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Effect of Administration of 5-(Phenylselenenyl)acyclouridine, an Inhibitor of Uridine Phosphorylase, on the Anti-tumor Efficacy of 5-Fluoro-2′-deoxyuridine against Murine Colon Tumor C26–10

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ABSTRACT. The effect of co-administration of 5-(phenylselenenyl)acyclouridine (PSAU), a new uridine phosphorylase (UrdPase, EC 2.4.2.3) inhibitor, on the efficacy of 5-fluoro-2′-deoxyuridine (FdUrd) was tested against murine colon C26–10 tumor xenografts. In contrast to our previous results with human tumors, co-administration of PSAU with FdUrd decreased instead of increasing the efficacy of FdUrd against tumor growth. However, co-administration of PSAU with FdUrd (300 mg/kg/day) protected the mice completely from the 83% mortality induced by the same dose of FdUrd alone. Enzyme studies indicated that UrdPase in colon C26–10 tumors is responsible for the catabolism of FdUrd to 5-fluorouracil (FUra), as colon C26–10 tumors do not have thymidine phosphorylase (dThdPase, EC 2.4.2.4). In contrast, colon C26–10 tumors had extraordinarily high UrdPase activity (300 μmol/min/mg protein), which was at least 200-fold higher than the highest UrdPase activity in any of the human xenografts we tested previously. Furthermore, the activities of UrdPase and orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) were 192- and 2-fold higher, respectively, while that of dihydrouracil dehydrogenase (EC 1.3.1.2) was 1000-fold lower in the tumor than in the host liver. It is suggested that FdUrd exerts its anticancer effects against colon C26–10 tumors mainly through the catabolism of FdUrd to FUra by UrdPase, which then could be anabolized to 5-fluorouridine 5′-monophosphate (FUMP) by OPRTase and ultimately to other toxic 5-fluorouridine nucleotides, hence inducing the observed FdUrd toxic effects. Co-administration of PSAU with FdUrd inhibited UrdPase and the catabolism of FdUrd to FUra. This would result in the observed reduction of the antitumor efficacy of FdUrd. In addition, the increase in plasma uridine concentration induced by PSAU as well as the catabolism of FUra by the high dihydrouracil dehydrogenase activity in the liver also may have circumvented any residual FUra toxic effects against the host. These results clearly demonstrate that the anticancer efficacy of the combination of UrdPase inhibitors and FdUrd is not general and is dependent largely on the type of tumor under treatment and the mode of FdUrd metabolism in these tumors. BIOCHEM PHARMACOL **60**;5:687–692, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. 5-(phenylselenenyl)acyclouridine; uridine phosphorylase; inhibitor; chemotherapy; FdUrd

The pyrimidine nucleoside analogue FdUrd¶ is used for the treatment of various human solid tumors, including hepatic metastases of advanced gastrointestinal adenocarcinomas, advanced ovarian cancer, advanced breast cancer, and squamous cell carcinoma of the head and neck [1]. However, the rapid catabolism of FdUrd to the less effective and more toxic nucleobase, FUra, limits the advantage of using FdUrd over FUra.

UrdPase (EC 2.4.2.3) and dThdPase (EC 2.4.2.4) are the two pyrimidine nucleoside phosphorylases responsible for the catabolism of FdUrd to FUra. dThdPase is the major enzyme responsible for the cleavage of FdUrd to FUra in tissues that contain both enzymes [2–5]. However, many types of neoplasia are deficient or have very low dThdPase activity [2–4, 6–18], and in such tissues FdUrd is degraded

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¶ *Abbreviations*: DHUDase, dihydrouracil dehydrogenase; dThdPase, thymidine phosphorylase; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FdUrd, 5-fluoro-2′-deoxyuridine; FUMP, 5-fluorouridine 5′-monophosphate; FUra, 5-fluorouracil; FUrd, 5-fluorouridine; OMP, orotate 5′-monophosphate; OPRTase, orotate phosphoribosyltransferase; PSAU, 5-(phenylselenenyl)acyclouridine; and UrdPase, uridine phosphorylase.

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by UrdPase [2, 3, 15, 18]. Therefore, UrdPase inhibitors could be used in combination with FdUrd in cancer chemotherapy to prevent FdUrd cleavage in such tumors. This combination would enhance the selective toxicity of FdUrd against tumors because the host tissues contain dThdPase and are capable of degrading FdUrd [2–4, 18].

A series of acyclouridine derivatives were developed as specific inhibitors of UrdPase [2, 3, 19–23]. It was shown that these compounds significantly inhibit the cleavage of FdUrd in extracts of tumors that lack or have very low dThdPase activity [2, 4, 15, 18]. These compounds were shown to enhance the efficacy of FdUrd *in vivo* and *in vitro* against human tumors [15, 18]. We recently synthesized PSAU, as a potent and specific inhibitor of UrdPase [22]. PSAU is lipophilic, and as such, its access to the liver and intestine, the main organs involved in uridine catabolism [24–29], would be facilitated.

In the present study, we tested the effect of PSAU on the efficacy of FdUrd against murine colon tumor C26–10, a commonly used tumor in studies on chemotherapy with 5-fluorouridines. In addition, the profile of important enzyme activities involved in FdUrd metabolism in tumor xenografts also was investigated, to understand the response of these tumors to the combination of PSAU and FdUrd.

MATERIALS AND METHODS

Chemicals

[2-^{14}C]Uridine (56 Ci/mol), [2-^{14}C]thymidine (56 Ci/mol), [2-^{14}C]FdUrd (56 Ci/mol), [6-^{14}C]uracil (55 Ci/mol), and [2-^{14}C]orotic acid (55 Ci/mol) were purchased from Moravek Biochemicals, Inc. Silica gel G/UV_{254} TLC polygram plates were obtained from Fisher Scientific. CEL 300/UV_{254} cellulose and 300 PEI/UV_{254} polyethyleneimine–cellulose polygram TLC plates were from Brinkmann Instruments, Inc. Bovine γ-globulin and dye reagent for protein assays were from Bio-Rad Laboratories. PSAU was synthesized as previously described [22]. All other chemicals were purchased from the Sigma Chemical Co.

Animals

Female Balb/c and CD-1 mice (18–20 g) were obtained from Charles River Laboratories and housed five/cage with water and food *ad lib.* under a normal light cycle (light, 6:00 a.m. to 6:00 p.m.; dark, 6:00 p.m. to 6:00 a.m.) according to an institutionally approved animal protocol.

Cell Line

Murine colon tumor C26–10 was provided by Dr. G. J. Peters, Department of Oncology, Free University Hospital, and was maintained *in vivo* in Balb/c mice. It is clone 10 from the murine colon tumor C-26, a chemically induced undifferentiated murine adenocarcinoma [30]. C26–10 does not produce cachexia, as the parent strain does, when implanted in Balb/c mice [31].

Administration of Drugs

PSAU and FdUrd were freshly prepared before use. They were dissolved in warm normal saline solution (0.9% NaCl containing 10% DMSO) with vortexing, sterilized by using 0.22-μm membrane filters, and injected i.p. at 0.1 mL/10 g. Control mice received the carrier solution (0.9% saline with 10% DMSO). To avoid a possible circadian variation in UrdPase and DHUDase (EC 1.3.1.2) activities [32, 33], all mice were injected at the same time (between 8:30 and 9:00 a.m.).

Toxicity of PSAU in Mice

The toxicity of PSAU was evaluated in groups of non-tumor bearing female CD-1 mice (20–25 g). Three groups of CD-1 mice (five mice/group) were injected i.p. with PSAU at 15, 25, and 50 mg/kg/day for 5 consecutive days. The mice were observed for a 30-day period.

Chemotherapeutic Efficacy of FdUrd and PSAU against C26–10

On day 0, colon tumor C26–10 was dissected from Balb/c mice, cut into 1- to 2-mm^3 pieces, and implanted s.c. in female Balb/c mice in an area just above the right inguinal region, using a trocar. FdUrd (100, 200, and 300 mg/kg) and/or PSAU (60 mg/kg) were injected weekly starting from day 5 after tumor implantation for 2 consecutive days/week for 3 weeks. The mice were monitored for tumor growth, toxicity (weight loss), and mortality for a 28-day period. Tumor weight was used to evaluate the effect of drugs on tumor growth. Tumor weight was calculated as the [long diameter (mm)] × [short diameter (mm)]^2/2.

Enzyme Studies

Buffer A was 20 mM potassium phosphate (pH 8), and Buffer B was 50 mM Tris–Cl (pH 7.4). Buffer A was used when assaying the activities of pyrimidine nucleoside phosphorylases and DHUDase. Buffer B was used when the activities of uridine kinase, thymidine kinase, and OPRTase (EC 2.4.2.10) were assayed.

PREPARATION OF EXTRACTS. Mouse liver and C26–10 tumor were homogenized in 3 vol. of the appropriate homogenization buffer at 4° using a Brinkmann Polytron homogenizer (Brinkmann Instruments), and the homogenate was centrifuged at 105,000 *g* for 1 hr at 4°. The supernatant fluid (cytosol) was collected and used as the source of enzyme.

ENZYME ASSAYS. All enzyme assays were performed using 25 μL of enzyme preparation in a final mixture volume of 50 μL. The reaction mixture was incubated at 37° under conditions where activity was linear with time and enzyme concentration. Reactions were started by the addition of

enzyme, and were stopped by boiling in a water bath for 2 min followed by freezing for 20 min. Precipitated proteins were removed by centrifugation, and 10 μL of the supernatant was spotted on the appropriate TLC plate to separate the substrate from the product(s). The amount of radioactivity in the substrate and product(s) was determined on a percentage basis, using a Berthold LB-2821 Automatic TLC-Linear Analyzer (Wallac Inc.).

PYRIMIDINE NUCLEOSIDE PHOSPHORYLASES. The activity was measured by following the formation of [2-^{14}C]nucleobase (uracil, thymine, or FUra) from their respective [2-^{14}C]nucleosides (uridine, thymidine, or FdUrd). The assay mixture contained 0.5 mM substrate (2.24 Ci/mol), 20 mM potassium phosphate (pH 8), 1 mM EDTA, and 1 mM dithiothreitol. After termination of the reaction, freezing, and centrifugation, 10 μL of the supernatant was spotted on silica gel TLC plates and developed in chloroform:methanol:acetic acid (90:5:5, by vol.) for 1 hr to separate the nucleobase (product) from the nucleoside (substrate) [4]. The R_f values were: uridine and FUrd, 0.07; thymidine, 0.14; uracil and FUra, 0.43; and thymine, 0.62.

PYRIMIDINE NUCLEOSIDE KINASES. The activity was measured by following the formation of the nucleotides UMP, dTMP, and FdUMP from their respective [2-^{14}C]nucleosides (uridine, thymidine, or FdUrd). The assay mixture contained 0.5 mM substrate (2.24 Ci/mol), 50 mM Tris–Cl (pH 7.4), 5 mM $MgCl_2$, 2.5 mM ATP, 25 mM NaF, 0.5 mM PSAU, 5 mM creatine phosphate, and creatine phosphokinase [34]. After termination of the reaction, freezing, and centrifugation, 10 μL of the supernatant was spotted on silica gel TLC plates and developed in chloroform:methanol:acetic acid (86:24:6, by vol.) until 1 inch from the top. The R_f values were: uridine and FdUrd, 0.78; UMP and FdUMP, 0.08; thymidine, 0.85; and dTMP, 0.13.

OPRTASE. The activity was measured by following the formation of OMP, orotidine, UMP, and uridine and uracil from [6-^{14}C]orotate. The assay mixture contained 15 μM orotate (55 Ci/mol), 50 mM Tris–Cl (pH 7.4), 5 mM $MgCl_2$, 2.5 mM phosphoribosyl pyrophosphate, and 1 mM dithiothreitol. After termination of the reaction, freezing, and centrifugation, 10 μL of the supernatant was spotted on prewashed polyethyleneimine–cellulose TLC plates. The plates were first developed in water (up to 10 cm) to separate the nucleosides that migrate with the front from the nucleotides that remain at the origin. Then the plates were dried and redeveloped overnight in 0.2 M LiCl to separate the nucleotides. The R_f values were: OMP, 0.16; UMP, 0.51; orotate, 0.62; orotidine, 0.77; and uridine and uracil, 0.95. The activity of OPRTase was measured as the sum of the products OMP, orotidine, UMP, and uridine and uracil [34].

DHUDASE. The activity was measured by following the formation of dihydrouracil, carbamyl β-alanine, and β-alanine from [6-^{14}C]uracil. The assay mixture contained 10 μM uracil (55 Ci/mol) or FUra (58 Ci/mol), 20 mM potassium phosphate (pH 8), 5 mM $MgCl_2$, 2 mM dithiothreitol, and 50 μM NADPH. After termination of the reaction, freezing, and centrifugation, 10 μL of the supernatant was spotted on cellulose TLC plates, and the plates were developed overnight in the top phase of a mixture of *n*-butanol:H_2O:ammonium hydroxide (90:45:15, by vol.). The R_f values were: dihydrouracil, 0.46; uracil, 0.23; carbamyl β-alanine plus β-alanine, 0.09. DHUDase activity was measured as the sum of the products dihydrouracil, carbamyl β-alanine, and β-alanine [35].

PROTEIN DETERMINATION. Protein concentrations were determined spectrophotometrically by the method of Bradford [36] using bovine γ-globulin as a standard.

TABLE 1. Toxicity of PSAU in mice

Dose (mg/kg)	Day 30 weight (g)
0	25.5 ± 1.2*
15	24.5 ± 0.9
25	24.8 ± 1.4
50	26.5 ± 1.8

PSAU was injected i.p. for 5 consecutive days. Mice were monitored daily for a 30-day period for weight loss and mortality.
*Values are means ± SD of weights from 5 mice.

Statistical Analysis

Student's *t*-test was used to determine the degree of significance between the different parameters in treated and untreated animals.

RESULTS

The results in Table 1 show that PSAU alone at 15, 25, and 50 mg/kg × 5 days produced no toxicity or mortality in mice. Similarly, at 60 mg/kg/day × 2/week × 3, PSAU had no toxic effect on the growth of colon C26–10 tumor *in vivo* (Table 2).

Table 2 shows the results of administering different doses of FdUrd alone and in combination with PSAU (60 mg/kg/day × 2/week × 3). Administration of FdUrd alone at doses of 100, 200, and 300 mg/kg/day × 2/week × 3 produced a significant reduction in tumor weight to 70, 82, and 87%, respectively, of the control tumors at day 10. The reduction in tumor size continued, and the tumor weight on day 17 was reduced further by 89, 93, and 97%, respectively, relative to the control group, achieving total remission in 20, 17, and 67% of the mice, respectively. On day 24, total remission was observed in all mice treated with 200 and 300 mg/kg/day × 2/week × 3 and 40% remission in the group receiving the lowest dose of FdUrd (100 mg/kg/day × 2/week × 3) with a reduction of tumor size by 94% relative to the control group.

Co-administration of PSAU at 60 mg/kg/day × 2/week

TABLE 2. Effect of co-administration of PSAU on the efficacy of FdUrd against murine colon tumor C26-10 growth in Balb/c mice

Drugs (mg/kg)		Day 10				Day 17				Day 24				Day 28			
PASU	FdUrd	Tumor weight (mg)	% Inhibition*	% Dead†	% Cure‡	Tumor weight (mg)	% Inhibition	% Dead	% Cure	Tumor weight (mg)	% Inhibition	% Dead	% Cure	Tumor weight (mg)	% Inhibition	% Dead	% Cure
0	0	62.5 ± 17.5§	0	0	0	583.3 ± 64.2	0	0	0	690.3 ± 232.6	0	17	0	702.2 ± 256.1	0	17	0
0	100	18.7 ± 5.3‖	70	0	0	62.4 ± 28.4‖	89	0	20	44.4 ± 28.4‖	94	0	40	142.5 ± 70.9‖	80	0	33
0	200	11.5 ± 2.7‖	82	0	0	42.2 ± 17.5‖	93	0	17	0‖	100	0	100	0‖	100	0	100
0	300	8.1 ± 2.0	87	0	0	15.5 ± 11.1‖	97	0	67	0‖	100	0	100	0‖	100	83	100
60	0	74.0 ± 18.2	0	0	0	665.7 ± 181.9	0	0	0	844.0 ± 223.9	0	0	0	915.9 ± 123.7	0	0	0
60	100	60.2 ± 11.7	4	0	0	453.5 ± 85.6	22	0	0	542.0 ± 86.0	21	0	0	752.5 ± 194.7	0	0	0
60	200	32.7 ± 11.6	48	0	0	188.5 ± 33.8	68	0	0	238.8 ± 91.6	65	0	0	311.5 ± 108.1	56	0	17
60	300	11.6 ± 7.0	81	0	0	90.5 ± 35.3	84	0	17	89.7 ± 39.1	87	0	33	164.2 ± 98.4	77	0	50

FdUrd and PSAU were injected i.p. on days 5 and 6. Injection was repeated for 2 consecutive days weekly for 3 weeks. Mice were monitored for weight loss, survival, and tumor volume for a period of 28 days.

*Percent tumor growth inhibition compared with the control untreated mice.

†Percent dead in the group.

‡Percent mice cured from the tumor in the group (total remission) on day 28.

§Values are mean tumor weight ± SEM from 5–6 tumor xenografts.

‖Significantly different from the group that received FdUrd plus PSAU ($P < 0.05$).

with different doses of FdUrd decreased the antitumor efficacy of FdUrd on colon C26–10 tumors, whereby at the end of the observation period (28 days), FdUrd at 200 and 300 mg/kg/day × 2/week × 3 plus PSAU at 60 mg/kg/day × 2/week × 3 induced a complete remission in only 17 and 50%, respectively, of the tumors in contrast to 100% in the group that did not receive PSAU. While PSAU reduced the antitumor efficacy of FdUrd at 300 mg/kg/day × 2/week × 3, it protected against FdUrd-induced host-toxicity. Table 2 shows that 83% of the mice treated with FdUrd (300 mg/kg/day × 2/week × 3) were dead by day 28, whereas no mortality was observed in mice treated with FdUrd plus PSAU.

Table 3 shows that phosphorolysis of uridine in colon C26–10 tumor was significantly higher (192-fold) than that present in the Balb/c mouse liver. PSAU (100 μM) inhibited this activity completely in both the liver and colon C26–10 tumor. PSAU also inhibited completely the phosphorolysis of thymidine and FdUrd in colon C26–10 but not in the liver. These results indicate that, unlike the liver, colon C26–10 tumor does not contain a distinct dThdPase but rather a UrdPase with activity towards thymidine and FdUrd as well as uridine. In mouse liver, the contribution of UrdPase to the phosphorolysis of thymidine and FdUrd was indicated by the 7.5 and 16.4% inhibition by PSAU.

Kinase activity towards uridine in the tumor was higher than in mouse liver (14-fold higher). However, the ratio of uridine kinase/UrdPase in mouse liver was 13.6-fold higher than in the tumor. Kinase activities towards thymidine and FdUrd were also higher in the tumors than in the liver. FdUrd was a better substrate than thymidine for the phosphorylase and kinase activity in colon C26–10 tumor. OPRTase activity was 1.9-fold higher in the colon C26–10 tumor than in the liver. DHUDase activity was significantly lower (1000-fold) in the tumor than in mouse liver.

DISCUSSION

In contrast to our previous results with human tumors [15, 18], the present study demonstrates that co-administration of a UrdPase inhibitor (PSAU) with FdUrd decreased instead of increasing the efficacy of FdUrd against tumor growth. However, co-administration of PSAU with FdUrd (300 mg/kg/day) protected the mice from the 83% mortality induced by the same dose of FdUrd alone (Table 2). The failure of PSAU to potentiate FdUrd antitumor efficacy in colon C26–10 tumor could be explained on the basis of the apparently peculiar metabolism of FdUrd in colon C26–10 tumor.

Like other human tumor xenografts [15, 18], the murine colon C26–10 tumors do not contain dThdPase, as indicated by the total inhibition of uridine, thymidine, and FdUrd phosphorolysis by PSAU, a specific UrdPase inhibitor [22]. However, colon C26–10 tumor had an unusually high UrdPase activity (300 μmol/min/mg protein) that was at least 200-fold higher than the highest UrdPase activity in

TABLE 3. Activities of FdUrd-metabolizing enzymes in colon C26-10 tumor and mouse liver and the effect of PSAU on pyrimidine nucleoside phosphorylase activities

Enzyme	Substrate	[mM]	Tumor	Mouse liver
Phosphorylase	Uridine	0.5	305,371 ± 13,605* (100)	1,593 ± 71 (100)
	Thymidine	0.5	8,054 ± 495 (100)	9,455 ± 296 (7.5)
	FdUrd	0.5	16,130 ± 1,050 (100)	9,255 ± 865 (16.4)
Kinase	Uridine	0.5	26,487 ± 3,963	1,877 ± 116
	Thymidine	0.5	818 ± 255	22 ± 5
	FdUrd	0.5	1,275 ± 138	59 ± 13
OPRTase	Orotate	0.05	2,162 ± 560	1,154 ± 52
DHUDase	Uracil	0.01	0.7 ± 0.7	860 ± 61

*Activity is expressed as the mean (pmol/min/mg protein) ± SD of three determinations. Percent inhibition by 100 μM PSAU is shown in parentheses.

any of the human xenografts that we tested previously [15, 18]. Murine colon C26–10 also had relatively high UrdPase and OPRTase, and low DHUDase activities when compared with those from mouse liver. The activities of UrdPase and OPRTase in the tumor were 192- and 2-fold higher than in the host liver, while the activity of DHUDase was at least 1000-fold lower in the tumor than in mouse liver (Table 3). From the enzyme studies and the results of the *in vivo* chemotherapy of colon C26–10 tumors with FdUrd, it appears that FdUrd exerts its anticancer effects on colon C26–10 tumors mainly through the catabolism of FdUrd by UrdPase to FUra, which then is anabolized to FUMP by OPRTase. The activities of both enzymes were quite high in the colon C26–10 tumor (Table 3). FUMP, when anabolized further to other toxic 5-fluorouridine nucleotides, would exert the toxic effects of FdUrd in this tumor. This suggestion is supported by the finding that co-administration of PSAU with FdUrd limited the toxicity of FdUrd, as indicated by the reduction in the antitumor efficacy of FdUrd and the protection from FdUrd-induced host-toxicity as the result of the inhibition of UrdPase and FdUrd catabolism to the more toxic FUra, thus preventing or limiting the toxicity of FdUrd in the tumor.

In addition, the increase in plasma uridine concentration induced by PSAU [37], as well as the greater catabolism of FUra, resulting from dThdPase activity in the liver, by the high hepatic DHUDase activity also may have circumvented any residual FUra toxic effects against the host. These findings are in agreement with the results of Iigo *et al.* [38], using the UrdPase inhibitor 2,2′-anhydro-5-ethyluridine. 2,2′-Anhydro-5-ethyluridine failed to exhibit any potentiating effect on FdUrd antitumor efficacy in murine mammary adenocarcinoma 755 in mice. In fact, it reduced the antitumor efficacy of FdUrd. On the other hand, 2,2′-anhydro-5-ethyluridine markedly potentiated the antitumor activity of FUrd against the tumor [38]. The authors concluded that the catabolism of FdUrd to FUra and then the anabolism of FUra to FUrd was the mechanism of the efficacy for FdUrd against these tumors.

In conclusion, the present results clearly demonstrate that the anticancer efficacy of the combination of UrdPase inhibitors and FdUrd is not general and is dependent largely on the type of tumor under treatment and the mode of FdUrd metabolism in the tumor.

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