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Involvement of Concentrative Nucleoside Transporter 1 in Intestinal Absorption of Trifluorothymidine, a Novel Antitumor Nucleoside, in Rats

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ABSTRACT

ααα-Trifluorothymidine (TFT), an anticancer nucleoside analog, is a potent thymidylate synthase inhibitor. TFT exerts its antitumor activity primarily by inducing DNA fragmentation after incorporation of the triphosphate form of TFT into the DNA. Although an oral combination of TFT and a thymidine phosphorylase inhibitor has been clinically developed, there is little information regarding TFT absorption. Therefore, we investigated TFT absorption in the rat small intestine. After oral administration of TFT in rats, more than 75% of the TFT was absorbed. To identify the uptake transport system, uptake studies were conducted by using everted sacs prepared from rat small intestines. TFT uptake was saturable, significantly reduced under Na^+-free conditions, and strongly inhibited by the addition of an endogenous pyrimidine nucleoside. From these results, we suggested the involvement of concentrative nucleoside transporters (CNTs) in TFT absorption into rat small intestine. In rat small intestines, the mRNAs coding for rat CNT1 (rCNT1) and rCNT2, but not for rCNT3, were predominantly expressed. To investigate the roles of rCNT1 and rCNT2 in TFT uptake, we conducted uptake assays by using *Xenopus laevis* oocytes injected with rCNT1 complementary RNA (cRNA) and rCNT2 cRNA. TFT uptake by *X. laevis* oocytes injected with rCNT1 cRNA, and not rCNT2 cRNA, was significantly greater than that by water-injected oocytes. In addition, in situ single-pass perfusion experiments performed using rat jejunum regions showed that thymidine, a substrate for CNT1, strongly inhibited TFT uptake. In conclusion, TFT is absorbed via rCNT1 in the intestinal lumen in rats.

Introduction

ααα-Trifluorothymidine (TFT; Fig. 1), an anticancer nucleoside analog, is a potent thymidylate synthase inhibitor similar to 5-fluorouracil. TFT exerts its antitumor activity primarily by inducing DNA fragmentation after the triphosphate form of TFT is incorporated into DNA (Emura et al., 2004; Temmink et al., 2005). Initial clinical studies showed promising antitumor activity of TFT, with >50% tumor shrinkage after bolus intravenous administration of TFT in patients with colorectal and breast cancers (Ansfield and Ramirez, 1971). However, TFT is rapidly degraded ($t_{1/2}$ 12–18 min) by thymidine phosphorylase (TP), and initial high plasma concentrations of TFT cause significant bone marrow toxicity (Ansfield and Ramirez, 1971). Therefore, intravenous administration of TFT cannot be performed in clinical anticancer chemotherapy (Dexter et al., 1972). However, a combination of TFT and 5-chloro-6-(2-iminopyrrolidin-1-yl)methyl-2,4(1*H*,3*H*)-pyrimidinedione hydrochloride, a competitive inhibitor of TP (TPI) without any intrinsic antitumor activity, at a molecular ratio of 1:0.5, results in retention of the effective concentration of TFT in the blood for a prolonged period after oral administration. An oral formulation of a combination of TFT and TPI has been clinically developed (Hong et al., 2006; Overman, et al., 2008).

The small intestine is the primary absorption site for many orally administered drugs. Intestinal absorption occurs mainly via two mechanisms: passive diffusion and carrier-mediated transport. Carrier-mediated transport plays an important role in small intestinal absorption of some drugs, especially those with low permeability and high solubility such as TFT. Nucleoside transporters (NTs) that are also expressed on the surfaces of epithelial cells in the intestine have been characterized in the carrier-mediated transport of

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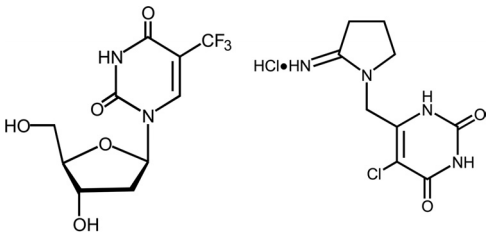
ABBREVIATIONS: TFT, ααα-trifluorothymidine; NT, nucleoside transporter; CNT, concentrative NT; hCNT, human CNT; rCNT, rat CNT; ENT, equilibrative NT; cRNA, complementary RNA; TP, thymidine phosphorylase; TPI, thymidine phosphorylase inhibitor; 2,4-DNP, 2,4-dinitrophenol; NaN_3, sodium azide; PCR, polymerase chain reaction; P_{eff}, effective permeability.



TFT TPI

Fig. 1. Structures of TFT (left) and TPI (right).

nucleoside analogs. These NTs include concentrative NTs (CNTs) and equilibrative NTs (ENTs). CNTs facilitate sodium-dependent uptake of substrates into cells, and three isoforms of CNTs (CNT1, CNT2, and CNT3) have been identified. CNT1 and CNT2 primarily act to translocate pyrimidine and purine nucleosides, respectively, via a sodium-dependent mechanism, whereas CNT3 shows broad substrate selectivity and the unique ability of translocating nucleosides via both sodium- and proton-coupled mechanisms (Smith et al., 2005). These transporters are involved in the membrane permeability of not only endogenous nucleosides but also some nucleoside analogs (Mackey et al., 1999; Lang et al., 2001; Ritzel et al., 2001); in addition, these transporters are involved in the absorption of drugs such as ribavirin (Patil et al., 1998) and mizoribine (Okada et al., 2006; Mori et al., 2008). CNTs are also predominantly expressed in various other mammalian tissues and cancer cells (Pennycooke et al., 2001; Lu et al., 2004; Govindarajan et al., 2007). Therefore, CNTs are known to be involved in not only the pharmacokinetics but also the clinical effect of nucleoside analogs (Maréchal et al., 2009). Furthermore, CNTs are expected to serve as prospective biomarkers for the clinical effect of nucleoside drugs.

The purpose of this study was to identify the mechanism of TFT absorption in the small intestine, because there is little information regarding TFT absorption. We investigated TFT absorption in the small intestine of rats and characterized the rat NT that accepts TFT as the substrate and identified the contribution of NTs in the small intestinal absorption of TFT that shows the type III character in the Biopharmaceutics Classification System (Amidon et al., 1995).

Materials and Methods

Materials. [^{14}C]TFT and unlabeled TFT were synthesized by Daiichi Pure Chemicals (Tokyo, Japan) and Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. [^{14}C]thymidine and [^{14}C]inosine were purchased from Moravek Biochemicals (Brea, CA). Unlabeled thymidine and inosine were obtained from Wako Pure Chemicals (Osaka, Japan). Uridine was purchased from Sigma (St. Louis, MO). [^{3}H]inulin was purchased from American Radiolabeled Chemicals (St. Louis, MO). 2,4-Dinitrophenol (2,4-DNP) and sodium azide (NaN_3) were purchased from Alfa Aesar (Karlsruhe, Germany) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Male Sprague-Dawley rats were purchased from Charles River Laboratories (Yokohama, Japan), and *Xenopus laevis* were purchased from Japan SLC (Hamamatsu, Japan). All other reagents and solvents were reagent or high-performance liquid chromatography grade.

Concentration of [^{14}C]TFT in Plasma. To evaluate the concentration of TFT in plasma, [^{14}C]TFT and TPI were dissolved in 0.5% hydroxypropylmethylcellulose solution and administered orally to rats by gavage. The concentrations of TFT and TPI in dosing solution were 50 mg/3.7 MBq/5 ml/kg and 23.6 mg/5 ml/kg, respectively. After oral administration, blood samples were collected from the abdominal aorta, and plasma was purified from each sample by centrifugation. The plasma was extracted with methanol, and the organic layer was dried under a nitrogen stream. The resultant residue was dissolved in the mobile phase (20 mM phosphate buffer, pH 7.2, and acetonitrile, 96:4) and injected into the radio high-performance liquid chromatography to evaluate the concentration of TFT in plasma.

Excretion in Urine, Feces, and Expired Air. To evaluate the concentration of total radioactivity in urine, feces, and expired air, [^{14}C]TFT and TPI were dissolved in 0.5% hydroxypropylmethylcellulose solution and administered orally to rats by gavage. The concentrations of [^{14}C]TFT and TPI in dosing solution were 50 mg/3.7 MBq/5 ml/kg and 23.6 mg/5 ml/kg, respectively. After oral administration, the rats were placed individually in metabolism cages to collect urine and feces separately at specific time points. Radioactive carbon dioxide in expired air was collected by using 20% 2-monoamine ethanol. Radioactivity in urine, expired air, and feces was determined by using a liquid scintillation counter.

Uptake Assays in Intestinal Everted Sacs. Uptake assays were conducted by using everted sacs (3 cm in length) prepared from the jejunum of male Sprague-Dawley rats by making a few modifications to a previously reported method (Nakashima and Tsuji, 1985; Kato et al., 2004). In brief, everted sacs were preincubated for 5 min in oxygenated Krebs-Ringer-bicarbonate buffer (Krebs buffer; 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$, pH 7.4) before the initiation of uptake experiments and incubated in a test solution at 37°C for 1 min. To prepare the test solution, [^{14}C]TFT and [^{3}H]inulin, a nonabsorbable marker, were added in oxygenated Krebs buffer. The uptake experiment was terminated by rinsing the everted sacs twice in ice-cold saline. Uptake into tissues was evaluated by determining the radioactivity with a liquid scintillation counter after solubilization of the sample using a Soluen-350 (PerkinElmer Life and Analytical Sciences, Waltham, MA) as a tissue solubilizer and 10 ml of Hionic-fluor (PerkinElmer Life and Analytical Sciences) as scintillation fluid.

Preparation of Total RNA, rCNT1 cDNA, and rCNT2 cDNA. Total RNA was extracted from rat intestinal mucosa by using RNA Later (Ambion, Austin, TX) and an RNeasy Mini Kit (QIAGEN, Valencia, CA). Total RNA was reverse-transcribed into cDNA by using the Takara STAR RT PCR kit (Takara, Shiga, Japan).

An rCNT1 (GenBank accession no. U10279.1) clone was obtained by amplifying the cDNA derived from rat small intestine mucosa by performing PCR with KOD Plus Polymerase (Toyobo Engineering, Osaka, Japan). A 5′ primer (5′-ATGGCAGACAACACACAGAG-3′) and a 3′ primer (5′-CCAGCTATGTGCAGACTGTG-3′) derived from the reported sequence of rat CNT1 (National Center for Biotechnology Information GenBank) were used. The PCR product was inserted into pCRII-TOPO (Invitrogen, Carlsbad, CA), and sequence analysis of the resulting pCRII-TOPO/rCNT1 vector was conducted by Hokkaido System Sciences (Hokkaido, Japan). The sequence of the amplified *CNT1* gene was confirmed to be identical to that in GenBank. Likewise, an rCNT2 (GenBank accession no. U66723) clone was obtained by amplifying cDNA using PCR with KOD Plus Polymerase (Toyobo Engineering). A 5′ primer (5′-CACCCAGCACATTCAGAGGA-3′) and a 3′ primer (5′-AGAACTGTCACGGAATGGCC-3′) were used. The PCR product was subcloned into pENTR/D-TOPO (Invitrogen) and introduced into pEF-DEST51 (Invitrogen) by using the Gateway LR Clonase Enzyme Mix (Invitrogen). Similar to rCNT1, sequence analysis of the pENTR/D-TOPO/rCNT1 vector was conducted by Hokkaido System Sciences, and the sequence of the amplified *CNT2* gene was confirmed to be identical to that in GenBank.

Uptake of TFT by *X. laevis* Oocytes. After linearization of the pCRII-TOPO vector/rCNT1 using PvuI (Toyobo Engineering), capped cRNA of rCNT1 was synthesized by using T7 RNA polymerase (Agilent Technologies, Santa Clara, CA). Likewise, after linearization of pEF-DEST51/rCNT2 using NaeI (Toyobo Engineering), cRNA of rCNT2 was also synthesized.

For uptake experiments, *X. laevis* oocytes were prepared by man-

ual dissection, and the samples were treated with collagenase A (Wako Pure Chemicals) in a calcium-free OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl, and 5 mM HEPES, pH 7.5). Oocytes were washed and peeled in modified Barth's solution [88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 10 mM HEPES, pH 7.4] and injected with 50 nl of cRNA or nuclease-free water. Oocytes were incubated for 3 days at 18°C in modified Barth's solution containing 50 μg/ml gentamycin.

Three days after cRNA injection, oocytes were transferred to ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) and preincubated at 25°C for 5 min. Uptake was initiated by replacing the solution with fresh ND96 solution containing labeled substrate at 25°C, and at the designated times, the oocytes were rinsed five to six times with ice-cold ND96 solution and dissolved with soluen-350. The associated radioactivity was measured by using a liquid scintillation counter.

In Situ Single-Pass Perfusion Method. Male Sprague-Dawley rats that had fasted overnight with free access to water were used for this procedure. Under anesthesia with sodium pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan), the upper jejunum region in rats was perfused with a test solution containing 0.4 μM [^{14}C]TFT and [^{3}H]inulin, a nonabsorbable marker, in saline (Otsuka Pharmaceutical Industry, Naruto, Japan). Perfusion was carried out with a constant-infusion pump at a flow rate of 0.4 ml/min, and the perfusate was maintained at 37°C. After 20 min, to ascertain whether steady state had been achieved, three samples were collected at 5-min intervals. Residual substrate concentrations of these samples were measured with a liquid scintillation counter. The steady-state intestinal effective permeability (P_{eff} cm/s) was calculated according to the following formula: $P_{eff} = [-Q \times \ln (C_{out}/C_{in})]/2(\pi)rL$, where Q is the flow rate (0.4 ml/min), C_{in} and C_{out} are the inlet and outlet perfusate concentrations, respectively, and the outlet concentration was corrected for water flux with [^{3}H]inulin. $2(\pi)rL$ is the cylindrical surface area of the intestinal segment with a length L (approximately 15 cm) and r (0.2 cm) as the intestinal radius.

Statistical Analysis. Data are expressed as mean ± S.D. or means ± S.E.M. Student's t test was used for paired variates. An overall $p < 0.05$ was considered significant.

Results

In Vivo Absorption of TFT in Rats. As shown in Fig. 2, the plasma concentration of TFT was measured after oral administration of [^{14}C]TFT and TPI to fasting rats. The T_{max} values and $t_{1/2}$ values of [^{14}C]TFT were 0.25 and 0.49 h, respectively. Moreover, after oral administration of [^{14}C]TFT and TPI, accumulated radioactivity excreted in the urine, feces, and expired air was measured by using a liquid scintillation counter. As shown in Table 1, 59.8, 19.7, and 15.6% of radioactivity (percentage of dose) was excreted in the urine, feces, and expired air, respectively, within 24 h after dosing, and total radioactivity excretion was 95.0% of the dosed radioactivity within 24 h after dosing. On the basis of these results, the absorption ratio of TFT in rats was estimated to be more than 75% of the dose.

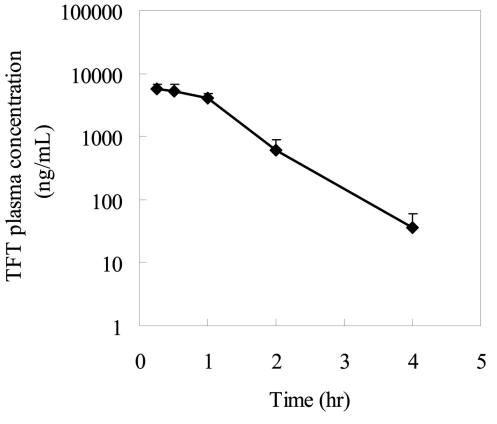


Fig. 2. Concentration of TFT in the plasma after single oral administration of 50 mg/kg [^{14}C]TFT + 23.6 mg/kg TPI to fasting male rats. Data represent the mean (S.D.) (n = 4).

Uptake of TFT in the Everted Sacs. To clarify the uptake mechanisms from the small intestinal lumen in rats, uptake studies using everted sacs were conducted. Uptake rates of TFT in the small intestine were saturable with regard to substrate concentration, as shown in Fig. 3a. An Eadie-Hofstee plot showed biphasic behavior in the small intestine, as shown in Fig. 3b, indicating the presence of high- and low-affinity uptake systems. To identify the active uptake transport system of TFT, the inhibitory effects of Na^+, pyrimidine nucleosides, 2,4-DNP, and NaN_3 on TFT uptake were evaluated. Pyrimidine nucleosides were endogenous substrates of nucleoside transporters such as CNTs, and NaN_3 and 2,4-DNP were Na^+-K^+ ATPase inhibitors. As shown in Fig. 4, TFT uptake significantly decreased under Na^+-free conditions and was strongly inhibited by the addition of thymidine, uridine, NaN_3, and 2,4-DNP.

Uptake of TFT by *X. laevis* Oocytes. To confirm that transport mediated by carrier proteins plays a role in TFT uptake in the small intestine, uptake studies using *X. laevis* oocytes injected with small intestinal total RNA were conducted. Figure 5 shows the results of experiments measuring TFT uptake into oocytes injected with total RNA prepared from rat small intestine. TFT uptake by *X. laevis* oocytes injected with rat small intestine total RNA was five times greater than that by water-injected oocytes; uptake after the addition of 1 mM thymidine was significantly lower than that in water-injected oocytes. This suggested that nucleoside transporters could play a role in the uptake of TFT in the rat small intestine.

To investigate the roles of rCNT1and rCNT2 in TFT uptake, uptake assays were conducted using *X. laevis* oocytes injected with rCNT1 cRNA or rCNT2 cRNA. Figure 6 shows the results of experiments measuring the uptake of thymidine and TFT in the oocytes injected with rCNT1 cRNA and water. TFT uptake into oocytes injected with rCNT1 cNRA was significantly greater than that into oocytes injected with water, and uptake of TFT by oocytes injected with rCNT1 was comparable with that of oocytes injected with thymidine. Figure 7 shows the results of the experiments measuring the uptake of inosine or TFT in oocytes injected with rCNT2 cRNA. Uptake of inosine, the endogenous substrate for rCNT2, by *X. laevis* oocytes injected with rCNT2 cRNA was significantly greater than that by water-injected oocytes, but TFT uptake of both groups of oocytes was the same.

TABLE 1

Cumulative excretion of radioactivity in the urine, feces, and expired air as $^{14}CO_2$ after single oral administration of 50 mg/kg [^{14}C]TFT and 23.6 mg/kg TPI to nonfasting male rats

Data represent the mean (S.D.) (n = 4).

Time	Excretion of Radioactivity			
	Urine	Feces	Expired Air	Total
	% of dose			
0–24 h	59.8 ± 4.7	19.7 ± 2.8	15.6 ± 2.5	95.0 ± 1.1

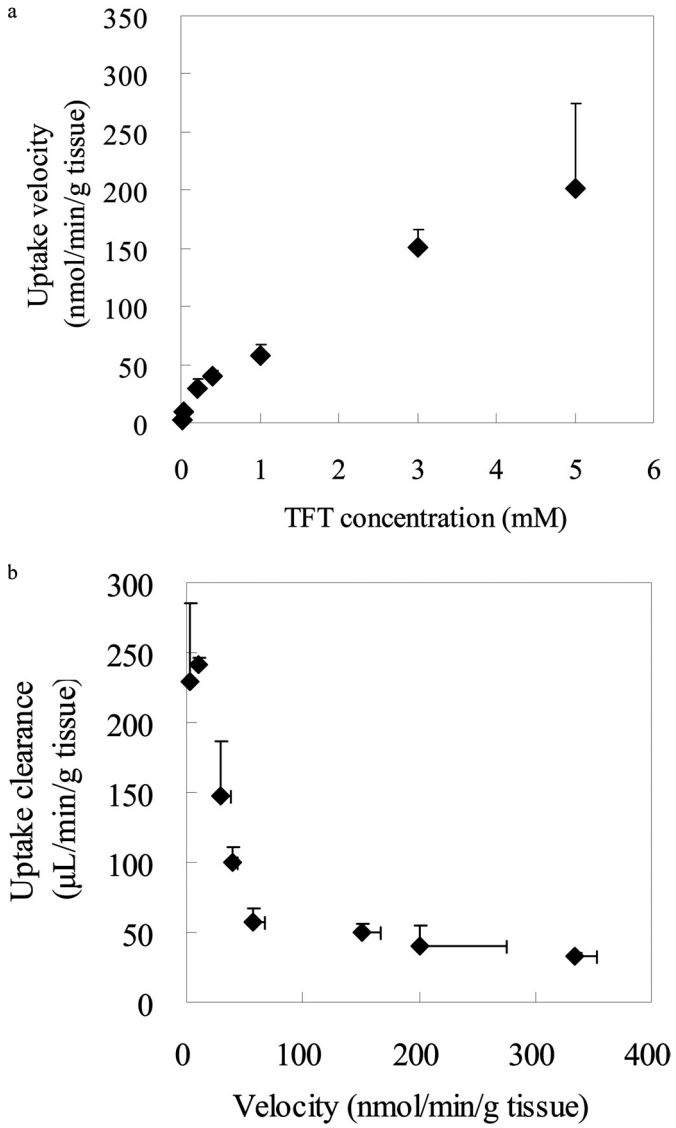


Fig. 3. Concentration-dependent uptake of TFT in everted sacs of the rat intestine. TFT uptake was evaluated for 1 min at 37°C; TFT concentration ranged from 10 μM to 5 mM. Michaelis-Menten plot (a) and Eadie-Hofstee plot (b) analyses are shown for TFT uptake in the everted sacs. Data represent the mean (S.D.) (n = 3).

Moreover, kinetic analysis of the concentration-dependent profile of the uptake velocity by oocytes injected with rCNT1 cRNA is shown in Fig. 8, and the estimated K_m value of TFT uptake mediated by rCNT1 was 26.9 μM. The inhibitory effects of thymidine and inosine on TFT uptake by *X. laevis* oocytes injected with rCNT1 were investigated; TFT uptake was strongly inhibited by thymidine, but not by inosine (Fig. 9).

Estimation of TFT Absorption by Using the In Situ Single-Pass Perfusion Technique. To confirm the contribution of rCNT1 in the intestinal absorption of TFT, the inhibitory effect of thymidine on TFT uptake was estimated by using the in situ single-pass perfusion method. As shown in Fig. 10, the values of P_{eff} in the absence and presence of thymidine were 1.02×10^{-4} and 0.54×10^{-4} cm/s, respectively. The value of P_{eff} significantly decreased to approximately 50% after the addition of 1 mM thymidine.

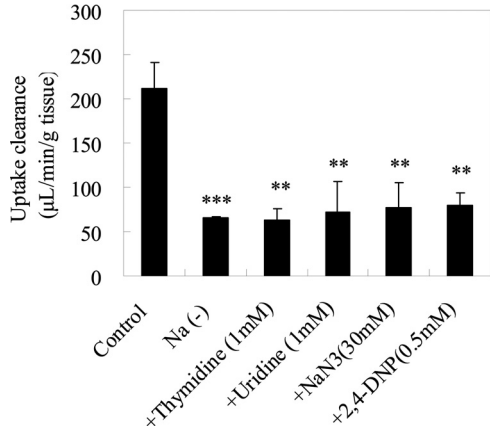


Fig. 4. Inhibitory effect of Na^+, thymidine, uridine, 2, 4-DNP, and NaN_3 on TFT uptake in the everted sacs of the rat intestine. The uptake of TFT (100 μM) was evaluated for 1 min at 37°C. Na^+-free buffer contained 118 mM choline chloride and 25 mM choline bicarbonate instead of NaCl and $NaHCO_3$, respectively. Control value means uptake clearance of TFT in test solution prepared from Krebs buffer containing Na^+. Data represent the mean (S.D.) (n = 3). **, $p < 0.01$, and ***, $p < 0.001$ compared with control.

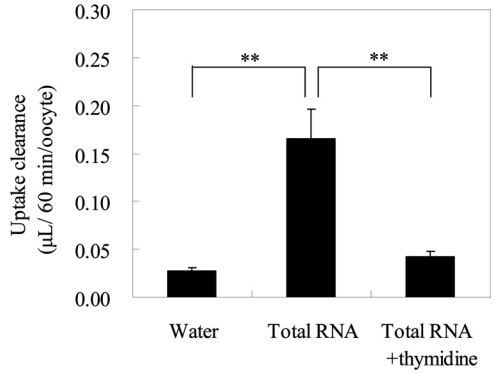


Fig. 5. TFT uptake by *X. laevis* oocytes injected with total RNA of rat small intestines. *X. laevis* oocytes were injected with 50 nl of nuclease-free water or total RNA (200 ng/oocyte) solution. Uptake of [^{14}C]TFT (8 μM) was evaluated for 60 min at 25°C. Data represent the mean (S.E.M.) (n = 3). In the experiment, 1 mM thymidine was used as an inhibitor. **, $p < 0.01$.

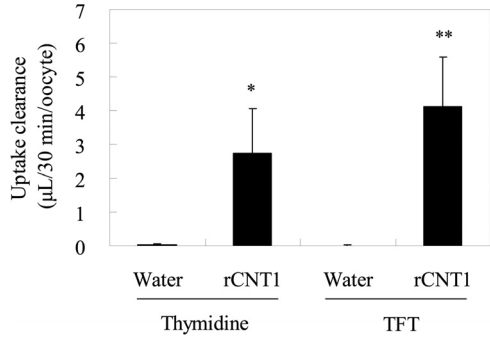


Fig. 6. TFT uptake by *X. laevis* oocytes injected with rCNT1 cRNA. *X. laevis* oocytes were injected with 50 nl of nuclease-free water or rCNT1 cRNA (25 ng/oocyte). The uptake of [^{14}C]thymidine (8 μM) and [^{14}C]TFT (6 μM) were evaluated for 30 min at 25°C. Data represent the mean (S.E.M.) (n = 3–4).*, $p < 0.05$ and **, $p < 0.01$ compared with water.

Discussion

TFT is a pyrimidine nucleoside analog currently being developed as an oral anticancer agent in combination with TPI. In the present study, we characterized the membrane

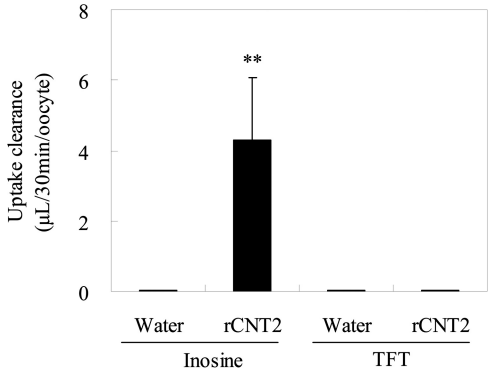


Fig. 7. TFT uptake by *X. laevis* oocytes injected with rCNT2 cRNA. *X. laevis* oocytes were injected with 50 nl of nuclease-free water or rCNT2 cRNA (25 ng/oocyte). The uptake of [^{14}C]inosine (8 μM) and [^{14}C]TFT (7 μM) were evaluated for 30 min at 25°C. Data represent the mean (S.E.M.) (n = 3–4). **, $p < 0.01$ compared with water.

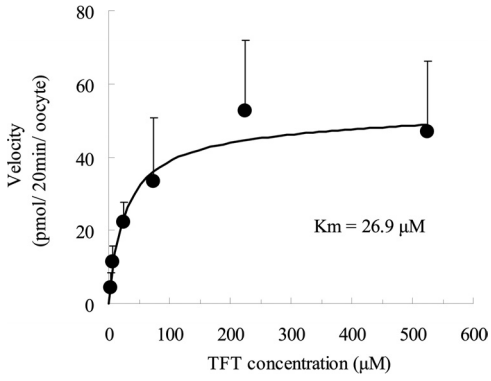


Fig. 8. Concentration-dependent uptake of TFT by *X. laevis* oocytes injected with rCNT1 cRNA. *X. laevis* oocytes were injected with 50 nl of rCNT1 cRNA (25 ng/oocyte). The uptake of [^{14}C]TFT was evaluated for 20 min at 25°C. Data represent the mean (S.D.) (n = 6–8). The K_m value of TFT for rCNT1 was calculated by Multi (Excel program; Microsoft, Redmond, WA).

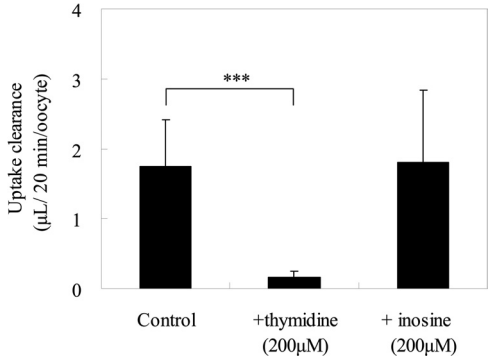


Fig. 9. Inhibitory effects of thymidine and inosine on TFT uptake by *X. laevis* oocytes injected with rat CNT1. *X. laevis* oocytes were injected with 50 nl of rCNT1 cRNA (25 ng/oocyte). The uptake of [^{14}C]TFT (8 μM) was evaluated for 20 min at 25°C. Data represent the mean (S.D.) (n = 5–6). ***, $p < 0.001$ compared with control. The uptake clearance of TFT in the absence of inosine and thymidine was used as a control value.

carrier protein of TFT and determined the contribution of the carrier protein on the small intestinal absorption of TFT in rats.

Although TFT was well absorbed in rats (>75% of the dose) after oral administration, the TFT uptake by the everted sacs was in a concentration-dependent saturable manner, suggesting that some active transport system may play a role in

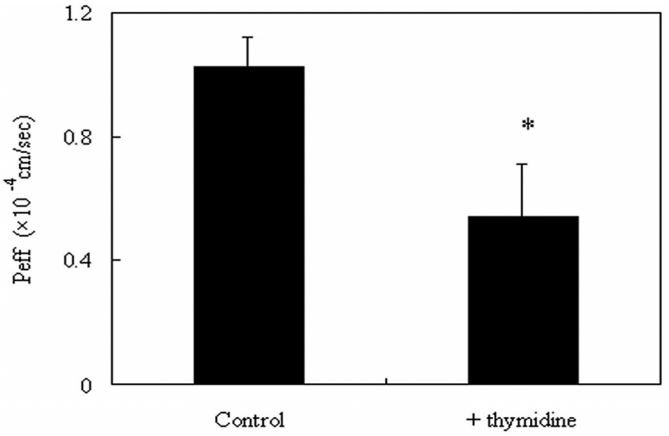


Fig. 10. Inhibitory effect of thymidine on TFT uptake based on the in situ single-pass perfusion method. The uptake of TFT (0.4 μM) from rat small intestine was evaluated at 37°C. Data represent the mean (S.E.M.) (n = 3). The value of control was P_{eff} of 0.4 μM TFT in the absence of thymidine. *, $p < 0.05$ compared with control value.

the small intestinal absorption of TFT. Furthermore, CNTs were thought to be involved in TFT absorption in small intestinal tissues of the rat because TFT was taken up by the everted sacs of the small intestine via a sodium-dependent mechanism. Furthermore, thymidine, which is a substrate for rCNTs, strongly inhibited the uptake of TFT into small intestinal tissues of everted sacs and into *X. laevis* oocytes injected with total RNA of the rat small intestine. A previous report (Lu et al., 2004) indicated that rCNT1 and rCNT2 mRNAs were expressed predominantly in the small intestine of rats, but rCNT3 mRNA was not detected; therefore, we performed uptake assays using *X. laevis* oocytes injected with rCNT1 cRNA and rCNT2 cRNA to investigate the substrate specificity of TFT for these intestinal transporters. Our results showed that TFT is a preferred substrate for rCNT1 but not for rCNT2. Furthermore, to investigate the contribution of rCNT1 in small intestinal absorption of TFT in rats, we performed in situ single-pass perfusion experiments using thymidine as an inhibitor of rCNT1. TFT uptake into the small intestinal tissues significantly decreased to approximately 50% after the addition of 1 mM thymidine. This result suggested that rCNT1 contributes to TFT absorption in the small intestinal lumen. Although the inhibitory effect of thymidine in in situ single-pass perfusion experiments is comparable with that in everted sac experiments, the inhibitory effect of thymidine was different from that in the uptake assay using *X. laevis* oocytes injected with rCNT1 cRNA. Therefore, other transport systems such as other transporters or passive diffusion might be involved in the small intestinal absorption of TFT in rats.

Because CNT1 has a wide distribution in various mammalian tissues and cancer cells (Pennycooke et al., 2001; Lu et al., 2004; Govindarajan et al., 2007), it might be involved not only in the pharmacokinetics but also in the anticancer effect and adverse effects of TFT. Nucleoside transporters have been reported to be involved in the clinical and adverse effects of nucleoside analog drugs. For instance, a previous report suggested that the mitochondrial toxicity of the antiviral drug fialuridine, a substrate for ENT1, was closely related to ENT1 expression in the mitochondrial membrane (Lai et al., 2004). A nucleoside transporter has been reported to be involved in the antitumor effect of gemcitabine (Achiwa

et al., 2004), and patients with pancreatic adenocarcinoma who show high human ENT1 and hCNT3 protein expression exhibit significantly longer survival after adjuvant gemcitabine-based chemotherapy (Maréchal et al., 2009). Biomarkers such as hCNT3 and human ENT1 should be prospectively evaluated in patients receiving gemcitabine-based adjuvant therapy. In addition, in Waldenstrom's macroglobulinemia and small lymphocytic lymphoma, patients with low levels of hCNT1 expression showed inferior clinical response to 2-chloro-2′-deoxyadenosine-based therapy, and the level of hCNT1 expression was suggested to be useful in predicting the response to nucleoside analogs such as 2-chloro-2′-deoxyadenosine known to be taken up via hCNT1 (Rabascio et al., 2010). In this study, we found that TFT is a preferred substrate for rCNT1. TFT could be a substrate for hCNT1 because rCNT1 and hCNT1 are homologous (Ritzel et al., 1997). Granulocytopenia was the main adverse effect in humans after oral administration of a combination of TFT and TPI (Hong et al, 2006). The expression of hCNT1 in bone marrow-derived cells was confirmed (Rabascio et al., 2010), and hCNT1 may be involved in the adverse effects of TFT. Thus, CNT1 might be involved in not only the anticancer effect but also the adverse effects of TFT in humans. Our results indicate the potential of CNT1 as a biomarker of TFT in clinical trials.

In conclusion, we showed that TFT is well absorbed after oral administration, TFT is the preferred substrate for rCNT1, and CNT1 clearly plays a role in TFT absorption in the intestinal lumen of rats.

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Authorship Contributions

Participated in research design: Okayama, Yoshisue, Kuwata, Komuro, and Nagayama.

Conducted experiments: Okayama, Yoshisue, and Kuwata.

Performed data analysis: Okayama.

Wrote or contributed to the writing of the manuscript: Okayama, Yoshisue, and Ohta.

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