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Structure and Activity of Specific Inhibitors of Thymidine Phosphorylase to Potentiate the Function of Antitumor 2′-Deoxyribonucleosides

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ABSTRACT. A new class of 5-halogenated pyrimidine analogs substituted at the 6-position was evaluated as competitive inhibitors of thymidine phosphorylase (TPase). The most potent member of the series was 5-chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1*H*,3*H*)-pyrimidinedione hydrochloride (TPI), which has an apparent K_i value of 1.7×10^{-8} M. TPI selectively inhibited the activity of TPase, but not that of uridine phosphorylase, thymidine kinase, orotate phosphoribosyltransferase, or dihydropyrimidine dehydrogenase. *In vitro* inhibition studies of TPI using a thymidine analogue, 5-trifluoromethyl-2′-deoxyuridine (F_3dThd), as the substrate demonstrated that F_3dThd phosphorolytic activity was inhibited markedly by TPI (1×10^{-6} M) in extracts from the liver, small intestine, and tumors of humans, from the liver and small intestine of cynomolgus monkeys, and from the liver of rodents, but not from the liver or small intestine of dogs or the small intestine of rodents, suggesting that the distribution of TPase differs between humans and animal species, and that TPI could contribute to the modulation of TPase in humans. When F_3dThd or 5-iodo-2′-deoxyuridine (IdUrd) was coadministered to mice with TPI at a molar ratio of 1:1, the blood levels of F_3dThd (or IdUrd) were about 2-fold higher than when F_3dThd (or IdUrd) was administered alone. In monkeys, the maximum concentration (C_{max}) and the area under the concentration–time curve (AUC) after oral F_3dThd alone were 0.23 μg/mL and 0.28 μg · hr/mL, respectively, but markedly increased to 15.18 μg/mL (approximately 70-fold) and 28.47 μg · hr/mL (approximately 100-fold), respectively, when combined with equimolar TPI. Combined oral administration of TPI significantly potentiated the antitumor activity of F_3dThd on AZ-521 human stomach cancer xenografts in nude mice. In conclusion, TPI may contribute not only to inhibition of TPase-mediated biological functions but also to potentiation of the biological activity of various 2′-deoxyuridine and thymidine derivatives by combining with them. BIOCHEM PHARMACOL **59**;10:1227–1236, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. thymidine phosphorylase; uridine phosphorylase; competitive inhibitor; 5-trifluoromethyl-2′-deoxyuridine; 5-iodo-2′-deoxyuridine

Two different pyrimidine nucleoside phosphorylases, TPase† (EC 2.4.2.4) and UPase (EC 2.4.2.3), have been identified and separated in some mammalian tissues [1–4]. These enzymes catalyze the reversible phosphorolysis of a number of natural and synthetic pyrimidine nucleosides according to the following equation:

Pyrimidine(deoxy)ribose + orthophosphate → pyrimidine base + (deoxy)ribose-1-phosphate.

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† *Abbreviations*: TPase, thymidine phosphorylase; TPI, 5-chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1*H*,3*H*)-pyrimidinedione hydrochloride; UPase, uridine phosphorylase; F_3dThd, 5-trifluoromethyl-2′-deoxyuridine; IdUrd, 5-iodo-2′-deoxyuridine; BrdUrd, 5-bromo-2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; PD-ECGF, platelet-derived endothelial cell growth factor; dThd, thymidine; Urd, uridine; 5-FU, 5-fluorouracil, DPDase, dihydropyrimidine dehydrogenase; OPRTase, orotate phosphoribosyltransferase; TK, thymidine kinase; BAU, 5-benzylacyclouridine; RTV, relative tumor volume; and IR, relative inhibition of tumor growth.

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UPase cleaves uridine and thymidine, as well as 2′-deoxyuridine and other pyrimidine nucleosides [5–7].

TPase, on the other hand, is thought to be specific for pyrimidine 2′-deoxyribonucleosides, such as FdUrd, F_3dThd, IdUrd, and BrdUrd, because pyrimidine ribonucleosides are not cleaved by this enzyme [6, 8–10]. The *in vivo* cytotoxicity of these chemotherapeutic agents is thought to be limited by cleavage of the nucleoside forms to the less active or inactive bases. Contrasting patterns of pyrimidine nucleoside phosphorylases in rodents and humans have been reported, and, unlike rodents, humans have high TPase activity and low UPase activity [11–14]. Therefore, it has been speculated that inhibitors of TPase might be useful as chemotherapeutic agents by enhancing the efficacy of these agents, which are cleaved by TPase. During the past 20 years, some 5′-substituted acyclouridines including BAU and 5′-benzyloxybenzyl acyclouridine [15–16] have been found to be pyrimidine nucleoside phosphorylase inhibitors. Unfortunately, these inhibitors mainly inhibit UPase, not TPase. Several other compounds have

been reported to inhibit TPase [17–21]. However, the inhibitory potency of such compounds is too weak to prevent degradation of biologically important pyrimidine 2′-deoxyribonucleosides *in vivo*.

Human TPase has been purified, cloned, and shown to be identical to PD-ECGF [22, 23]. TPase is chemotactic and non-mitogenic for endothelial cells [24, 25], and its expression has also been shown to be linked to angiogenesis, metastasis, and advanced disease in esophageal, gastric, breast, bladder, ovarian, and lung cancer [26–31]. Thus, TPase inhibitors may serve as tools for the study of tumor vascularization, prognosis, and response to chemotherapy. In the present paper, we describe the inhibitory effects of synthetic compounds, including 5-halogenated-6-substituted pyrimidines, on human TPase and their biological activity *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals and Animals

The compounds with TPase inhibitory activity were synthesized at the Taiho Pharmaceutical Co., Ltd. [6-^{3}H]dThd (603.1 GBq/mmol), [2-^{14}C]dThd (2.0 GBq/mmol), and [5-^{3}H]Urd (925 GBq/mmol) were obtained from New England Nuclear, Moravek Biochemicals Inc., and Amersham Life Science, respectively. [6-^{3}H]F_3dThd (366.3 GBq/mmol) and [6-^{3}H]5-FU (854.7 GBq/mmol) were obtained from Moravek Biochemicals Inc. F_3dThd and 5-trifluoromethyluracil were purchased from the Yuki Gosei Kogyo Co., Ltd. and PCR Inc., respectively. IdUrd was a product of the Yamasa Co., Ltd. BAU was synthesized in our laboratory according to the method of Niedzwicki *et al.* [15]. All other chemicals used were commercially available products. Enzyme kinetic studies of TPI with TPase were carried out by using a commercially available recombinant human PD-ECGF (R & D Systems Inc.) that is biochemically and genetically identical to human TPase [22, 23].

Experimental Animals

Six-week-old male ICR mice and 5-week-old male BALB/c *nu/nu* nude mice were purchased from the Clea Japan Co. Male Donryu strain rats were obtained from Charles River Japan. Beagle dogs were supplied from CSK Research Park, Inc. Male and female cynomolgus monkeys, weighing between 2.90 and 3.65 kg (Life Science Lab.) were used. All animals were supplied *ad lib.* with a commercial diet and autoclaved water until use.

Preparation of Crude Enzyme Extracts

For the preparation of pyrimidine nucleoside phosphorylase, monkeys and dogs were killed with overdoses of Nembutal, and mice and rats were anesthetized with chloroform and exsanguinated by heart puncture. The liver and intestine of each animal were removed, and were homogenized in ice-cold 50 mM Tris–HCl (pH 8.0) containing 5 mM 2-mercaptoethanol. Then the homogenates were centrifuged at 105,000 *g* for 60 min at 4°, and the supernatant was used for the measurement of TPase and UPase activity. The crude enzyme extracts of human normal and tumor tissues were provided by Dr. Junichi Sakamoto (Clinical and Pharmacological Division, Aichi Prefectural Hospital). Preparation of crude human enzyme was also carried out as described above. Murine Yoshida sarcoma cells were also homogenized with 4 vol. of the same buffer. After centrifugation at 105,000 *g* for 60 min at 4°, the supernatant was used for the measurement of TK and OPRTase activity.

Purification of TPase from Human Placenta

TPase protein was partially purified from human placenta according to methods described previously [4, 32]. Briefly, lyophilized human placenta powder was suspended in buffer A (50 mM potassium phosphate, 5 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.0) and centrifuged at 9000 *g* for 20 min. The supernatant was dialyzed against buffer A for 16 hr and centrifuged at 105,000 *g* for 1 hr. The supernatant was fractionated between 30 and 60% ammonium sulfate saturation, and the fraction was dissolved in buffer A and dialyzed extensively against the same buffer. The dialyzed solution was applied to a DEAE–Sepharose column equilibrated with buffer A and eluted with a linear gradient of 0 to 0.4 M KCl in buffer A. This chromatographic procedure was repeated. Then the active fractions were dialyzed against buffer A and frozen at −80° until use. Under these conditions, the enzymes were stable with no loss of activity for over 6 months. Human placenta UPase was not bound to DEAE–Sepharose under these conditions, suggesting that TPase is separated easily from UPase by this procedure.

Assay of Pyrimidine Nucleoside Phosphorylase

In this experiment, dThd-, Urd-, and F_3dThd-phosphorolytic activity was measured by using [6-^{3}H]dThd, [5-^{3}H]Urd, and [6-^{3}H]F_3dThd, respectively, as substrates. The reaction mixture consisted of a total volume of 0.125 mL containing 100 mM potassium phosphate buffer (pH 7.4), 0.6 mM of one of the above substrates containing 9.25 KBq of radiolabeled compound, enzyme extracts, and inhibitor solution, and the mixture was incubated at 37° for 5 min. Next, the mixture was heated at 100° for 2 min in boiling water, and centrifuged at 1900 *g* for 5 min. A 10-μL sample of the supernatant was spotted on a silica gel TLC plate (Merck TLC plates with silica gel 60F_{254} precoated; 2 x 10 cm; thickness 0.25 mm) and developed with a mixture of chloroform, methanol, and acetic acid (17:3:1, by vol.). A mixture of 5 mM dThd and thymine (or Urd and uracil, or F_3dThd and 5-trifluoromethyluracil) was applied to the plate as a visible marker before the test samples. The thymine, uracil, or 5-trifluoromethyluracil spots were scraped into a vial and extracted with 4 M HCl

(0.1 mL), and after adding 10 mL of scintillator, their radioactivity was measured.

Assay of OPRTase

Murine Yoshida sarcoma cells were homogenized with 3 vol. of 50 mM Tris–HCl (pH 8.0) containing 10 mM β-mercaptoethanol, 5 mM KCl, and 10 mM $MgCl_2$ and centrifuged at 105,000 *g* for 60 min. The resultant supernatant was used as OPRTase. OPRTase activity was measured by the formation of 5-fluorouridine 5′-monophosphate (FUMP) from [6-^{3}H]5-FU, as described before [33].

Assay of TK

The above-described enzyme extracts were also used as a source of TK. TK activity was measured using [2-^{14}C]dThd as a substrate, according to the method of Tatsumi *et al.* [34].

Assay of DPDase

Rat liver was homogenized with 3 vol. of 50 mM Tris–HCl (pH 8.0) containing 10 mM β-mercaptoethanol, 5 mM KCl, and 10 mM $MgCl_2$, and centrifuged at 105,000 *g* for 60 min. The resultant supernatant was used as DPDase. DPDase activity was measured by the total formation of 2-fluoro-β-alanine, 2-fluoro-3-ureidopropionic acid, and 5-fluorodihydrouracil from [6-^{3}H]5-FU as described before [32].

Determination of F_3dThd or IdUrd in the Plasma of Mice and Monkeys

A 50 mg/kg sample of F_3dThd with or without equimolar TPI dissolved in 0.5% hydroxypropylmethylcellulose solution was administered orally to male ICR mice weighing about 25 g. The mice were killed at the times indicated, and their blood was removed rapidly and centrifuged to obtain the plasma. For IdUrd, the same dose with or without equimolar TPI also was administered to the mice, and the subsequent treatment was the same as for F_3dThd. In cynomolgus monkeys, 10 mg/kg of F_3dThd with or without equimolar TPI was administered orally, and blood was sampled from each monkey at 0.5, 1, 2, 4, and 8 hr after dosing. IdUrd and F_3dThd were used as the internal standards for the determinations of plasma F_3dThd and IdUrd concentrations, respectively. About 0.2 to 0.4 mL of plasma containing 1 μg/mL of internal standards (mice) or 5 μg/mL (monkeys) was passed through a AG50W-X4 cation exchange resin column (1.0 i.d. × 5 cm), and the column was washed twice with 2 mL of 0.1 N HCl. Next, a mixture of 3 vol. of 25 mM carbonate buffer (pH 10.0) and 1 vol. of 1 M NaOH was added to the column to collect the F_3dThd, and the eluate was passed immediately through the same size AG1-X4 anion exchange resin column. The column was washed twice with 5 mL of distilled water, and then twice with 5 mL of methanol. Subsequently, 0.3 M acetic acid in absolute methanol (5 mL) was applied to the column, twice, and the eluate was collected carefully. Then the eluate was evaporated to dryness under a nitrogen gas flow at 50°, and the residue was dissolved in 200 μL of distilled water and passed through a 0.45-μm filter. The F_3dThd and IdUrd content of the filtrates was determined by reverse-phase HPLC (Gulliver HPLC System, Jasco Co., Ltd.). Then aliquots (40 μL) of the sample were applied to a Chemcosorb 300–5C18 column (4.6 i.d. × 250 mm, Chemco Co., Ltd.) under the following chromatographic conditions: mobile phase, 6% acetonitrile containing 0.01% trifluoroacetic acid; flow rate, 1.0 mL/min; monitoring wavelength, 260 nm (F_3dThd) or 270 nm (IdUrd). Under these conditions, the retention times for IdUrd and F_3dThd were 8.8 and 11.7 min, respectively.

Antitumor Experiments

Groups consisting of 6–7 nude mice were used. The human stomach tumor AZ-521 was supplied by the Cancer Research Foundation and maintained by implantation of 8-mm^3 fragments into the right axillae of nude mice weighing about 20 g. When the tumor volume of AZ-521 reached about 200 mm^3, drugs were administered orally to mice for 14 days, starting 9 days after the tumor implantation. Control mice received 0.5% hydroxypropylmethylcellulose solution alone. The antitumor effect of the drugs was measured by using the following equation: relative inhibition of the tumor growth (IR, %) = [1 − (mean RTV of drug-treated group/mean RTV of control group)] × 100.

RESULTS

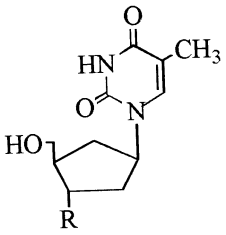
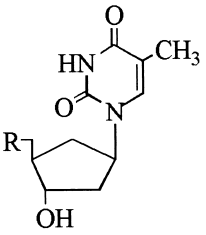
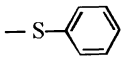
Inhibitory Effects of Thymidine Analogs Substituted at the 3′- or 5′-Position

To develop TPase specific inhibitors, we focused first on modifying the sugar portion (3′- or 5′-position) of deoxynucleoside analogs. As shown in Table 1, none of the 3′- and 5′-substituted 2′-deoxynucleosides tested inhibited TPase activity. About 100 pyrimidine derivatives substituted with alkyl, phenyl, and carbonyl residues instead of the deoxyribose moiety at the N-1 position also had no inhibitory activity at concentrations up to 0.1 mM (data not shown).

Inhibitory Activity of 6-Substituted 5-Chlorouracil Derivatives

Since Niedzwicki *et al.* [19] had reported that several 5-halogenated 6-aminouracils inhibit both TPase and UPase activities, and we had found that 5-chloro-6-aminouracil has regulatory activity on TPase-induced angiogenesis [35], we focused on the synthesis and inhibitory activity of 6-substituted 5-chlorouracil derivatives. Typical active TPase inhibitors are shown in Table 2. 5-Chloro-6-aminouracil inhibited the activity of both TPase and UPase,

TABLE 1. Inhibitory effect of 3′- and 5′-substituted thymidine on human TPase

R	TPase IC_{50} (M)*	R	TPase IC_{50} (M)*
			
−Cl	$>1.0 \times 10^{-4}$	$-O-\overset{O}{\overset{\|}{P}}-O-(CH_2)_9CH_3$ (O)	$>1.0 \times 10^{-4}$
$-NH_2$	$>1.0 \times 10^{-4}$	$-O-\overset{O}{\overset{\|}{P}}-(CH_3CH_2O)_2$ (O)	$>1.0 \times 10^{-4}$
$-N_3$	$>1.0 \times 10^{-4}$	$-NH_2$	$>1.0 \times 10^{-4}$
−CN	$>1.0 \times 10^{-4}$	$-\overset{H}{N}-\overset{O}{\overset{\|}{C}}-(CH_2)CH_3$	$>1.0 \times 10^{-4}$
$-OCH_2CH_3$	$>1.0 \times 10^{-4}$	$-N=\overset{H}{C}-N(CH_3)_2$	$>1.0 \times 10_{-4}$
$-O-\overset{O}{\overset{\|}{S}}-CH_3$ (O)	$>1.0 \times 10^{-4}$		
$-SCH_2CH_3$	$>1.0 \times 10^{-4}$		
$-S(CH_2)_7CH_3$	$>1.0 \times 10^{-4}$		
	$>1.0 \times 10^{-4}$		

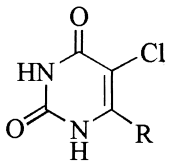
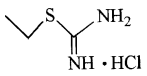
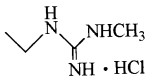
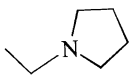
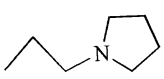
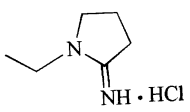
These assays were performed in duplicate at concentrations of 10 and 100 μM. A 0.6 mM concentration of [6−^{3}H]dThd was used as the substrate for the TPase reactions.
*Concentration of the compound required to inhibit TPase activity by 50%.

with IC_{50} values of 1.5×10^{-5} and 5.3×10^{-5} M, respectively. The introduction of the amidinothiomethyl and (1-methylguanidino)methyl at the 6-position of 5-chlorouracil increased the inhibitory effect on TPase (3.5×10^{-7} and 8.7×10^{-8} M, respectively) but not on UPase (6.1×10^{-5} and 2.3×10^{-4} M, respectively). Also, 6-[(1-pyrrolidinyl)methyl]-5-chlorouracil hydrochloride had relatively high inhibitory potency on TPase and UPase, with IC_{50} values of 2.2×10^{-6} and 8.6×10^{-6} M, respectively. It is especially noteworthy that the introduction of the (2-imino-1-pyrrolidin-1-yl)methyl group at the 6-position of 5-chlorouracil potentiated maximally the inhibitory activity on TPase alone (IC_{50}: 3.5×10^{-8} M), and this compound had no inhibitory effect on UPase.

Inhibitory Effect of 5-Substituted 6-[(2-Iminopyrrolidin-1-yl)methyl] uracil on TPase

The effect of substitution of various halogens at the 5-position of 6-[(2-iminopyrrolidin-1-yl)methyl] uracil on

TABLE 2. Inhibitory effect of 6-substituted 5-chlorouracil on human TPase or UPase

R	TPase IC_{50} *M)*	UPase IC_{50} (M)
		
$-NH_2$	1.5×10^{-5}	5.3×10^{-5}
	3.5×10^{-7}	6.1×10^{-5}
	8.7×10^{-8}	2.3×10^{-4}
	2.2×10^{-6}	8.6×10^{-6}
	8.2×10^{-5}	ND†
	3.5×10^{-8}	$>1.0 \times 10^{-3}$

These assays tested at least three concentrations of each compound. A 0.6 mM concentration of [6−^{3}H]dThd and [5−^{3}H]Urd was used as the substrate for the TPase and UPase reactions, respectively.
*Concentration of the compound required to inhibit enzyme activity by 50%.
†Not determined.

TABLE 3. Inhibitory activity of 5-substituted 6-[(2-iminopyrrolidin-1-yl)methyl uracil on human TPase

R	TPase
$-CH_3$	1.2×10^{-7}
$-Cl$	3.5×10^{-8}
$-Br$	3.0×10^{-8}
$-I$	3.0×10^{-8}

These assays tested at least three concentrations of each compound. A 0.6 mM concentration of [6 − ^{3}H]dThd was used as the substrate for TPase.

*Concentration of the compound required to inhibit TPase activity by 50%.

TPase inhibitory activity was examined. As shown in Table 3, there were no changes in TPase inhibitory activity as a result of substituting other halogens, i.e. bromine and iodine, for the chlorine residue at the 5-position of 6-[(2-iminopyrrolidin-1-yl)methyl] uracil, but substitution of the chlorine with a methyl group at the 5-position of this compound reduced its TPase inhibitory activity. No inhibitory activity against UPase was observed at 1×10^{-3} M concentrations of these compounds (the results are shown in Table 4). Thus, 5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl] uracil (called TPI) was found to be one of the most potent and specific inhibitors of TPase.

Inhibitory Effects of TPI on Pyrimidine-Metabolizing Enzymes

Naturally occurring and synthetic pyrimidines and their nucleosides are metabolized by various enzymes such as TK, OPRTase, and DPDase. As shown in Table 4, TPI did not inhibit the activity of these enzymes.

Mode of TPI Inhibition of Recombinant Human TPase

The K_m value for dThd as substrate and the apparent K_i value for TPI as TPase inhibitor were determined by using a recombinant human TPase (PD-ECGF). A Lineweaver–Burk plot of the reaction (Fig. 1) showed TPI to be a competitive inhibitor with a K_i value of 1.7×10^{-8} M.

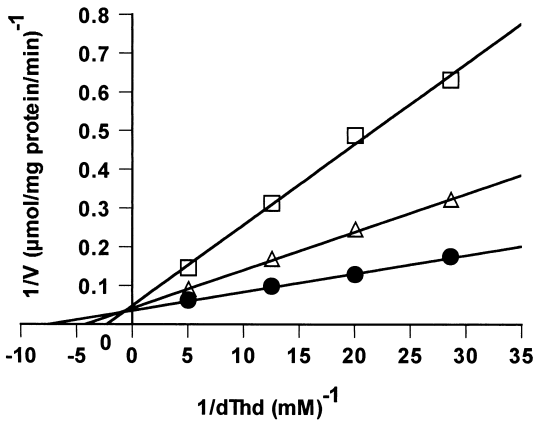


FIG. 1. Lineweaver–Burk plot of the TPI inhibition of recombinant human TPase. [6-^{3}H]dThd was used as a substrate for the TPase reaction. The reaction was carried out in the absence (●) and presence of 2×10^{-8} M (Δ) and 6×10^{-8} M (□) TPI, respectively. The Michaelis–Menten value (K_m) of TPase for dThd was 134 μM, which was almost the same as described previously [20].

Inhibition by TPI of F_3dThd Phosphorolytic Activity in Normal and Tumor Tissue from Animals In Vitro

The inhibitory potency of TPI on the phosphorolysis of F_3dThd, a cytotoxic nucleoside, was evaluated by using crude enzyme preparations from the liver and small intestine of rodents, dogs, monkeys, and humans, and from human colon cancer. As shown in Fig. 2, TPI (1×10^{-6} M) strongly inhibited F_3dThd phosphorolytic activity in preparations from mouse, rat, monkey, and human liver and from monkey and human intestine and human colon tumors, suggesting that TPase catalyzes the phosphorolysis of F_3dThd in those tissues. On the other hand, F_3dThd phosphorolytic activity in rodent intestine and dog liver and intestine was not inhibited by the addition of the same concentration of TPI. These results suggest that the phosphorolysis of F_3dThd is catalyzed mainly by UPase. We further compared the inhibitory effects of TPI and BAU, known as an inhibitor of UPase, on F_3dThd phosphorolytic activity in freshly obtained human breast and colon carcinomas from patients. The result showed that TPI but not BAU almost completely inhibited the phosphorolysis of F_3dThd in human tumors (Table 5).

TABLE 4. Inhibitory effect of TPI on pyrimidine-metabolizing enzymes

	Enzyme activities (nmol/mL/min)					
	TPI concentrations added (M)					
Enzyme	0	1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}	IC_{50} (M)
UPase	22.08	22.64	22.10	22.40	19.42	$> 1.0 \times 10^{-3}$
OPRTase	0.378	0.373	0.390	0.388	0.390	$> 1.0 \times 10^{-3}$
TK	4.24	4.17	4.18	4.15	4.34	$> 1.0 \times 10^{-3}$
DPDase	0.771	0.764	0.778	0.814	0.820	$> 1.0 \times 10^{-3}$

These assays were performed in duplicate.

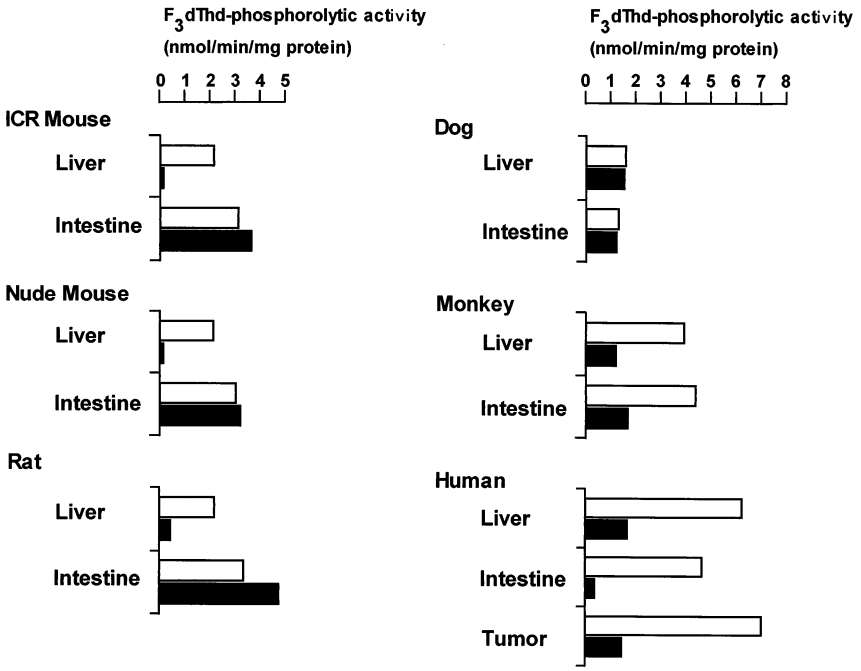


FIG. 2. Inhibitory effect of TPI on F_3dThd phosphorolytic activity in crude extracts from various mammalian tissues. [6-^{3}H]F_3dThd was incubated with enzyme extracts from rodent, dog, monkey, and human tissues in the presence (■) and absence (□) of 1×10^{-6} M TPI. Values are means of duplicate determinations.

Plasma F_3dThd and IdUrd Concentrations in Mice Following Oral Administration with or without TPI

Almost all antitumor drugs have been commonly administered i.v. to cancer patients. In the case of antitumor 2′-deoxyribonucleosides, as their functions are time-dependent, it is assumed that consecutive administration of these drugs may be more effective and reasonable than single-dose or intermittent administration. Therefore, F_3dThd and IdUrd with or without equimolar amounts of TPI were given orally to mice, and their plasma concentrations were determined. When F_3dThd alone (50 mg/kg) was administered, the plasma F_3dThd level peaked at a C_{max} of 16 μg/mL at 15 min, and rapidly declined thereafter with an AUC value of 13 μg · hr/mL. On coadministration of equimolar amounts of TPI with F_3dThd, however, its blood level increased markedly, with a T_{max} of 30 min, a C_{max} of 32 μg/mL, and an AUC value of 24 μg · hr/mL (Fig. 3, left panel). A similar additive effect of TPI on oral IdUrd was observed in mice (Fig. 3, right panel). The maximal plasma concentration of IdUrd significantly increased from 4.4 μg/mL with IdUrd alone to 10.1 μg/mL after IdUrd plus TPI. These enhancing effects of TPI on plasma 2′-deoxynucleoside levels demonstrated that it strongly inhibits the catabolism of 2′-deoxynucleosides in mouse liver *in vivo*.

TABLE 5. Effect of TPI and BAU on F_3dThd phosphorolytic activity in human cancer specimens.

	Breast Carcinoma*		Colon carcinoma*	
Inhibitor	nmol/mg/min	% of control	nmol/mg/min	% of control
None	5.549†		6.255	
+TPI	0.152	2.7	0.225	3.6
+BAU	5.022	90.5	6.199	99.1
+TPI + BAU	0.200	3.6	0.248	4.0

*Freshly isolated tumors from breast and colon cancer patients were used.
†Values are the means of duplicate measurements.

Plasma F_3dThd Levels in Monkeys after Administration of F_3dThd Alone and with TPI

In the former experiment (Fig. 2), phosphorolysis of cytotoxic 2′-deoxyribonucleosides in monkey liver and intestine was found to be catalyzed mainly by TPase. We therefore determined the plasma F_3dThd levels in monkeys following administration of oral F_3dThd (10 mg/kg) with or without equimolar amounts of TPI. As shown in Fig. 4, plasma F_3dThd levels were increased very markedly, with a C_{max} of 15 μg/mL, by coadministration of TPI, compared with F_3dThd administration alone, which yielded very low concentrations in monkey blood.

Antitumor Activity of F_3dThd Combined with or without TPI on Human Cancer Xenograft

Table 6 shows the results from an antitumor experiment comparing oral administration of F_3dThd alone and combined with equimolar TPI against the AZ-521 stomach cancer model in mice. TPI was found to augment the antitumor effect of F_3dThd, suggesting that this potentiation of the antitumor activity was based on the significant

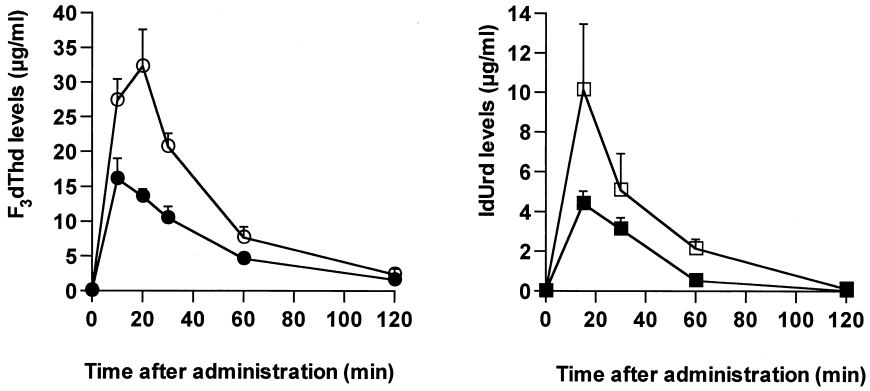


FIG. 3. Levels of plasma 2′-deoxyribonucleosides in mice following oral administration alone and combined with TPI. F_3dThd and IdUrd alone (● and ■, respectively) in doses of 50 mg/kg or in combination with an equimolar amount of TPI (○ and □, respectively) were administered orally to normal mice. Plasma F_3dThd and IdUrd concentrations were determined as described in Materials and Methods. Values are means ± SD for 6 mice.

elevation of plasma F_3dThd levels by combined administration of TPI and F_3dThd.

DISCUSSION

Inhibitors of pyrimidine nucleoside phosphorylases may serve as useful modulators by inhibiting the catabolism of pyrimidine nucleoside analogs such as FdUrd, F_3dThd, and IdUrd that possess chemotherapeutic activity. Pyrimidine phosphorylase inhibitors have been reported by several investigators. Baker and Kelley [36] reported finding that 5-benzyluracil inhibits the activity of uridine phosphorylase from Walker 256 cells with an IC_{50} value of 1.4×10^{-6} M. Niedzwicki *et al.* [15] also reported that certain 5-substituted acyclouridines, including 5-benzylacyclouridine and 5-benzyloxybenzylacyclouridine, potently inhibit UPase but not TPase. In addition, 6-aminothymine has been reported to have inhibitory activity against mouse liver TPase, with an IC_{50} value of 70 μM, and also to inhibit UPase activity to the same degree [18, 21].

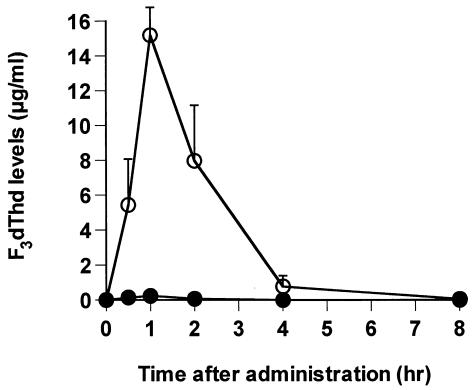


FIG. 4. Plasma F_3dThd levels in monkeys after administration of F_3dThd with or without TPI. Monkeys were given F_3dThd (10 mg/kg) orally, alone (●) or combined with an equimolar amount of TPI (○). At the times indicated, blood was sampled and centrifuged to obtain plasma. Values are means ± SD for 3 monkeys.

It has been suggested that there are distinct differences in the pyrimidine nucleoside phosphorylase of humans and rodent species in regard to the content and substrate specificity of these enzymes [1, 11–13]. Maehara *et al.* [37] reported that in rat and mouse liver the ratio of the Urd, dUrd, and dThd phosphorolytic activities is 10:7:1, whereas in human liver it is 1:30:20. TPase, therefore, seems to play a major role in the degradation of 2′-deoxyribonucleosides, such as FdUrd, F_3dThd, IdUrd, and BrdUrd, in human tissues [8–10, 38–40].

In the present studies, we evaluated the inhibitory effects of 5-halogenated pyrimidine analogs substituted at the 6-position on partially purified human TPase and found that the most potent inhibitor of the series was TPI. TPI also strongly inhibited the activity of recombinant human TPase (PD-ECGF) in a competitive manner with an apparent K_i value of 1.7×10^{-8} M when thymidine was the substrate, but it did not have any inhibitory effect on UPase, OPRTase, TK, or DPDase. These results suggested that the TPI developed by us has much stronger inhibitory potency against TPase than any other inhibitors reported to date. We used this TPI to investigate its ability to inhibit phosphorolysis of F_3dThd, an antitumor 2′-deoxyribonucleoside, in enzyme extracts from human, monkey, dog, and rodent tissues. The antitumor activity of F_3dThd had been tested in clinical trials and found to be active in patients with breast and colon cancer when 2.5 mg/kg was administered i.v. every 3 hr for 6 days, but not when about 30 mg/kg was given i.v. once daily for 5 days [41]. The phosphorolytic activity of F_3dThd in enzyme extracts from human liver, intestine, and tumor and from monkey liver and intestine was higher than that in murine liver and intestine and in dog liver and intestine. Under the above conditions, the addition of TPI (1×10^{-6} M) resulted in potent inhibition of F_3dThd phosphorolytic activity in enzyme extracts from human liver, intestine, and tumor, from monkey liver and intestine, and from rodent liver, but not from dog liver or intestine or from rodent intestine. These results indicate that the phosphorolysis of antitumor

TABLE 6. Enhancement of the antitumor activity of F_3dThd by combining it with TPI in nude mice transplanted with human gastric carcinoma (AZ-521)

Group	F_3dThd dose* (mg/kg/day)	N†	RTV‡	IR§ (%)	Body weight (g)		
					Day 0	Day 15	Gain‖
Control		12	9.12		21.5	25.7	4.2
F_3dThd alone	12.5	6	5.02	45.0	21.0	24.5	3.5
	25	6	4.93	45.9	21.5	26.1	4.6
	50	6	1.35	85.2	22.8	24.2	1.4
F_3dThd plus TPI	12.5	6	3.51	61.5	21.8	24.9	3.0
	25	6	2.45	73.2	19.8	24.3	4.5
	50	6	0.76	91.6	20.9	24.5	3.6

*F_3dThd alone or in combination with equimolar TPI was administered orally for 14 consecutive days.
†Number of animals.
‡RTV (relative tumor volume) values were calculated using the following equation: RTV = (mean tumor volume on day 15)/(mean tumor volume on day 0).
§IR values were calculated using the following equation:IR (%) = [1 − (mean RTV of drug-treated group)/(mean RTV of control group)] × 100.
‖Gain of body weight was calculated using the following equation: Gain of body weight (g) = (mean weight on day 15) − (mean weight on day 0).

2′-deoxyribonucleosides such as F_3dThd is catalyzed by TPase in the former tissues, and by UPase in the latter tissues, and that co-administration of TPI with F_3dThd would potentiate its antitumor activity. Recently, Liu *et al.* [42] reported the presence of an alternative phosphorolytic activity of pyrimidine nucleosides in human tumors, independent of TPase and UPase. Also, el-Kouni *et al.* [14] have suggested the presence of various forms of TPase in different human tissues. However, in our inhibitory experiment, F_3dThd phosphorolytic activity was inhibited almost completely by the addition of 1 μM TPI but not 10 μM BAU, suggesting that the phosphorolysis of F_3dThd is absolutely catalyzed by TPase but not UPase and that there is no presence of TPase isoforms insensitive to our TPI in human cancer tissues tested.

It is important to confirm the ability of TPI to enhance the biological function of 2′-deoxyribonucleosides *in vivo*. In general, almost all antitumor drugs have been given in i.v. form to cancer patients. Considering the functional mechanisms of antitumor nucleosides, which are fairly time-dependent, we assume that long-term consecutive administration of these drugs is more effective than single or intermittent dosing. Therefore, we selected oral administration of the antitumor nucleosides. When F_3dThd in combination with an equimolar amount of TPI was given orally to mice, their plasma F_3dThd concentrations increased about 2-fold over the concentration after oral F_3dThd alone, suggesting that TPI inhibits the degradation of F_3dThd in the liver (Fig. 3, left panel). Furthermore, we ensured the augmentation of the antitumor activity of F_3dThd by combining it with equimolar TPI (Table 6). These results would be brought about by the elevation of plasma F_3dThd levels in mice following administration of our TPI with F_3dThd. A similar result was obtained by co-administration of TPI with IdUrd, which is used clinically as a sensitizer for radiation therapy (Fig. 3, right panel). In monkeys, the plasma F_3dThd level after oral administration alone was very low, suggesting that F_3dThd is degraded rapidly to an inactive form by the liver and intestine TPase. However, the blood F_3dThd level was increased markedly by coadministration of TPI with F_3dThd (Fig. 4). Considering the similar behavior of TPase expression between human and monkey tissues, TPI may be effective in inhibiting the phosphorolysis of biologically active 2′-deoxyribonucleosides in cancer patients. Recently Furukawa *et al.* [43] reported that TPase is identical to PD-ECGF, and several papers have reported that expression of TPase (PD-ECGF) proteins is related to progression and metastasis of cancer in patients with stomach, ovarian, bladder, esophageal, non-small-cell lung, and breast cancers [26–31].

Furthermore, Miyadera *et al.* [35] reported that 5-chloro-6-aminouracil, an inhibitor of pyrimidine nucleoside phosphorylase, inhibited TPase-induced angiogenesis. The use of our potent TPI, therefore, may have a possibility of potent inhibition of TPase-induced angiogenesis and metastasis, in part, in cancer patients. Evaluation of the combination of TPI with antitumor 2′-deoxyribonucleosides is now in progress with a view to potentiating the antitumor activity of such drugs and to inhibiting the TPase-related angiogenetic events.

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