# Involvement of Concentrative Nucleoside Transporter 1 in Intestinal Absorption of Trifluridine Using Human Small Intestinal Epithelial Cells

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**ABSTRACT:**TAS-102, which is effective for refractory metastatic colorectal cancer, is a combination drug of anticancer trifluridine (FTD; which is derived from pyrimidine nucleoside) and FTD-metabolizing enzyme inhibitor tipiracil hydrochloride (TPI) at a molecular ratio of 1:0.5. To evaluate the intestinal absorption mechanism of FTD, the uptake and transcellular transport of FTD by human small intestinal epithelial cell (HIEC) monolayer as a model of human intestinal epithelial cells was investigated. The uptake and membrane permeability of FTD by HIEC monolayers were saturable, $Na^+$-dependent, and inhibited by nucleosides. These transport characteristics are mostly comparable with those of concentrative nucleoside transporters (CNTs). Moreover, the uptake of FTD by CNT1-expressing *Xenopus* oocytes was the highest among human CNT transporters. The obtained $K_m$ and $V_{max}$ values of FTD by CNT1 were 69.0 μM and 516 pmol/oocyte/30 min, respectively. The transcellular transport of FTD by Caco-2 cells, where CNT1 is heterologously expressed, from apical to basolateral side was greater than that by Mock cells. In conclusion, these results demonstrated that FTD exhibits high oral absorption by the contribution of human CNT1. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3146–3153, 2015
**Keywords:** trifluridine; FTD; intestinal absorption; nucleoside transporters; CNT1; cancer; intestinal epithelia; transcellular transport

## INTRODUCTION

TAS-102, which has been approved for the first time in Japan and has been used to treat refractory metastatic colorectal cancer, is an orally administered combination drug of trifluridine (FTD) and tipiracil hydrochloride (TPI) at a molecular ratio of 1:0.5. FTD is metabolized to triphosphate form by thymidine kinase, incorporated into DNA and exhibits antitumor activity.[1,2] However, FTD is inactivated by thymidine phosphorylase. Accordingly, a competitive inhibitor of thymidine phosphorylase TPI is coadministered with FTD as a combination drug to maintain the high-plasma concentration of FTD. Although FTD, which has anticancer activity, is a hydrophilic compound ($\log P = -0.452$, pH 7), it shows a good absorption after oral administration based on our mass balance study in human (unpublished observation). So, it is considered that certain transporters for nucleosides may contribute to high permeability of FTD across the plasma membrane of intestinal epithelial cells. Previously, it has been suggested that FTD is a substrate of rat-concentrative nucleoside transporter, Cnt1, and it may contribute to intestinal absorption in rats.[3] The CNT family (SLC28A transporters) in human consists of three members (CNT1, CNT2, and CNT3). Among them, CNT1 and CNT2 selectivity transports pyrimidine and purine nucleosides, respectively.[4,5] CNT3 broadly accepts pyrimidine and purine nucleosides as substrates and transports them.[6] It has also been reported that CNT1, CNT2, and CNT3 are expressed in human gastrointestinal tract.[6,7] Moreover, immunohistochemical study demonstrated that CNT1 and CNT2 were localized at the apical membrane of enterocytes.[8] In addition, isolated human intestinal brush-border membrane vesicles exhibited transport activity that is comparable with CNT1 and CNT2.[9] Although it has been reported that Cnt1 is likely involved in FTD absorption in rats, it is also reported that there is species difference in substrate specificity between human and rat CNT2 using synthetic nucleoside analogs.[10] Accordingly, in the present study, the intestinal absorption mechanism of FTD in human was investigated by postulating that FTD is absorbed by CNT(s).

Until now, variable methods have been used for estimating human oral absorption, including Ussing chamber model,[11,12] intestinal perfusion technique,[13] Caco-2 cells,[14] and others. However, in the present study, we selected human small intestinal epithelial cell (HIEC) mainly as an *in vitro* model of human intestinal epithelial cells, as HIEC cells were recently characterized to form monolayer, and maintain P-gp and BCRP in a similar manner to and higher activity of nucleoside transporters than Caco-2 cells.[15] In addition, nucleoside transporter-expressing *Xenopus* oocytes and CNT1-stably expressing Caco-2 cells established in the present study were also used to clarify intestinal absorption mechanism of FTD in human.

**Abbreviations used:** FTD, trifluridine; TPI, tipiracil hydrochloride; CNT, concentrative nucleoside transporter; HIEC, human small intestinal epithelial cell; AUC, area under the plasma concentration–time curve; $C_{max}$, maximum plasma concentration.

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## MATERIALS AND METHODS

### Materials

[$^{14}$C]FTD was synthesized by Moravek Biochemicals (Brea, California). HIECs and Caco-2 cells were obtained from Cell Systems (Kirkland, Washington) and the American Type Culture Collection (Rockville, Virginia), respectively. Dulbecco's modified Eagle's medium (DMEM), DMEM mixed 1:1 with Ham's F-12 (DMEM/F12), fetal bovine serum (FBS), 0.25% trypsin-ethylenediaminetetraacetic acid, and penicillin-streptomycin were obtained from Life Technologies (Carlsbad, California). Bovine pituitary extract was purchased from Kohjin Bio (Saitama, Japan). Recombinant human insulin and epidermal growth factor were purchased from Sigma–Aldrich (St. Louis, Missouri). Fibrillar collagen-coated 24-well inserts were purchased from BD Gentest (Woburn, Massachusetts). Twenty-four-well membrane inserts were obtained from Corning (Tewksbury, Massachusetts). The plasmid cDNAs of CNT1, CNT2, and CNT3 were purchased from OriGene Technologies (Rockville, Maryland). *Xenopus laevis* were purchased from Hamamatsu Seibutsu Kyozai (Shizuoka, Japan). All compounds for the transport assay were purchased from Sigma–Aldrich and Wako Pure Chemical Industries (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

### Cell Culture

Human small intestinal epithelial cells were cultured according to the previous report with a slight modification.[15] Briefly, HIECs were maintained on type I collagen-coated culture dishes in DMEM/F-12 supplemented with 10% FBS, 1% GlutaMAX, 10 μM dexamethasone, 1 μg/mL insulin, 20 ng/mL epidermal growth factor, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Caco-2 cells were grown on culture dishes in DMEM with 4.5 g/L glucose, supplemented with 10% FBS, 1×non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. Both cell lines were cultured at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

### Uptake Experiments

Caco-2 cells and HIECs were seeded onto type I collagen-coated 24-well plates at a density of $3 \times 10^5$ or $1 \times 10^5$ cells per well and cultured for 3 weeks or 1 week, respectively. The culture medium for HIECs was supplemented with 50 μg/mL bovine pituitary extract. During the cultivation, the medium was changed every other day. For uptake studies, initially the growth medium was aspirated and the cells were washed with 250 μL of transport buffer (125 mM NaCl or 125 mM choline chloride, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 5.6 mM glucose, and 25 mM HEPES, pH 7.4) at 37°C. Uptake was initiated by adding 250 μL of transport buffer containing [$^{14}$C]FTD. At a designated time, the uptake was terminated by washing two times with 1 mL ice-cold transport buffer. To solubilize the cells, 400 μL of 0.5 N NaOH was added to each well and the plate was left at room temperature for overnight. After neutralization with 200 μL of 1 N HCl, cells were transferred to a plastic tube and mixed with liquid scintillation cocktail Insta-Gel Plus (Perkin Elmer, Waltham, Massachusetts), and then the radioactivity was quantified with a liquid scintillation counter. Cellular protein content was determined using BCA[16] with bovine serum albumin as the standard.

### Establishment of Caco-2 Cells Stably Expressing CNT1

Caco-2 cells were cultured in the humidified atmosphere (95%) with 5% $CO_2$ at 37°C in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1×non-essential amino acids. The cDNA of CNT1 was subcloned into the *XbaI* and *NotI*-cut mammalian expression vector pcDNA3.1/Hygro (+) (Life Technologies). Caco-2 cells were transfected with pcDNA3.1/Hygro (+) containing CNT1 cDNA or vector alone (Mock cells) using FuGENE®HD reagent according to the manufacturer's instructions. After the transfection, single colonies were picked up and cultured in DMEM supplemented with 200 μg/mL hygromycin B for selection.

### Bidirectional Transport Experiments

Human small intestinal epithelial cells and Caco-2 cells transfected with CNT1 were seeded onto 0.31 $cm^2$ polycarbonate filter chamber at a density of $1 \times 10^5$ and $1.5 \times 10^5$ cells per well, respectively. The transcellular transport of [$^{14}$C]FTD was measured using monolayer cultures grown in chamber. Prior to transport experiments, the cells were incubated in transport buffer for 10 min at 37°C. After pre-incubation, transport experiments were started by replacing the transport buffer in either the apical or basolateral compartment with transport buffer containing [$^{14}$C]FTD. Thereafter, an aliquot of sample was taken from the other side of addition of [$^{14}$C]FTD at 0.5, 0.75, 1, and 2 h. For measurement of cellular accumulation of [$^{14}$C]FTD after 2 h incubation, the cell monolayer was rinsed in each side three times with ice-cold transport buffer and the filter was detached, followed by solubilization with 400 μL of 0.5 N NaOH at room temperature for overnight. After neutralization with 200 μL of 1 N HCl, the solubilized cells were mixed with liquid scintillation cocktail to quantify radioactivity with a liquid scintillation counter. Cellular protein content was determined using BCA[16] with bovine serum albumin as the standard.

### Uptake Experiments with *Xenopus* Oocytes Expressing CNT1, CNT2, and CNT3

Uptake experiments were conducted with *Xenopus laevis* oocytes that had been microinjected with complementary RNA (cRNA) of CNT1, CNT2, or CNT3, synthesized *in vitro* using T7 RNA polymerase (Ambion, Austin, Texas), as described previously.[17] Briefly, defolliculated oocytes were injected with 50 nL of the cRNA solution (0.5 μg/μL) or water, and then incubated for 3 days at 18°C 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] containing 50 μg/mL gentamicin. For the uptake studies, oocytes expressing CNT1, CNT2, or CNT3 were transferred to a 24-well culture plate and pre-incubated in uptake buffer (96 mM NaCl or 96 mM choline chloride, 2 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) for 5 min at room temperature. To initiate uptake, the uptake buffer was completely replaced with uptake buffer containing [$^{14}$C]FTD, and the oocytes were incubated for designated time at room temperature. The uptake was terminated by washing the oocytes five times with ice-cold uptake buffer. To determine the uptake of [$^{14}$C]FTD, the oocytes were solubilized in SOLVABLE (Perkin Elmer) and mixed with liquid scintillation cocktail Hionic-Fluor (Perkin Elmer), and then the radioactivity was quantified with a liquid scintillation counter.

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