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ORIGINAL ARTICLE

Potent combination therapy for human breast tumors with high doses of 5-fluorouracil: remission and lack of host toxicity

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Abstract
Purpose The purpose of this investigation was to evaluate the effectiveness of oral 5-(phenylthio)acyclouridine (PTAU) in reducing 5-fluorouracil (FUra) host toxicity and enhancing its chemotherapeutic efficacy against human breast tumors. PTAU is a potent and specific inhibitor of uridine phosphorylase (UP, EC 2.4.2.3), the enzyme responsible for uridine catabolism.
Methods SCID mice bearing MDA-MB-468 and MCF-7 human breast tumors were injected intraperitoneally with FUra (50, 200 or 300 mg/kg) on days 17, 24, and 31 after tumor cell inoculation. PTAU (120 mg/kg), uridine (1,320 mg/kg), or their combination was administered orally two or 4 h after FUra injection. Another four administrations of PTAU plus uridine were given every 8 h after the first treatment with PTAU plus uridine. Survival and body weight were used to evaluate host toxicity. Tumor weight was used to evaluate the efficacy of the drugs on tumor growth. The mice were monitored for 38 days.
Results Administration of the maximum tolerated dose (50 mg/kg) of 5-fluorouracil (FUra) to SCID mice bearing human breast MDA-MB-468 and MCF-7 adenocarcinoma tumor xenografts reduced tumor weight by 59 and 61%, respectively. Administration of 200 mg/kg FUra resulted in 100% mortality. Oral administration of uridine (1,320 mg/kg) alone, 2 h following the administration of 200 mg/kg FUra, did not rescue from FUra host toxicity as all the mice died. Administration of 120 mg/kg PTAU resulted in partial rescue from this lethal dose of FUra as 38% of inoculated mice survived and the tumor weights were reduced by approximately 67%. Coadministration of PTAU plus uridine resulted in complete rescue from the toxicity of FUra. All of the mice survived, and MDA-MB-468 and MCF-7 tumor weights were reduced by 97% and total remission, respectively. Doubling the FUra treatment dose to 400 mg/kg in the MDA-MB-468 inoculated mice, with the administration of the adjuvant combination treatment of PTAU plus uridine, was unsuccessful in rescuing from FUra toxicity as all the mice died. Lowering the dose of FUra to 300 mg/kg, under the same conditions, resulted in 67% mice survival, and the MCF-7 tumor weights were reduced by 100%. Treatment with uridine alone did not protect from FUra toxicity at 200, 300, and 400 mg/kg as all of the mice died. At the higher dose of 300 and 400 mg/kg FUra, PTAU alone had no rescuing effect. There was no significant difference between MDA-MB-468 and MCF-7 in their response to the different regimens employed in this study in spite of the fact that MDA-MB-468 is estrogen receptor negative while MCF-7 is estrogen receptor positive.
Conclusions The present results demonstrate that the combination of PTAU plus uridine represents an exceptionally efficient method in increasing FUra chemotherapeutic efficacy while minimizing its host toxicity. The efficiency of the PTAU plus uridine combination can be attributed to the extraordinary effectiveness of this combination treatment in raising and maintaining higher levels of uridine in vivo (Al Safarjalani et al. in Cancer Chemo Pharmacol 55:541–551, 2005). Therefore, the combination of PTAU plus uridine can provide a better substitute for the massive doses of uridine necessary to rescue or protect from FUra host-toxicities, without the

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toxic side effects associated with such doses of uridine. The combination may also allow the escalation of FUra doses for better chemotherapeutic efficacy against human breast carcinoma, with the possibility of avoiding FUra host-toxicities. Alternatively, the combination of PTAU and uridine may be useful as an antidote in the few cases when cancer patients receive a lethal overdose of FUra.

Keywords 5-(Phenylthio)acyclouridine · Uridine phosphorylase inhibitor · 5-Fluorouracil · Combination chemotherapy · Uridine · Lack of toxicity

Abbreviations

FUra	5-Fluorouracil
HPMC	Hydroxypropyl methylcellulose
MTD	Maximum tolerated dose
PTAU	5-(Phenylthio)acyclouridine
UP	Uridine phosphorylase [EC 2.4.2.3]

Introduction

The pyrimidine nucleoside, uridine, has been used successfully as a "protective" and/or "rescuing" agent against host toxicity by the anti-cancer drug 5-fluorouracil (FUra) [1–5] without interfering with its chemotherapeutic efficacy. However, because of its rapid clearance [6–15], it is necessary to administer substantial doses of uridine (10–12 g/m^2) [8] to attain and sustain the high plasma uridine concentrations (70 μM) [16], required to achieve the protective or rescuing effects. Unfortunately, such large doses of uridine also produce dose limiting side effects (e.g., phlebitis, pyrogenic reactions and diarrhea, high fever, cellulitis, and superior vena cava syndrome) [8, 10–12, 17–20].

Most of these side effects are not induced by uridine per se but rather by the accumulation of uridine catabolites [18, 21]. Therefore, it seems unlikely that combining FUra with uridine alone will improve the therapeutic index of FUra to a significant extent. Prodrugs of uridine (e.g., 2′,3′,4′-triacetyluridine or PN401) were synthesized to overcome the short half-life of uridine [14, 15, 22]. However, such prodrugs also suffer from rapid degradation [14, 15, 23]. Hence, high doses must still be administered [14, 15, 22–25] making the use of uridine prodrugs as single agents impractical in the clinic. Therefore, alternative approaches to increase uridine bioavailability to the required concentrations must be sought.

Uridine is maintained in rigorous homeostasis at a concentration of 1–5 μM in the plasma of various species [6, 14, 26, 27]. However, plasma uridine half-life is approximately 2 min [26]. Hence, the turnover of plasma uridine must be rapid and efficient. Indeed, more than 90% of the circulating uridine is catabolized in a single pass through the liver by the activity of hepatic uridine phosphorylase (UP, EC 2.4.2.3), while constant amounts of uridine are synthesized de novo and released into the hepatic vein blood [28, 29]. Less than 2% of the uridine metabolized by the liver is salvaged and recovered in the uracil nucleotide pool in tissues of whole animals [27, 30–32], perfused rat liver [6, 13], or isolated liver cells [31]. The remainder is rapidly catabolized to products beyond uracil in the pyrimidine catabolic pathway [13, 28, 33].

An approach to maintain a high uridine concentration over a prolonged period is the use of UP inhibitors to block the rapid catabolism of uridine to uracil. Inhibition of uridine catabolism by UP inhibitors would lead to increased plasma uridine concentrations as a result of the continuous de novo biosynthesis of uridine in the liver. Indeed, UP inhibitors have been used to increase the concentration and half-life of plasma uridine [13–16, 20, 28, 34–36] and the salvage of uridine by various tissues [16, 20, 29, 37]. Inhibition of uridine catabolism also prevented the toxic side effects associated with high doses of uridine that resulted from the accumulation of uridine catabolites [18]. However, the effectiveness and bioavailability of the currently available UP inhibitors are limited by metabolism and inadequate pharmacokinetic properties [13, 15, 22, 35, 36, 38].

In order to overcome the limitations of these UP inhibitors, 5-(phenylthio)acyclouridine (PTAU) was designed as a lipophilic inhibitor of UP [39] and as such has better access to the liver and intestine, the main organs involved in uridine catabolism. PTAU is neither toxic nor metabolized (100% oral bioavailability), has excellent pharmacokinetic properties, and is far superior to the previously known UP inhibitors in improving the oral bioavailability of uridine [40–42].

In the present study, we tested the effect and time of administration of oral PTAU alone or in combination with uridine on the host toxicity and chemotherapeutic efficacy of FUra in SCID mice bearing human breast xenografts.

Materials and methods

Chemicals

Hydroxypropyl methylcellulose (HPMC) and other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo). PTAU was synthesized as previously described [39].

Mice

Female SCID mice (18–22 g) were obtained from Fredrick Cancer Research (Fredrick, MD) and housed 5/cage with water and food ad libitum under a normal light cycle (light, 0600–1800 h; dark, 1800–0600 h) according to an institutionally approved animal protocol.

Cell Line

MDA-MB-468 and MCF-7 were obtained from American Type Culture Collection, Rockville, MD. MDA-MB-468 and MCF-7 cells are human breast adenocarcinomas. These are two epithelial cell lines that are heterogeneous and produce moderately to poorly differentiated adenocarcinomas when inoculated subcutaneously into nude mice. The MDA-MB-468 cell lines were maintained at 37°C in growth medium (Leibovitz's L-15 with 2 mM L-glutamine and supplemented with 10% fetal bovine serum) in plastic tissue culture flasks (75 cm^2) in a humidified incubator under 5% CO_2 and 95% air. The MCF-7 cell lines were maintained under the same conditions in growth medium (EMEM medium with Earle's BSS and 2 mM L-glutamine modified to contain 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/L bicarbonate and supplemented with 0.01 mg/mL bovine insulin, and 10% fetal bovine serum) in plastic tissue culture flasks (75 cm^2) in a humidified incubator under 5% CO_2 and 95% air. These two lines were chosen because these tumors have estrogen receptor differences. MDA-MB-468 xenografts are estrogen receptor negative, while the MCF-7 xenografts are estrogen receptor positive (as described in the product information sheet by the American Type Culture Collection). This ensures that the results are not specific to one type of tumor. Furthermore, these two cell lines have been used extensively for antitumor drug screening in vitro and in vivo.

Administration of drugs

PTAU alone or combined with uridine was mixed well with HPMC powder in hot water (80°C) and homogenized thoroughly using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The final concentration of HPMC was 0.75%. The drug solution was vortexed well before and periodically during dosing. HPMC was preferred over the commonly used methylcellulose because the latter must be cooled to 10°C in order to hydrate completely [14, 22, 40–42]. Drugs were administered orally (0.1 mL/10 g) using 18G intubation needles (Popper and Sons, Inc., New Hyde Park, NY). FUra was dissolved in normal saline solution (0.9% NaCl) and injected intraperitoneally at 0.1 mL/10 g. Control mice received the carrier solution (0.9% saline or 0.75% HPMC). To avoid a possible circadian variation in the activities of the major enzymes involved in FUra metabolism (UP, orotate phosphoribosyltransferase [EC 2.4.2.10] and dihydrouracil dehydrogenase [EC 1.3.1.2]) [43, 44], all mice were treated at the same time starting at 1:00 P.M.

Host toxicity and chemotherapeutic studies

In a previous study, we found that oral administration of PTAU at 120 mg/kg alone or in combination with uridine at 1,320 mg/kg was exceptionally effective in elevating and sustaining high plasma uridine concentrations [40–42]. Therefore, these doses of PTAU, uridine, or their combination were used in the present study to evaluate their effect on the host toxicity of FUra in the SCID mouse xenograft model. Furthermore, PTAU alone or in combination with uridine was administered after FUra administration to evaluate the significance of time of administration of the rescue regimen.

Mice were divided into groups (8 mice/group). To establish the MDA-MD-468 human breast xenografts, cultured cells were harvested on day 0 from the monolayer cultures, washed three times, and then resuspended in a serum-free Leibovitz's medium:Matrigel basement membrane matrix (2:1), and injected s.c. ($10x10^6$ cells, total volume 0.2 mL) into the right upper back area of the SCID mice. To establish the MCF-7 human breast xenografts, each of the SCID mice was implanted s.c. on the lateral side of the neck using a 10-gauge precision trochar (MP-182; Innovative research of America, Sarasota, FL) with a 60-day release estrogen pellet (SE-121, 1.7 mg of 17β-estradiol/pellet; Innovative research of America, Sarasota, FL). In the same manner, cultured MCF-7 cells were harvested from the monolayer cultures, washed three times, then resuspended in a serum-free EMEM medium, and injected s.c. (10×10^6 cells, total volume 0.2 mL) into an area just at the upper right back of the SCID mice. The tumor cell injections took place on day 0.

According to their groups, mice were injected intraperitoneally with FUra (200, 300 or 400 mg/kg) on days 17, 24, and 31 after tumor cell inoculation. PTAU (120 mg/kg), uridine (1,320 mg/kg), or their combination was administered orally 2 h after FUra injection. Another four administrations of PTAU plus uridine were given every 8 h after the first treatment with PTAU plus uridine. Survival and body weight were used to evaluate host toxicity. Tumor weight ([the long diameter (mm)] $\times$ [short diameter (mm)]^2/2) was used to evaluate the efficacy of the drugs on tumor growth. The mice were monitored for 38 days.

Results and discussion

Previous studies have established that the administration of UP inhibitors and/or uridine should occur after the administration of FUra [1–3, 16, 45, 46]. Therefore, we tested the effect of PTAU, uridine, and their combination 2 h following FUra administration. Tables 1 and 2 show the effect of PTAU (120 mg/kg), uridine (1,320 mg/kg), and their combination on host toxicity and chemotherapeutic efficacy of FUra at 200, 300 and 400 mg/kg. The maximum tolerated dose of FUra (50 mg/kg) in SCID mice [47] was included as a reference for the chemotherapeutic efficacy of FUra.

The results in Tables 1 and 2 indicate that as expected PTAU alone did not cause any host toxicity confirming our previous results [40–42]. FUra at the maximum tolerated dose (50 mg/kg) also did not cause any mortality and reduced MDA-MB-468 and MCF-7 tumor weights by 59% (Table 1) and 61% (Table 2), respectively. Increasing the dose of FUra to 200 mg/kg or above was accompanied by a 100% mortality (all mice were dead by day 38). Administration of PTAU alone 2 h following the administration of 200 mg/kg FUra resulted in partial rescue from this lethal dose of FUra as 38% of mice survived and reduced MDA-MB-468 and MCF-7 tumor weights by approximately 58 and 62%, respectively (Tables 1, 2). Administration of uridine alone under the same conditions did not protect from FUra host toxicity as all mice died by day 38.

On the other hand, coadministration of PTAU with uridine 2 h following the administration of 200 mg/kg FUra resulted in a better outcome than either PTAU or uridine alone as it rescued all the mice (100% survival and no appreciable weight loss at day 38) from this lethal dose of FUra (Tables 1, 2). In addition, administration of uridine plus PTAU 2 h after FUra treatment had a greater chemotherapeutic effect than that achieved by the maximum tolerated dose of FUra (50 mg/kg) and reduced MDA-MB-468 tumor weights by 97% with a complete remission of the MCF-7 (Tables 1, 2). We also used the 2-h delayed regimen to test the efficacy of PTAU, uridine, and the combination, on rescuing from a higher dose of FUra (300 mg/kg). Table 2 shows that neither uridine nor PTAU alone had any rescuing effect (0% survival) from this high dose of FUra. On the other hand, the use of PTAU plus uridine combination reduced the tumor weight to a remission state although the tumor remission was accompanied by 33% mortality (Table 2). Table 1 similarly demonstrates that a higher dose of 400 mg/kg FUra with uridine or PTAU alone or in combination had no rescuing effect either (0% survival). The results in Tables 1 and 2 suggest that there is no difference between MDA-MB-468 and MCF-7 in their response to the different regimens

Table 1 Effect of 5-(phenylthio)acyclouridine (PTAU) alone or in combination with uridine on the chemotherapeutic efficacy and host toxicity of 5-FUra (200 and 400 mg/kg) in SCID mice bearing human breast tumor MDA-MB-468 xenografts

Drug (mg/kg)			Tumor weight (mg)	%T/C[b]	% survival at day			Day of first death	Body weight (g) at day 38
FUra	PTAU	Uridine			24	31	38		
0	0	0	1,392 ± 273[a]	100	100	100	100	–	25.5 ± 1.1
0	0	1,320	1,328 ± 202	95	100	100	100	–	25.2 ± 0.8
0	120	0	1,229 ± 522	88	100	100	100	–	24.1 ± 1.4
50[c]	0	0	487 ± 189	35	100	100	100	–	25.4 ± 0.6
200	0	0	–	–	13	0	0	24	–
200	0	1,320	–	–	88	50	0	24	–
200	120	0	504 ± 88[d]	36	100	88	38	31	25.8 ± 0.2
200	120	1,320	40 ± 7[d,e]	3	100	100	100	–	24.8 ± 1.4
400	0	0	–	–	0	0	0	24	–
400	0	1,320	–	–	0	0	0	24	–
400	120	0	–	–	0	0	0	24	–
400	120	1,320	–	–	29	0	0	24	–

PTAU alone or in combination with uridine was administered 2 h after FUra administration

[a] Mean tumor weight ± SD from 5 to 8 tumors

[b] %T/C, percentage of tumor weight in treated mice/tumor weight in untreated control mice

[c] Maximum tolerated dose of FUra in SCID mice

[d] Significantly different ($P < 0.05$) from that obtained by untreated controls

[e] Significantly different ($P < 0.05$) from that obtained by the maximum tolerated dose of FUra (50 mg/kg)

Table 2 Effect of 5-(phenylthio)acyclouridine (PTAU) alone or in combination with uridine on the chemotherapeutic efficacy and host toxicity of 5-FUra (200 and 300 mg/kg) in SCID mice bearing human breast tumor MCF-7 xenografts

Drug (mg/kg)			Tumor weight (mg)	%T/C[b]	% survival at day			Day of first death	Body weight (g) at day 38
FUra	PTAU	Uridine			24	31	38		
0	0	0	880 ± 48[a]	100	100	100	100	–	21.3 ± 2.1
0	0	1,320	933 ± 186	100	100	100	100	–	25.0 ± 3.2
0	120	0	967 ± 163	100	100	100	100	–	25.4 ± 3.4
50[c]	0	0	343 ± 53[d]	39	100	100	100	–	22.0 ± 3.7
200	0	0	–	–	0	0	0	24	–
200	0	1,320	–	–	38	13	0	24	–
200	120	0	333 ± 58[d]	38	100	84	38	35	25.5 ± 4.1
200	120	1,320	0[d,e]	0	100	100	100	–	26.2 ± 2.4
300	0	0	–	–	25	0	0	24	–
300	0	1,320	–	–	100	0	0	28	–
300	120	0	–	–	100	0	0	28	–
300	120	1,320	0[d,e]	0	100	100	67	38	25.6 ± 3.0

PTAU alone or in combination with uridine was administered 2 h after FUra administration

[a] Mean tumor weight ± SD from 5 to 8 tumors

[b] %T/C, percentage of tumor weight in treated mice/tumor weight in untreated control mice

[c] Maximum tolerated dose of FUra in SCID mice

[d] Significantly different ($P < 0.05$) from that obtained by untreated controls

[e] Significantly different ($P < 0.05$) from that obtained by the maximum tolerated dose of FUra (50 mg/kg)

employed in this study despite the fact that the tumors have different estrogen receptor properties. These results demonstrate that the combination of PTAU plus uridine is quite promising in protecting from FUra host toxicity. The combination of PTAU plus uridine also allowed the escalation of the maximum tolerated dose of FUra from 50 to 200 mg/kg. This delayed rescue from host toxicity is time dependent and appeared to be optimum at 2 h post-FUra administration. Similar results were obtained when such combinations were used against colon tumors [46]. Further adjustments of the PTAU plus uridine regimen may yield even better results.

The mechanism by which PTAU plus uridine combination protect from FUra host toxicity, while maintaining its efficacy against the tumors, is attributed to the exceptional effectiveness of this combination in elevating and sustaining high uridine concentrations in vivo. Metabolic and pharmacokinetic studies [13–15, 22, 35, 38, 40, 42, 46] established PTAU as the best of all known uridine phosphorylase inhibitors in increasing the efficacy and reducing the toxicity of FUra in chemotherapy. All the other inhibitors suffer from limited potency, low bioavailability, and/or poor pharmacokinetic properties [13, 15, 22, 35, 36, 38, 48].

In summary, the combination of PTAU plus uridine provides a potentially powerful, practical, and less invasive modulation regimen which may meet all the desired criteria to increase the chemotherapeutic doses of FUra without encountering its renowned host toxicity. The combination of PTAU plus uridine allowed the escalation of the maximum tolerated dose of FUra by fourfold (from 50 to 200 mg/kg). This increase in the administered dose of FUra resulted in 97% reduction to total remission of the tumors without any apparent host toxicity. Therefore, the combination of PTAU plus uridine can provide a better substitute for the massive doses of uridine necessary to rescue or protect from FUra host-toxicities, without the toxic side effects associated with such doses of uridine. The combination may also allow the escalation of FUra doses for better chemotherapeutic efficacy. Alternatively, the combination of PTAU and uridine may be useful as an antidote in the few cases when cancer patients receive a lethal overdose of FUra.

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