INTESTINAL TRANSPORT OF IRINOTECAN IN CACO-2 CELLS AND MDCK II CELLS OVEREXpressing EFFLUX TRANSPORTERS PGP, CMOAT, AND MRP1

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(Received October 15, 2001; accepted March 18, 2002)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

Irinotecan (CPT-11) is a water-soluble camptothecin (CPT) derivative that has been recently approved in the United States for patients as a first-line therapy in advanced colorectal cancer. Phase I clinical trials using oral CPT-11 have shown poor and variable oral bioavailability. The present study was designed to investigate the intestinal absorption and efflux mechanisms of CPT-11 using in vitro cell culture models, Caco-2 cells, and engineered Madine-Darby canine kidney (MDCK) II cells overexpressing P-glycoprotein (Pgp), canalicular multispecific organic anion transporter (cMOAT), and multidrug resistance-associated protein (MRP1). The intestinal absorptive and secretory transport of CPT-11 was investigated using Caco-2 cell monolayers. Secretory transport was concentration-dependent and saturable. The secretory efflux permeability (P_{se}) of CPT-11 decreased with decreasing temperature, with an estimated activation energy of 19.6 ± 2.9 kcal/mol suggesting the involvement of active transporters. The involvement of potential secretory transporters was further characterized in MDCK II cells. The secretory efflux carrier permeability (P_{se}) was -4- and -2-fold greater in MDCK II/Pgp and MDCK II/cMOAT cells than that in MDCK II/wild-type cells. Furthermore, the secretory efflux P_{se} of CPT-11 was significantly decreased by Pgp inhibitors, elacridar (GF120918) (IC_{50} = 0.38 ± 0.06 μM) and verapamil (IC_{50} = 234 ± 48 μM) in MDCK II/Pgp cells and by cMOAT inhibitor 3-[[3-[2-[7-chloro-2-quinolinyl][ethyl]phenyl]-(3-dimethylamino-3-oxopropyl)]-thio]-methyl-thio) propanoic acid (MKS571) (IC_{50} = 469 ± 60 μM) in MDCK II/cMOAT cells. Overall, the current study suggests that Pgp and cMOAT are capable of mediating the efflux of CPT-11 in vitro. Since both Pgp and cMOAT are expressed in the intestine, liver, and kidney, it is likely that these efflux transporters play a significant role limiting the oral absorption and disposition of this important anticancer drug.

Irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy camptothecin (CPT-11), is a potent anticancer drug with a broad spectrum of anticancer activity (Slichenmyer et al., 1993). It is a prodrug and is converted in vivo by carboxylesterases to its active metabolite, SN-38 (Ahmed et al., 1999). CPT-11 has been recently approved in the United States for first-line therapy of patients with advanced colorectal cancer (Food and Drug Administration, 2000). Despite extensive evaluation of CPT-11 in a variety of dosing schedules, clinical testing has been restricted to the intravenous route of administration (Thompson et al., 1997). Both preclinical and clinical data have suggested that the efficacy of CPT-11 is highly schedule-dependent and low dose protracted schedules of daily administration of CPT-11 are more efficacious and less toxic than the high dose shorter schedules of administration. This can be explained by the mechanism of action for CPT-11. CPT-11, a topoisomerase I inhibitor, targets the topoisomerase of tumor cells in S-phase of cell cycle (Del Bino et al., 1991). Thus, dosing drug over a prolonged period will be more efficacious, especially in tumors that have only a small proportion of S-phase cells.

Oral administration may be especially well suited for frequent or protracted dosing of CPT-11 to achieve an improved therapeutic ratio. Low gastric pH may favor retention of CPT-11 in the active lactone ring configuration (Hertzberg et al., 1989). The high concentrations of carboxylesterases in the intestine and liver may promote presystemic conversion of CPT-11 to SN-38, which is 300- to ~1000-fold more active than CPT-11 (Kawato et al., 1991). Oral administration is also more convenient and cost-effective for frequent or protracted dosing than i.v. administration. In a Phase I clinical trial of orally administered CPT-11, good efficacy and toxicity profiles have been observed (Drengler et al., 1999). However, low and variable oral bioavailability of CPT-11...
was observed potentially limiting the viability of oral delivery due to its narrow therapeutic window.

It is generally believed that the low and variable oral bioavailability may be explained by physiochemical factors (i.e., solubility, and dissolution) as well as physiological factors (i.e., intestinal absorption, efflux, and the first pass metabolism). However, physiochemical factors are less likely to play a crucial role since the low and variable oral bioavailability occurs among other CPT analogs with diverse physicochemical properties (Gupta et al., 2000b). Therefore, physiological factors such as intestinal absorption and secretion, as well as first pass metabolism, are of scientific importance and should be investigated to fully understand the mechanism responsible for the low and variable oral absorption of CPT-11.

CPT-11 is metabolized by carboxylesterases and cytochrome P450 3A (Danks et al., 1999). Due to abundance of these enzymes in the intestine and liver, first pass metabolism may also contribute significantly to the low and variable oral absorption of CPT-11 (Wacher et al., 1998). The impact of first pass metabolism on the oral absorption of CPT-11 is currently being investigated in our laboratory.

Over the past decade, there has been rapid progress in the understanding of drug transporting and metabolizing systems. A multitude of transporters have been implicated in the efflux of drugs of the camptothecin class (camptothecin, topotecan, and irinotecan) including Pgp, canalicular multispecific organic anion transporter (cMOAT; Sugiyama., 1998), multidrug resistance-associated protein (MRP1; Chu et al., 1999b), and more recently breast cancer resistance protein (Schellens et al., 2000). Mostly, all the investigations have been carried out to explain the phenomenon of multidrug resistance in cancer cells. It is currently unknown whether the intestinal absorption and secretion contribute to the low and variable bioavailability of CPT-11. The active efflux of CPT-11 and SN-38 has been observed in drug-resistant tumor cells and in canalicular membrane vesicles (CMVs) in vitro as well as in rats in vivo. For example, in drug-resistant tumor cells overexpressing Pgp, the cellular accumulation of CPT-11 and SN-38 are reduced (Yang et al., 1995), and the reduction of cellular accumulations can be reversed by Pgp reversal agents (Jansen et al., 1998). In rat and human bile CMVs, Pgp, and cMOAT have been demonstrated to mediate the efflux of CPT-11 and SN-38 (Chu et al., 1998). The involvement of Pgp and cMOAT in the efflux of CPT-11 and SN-38 has been further demonstrated in wild-type rats (Gupta et al., 1996; Chu et al., 1999a) and rats defective in cMOAT in vivo. Due to the intestinal expression of Pgp and cMOAT, we postulate that CPT-11 may be secreted by Pgp and/or cMOAT into the intestine, and this may be responsible for the low oral absorption of CPT-11. Moreover, variation in the intestinal expression of Pgp and/or cMOAT could cause variable oral absorption of CPT-11. In addition, several reports have been published in which overlapping substrate specificity between Pgp and MRP is described (Gottesman et al., 1996; Marbeuf-Gueye et al., 1998; Borst et al., 1999). Additionally, due to similar substrate specificity of MRP and cMOAT, it is possible that CPT-11 and its metabolites can be substrates for MRP1.

To develop a mechanistic basis for understanding the oral absorption of CPT-11, characterization of its intestinal transport mechanism and contribution of putative secretory/efflux transporters is imperative. The present study was designed to investigate the intestinal absorption and efflux mechanisms of CPT-11 using in vitro cell culture models, Caco-2 cells and engineered MDCK II cells overexpressing Pgp, cMOAT, and MRP1.

**Experimental Procedures**

**Materials.** Camptothecin, etoposide and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). Irinotecan was provided by Dr. McGovren (Pharmacia & Upjohn Diagnostics, Kalamazoo, MI). GF120198 was provided by Dr. Polli (Glaxo Wellcome Inc., Research Triangle Park, NC); MK571 was provided by Dr. Koch (Merck & Co. Inc., Rahway, NJ). All other materials were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma-Aldrich and were used as received.

**Cell Culture.** Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) and frozen at −80°C. Four weeks prior to the experiment, passage number 20 cells were cultured at 37°C/5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 2% penicillin-streptomycin. After harvesting at 90% confluency, the cells were seeded on Snapwell polycarbonate filter inserts (Corning Inc., Acton, MA) at a density of 45,000 cells/well and grown for 21 days before mounting on side-by-side Ussing diffusion chambers (Harvard Apparatus Inc., Holliston, MA).

MDCK II/WT, MDCK II/Pgp, MDCK II/cMOAT, and MDCK II/MRP1 cells were provided by Dr. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and frozen at −80°C. Four weeks prior to the experiment, passage number 10 cells were cultured at 37°C/5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 2% penicillin-streptomycin. After harvesting at 90% confluency, the cells were seeded on Transwell polycarbonate filter inserts (Corning Inc.) at a density of 665,000 cells/cm² and grown for 3 days before diffusion study.

**Diffusion Studies.** Drug diffusion across Caco-2 cells was conducted in side-by-side Ussing diffusion chambers. Chambers were prewarmed to 37°C before mounting Snapwell polycarbonate filter inserts with Caco-2 cells grown for 21 days. The apical (AP) and the basolateral (BL) compartments were filled with appropriate buffers (the AP buffer was MES Ringer’s buffer, pH 6.5, and the BL buffer was Ringer’s glucose buffer pH 7.4, 290 mOsm/kg). The fluid in the chambers was circulated using a gas lift mechanism with 5% CO2 and 95% O2 and at a flow rate of 10 ml/min during diffusion study. Drug diffusion across MDCK II cells was conducted in Transwell plates that were placed on an orbital shaker; the plates were shaken at 40 rpm during diffusion study to minimize the influence of aqueous boundary layer. Both the AP and the BL compartments were filled with Hanks’ balanced salts buffer (pH 7.4, 10 mM Heps). The drug solution was placed in the donor chamber and samples were taken from the receptor chamber at 30, 45, 60, 75, 90, and 105 min followed by an immediate replacement of the same volume of prewarmed buffer. Samples were analyzed by HPLC immediately. The cellular integrity was evaluated before and after experiment by measuring the transepithelial electrical resistance and [14C] mannitol transport. The transepithelial electrical resistance measurements in Caco-2 cells indicated values of 300 to 350 Ωcm². The permeability of mannitol was determined to be <1*10⁻⁶ cm/s. These results indicated that the cell monolayers were not leaky (i.e., monolayer integrity was high).

**Concentration Dependence Study.** Concentration dependence study was carried out by investigating the transport of CPT-11 at concentrations ranging from 1.0 to 500 μM. The effective permeability (Pₑₑₑₑ) (cm/s) was determined using eq. 1:

\[
Pₑₑₑₑ = \frac{Vₑ × A × Cₑ}{dC/dt}
\]

Where \(Vₑ\) is the volume of the receptor chamber, \(A\) is the surface area of the filter, \(Cₑ\) is the initial drug concentration, and \(dC/dt\) is the linear slope of the drug concentration in receptor chamber versus time after correcting dilution. Using a nonlinear regression (Scientist v2.01; Micromath Software; Salt Lake City, UT), kinetic values were determined using eq. 2:

\[
Pₑₑₑₑ = Pₑ × (1 + C/Kₐₕₐₙ) + Pₐₙ
\]

Where \(Pₑ\) is the carrier permeability (\(=P_{max}/Kₘₐₜₐₙ\)), \(Kₐₕₐₙ\) is the Michaelis-Menten constant, and \(Pₐₙ\) is the nonsaturable passive diffusion permeability. A weighted fit was performed using 1/S.D. as the weighting factor.

**Temperature Dependence.** The efflux transport of CPT-11 was investigated at a concentration of 10 μM and at the following temperatures: 5, 10, 15, 25, and 37°C. An Arrhenius plot of log \(Pₑₑₑₑ\) versus 1/\(T\) was constructed where \(T\) = absolute temperature (°C + 273.16°C). Using a least square linear regression, activation energy \(*Eₐ*\) was determined using eq. 3:
Protein (10 µM) was size-fractionated in a polyacrylamide gel containing 7% SDS and transferred to a nitrocellulose membrane. The blots were incubated with antibodies C219 or MRPr1 and subsequently incubated with anti-mouse secondary antibodies. SDS and transferred to a nitrocellulose membrane. The blots were incubated with antibodies C219 or MRPr1 and subsequently incubated with anti-mouse secondary antibodies. The expression of cMOAT was detected using RT-PCR. Total RNA was isolated from MDCK II cells after culturing for 4 days with the Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized in a 20-µl reaction volume containing 3 µg of total RNA, 2 µmol of reverse primer, 10 mM dithiothreitol; 0.5 mM dATP, dCTP, dGTP, and dTTP; and 200 units of Taq and 2.5 units of Taq polymerase.

### Inhibition Studies

The inhibition of efflux of CPT-11 (10 µM) across Caco-2 cells was investigated in the presence of a common Pgp substrate etoposide (200–300 µM). To determine the kinetics of inhibition, the efflux $P_{\text{eff}}$ of CPT-11 was determined at various substrate concentrations (0.2–1.0 µM) and two fixed inhibitor concentrations (200 and 300 µM). The substrate concentrations were measured in the apical chamber (BL-AP) study for all secretory transport inhibition studies. Lineweaver-Burk plot was constructed with the reciprocal of flux $(1/P_{\text{eff}})$ versus the reciprocal of substrate concentration $(1/C)$ to determine the type of inhibition. The inhibition constant ($K_i$) was determined from a secondary plot of the slope of the Lineweaver-Burk plots versus the corresponding inhibitor concentration. The intersection of the least square linear regression line with the x-axis gives the value of $K_i$. The inhibition of efflux of CPT-11 (10 µM) across MDCK II/Pgp or MDCK II/cMOAT cells was investigated in the presence of etoposide (200–400 µM), verapamil (200–400 µM), and GF120918 (0.2–0.4 µM) or cMOAT inhibitor MK571 (200–400 µM).

### HPLC Analysis

Samples were analyzed by modified reversed phase HPLC method described previously (Gupta et al., 1994). For samples obtained from diffusion study in Caco-2 cells, 450 µl of sample was acidified with 250 µl of 0.1 N HCl followed by addition of 10 µl of 10 µM CPT (internal standard). For samples obtained from diffusion study in MDCK II cells, 100 µl of sample was acidified with 400 µl of 0.1 N HCl followed by addition of 10 µl of 10 µM CPT (internal standard). Samples were acidified before injection onto HPLC column to ensure conversion to lactone form. HPLC analysis of samples was performed using a C$_{18}$ column (µBondapack, 10 µm, 3.9 x 300 mm; Waters Corp., Milford, MA) preceded by a C$_{18}$ µBondapack guard column. The mobile phase was a mixture of 35% acetonitrile with 65% aqueous buffer containing 100 mM potassium dihydrogen phosphate and 1 mM sodium heptane sulfonate (pH 4.0). The flow rate was maintained at 1 ml/min. CPT-11 was detected by Shimadzu RF-551 fluorescence detector (Shimadzu Scientific Instruments, Inc., Columbia, MD) with $\lambda_{ex}$ at 359 nm and $\lambda_{em}$ at 536 nm. Standard curve of CPT-11 was linear within the range of 0.01 to 1.0 µM ($r = 0.99$).

### Immunoblotting and RT-PCR

The expression of Pgp was detected using Western blot analysis. MDCK II cells were harvested after culturing for 4 days. Protein (10 µg) was size-fractionated in a polyacrylamide gel containing 7% SDS and transferred to a nitrocellulose membrane. The blots were incubated with antibodies C219 or MRPr1 and subsequently incubated with anti-mouse or anti-rat. Pgp was detected with an enhanced chemiluminescence system. The expression of cMOAT was detected using RT-PCR. All statistical tests were performed using Jandel SigmaStat version 2.0 (Jandel Scientific Corp., San Rafael, CA). A minimal P-value of 0.05 was used as the significance level for all tests. One-way analysis of variance and Tukey tests were performed on permeability data. All data are reported as the mean ± S.D. unless otherwise noted. Weighted nonlinear regression was performed using Micromath Scientist v2.01 with a weighing factor of 1/S.D.

### Results

**Absorptive and Secretory Transport of CPT-11 across Caco-2 Cells.** AP to BL and BL to AP transport of CPT-11 was investigated at concentrations ranging from 1.0 to 500 µM. It was found that secretory permeability ($P_{\text{sec}}$) was concentration-dependent, but saturation was only partially reached at the highest achievable concentration of CPT-11. The secretory $P_{\text{eff}}$ (BL-AP) of CPT-11 decreased from 13.4 ± 1.3 x 10$^{-6}$ to 2.0 ± 0.4 x 10$^{-6}$ cm/s with increasing concentration from 1.0 to 500 µM (Fig. 1A). Michaelis-Menten parameters $K_m$, $P_{\text{ss}}$, and $P_{m}$ were estimated to be 116.1 ± 15.9 µM, 13.2 ± 0.7 x 10$^{-6}$ cm/s, and 0.17 ± 0.04 x 10$^{-6}$ cm/s (Table 1), implying a low affinity and high capacity intestinal secretory transport system for CPT-11. No concentration dependence or saturation was observed for the absorptive transport of CPT-11 at concentrations up to 500 µM (Fig. 1A), suggesting that passive diffusion dominates the absorptive-transport behavior of CPT-11. The secretory efflux transport of CPT-11 was ~10- to 100-fold greater than its absorptive transport at concentrations ranging from 1.0 to 500 µM (Fig. 1B), suggesting that specialized mechanisms are involved in the secretory transport of CPT-11.

**Temperature Dependence of the Secretory Transport of CPT-11 across Caco-2 Cells.** To determine whether the efflux of CPT-11 occurs by an active process (i.e., an energy-dependent process), the secretory $P_{\text{eff}}$ (BL-AP) was determined at various temperatures ranging from 5° to 37°C. It was found that the secretory $P_{\text{eff}}$ (BL-AP) of CPT-11 decreased with decreasing temperature. An Ar-
energy of activation ($E_a$) was estimated to be 19.6 kcal/mol.

The concentration and temperature dependence studies suggested that the intestinal efflux of CPT-11 is likely to be mediated by active transporters. Therefore, the potential efflux transporters involved in the efflux of CPT were further characterized. Previous studies have shown that Pgp is able to efflux CPT-11 in drug-resistant carcinoma cells and canalicular membrane vesicles (Jansen et al., 1998). Therefore, the efflux transport of CPT-11 was investigated in the presence of etoposide, a common mixed mechanism efflux inhibitor, at various substrate and inhibitor concentrations (Fig. 3). The transport of etoposide across Caco-2 has been studied previously in this laboratory, and the $K_m$ was estimated to be $\sim$213 $\mu$M. Thus, the inhibitory effects of etoposide were investigated at concentrations around its $K_m$ in the present study. It was found that the efflux of CPT-11 was competitively inhibited by etoposide (Fig. 3A) with an estimated $K_i$ of 259.3 $\pm$ 57.4 $\mu$M (Fig. 3B). This implied that Pgp might be involved in the efflux of CPT-11. Since the inhibition of CPT-11 efflux is relatively weak, it was suspected that the efflux of CPT-11 might be mediated by multiple transporters including Pgp. Previous studies have reported that cMOAT, an efflux transporter, may synergistically work with Pgp to efflux CPT-11 in the liver (Gupta et al., 1996; Chu et al., 1998; Sugiyama et al., 1998). Since cMOAT is also expressed in the intestine, it was postulated in the present study that cMOAT may also efflux CPT-11 during the intestinal absorptive process of CPT-11.

Immunoblotting and RT-PCR. The RT-PCR and Immunoblot confirm the presence of relevant transporters in Caco-2 cells and the overexpression of individual transporters in MDCK II cells (Fig. 4, A and B).

Absorptive and Secretory Transport of CPT-11 across Engineered MDCK II Cells. In an attempt to fully elucidate the involvement of the efflux transporters in the efflux of CPT-11, the transport of CPT-11 was investigated in polarized MDCK II cells transfected with Pgp, cMOAT, and MRP1 genes. In MDCK II/WT cells, the secretory transport of CPT-11 was concentration-dependent and saturable whereas the absorptive transport appeared to be passive diffusion, which is similar to the transport pathway of CPT-11 in Caco-2 cells (Fig. 5A). The secretory transport of CPT-11 was partially saturated at the highest drug concentration (400 $\mu$M). The secretory $P_{\text{eff}}$ of CPT-11 decreased from $6.4 \pm 0.4 \times 10^{-6}$ to $1.1 \pm 0.1 \times 10^{-6}$ cm/s at concentrations ranging from 1.0 to 400 $\mu$M. The secretory $P_e$ was estimated to be $6.0 \pm 0.4 \times 10^{-6}$ cm/s, which is $\sim$7.5-fold higher than the absorptive $P_{\text{eff}}$ for CPT-11 (Table 1). The values of secretory $P_e$ (BL-AP) and $K_m$ were comparable for CPT-11 between Caco-2 and MDCK II/WT cells (Table 1).

In MDCK II/Pgp, MDCK II/cMOAT, and MDCK II/MRP1 cells, the absorptive transport of CPT-11 also appeared to be passive whereas the secretory transport was concentration-dependent and saturable from 1.0 to 400 $\mu$M (Fig. 5, B-D). The secretory $P_{\text{eff}}$ (AP-BL) decreased from $21.5 \pm 1.5 \times 10^{-6}$ to $1.4 \pm 0.4 \times 10^{-6}$ cm/s across MDCK II/Pgp cells, from $10.5 \pm 0.9 \times 10^{-6}$ to $1.2 \pm 0.4 \times 10^{-6}$ cm/s across MDCK II/cMOAT, and from $5.6 \pm 0.4 \times 10^{-6}$ to $1.0 \pm 0.2 \times 10^{-6}$ cm/s across MDCK II/MRP1 cells. The absorptive $P_{\text{eff}}$ (AP-BL) was less than $1.0 \times 10^{-6}$ cm/s across MDCK II/Pgp, MDCK II/cMOAT, and MDCK II/MRP1 cells. The secretory $P_e$ (BL-AP) was $\sim$4- and $\sim$2-fold greater for CPT-11 across MDCK II/Pgp and MDCK II/cMOAT compared to that across MDCK II/WT cells, suggesting that both Pgp and cMOAT are capable of effluxing CPT-11 (Table 1). However, the secretory $P_e$ (BL-AP) was approximately equal for CPT-11 across MDCK II/WT and MDCK II/MRP1 cells. The value of $K_m$ was $\sim$2-fold lower for CPT-11 across MDCK II/Pgp than across MDCK II/cMOAT, indicating that Pgp had a higher affinity for CPT-11 than cMOAT. This is consistent with a previous efflux study in CMVs that Pgp is probably the high affinity component whereas cMOAT is the low affinity transporter for CPT-11 (Chu et al., 1999a).

Inhibition of Efflux Transport of CPT across Engineered MDCK II Cells. The transport of CPT-11 across MDCK II/Pgp or MDCK II/cMOAT cells was investigated in the presence of Pgp inhibitors, etoposide, verapamil, and GF120918 or cMOAT inhibitor.

**Table 1**

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<th>Caco-2</th>
<th>MDCK II/WT</th>
<th>MDCK II/Pgp</th>
<th>MDCK II/cMOAT</th>
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<td><strong>AP to BL</strong></td>
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<td>$P_e$ (cm/s $\times 10^5$)</td>
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<td>$P_m$ (cm/s $\times 10^0$)</td>
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<tr>
<td><strong>BL to AP</strong></td>
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</tr>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>116.1 $\pm$ 15.9</td>
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<td>$P_e$ (cm/s $\times 10^5$)</td>
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<td>$P_m$ (cm/s $\times 10^0$)</td>
<td>0.17 $\pm$ 0.04</td>
<td>N.D.</td>
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N.D. Not determinable.

**Fig. 2. Temperature dependence of irinotecan efflux transport across Caco-2 cells.**

Plot of logarithmic effective permeability (mean $\pm$ S.D.) and the best fit line versus the reciprocal of absolute temperature. Each point represents the mean ($\pm$ S.D.) for at least three observations. Activation energy ($E_a$), was determined based on the following equation: slope $= -E_a / R$, where $R$ = gas constant = 0.001987 kcal/deg mol.
MK571. We have presented the inhibition data in terms of IC\textsubscript{50} values defined as the concentration of inhibitor corresponding to 50% change in permeability. The secretory P\textsubscript{eff} (BL-AP) of CPT-11 was reduced by GF120918 (IC\textsubscript{50} = 0.38 ± 0.06 μM), verapamil (IC\textsubscript{50} = 234 ± 48 μM), and etoposide (IC\textsubscript{50} = 1185 ± 190 μM) in MDCK II/Pgp cells and by MK571 (IC\textsubscript{50} = 469 ± 60 μM) in MDCK II/cMOAT cells (Fig. 6; Table 2). Meanwhile, the absorptive P\textsubscript{eff} (AP-BL) was increased by GF120918 (IC\textsubscript{50} = 0.21 ± 0.05 μM), verapamil (IC\textsubscript{50} = 29 ± 6 μM), and etoposide (IC\textsubscript{50} = 468 ± 56 μM) in MDCK II/Pgp cells and by MK571 (IC\textsubscript{50} = 49.8 ± 13.8 μM) in MDCK II/cMOAT cells (Fig. 7). This further confirmed involvement of Pgp and cMOAT in the efflux of CPT-11.

Discussion

The oral delivery of CPT-11 has been evaluated in Phase I clinical trials (Drengler et al., 1999) resulting in encouraging efficacy and toxicity profiles. However, the low (<20%) and variable (50% variation in area under the curve) bioavailability significantly diminishes its potential for oral delivery. Since low and variable oral bioavailability occurs among all CPT analogs and their physicochemical properties vary widely, these factors are less likely to play a crucial role in the oral absorption of CPT-11 (Gupta et al., 1998; Verschraegen et al., 1998). Instead, physiological factors, such as intestinal absorption/secretion and first pass metabolism, are more likely to account for the low and variable oral absorption of CPT-11. To determine the extent of the contribution of intestinal transport to the

**Fig. 3.** Lineweaver-Burk plot (A) of inhibition of irinotecan efflux across Caco-2 cells at fixed etoposide concentration of 0 (□), 200 (●), and 300 μM (▲); plot of flux (1/P\textsubscript{eff} *C) (mean ± S.D.) and the best-fit line versus the reciprocal of substrate concentration; each point represents the mean (± S.D.) for at least three observations; the inhibition constant (K\textsubscript{i}) was determined from a secondary plot (B) of the slopes of Lineweaver-Burk plots for various inhibitor concentrations.

**Fig. 4.** Overexpression of Pgp and cMOAT in MDCK II cells. A, Western blot analysis of Pgp; B, RT-PCR analysis of cMOAT.

**Fig. 5.** Transport of irinotecan across MDCK II/WT (A), MDCK II/Pgp (B), MDCK II/cMOAT (C), and MDCK II/MRP1 (D) for absorption (●) and secretory (▲).

Plot of effective permeability (mean ± S.D.) and the best-fit line versus logarithmic concentration. Each point represents the mean (± S.D.) for at least three observations. Michaelis-Menten parameters were determined using a weighted (1/S.D.) nonlinear regression and are reported in Table 1.
CPT-11 efflux in the liver (Sugiyama et al., 1998). Since Pgp and cMOAT are also expressed in the intestine, we postulated that CPT-11 might be effluxed by Pgp and/or cMOAT in the intestine and may be responsible for the low oral absorption of CPT-11. Recently, other investigators reported active transport of CPT-11 in Caco-2 cells, which are in agreement with our results (Yamamoto et al., 2001), but Caco-2 cells are known to express multiple transporters (Taipalensuu et al., 2001), making the interpretation of transport data challenging. These studies do not identify the specific transporters involved in the secretion of CPT-11. Therefore, the present study was designed to further characterize the interaction of CPT-11 with Pgp and/or cMOAT in vitro. Since the expression level of Pgp and cMOAT in MDCK II/Pgp and MDCK II/cMOAT cells, respectively, is much higher than that of endogenous Pgp and cMOAT present in MDCK II/WT cells, the interaction of CPT-11 with Pgp and cMOAT was further investigated in these two cell lines. It was found that the efflux $P_{\text{eff}}$ of CPT-11 was $\sim 4$-fold greater in MDCK II/Pgp cells and $\sim 2$-fold greater in MDCK II/cMOAT cells compared with that in MDCK II/WT cells. The increase in $P_{\text{eff}}$ correlates well with the increased expression levels of Pgp and cMOAT in MDCK II/Pgp and MDCK II/cMOAT cells relative to MDCK II/WT cells, suggesting that the efflux of CPT-11 is likely proportional to the amount of efflux transporters. Preliminary data from our lab has indeed found significant correlation between the expression of efflux transporters in rat and human intestine with CPT-11 secretory transport (Guo A, et al., 2001). The data obtained in the present study is also in consonance with cytotoxicity $LD_{50}$ values obtained in these MDCK II cell lines (unpublished results) correlating further the acquisition of resistance to secretory transport. The MDCK II cells overexpressing Pgp and cMOAT acquired resistance to CPT-11 reflected by an increase in the $LD_{50}$ value. If MRP1 were involved in transporting CPT-11 then the absorptive (AP-BL) transport would be greater than secretory (BL-AP) transport. The permeability values obtained with MDCK/MPR1 cells are approximately similar to those obtained in MDCK II/wild-type cells, and additionally the flux was higher in BL-AP direction than AP-BL direction. We hypothesize that due the expression of endogenous canine Pgp and cMOAT (Conrad et al., 2001) in MDCK II/WT cells (Fig. 4), and since the substrate specificities of Pgp and MRP1 and cMOAT and MRP1 are known to overlap (Gottesman et al., 1996; Marbeuf-Gueye et al., 1998; Borst et al., 1999), the efflux of CPT-11 across MDCK II/WT observed in the present study is more likely to be mediated by endogenous Pgp rather than by MRP1. Various investigators have reported active efflux of CPT-11 and its metabolites in nonintestinal cell lines like KB-derived cell lines (Chen et al., 1999) and have implicated MRP1 in its transport. The basolateral location of MRP1 makes interpretation of efflux transport data challenging since MRP1 is an absorptive efflux transporter whereas Pgp and cMOAT are secretory efflux transporters. Our results do not conclusively implicate MRP1, but this does not preclude its involvement in efflux of CPT-11. In addition, there is a possibility that MRP1 is a weak transporter for CPT-11, and its absorptive function was masked by the effective efflux mediated by Pgp and cMOAT in MDCK II cells. For confirmation, studies in membrane vesicles isolated from intestinal tissue are warranted to isolate the specific membrane domain-associated events.

To further study the involvement of Pgp and cMOAT in the efflux of CPT-11, the efflux transport of CPT-11 was investigated in the presence of efflux transporter inhibitors. We have presented the inhibition data in terms of $IC_{50}$ values defined as the concentration of inhibitor corresponding to 50% change in permeability. The secretory $P_{\text{eff}}$ (BL-AP) of CPT-11 was reduced by Pgp inhibitors GF120918 ($IC_{50} = 0.38 + 0.06 \mu M$) and verapamil ($IC_{50} = 234 + 48 \mu M$) in

Our recent findings suggest that the parent camptothecin is absorbed by an active carrier-mediated process (Gupta et al., 2000a). In the present study, it was observed that the absorptive transport of CPT-11 was not concentration-dependent, suggesting the involvement of passive membrane diffusion as the dominating process. The absorptive $P_{\text{eff}}$ was estimated to be $1.3 \pm 0.2 \times 10^{-7} \text{ cm/s}$, which is well below the suggested threshold value ($1.0 \times 10^{-5} \text{ cm/s}$) for poorly absorbed compounds (Rubas et al., 1993) and consistent with the low oral bioavailability of CPT-11 observed in clinical trials. In contrast to absorptive transport, the secretory transport appeared to be concentration-dependent and saturable suggesting the involvement of a transporter or multiple transporters. The efflux of CPT-11 was competitively but relatively weakly inhibited by a common Pgp substrate, etoposide ($K_i = 215.4 \pm 39.9 \mu M$), suggesting involvement of multiple efflux transporters including Pgp in the efflux of CPT-11. The activation energy for efflux transport was $\sim 5$-fold greater than 4 kcal/mol (a threshold value for active transporter-mediated process), suggesting that the secretion of CPT-11 is an active and energy-dependent process (Hidalgo and Borchardt, 1990).

Directional studies showed that the secretory $P_{\text{eff}}$ of CPT-11 is 10- to 100-fold greater than its absorptive $P_{\text{eff}}$, suggesting that the efflux of CPT-11 may limit the oral absorption of CPT-11. Previous studies have reported that both Pgp and cMOAT may be involved in the low and variable oral absorption of CPT-11, the absorptive and secretory transport mechanisms of CPT-11 were investigated in the present study. In this study, the macroscopic processes (i.e., absorptive and secretory transport independent of specific mechanism) were investigated using Caco-2 cells, a well established cell culture model (Artursson et al., 2001) for the study of drug intestinal absorption and secretion. To study the involvement of specific transporters in the absorptive or secretory efflux of CPT-11, MDCK II cells overexpressing efflux transporters Pgp, cMOAT and MRP1 were used. Detailed kinetic analysis of individual transporters involved was carried out using these in vitro models. This has lead to mechanistic understanding of the process of CPT-11 oral absorption and impact of transporters on oral bioavailability.

Fig. 6. Inhibition of irinotecan efflux transport across MDCK II/Pgp cells in the presence of etoposide (A), verapamil (B), GF120918 (C), and across MDCK II/ cMOAT cells in the presence of MK571 (D).

Each point represents the mean (±S.D.) for at least three observations.
Increased significantly by Pgp inhibitors GF120918 (IC50 0.05 μM) diffusing into cytosol. IC50 values observed in the present study favors that it directly interacts with drug within the membrane environment and moves drug from the inner to the outer leaflet of the bilayer before it is extruded more efficiently. One mechanism of action proposed for Pgp is from the AP side than from the BL side, therefore reducing drug inhibitor molecules can access the efflux transporters more readily.

In summary, the present study provides a rationale for the poor absorption of CPT-11 observed in clinical trials. Furthermore, since the current results implicate Pgp and cMOAT in the oral absorption of CPT-11, variable expression of these transporters in the intestine may also be responsible for the high variability observed in clinical trials. Due to the expression of Pgp and cMOAT in the liver and kidney, it is possible that first pass metabolism may contribute significantly to the low oral absorption of CPT-11 (Zamboni et al., 1998). Moreover, it has been reported that efflux transporters and metabolism enzymes may be coordinately up-regulated upon treatment with drugs (Schuetz et al., 1996), which may potentiate the first pass effect synergistically. Since CPT-11 is eliminated through both biliary and urinary routes, Pgp and cMOAT in the liver and kidney may also influence the oral bioavailability of CPT-11 to certain extent. Therefore, the variable oral absorption of CPT-11 is likely related with the variation in the expression levels of efflux transporters and/or metabolic enzymes in the intestine, liver, and kidney.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>MDCK II/Pgp (Mean ± S.D.)</th>
<th>MDCK II/cMOAT (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorption</td>
<td>Efflux</td>
</tr>
<tr>
<td>GF120918</td>
<td>0.21 ± 0.05</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>Verapamil</td>
<td>29 ± 6</td>
<td>234 ± 48</td>
</tr>
<tr>
<td>Etoposide</td>
<td>468 ± 56</td>
<td>1185 ± 190</td>
</tr>
<tr>
<td>MK-571</td>
<td>N.E.</td>
<td>N.E.</td>
</tr>
</tbody>
</table>

\*IC50 is defined as the concentration of inhibitor corresponding to 50% of change in permeability.

N.E., No effect.
N.D., Not determinable.

**Acknowledgments.** We thank Drs. Borst, Koch, and Polli for generously providing the MDCK II cell lines, MRP inhibitor MK-571, and the Pgp inhibitor GF120918, respectively. We also thank Melissa Pao and Jennifer Thomas for assistance with HPLC analysis.

**References**


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