antagonized the growth-inhibitory activity of FUra in certain cell lines (L5178Y, L1210, P388, and Sarcoma 180) but not in others (BWS147,4 Walker 256, and HeLa) (19). This difference in susceptibility to protection by HPP may reflect a difference in the balance between alternative pathways of FUra activation in these cell lines. We now show that HPP can protect normal tissues in mice and rats against lethal doses of FUra. Studies with transplantable tumors indicate the potential of the HPP-FUra combination to increase the therapeutic index of FUra. Preliminary reports of these observations have been made (17, 18).

MATERIALS AND METHODS

Experimental Animals. Female BALB/c × DBA/2 F1 (hereafter called CD2F1), DBA/2, and CD1 mice, male CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.), and female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were used at 10 to 18 weeks of age; male Fischer rats (Charles River) were between 80 and 110 g. Animals were maintained on Purina laboratory chow and water ad libitum.

Drugs and Schedules. FUra and HPP were obtained from Sigma Chemical Co., St. Louis, Mo. Oxypurinol was the kind gift of Dr. Donald J. Nelson, Burroughs Wellcome and Co., Research Triangle Park, N. C. [6-3H]dUrd (24.2 Ci/mmol) was obtained from New England Nuclear, Boston, Mass.

Solutions of HPP and FUra were prepared in 0.9% NaCl solution, adjusted to pH 7, and sterile filtered; HPP precipitated from stock solutions (approximately 2 mg/ml) at room temperature and was redissolved at 60° prior to injection. All drugs were administered i.p. in volumes of 0.5 ml/mouse; the dosage was based on the average weight of the mice in the group. Rats were given drug according to their individual weights on a daily basis. Control animals received 0.9% NaCl solution. Unless otherwise noted, HPP was administered 2 days before starting the FUra schedule; thereafter, HPP was given 1 hr before each FUra dose.

Toxicity and Antitumor Studies. Drug toxicity was monitored as appearance, weight loss, and lethality, i.e., mean day of death. Sections of the gastrointestinal tract were fixed in formalin and stained with hematoxylin and eosin. Survival of in situ CD-1 mouse marrow stem cells following FUra was measured using the technique of Gordon (10) as modified by Marsh (15). Briefly, bone marrow cells flushed from the femurs of 5 mice 1 day after the last day of treatment were pooled, counted, and suspended in an agar-containing medium; 1.5 x 10^6 cells were introduced into diffusion chambers, and the chambers were implanted in the peritoneal cavity of CD-1 mice. One

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2 To whom requests for reprints should be addressed.
3 The abbreviations used are: FUra, 5-fluorouracil; HPP, allopurinol; dUrd, deoxyuridine.
4 P. M. Schwartz, unpublished observation.

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week later, colonies that had formed in the chamber were scored.

P388 ascites tumor was passaged weekly in DBA/2 mice. CD2F, mice were given approximately 1 x 10^6 P388 ascites tumor cells i.p. on Day 0; the mean day of death was 10.7 days. Antitumor activity was measured as an increase in life span.

Solid Colon Tumor 38 was obtained through Dr. T. Corbett of Southern Research Institute, Birmingham, Ala. (3, 11). This tumor was passaged every 30 to 40 days in C57BL/6 mice as an s.c. implantation of either a tumor brie or a tumor fragment. Tumor weight was estimated by caliper measurements of length (L) and width (W) in mm and by the formula, weight (mg) = L x W^2/2.

Incorporation of [6-3H]dUrd into the Acid-insoluble Fraction of Mouse Bone Marrow Cells in Vitro. [6-3H]dUrd incorporation into the acid-insoluble fraction of bone marrow cells flushed from femurs of C57BL/6 mice (approximately 2.4 x 10^6 cells/femur) was measured in vitro. Cells (4 x 10^6 cells/ml, 12 ml) were incubated in Fisher’s medium containing 10% horse serum, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Calbiochem-Behring Corp., La Jolla, Calif.), and 200 units of heparin (Upjohn Co., Kalamazoo, Mich.) at 37°C. Cultures were treated with HPP (5 x 10^-4 M) or oxypurinol (5 x 10^-8 M) for 1 hr prior to addition of FUra (5 x 10^-4 M). Three hr after addition of FUra, 0.5 μCi of [6-3H]dUrd per ml (2 x 10^-9 M) were added, and incubation was continued for 1 hr prior to precipitation with 1 N perchloric acid. The acid-insoluble material was washed 3 times with cold 1 N perchloric acid and hydrolyzed at 70°C for 60 min in 0.5 N perchloric acid. Radioactivity in the hydrolysate after centrifugation was determined in Aquasol (New England Nuclear), and data were expressed as cpm [6-3H]dUrd incorporated into cold acid-soluble material per 10^6 cells.

RESULTS

Effect of HPP on FUra Toxicity. CD2F, mice (6/group) received 20, 30, or 40 mg of FUra/kg/day for 8 days. Other animals were given HPP (50 mg/kg/day) plus FUra doses of 40, 50, or 60 mg/kg/day for 8 days. Toxic deaths were observed within 4 days of the final dose of FUra. Changes in weight and survival were recorded for a total of 35 days (Chart 1). FUra alone (40 mg/kg/day for 8 days) resulted in 100% lethality. However, all animals pretreated with HPP survived this dose of FUra. The approximate 10% lethal dose of FUra was almost doubled, from 22 to 42 mg/kg/day by the addition of HPP.

The administration of HPP (50 mg/kg/day) also permitted a toxic daily dose of FUra (30 mg/kg) to be given for an extended time period to CD2F, mice. Mice given daily FUra (30 mg/kg/day) alone lost about 25% of their initial body weight by Day 7; the combination of HPP plus FUra did not produce a comparable weight loss until Day 12.

HPP also reduced the rate of weight of loss and lethality of FUra to Fischer rats. Six rats given FUra (50 mg/kg/day) alone for 8 days died on Days 9 to 12 and had a 35% weight loss on Day 8. Five of 6 rats survived this same schedule of FUra given with HPP (60 mg/kg/day) starting 2 days before and 1 hr before each FUra dose. One of 6 rats on the HPP-FUra schedule lost a maximum of about 18% of its initial weight, failed to regain weight, and died on Day 18. The maximum weight loss of the survivors was 11 g (10%), and they regained weight at the same rate as controls after treatment was discontinued. Control rats or those given HPP (60 mg/kg/day for 10 days) alone gained an average of 95 g above their initial weight (104 g) in 20 days.

Correlates of HPP Modification of Toxicity. A limiting toxicity of FUra in rodents is gastrointestinal damage with associated diarrhea. Histological examination of sections of the small intestine and colon by light microscopy revealed damage to the crypts and villi of mice receiving FUra (30 mg/kg/day for 7 days) (Fig. 1). The disruption of cellular architecture seen in these mice was essentially absent in animals given the same schedule of FUra with HPP (50 mg/kg/day).

It was also possible to demonstrate both biochemically and with a bone marrow colony assay that HPP protected against FUra-associated damage to bone marrow cells. In CD-1 mice treated with FUra (30 mg/kg/day for 7 days), there was a 5% survival of clonogenic cells recovered from the femurs of these mice; prior administration of HPP (50 mg/kg/day for 8 days) increased survival to 35% (Table 1). Unlike CD2F, or C57BL/6 mice, CD-1 mice given an 8-day schedule of FUra (50 mg/kg/day) lost 15% body weight; there was no reduction in the survival of bone marrow cells taken from CD-1 animals.

Biochemical evidence for protection of bone marrow cells against FUra toxicity was obtained in vitro. The incorporation of [6-3H]dUrd into DNA of a suspension of marrow cells exposed to 5 x 10^-4 M FUra for 3 hr is reduced by 54% of that in control cells. HPP (5 x 10^-4 M) did not protect against the FUra reduction of incorporation of [6-3H]dUrd into DNA, whereas preincubation of cells with oxypurinol (5 x 10^-4 M) eliminated this FUra effect (Chart 2). That HPP was relatively ineffective as the protecting agent in vitro is presumably because the xanthine oxidase activity needed to convert HPP to oxypurinol is relatively low in marrow suspensions.

Effect of HPP on FUra Antitumor Activity. An initial study was made of the effect of combinations of FUra and HPP on the growth of P388 tumor. This lymphoblast line in culture was protected against FUra toxicity by HPP and might not be

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Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells/femur (×10^5)</th>
<th>Colonies/chamber (%)</th>
<th>Cell survival (%)</th>
<th>Femur fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.9% NaCl solution)</td>
<td>22.7 ± 3.3</td>
<td>43.4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HPP (50 mg/kg/day for 8 days)</td>
<td>17.3 ± 2.4</td>
<td>52.8 ± 4.4</td>
<td>121</td>
<td>93</td>
</tr>
<tr>
<td>FUra (30 mg/kg/day for 7 days)</td>
<td>5.6 ± 0.5</td>
<td>2.0</td>
<td>4.6</td>
<td>1.1</td>
</tr>
<tr>
<td>HPP (50 mg/kg/day for 8 days) + FUra (30 mg/kg/day for 7 days)</td>
<td>11.6 ± 1.4</td>
<td>15.3 ± 1.4</td>
<td>35.1</td>
<td>17.1</td>
</tr>
</tbody>
</table>

* Colonies per chamber as a percentage of control.
* Number of colony-forming cells per femur as a percentage of control.
* HPP given 1 day before and 1 hr before each FUra dose.

**HPP Modification of FUra Toxicity**

**Fig. 1.** Intestinal sections from mice. A, control; B, FUra (30 mg/kg/day on Days 3 to 9); C, HPP (50 mg/kg/day on Days 1 to 9) and FUra (30 mg/kg/day on Days 3 to 9). H&E, ×100.

Treatment with FUra (20 mg/kg/day) for 8 days caused no toxic deaths and increased the life span of animals by 120% (data not shown). Early deaths from drug toxicity were observed if the FUra dose was increased to 30 mg/kg/day and continued for 6 days (1 death in 6), 7 days (4 deaths in 6), or 8 days (6 deaths in 6). Treatment with HPP (50 mg/kg/day) 1 day prior to and 1 hr before the administration of FUra (30 mg/kg/day) markedly reduced toxicity, as indicated by fewer early deaths. However, the HPP-FUra combination did not increase the average life span of P388 tumor-bearing mice beyond that observed with FUra alone.

Since FUra has some activity against intestinal neoplasms in humans, it was of interest to evaluate it in combination with HPP in a murine model sensitive to FUra (3, 11). Mice were inoculated with Colon Tumor 38 and given 0.9% NaCl solution or HPP (50 mg/kg/day) starting 1 day after inoculation; tumors were palpable by Day 16, and tumor size doubled about every 7 days to give 9- to 10-g tumors by Day 60. Several dosages and schedules of FUra were compared in HPP-FUra combination regimens. Toxic deaths were defined as those occurring before Day 20 and accompanied by more than 20% weight loss; antitumor activity was expressed as the delay in tumor expected to display an increased therapeutic index (19). The mean day of death of P388-bearing control or HPP-treated (50 mg/kg/day for 8 days) mice was 10 to 11 days (Chart 3).
growth to a 1-g tumor size, as described by Corbett et al. (3).

Administration of HPP allows extension of the maximally tolerated dosage of FUra (30 mg/kg/day) from 4 to 9 days (Table 2). A 6-day schedule of FUra which caused 50% of mice to die from toxic deaths resulted in a 22-day delay in tumor growth to 1 g in the remaining animals. This same delay in tumor growth can be achieved by a 9-day schedule of HPP plus FUra with no toxic deaths. Continued administration of FUra (30 mg/kg/day) and HPP beyond 9 days did not result in any greater delay in tumor growth.

A weekly cycle of HPP (50 mg/kg/day for 5 days) plus FUra (30 mg/kg/day for 5 days) therapy was repeated for 8 weeks with 16% weight loss and no toxic deaths (data not shown). This regimen resulted in a 23-day delay of tumor growth to 1 g; the tumor grew despite continued treatment.

A direct comparison was made of the antitumor effect of FUra and the FUra-HPP combination at equitoxic doses given for the same number of days (Table 2). Approximately twice as large a FUra dose could be tolerated as had been seen in previous studies with normal mice (Chart 1). A minimally toxic dose of FUra alone (20 mg/kg/day) for 8 days (20% weight loss and no toxic deaths) gave a 16-day delay in growth of tumors to 1 g. An equitoxic schedule with FUra (40 mg/kg/day for 8 days) plus HPP delayed tumor growth to 34 days. Thus, improvement in the therapeutic index was achieved with appropriate scheduling of combinations.

**DISCUSSION**

Previous reports from this laboratory have shown that the cytotoxicity of FUra to a number of cell lines in culture can be antagonized by prior exposure of the cells to HPP (17–19). It is proposed that the orotate accumulation within the cell consequent to inhibition of orotidine 5’-monophosphate decarboxylase by an HPP metabolite (oxypurinol ribonucleotide) (6, 7) competes with the activation of FUra via an orotate phosphoribosyltransferase, as suggested from in vitro studies by Reyes and Guganig (16). However, in some cell lines, protection from FUra toxicity by HPP was not observed. In one case, a BW5147 lymphoblast cell line, oxypurinol but not HPP was capable of antagonizing FUra growth inhibition, an indication that these cells lack sufficient xanthine oxidase activity to convert HPP to oxypurinol. In Walker 256 cells, neither HPP nor oxypurinol could prevent FUra cytotoxicity (19). In this cell line, FUra may be activated predominantly by the alternate 2-step uridine phosphorylase-uridine kinase pathway, and this may explain the insensitivity of the cell line to HPP protection.

These observations in cell culture were extended to animal model systems to determine if HPP could reduce FUra toxicity to normal host tissue selectively and thereby provide a means to increase the therapeutic index of FUra against certain tumors. The mouse was used to study the effect of HPP on FUra toxicity, since earlier work by Goldsmith et al. (9) indicated its usefulness as a predictive model. More recently, Harrison et al. (12) have indicated that the day of onset, severity, and recovery of hematopoietic depression and changes in gastrointestinal mucosa of BD2F mice were comparable to those seen in patients treated with FUra; they also concluded that the mouse was a good model of FUra toxicity but was more sensitive than humans.

In the current study, bone marrow and gastrointestinal toxicity of FUra in mice were reduced by prior and concomitant administration of HPP. A similar mechanism to that suggested from cell culture data may also account for HPP protection of these normal host tissues. The observation that oxypurinol but not HPP was capable of preventing FUra toxicity to mouse bone marrow cells in vitro suggests that conversion of HPP to its xanthine oxidase metabolite oxypurinol is necessary, as was observed with BW5147 lymphoblasts, which are apparently deficient in this enzyme. Since HPP is rapidly oxidized to oxypurinol in the gastrointestinal tract and liver (14), distribution of this metabolite to tissues lacking xanthine oxidase, such as bone marrow, may explain the protection of these sites from
FURA toxicity in vivo.

Means of selectively reducing the untoward effects of FURA in patients are needed, since therapy with FURA is limited by severe toxicity, including stomatitis, diarrhea, vomiting, leukopenia, and thrombocytopenia. The use of aggressive schedules of FURA in which some degree of toxicity is accepted has been supported by several investigators as a means to increase the marginal response rate of FURA against colorectal cancer (1). Therefore, the use of high-dose FURA schedules with HPP was explored with experimental animal models in an attempt to effect increased antitumor activity without a comparable increase in toxicity. With equitoxic regimens of FURA or FURA plus HPP, an increase in the therapeutic index was observed in the treatment of murine Colon Tumor 38 by the combination therapy. A mechanism to explain an increased, selective antitumor activity of FURA in this model might be the differential distribution of HPP, FURA, and metabolites in normal versus neoplastic tissue; the accumulation of orotate may also vary in normal versus tumor tissues. Brockman et al. (2) reported that the activity of FURA phosphoribosyltransferase was 20 times greater in Colon Tumor 38 tissues than in normal colon mucosa; this could account for different rates of FURA metabolism in these tissues subsequent to HPP induction of intracellular orotate levels. Another possible mechanism to explain the selectivity of HPP-FURA combination is that FURA is predominantly activated via the 2-step 5-fluorouridine pathway in Colon Tumor 38 cells, and, thus, elevated levels of orotate may not interfere as much with FURA metabolism and subsequent toxicity in this tumor.

Clinical trials of HPP plus FURA have been recently initiated by Fox et al. (5) based on our reports of cell culture and animal studies (17–19). HPP reduced FURA toxicity and permitted escalation of the dose of FURA with retention of antitumor activity. These preliminary clinical studies have been confirmed by Howell (5) and Cadman. (6) In these clinical studies, doses of HPP used with FURA are within the range of those used clinically for the treatment of hyperuricemia and gout and have been shown to cause the excretion of orotate and orotidine. The urinary excretion of orotate is variable in nongouty patients treated with HPP and is known to be modified by dietary pyrimidines (20); however, genetic factors may also control the response to this inhibition of the final step in pyrimidine synthesis de novo. To what extent orotate excretion reflects tissue levels of this metabolite in humans has not been investigated, but this may be of particular relevance to the toxicity and antitumor activity of FURA.

Current studies in this laboratory are directed toward establishing a correlation between alterations in pyrimidine metabolism in humans and the clinical response to the HPP-FURA combination. It is hoped that such data will provide a guide to optimizing dose schedules and to aid in the selection of patients most likely to benefit from this treatment. Not only can the toxicity of currently used doses of FURA be reduced, but, more importantly, FURA dosage may be elevated to increase the antitumor effect against marginally sensitive neoplasms.

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