Cancer Chemother Pharmacol (2005) 55: 420–424
DOI 10.1007/s00280-004-0937-4

ORIGINAL ARTICLE

Tatsuya Itoh · Shirou Itagaki · Yoshinobu Sumi
Takeshi Hirano · Isao Takemoto · Ken Iseki

Uptake of irinotecan metabolite SN-38 by the human intestinal cell line Caco-2

Received: 21 April 2004 / Accepted: 6 July 2004 / Published online: 23 November 2004

Abstract *Purpose*: The aim of this study was to investigate the transport mechanisms of transporters that contribute to the intestinal uptake of 7-ethyl-10-hydroxycamptothecin (SN-38). *Methods*: Human intestinal epithelial Caco-2 cells were used to investigate the mechanistic basis of transepithelial uptake of SN-38. We investigated the characteristics of SN-38 uptake into Caco-2 cells. The effects of baicalin and sulfobromophthalein (BSP) on the uptake of SN-38 by Caco-2 cells were examined. *Results*: Uptake of SN-38 was significantly reduced at 4°C. Baicalin inhibited the uptake of SN-38 in a concentration-dependent manner. BSP significantly reduced the uptake of SN-38. However, probenecid, pravastatin and grepafloxacin did not affect the uptake of SN-38. *Conclusions*: The results suggest that a specific transport system mediates the uptake of SN-38 across the apical membrane in Caco-2 cells.

Keywords Irinotecan · Diarrhea · Caco-2 cells · Baicalin

Introduction

Irinotecan hydrochloride, 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxy-camptothecin (CPT-11), is a synthetic derivative of the plant alkaloid camptothecin, which has demonstrated pronounced antitumor activity. [7]. Unlike other clinically used camptothecin analogs, CPT-11 is a prodrug with very little inherent antitumor activity that needs to be hydrolyzed by a carboxylesterase to form the active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) [15]. SN-38 subsequently undergoes glucuronic acid conjugation to form the corresponding glucuronide, SN-38 glucuronide (SN-38-Glu) [3].

The major dose-limiting toxicity after administration of CPT-11 is severe diarrhea that is often unresponsive to common antidiarrheal agents [1]. There is no generally accepted prophylactic treatment for the delayed-type diarrhea. Many pharmacokinetic analyses in humans have been performed to predict the incidence of delayed-type diarrhea, with conflicting results. The SN-38-Glu excreted in the gut via bile is hydrolyzed to SN-38 by beta-glucuronidase, and consequently it impairs the gut [16]. Takasuna et al. examined the possible inhibitory effect of a Chinese herbal medicine (Hange-Shashin-To; Tsumura Company, Tokyo, Japan) on CPT-11-induced chronic diarrheal symptoms and verified the efficacy of the medicine, which may be based on a competitive inhibition of beta-glucuronidase by baicalin, a major component of the medicine [19].

Recently, we have shown that sulfobromophthalein (BSP) inhibits the secretion of SN-38-Glu into the gastrointestinal lumen but does not affect the plasma concentration of the active metabolite SN-38 [9]. Moreover, it has been reported that coadministration of probenecid with a reduced dose of CPT-11 potently reduces CPT-11-induced late-onset toxicity in gastrointestinal tissues [6]. However, little is known about the transport mechanisms or transporters that contribute to the intestinal absorption of SN-38. The human colon adenocarcinoma cell line Caco-2 has been used as a model in which to study intestinal absorption or secretion of various drugs [5]. This cell line spontaneously differentiates in culture into polarized cell monolayers with many enterocyte-like properties of transporting epithelia. Caco-2 cells retain various transporters expressed in the intestine. Using

T. Itoh · I. Takemoto
Department of Pharmacy,
Sapporo Social Insurance General Hospital,
Chuo 2-jo, 6-chome, Atsubetsu-ku,
Sapporo 004-8618, Japan

S. Itagaki · Y. Sumi · T. Hirano · K. Iseki (✉)
Department of Clinical Pharmaceutics and Therapeutics,
Graduate School of Pharmaceutical Sciences,
Hokkaido University, Kita 12-jo, Nishi 6-chome,
Kita-ku, Sapporo 060-0812, Japan
E-mail: ken-i@pharm.hokudai.ac.jp
Tel.: +81-11-7063770
Fax: +81-11-7063770

this model, a number of studies have been performed to characterize the intestinal transport mechanism.

In this study, we investigated the characteristics of SN-38 uptake into Caco-2 cells. The effects of baicalin and BSP on the uptake of SN-38 by Caco-2 cells were also investigated.

Materials and methods

Chemicals

All chemicals and reagents used were of analytical grade. Baicalin was purchased from Wako Pure Chemicals (Osaka, Japan). BSP was obtained from Sigma (St Louis, Mo.). Pravastatin was kindly supplied by Sankyo (Tokyo, Japan). Grepafloxacin was kindly supplied by Otsuka Pharmaceutical (Tokyo, Japan). SN-38 was kindly supplied by Daiichi Pharmaceutical (Tokyo, Japan). SN-38 was dissolved in DMSO (2% w/v final concentration) due to its hydrophobic property and poor solubility in water. The upper limit of SN-38 concentration was 25 μ*M*.

Cell culture

Caco-2 cells obtained form American Type Culture Collection (Rockville, Md.) were maintained in plastic culture flasks (Falcon, Becton Dickinson, Lincoln Park, N.J.) as described previously [8]. For the uptake study, Caco-2 cells were seeded at a cell density of 6×10^5 cells/cm^2 on six-well plastic plates (Corning Costar, Cambridge, Mass.). The cell monolayers were fed with fresh growth medium every 2 days and were then used at 4–6 days for the uptake experiments.

Uptake studies

SN-38 uptake was measured using monolayer cultures grown in six-well plastic plates. The incubation medium used for the uptake study was a modified Hank's balanced salt solution (HBSS), pH 7.4, containing 137 m*M* NaCl, 5.4 m*M* KCl, 1.3 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 0.4 m*M* KH_2PO_4, 0.3 m*M* NaH_2PO_4, 25 m*M* D-glucose and 10 m*M* HEPES/Tris. After removal of the growth medium, cells were preincubated at 37°C for 15 min with 1 ml incubation medium. After removal of the medium, 1 ml incubation medium containing SN-38 was added. The monolayers were incubated for stated times at 37°C or 4°C. For inhibition studies, monolayers were incubated with 1 ml incubation medium containing SN-38 (25 μ*M*) for 10 min at 37°C in the presence or absence of various inhibitors. Each cell monolayer was washed rapidly twice with an ice-cold incubation medium at the end of the incubation period. The cells were solubilized with 0.5 ml 1 *N* NaOH and neutralized with 0.5 ml 1 *N* HCl. After centrifugation of the mixture (5000 *g* for 2 min), a part of the upper aqueous layer (100 μl) was transferred to a fresh tube and 100 μl 50 m*M* monobasic potassium phosphate (pH 2.5) was added. After vortexing briefly, the sample was left overnight at 37°C.

Analytical procedures

An HPLC system equipped with a fluorescence detector was used to determine SN-38 as described previously [10]. The column was a C8 column (250×4.5 mm, 5 μm; GL Sciences). A mobile phase consisting of (50 m*M* monobasic potassium phosphate, pH 2.5, 7 m*M* tetrabutylammonium bromide)/acetonitrile (70:30, v/v) was used. The column temperature and flow rate were 40°C and 0.8 ml/min, respectively. The fluorescence detector (F1000; Hitachi) was operated at excitation and emission wavelengths of 355 nm and 515 nm, respectively. The lower limit of quantitation for SN-38 was 50 pmol/ml. Protein concentrations were measured by the method of Lowry et al. with bovine serum albumin as a standard [13]. Student's *t*-test was used for statistical analysis, and a P values <0.05 were considered significant.

Results

Uptake of SN-38 by Caco-2 cells

In the first part of this study, the uptake of SN-38 by Caco-2 cell monolayers was measured. Figure 1 shows the time courses of the uptake of SN-38. The uptake of SN-38 increased linearly over a period of 15 min and was markedly lower at 4°C than at 37°C. SN-38 uptake

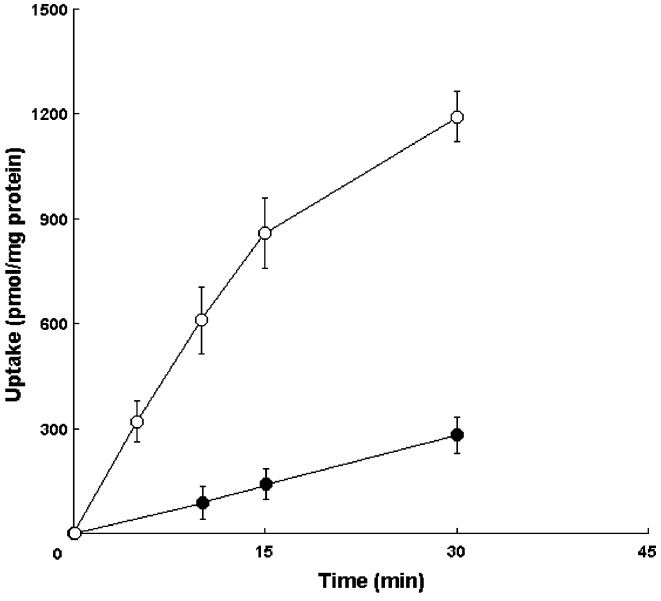

Fig. 1 Uptake of SN-38 by Caco-2 cells. Cells were incubated for the indicated periods at 37°C (*open circles*) or 4°C (*closed circles*) with SN-38 (25 μ*M*). Each value is the mean with SD of three determinations

at 10 min was used to determine concentration-dependence and effects of various inhibitors. The concentration-dependence of the uptake of SN-38 was determined. However, the uptake of SN-38 was not saturated up to 25 μ*M* (data not shown).

Effect of baicalin on the uptake of SN-38 by Caco-2 cells

The effect of baicalin on the uptake of SN-38 was examined. As shown in Fig. 2, baicalin reduced the uptake of SN-38 in a concentration-dependent manner. The highest inhibition (60%) was observed at 1 m*M*.

Effects of various compounds on the uptake of SN-38 by Caco-2 Cells

The effects of various compounds on the uptake of SN-38 by Caco-2 cells were determined. It has been reported that cotreatment of CPT-11 with BSP and probenecid decreases CPT-11-induced chronic diarrheal symptoms [6, 9]. As shown in Table 1, BSP significantly reduced the uptake of SN-38. However, probenecid had no effect on the uptake of SN-38. Several studies have suggested the involvement of specific transporters in intestinal absorption of organic acids [11]. Pravastatin, an inhibitor of organic anion transporting polypeptide (OATP), and grepafloxacin, a new quinolone transport system substrate, do not affect the uptake of SN-38 [12, 23]. Moreover, probenecid and pravastatin are known to be monocarboxylate transporter (MCT) inhibitors [4, 21].

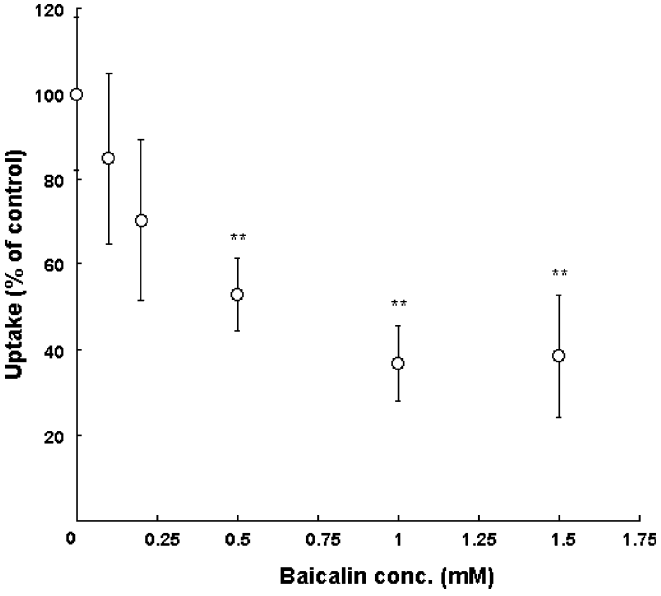

Fig. 2 Inhibitory effects of baicalin on the uptake of SN-38 by Caco-2 cells. The uptake of SN-38 (25 μ*M*) by Caco-2 cells was determined in the presence or absence of baicalin at defined concentrations. Each value is the percentage of the uptake in the absence of baicalin, and is the mean with SD of three or four determinations. $^{**}P < 0.01$, vs absence of baicalin

Table 1 Effects of various compounds on the uptake of SN-38 by Caco-2 cells. Cells were incubated with SN-38 (25 μ*M*) for 10 min at 37°C in the presence or absence of inhibitors. Each value is the mean with SD of three to five determinations

Compound	Concentration (m*M*)	Relative uptake (% of control)
Control		100 ± 18.9
BSP	0.05	95.6 ± 6.70
	0.2	40.2 ± 9.95**
	1	34.1 ± 7.26**
Probenecid	1	86.8 ± 13.2
Pravastatin	1	89.9 ± 31.4
Grepafloxacin	1	98.1 ± 28.4

$^{**}P < 0.01$, vs control.

Discussion

Due to the unpredictable severe diarrhea observed in patients treated with CPT-11, the clinical use of this anticancer agent has remained limited [1]. It has been proposed that the severe gastrointestinal toxicity results from exposure of intestinal tissues to SN-38, due to its biliary excretion and/or deconjugation of SN-38-Glu [3, 20]. Therefore, it is considered possible to prevent CPT-11-induced delayed diarrhea by using an inhibitor of beta-glucuronidase [15]. Hange-Shashin-To contains the beta-glucuronidase inhibitor, baicalin. It has been reported that Hange-Shashin-To can prevent diarrhea caused by CPT-11 [17, 18]. However, little is known about the transport mechanisms that contribute to the intestinal absorption of SN-38.

Drug discovery, development and targeting require knowledge of transporters that play a role in the disposition of a drug and its subsequent effects. In the current study, we obtained important data that provide an insight into the pathogenesis, and thus means for preventing, CPT-11-induced delayed-type diarrhea. In the first part of this study, SN-38 uptake by Caco-2 cell monolayers was measured. The fact that uptake of SN-38 was significantly reduced at 4°C suggests the involvement of a transporter or a carrier-mediated process. We examined the effect of baicalin on the uptake of SN-38 by the human intestinal cell line Caco-2. Baicalin inhibited the uptake of SN-38 in a concentration-dependent manner, suggesting that baicalin inhibits not only microbial beta-glucuronidase activity but also intestinal absorption of SN-38. It is possible that both inhibitions are responsible for the clinical usefulness of Hange-Shashin-To.

It has been reported that cotreatment of CPT-11 with BSP and probenecid decreases CPT-11-induced chronic diarrheal symptoms [6, 9]. We examined the effect of BSP and probenecid on the uptake of SN-38. BSP inhibited the SN-38 uptake, suggesting that BSP inhibits not only the secretion of SN-38-Glu into the gastrointestinal lumen but also the intestinal absorption of SN-38. On the other hand, no effect of probenecid was observed. A number of foreign weak electrolytes are

thought to penetrate the intestinal mucosal barrier by passive diffusion of the nonionized drug species across a lipoidal membrane according to the pH-partition theory [11]. However, the rate of diffusion of monocarboxylate forms of SN-38 is greatly increased between pH 7.0 and pH 8.0 [2]. Participation of transporter-mediated intestinal absorption has also been demonstrated both directly and indirectly for organic anion compounds. Several monocarboxylates have been reported to be absorbed via MCTs [11, 22]. We examined the contribution of MCTs to the uptake of SN-38. Pravastatin, a MCT inhibitor, do not affect SN-38 uptake [21]. Moreover, probenecid is known to be an MCT inhibitor [4]. These results suggest that MCT does not contribute to absorption of SN-38.

Grepafloxacin is well absorbed from the intestine after oral administration. Its physicochemical features are incompatible with rapid absorption by passive diffusion, suggesting that grepafloxacin is absorbed via a specific transport system(s) in the intestine. It has been reported that grepafloxacin uptake is mediated by a specific transport system distinct from those for organic anions, amino acids, dipeptides and monocarboxylates [23]. We examined the contribution of this transport system to the uptake of SN-38. Grepafloxacin had no effect on the uptake of SN-38, suggesting that the grepafloxacin transport system does not contribute to absorption of SN-38. It is known that mRNAs of OATPs are expressed in human intestine. However, only little information is available on intestinally expressed OATPs in humans [11, 22]. It has recently been shown that OATP-B (SLC21A9) plays a role in the absorption of anionic compounds across the apical membrane of human intestinal epithelial cells [12]. In our study, pravastatin did not affect SN-38 uptake, suggesting no contribution of OATP-B.

Information on the functional characteristics of drug transporters is important for improvements in drug delivery or drug design by targeting specific transporter proteins [14, 22]. It is thought that regulation of the functions of transporters will enable development of highly efficient drugs with ideal pharmacokinetic profiles and that approaches using intentional drug–drug interactions (positive drug interactions) may become more important in the future. It is possible that coadministration of an intestinal SN-38 transporter inhibitor with CPT-11 will reduce the late-onset diarrhea that occurs during treatment with CPT-11. Thus, strategic application of intestinal SN-38 transporter inhibitors may lead to more effective oral chemotherapy with CPT-11. The expression system of transporters would be an efficient tool for screening the activity of individual transport processes. However, this transport system has not yet been elucidated at the molecular level. Further studies are needed to elucidate the mechanism of intestinal uptake of SN-38.

In conclusion, our results suggest that a specific transport system mediates the uptake of SN-38 across the apical membrane in Caco-2 cells. Baicalin and BSP inhibit this transporter. Inhibition of this transporter would be a useful means for reducing late-onset diarrhea.

Acknowledgements We thank Dr. S. Miyauchi for his advice on the experimental technique. This work was supported in part by grants from the Akiyama Foundation and the Japan Research Foundation for Clinical Pharmacology.

References

1. Abigerges D, Armand JP, Chabot GG, Da Costa L, Fadel E, Cote C, Herait P, Gandia D (1994) Irinotecan (CPT-11) high-dose escalation using intensive high-dose loperamide to control diarrhea. J Natl Cancer Inst 86:446–449
2. Akimoto K, Kawai A, Ohya K (1994) Kinetic studies of the hydrolysis and lactonization of camptothecin and its derivatives, CPT-11 and SN-38, in aqueous solution. Chem Pharm Bull 42:2135–2138
3. Atsumi R, Suzuki W, Hakusui H (1991) Identification of the metabolites of irinotecan, a new derivative of camptothecin, in rat bile and its biliary excretion. Xenobiotica 21:1159–1169
4. Enerson BE, Drewes LR (2003) Molecular features, regulation, and function of monocarboxylate transporters: implications for drug delivery. J Pharm Sci 92:1531–1544
5. Hidalgo IJ, Raub TJ, Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 96:736–749
6. Horikawa M, Kato Y, Sugiyama Y (2002) Reduced gastrointestinal toxicity following inhibition of the biliary excretion of irinotecan and its metabolites by probenecid in rats. Pharm Res 19:1345–1353
7. Houghton PJ, Cheshire PJ, Hallman JC, Bissery MS, Mathieu-Boue A, Houghton JA (1993) Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxy-camptothecin against human tumor xenografts: lack of cross-resistance in vivo in tumors with acquired resistance to the topoisomerase I inhibitor 9-dimethylaminomethyl-10-hydroxycamptothecin. Cancer Res 53:2823–2829
8. Itagaki S, Sugawara M, Kobayashi M, Miyazaki K, Iseki K (2003) Mechanism of active secretion of phenolsulfonphthalein in the liver via MRP2 (abcc2), an organic anion transporter. Drug Metab Pharmacokinet 18:238–244
9. Itoh T, Itagaki S, Sasaki K, Hirano T, Takemoto I, Iseki K (2004) Pharmacokinetic modulation of irinotecan metabolites by sulfobromophthalein. J Pharm Pharmacol 56:809–812
10. Itoh T, Takemoto I, Itagaki S, Sasaki K, Hirano T, Iseki K (2004) Biliary excretion of irinotecan and its metabolites. J Pharm Pharm Sci 7:13–18
11. Katsura T, Inui KI (2003) Intestinal absorption of drugs mediated by drug transporters: mechanisms and regulation. Drug Metab Pharmacokinet 18:1–15
12. Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I (2003) Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. J Pharmacol Exp Ther 306:703–708
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
14. Mizuno N, Sugiyama Y (2002) Drug transporters: their role and importance in the selection and development of new drugs. Drug Metab Pharmacokinet 17:93–108
15. Narita M, Nagai E, Hagiwara H, Aburada M, Yokoi T, Kamataki T (1993) Inhibition of beta-glucuronidase by natural glucuronides of kampo medicines using glucuronide of SN-38 (7-ethyl-10-hydroxycamptothecin) as a substrate. Xenobiotica 23:5–10

16. Rivory LP, Bowles MR, Robert J, Pond SM (1996) Conversion of irinotecan (CPT-11) to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by human liver carboxylesterase. Biochem Pharmacol 52:1103–1111
17. Sakata Y, Suzuki H, Kamataki T (1994) Preventive effect of TJ-14, a kampo (Chinese herb) medicine, on diarrhea induced by irinotecan hydrochloride (CPT-11). Jpn J Cancer Chemother 21:1241–1244
18. Satoh H, Ishikawa H, Murakami O, Yamashita Y, Maeno T, Kamma H, Ohtsuka M, Hasegawa S (1999) Reduction by hange-shashin-to (TJ-14), a herbal medicine on gastrointestinal tract complications induced by CPT-11 containing chemotherapy. Cancer Res Ther Control 7:321–323
19. Takasuna K, Kasai Y, Kitano Y, Mori K, Kobayashi R, Hagiwara T, Kakihata K, Hirohashi M, Nomura M, Nagai E, Kamataki T (1995) Prospective effect of Kampo medicines and baicalin against intestinal toxicity of a new anticancer camptothecin derivative, irinotecan hydrochloride (CPT-11), in rats. Jpn J Cancer Res 86:978–984
20. Takasuna K, Hagiwara T, Hirohashi M, Nomura M, Nagai E, Yokoi T, Kamataki T (1996) Involvement of b-glucuronidase in intestinal microflora in the intestinal toxicity of the antitumor camptothecin derivative irinotecan hydrochloride (CPT-11) in rats. Cancer Res 56:3752–3757
21. Tamai I, Takanaga H, Maeda H, Sai Y, Ogihara T, Higashida H, Tsuji A (1995) Participation of a proton-cotransporter, MCT1, in the intestinal transport of monocarboxylic acids. Biochem Biophys Res Commun 214:482–489
22. Tsuji A (2002) Transporter-mediated drug interactions. Drug Metab Pharmacokinet 17:253–274
23. Yamaguchi H, Yano I, Saito H, Inui K (2001) Transport characteristics of grepafloxacin and levofloxacin in the human intestinal cell line Caco-2. Eur J Pharmacol 431:297–303