Characterization of the Regional Intestinal Kinetics of Drug Efflux in Rat and Human Intestine and in Caco-2 Cells

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Purpose. The aim of the present study was to investigate the transport kinetics of intestinal secretory processes in the jejunum, ileum and colon of rats and humans and in Caco-2 cells, *in vitro*.

Methods. Etoposide, vinblastine sulphate and verapamil hydrochloride were chosen as model substrates since they have been reported to undergo efflux in various other tissues. The concentration dependence, inhibition, directionality, temperature dependence, proton/sodium dependence, and ATP dependence of efflux were studied using side-by-side diffusion chambers and brush border membrane vesicles (BBMVs). Intestinal tissue from rats and humans and Caco-2 cells (passage no. 26) were used. Directional steady state effective permeabilities were calculated from drug appearance in the apical (AP) or basolateral (BL) chambers. Kinetic studies were carried out by investigating substrate efflux at concentrations ranging from 0.2 μM to 1000 μM. Since substrate efflux may be a result of more than one transporter, the hybrid efflux Km (Michaelis-constant), Pc (carrier-mediated permeability), and Pm (passive permeability) were determined as a function of intestinal region. Inhibitor studies were performed using quinidine (0.2 mM), a mixed inhibitor of P-glycoprotein (Pgp) and Multidrug Resistance-Associated Protein (MRP), and Leukotriene C_4 (100 nM), an inhibitor of MRP and the canalicular multispecific organic anion transporter (cMOAT). Temperature dependent efflux was determined by investigating the BL to AP transport at temperatures ranging from 3°C to 37°C. Energies of activation (Ea) were determined from an Arrhenius analysis. Sodium, proton, and ATP dependence were determined using BBMVs. Immunoquantitation of Pgp, MRP and Lung Resistance Protein (LRP) in Caco-2 cells were carried out using Western blot analysis.

Results. Active efflux of all substrates was observed in all regions of rat and human intestine and in Caco-2 cells. Directionality was observed with BL to AP transport exceeding AP to BL transport. The BL to AP/AP to BL permeability ratio, the efflux ratio, ranged from 1.4 to 19.8. Ileal efflux was significantly higher ($p < 0.001$) than in other regions. Kinetic studies revealed that hybrid efflux Km values ranged from 4 to 350 μM. In some cases, efflux was not saturable due to the solubility limits of the compounds utilized in this study. In presence of inhibitors, efflux ratios approached 1. BL to AP transport was temperature dependent in rat ileum for all substrates. Ea of intestinal efflux was found to be 11.6, 8.3, and 15.8 kcal/mole for etoposide, vinblastine and verapamil, respectively, suggesting an active, energy-dependent efflux mechanism. Substrate efflux was not sodium or proton dependent but was dependent on ATP. Using Western blot analysis the presence of Pgp, MRP, and LRP was demonstrated in Caco-2 cells and the amount of each transport protein varied as a function of passage number.

Conclusions. Using multiple putative efflux substrates, the current results demonstrate that intestinal efflux was regionally dependent, mediated by multiple efflux transporters, the Km's were in the micromolar range, and involved an energy dependent mechanism(s).

KEY WORDS: intestinal transport kinetics; drug efflux; jejunum; ileum; colon; Caco-2 cells; vinblastine sulphate; verapamil hydrochloride; etoposide.

INTRODUCTION

Drug resistance to chemotherapeutic agents is a major obstacle in treatment of many human malignancies such as AIDS and cancer. Multidrug resistance (MDR) has been found to be an important and common cause of drug resistance in experimental systems. One definition of MDR is the ability of cells exposed to a single cytotoxic agent to develop resistance to a broad range of structurally and functionally unrelated drugs due to enhanced drug efflux. MDR is mediated by the increased expression of energy-dependent drug efflux pumps such as P-glycoprotein (Pgp), MDR-associated protein (MRP1) and lung resistance protein (LRP) in cancer cells (1–3). In normal human tissues, Pgp (170-kDa protein) is found in kidney, adrenal, brain vessels, muscle, lung, pancreas, liver, intestine, placenta, testis and stomach (4). The distribution of MRP1 (190-kDa protein) overlaps significantly with Pgp and is expressed in the kidney, adrenal, lung, pancreas, muscle, intestine, thyroid and prostate. Interestingly, MRP1 is not expressed in the liver (5). cMOAT (i.e., cMRP or MRP2), responsible for the active excretion of amphipathic anionic conjugates formed by phase II conjugation into bile, is also found in the intestine (6,7). LRP (110-kDa protein) is found in kidney, adrenal, heart, lung, muscle, thyroid, prostate, bone marrow, testis and digestive tract (5,8). The roles of cMOAT, LRP, MRP and Pgp as efflux transporters in normal tissues such as the intestine and blood-brain barrier is controversial and the subject of numerous ongoing investigations.

The clinical importance of intestinal secretory transport remains controversial although a few reports support the contention that oral bioavailability (BA) may be reduced by active intestinal secretion for some drugs. For example, it has been suggested that significant decreases in BA resulting from intestinal cytochrome P450 (CYP) 3A-mediated metabolism of drugs such as cyclosporine are complemented by active secretion mediated by Pgp (9). Also, the low oral BA of CYP 3A substrates, such as tacrolimus, has been ascribed to the inhibition of intestinal metabolism or to the inhibition of Pgp-mediated efflux (10).

Although numerous investigators have demonstrated polarized vectorial efflux transport of numerous compounds through models of intestinal transport (11–15), a detailed, mechanistic and kinetic investigation of intestinal secretory transport has not yet been reported. In order to begin assessing the effect of intestinal efflux on oral BA, an understanding of the saturability and capacity of these systems and the regional dependence of their activity is required. The aim of the present study was to investigate the mechanisms and kinetics of intestinal secretory transport processes in the jejunum, ileum and colon of rats, humans and in Caco-2 cells.

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MATERIALS AND METHODS

Materials

Pgp, MRP and LRP monoclonal antibodies, C219, MRPr1 and LRP-56 were obtained from Signet (Dedham, MA). ^{3}H-etoposide and ^{3}H-vinblastine sulphate were obtained from Moravek Biochemicals (Brea, CA). ^{3}H-verapamil hydrochloride was obtained from American Radiolabeled Chemicals (St. Louis, MO). All other materials were obtained from Fisher Scientific (Fair Lawn, NJ) or Sigma Chemical Co. (St. Louis, MO) and were used as received.

Cell Culture

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD) at passage 18. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 5% penicillin (100 U/mL) and streptomycin (100 mg/mL), in an atmosphere of 95% air and 5% CO_2 at 37°C. Stock cells were passaged (passage 25-90) every three to five days by trypsinizing cells at 90% confluency using 0.05% trypsin and 0.053 mM EDTA at 37°C for 10 min. To seed cells onto the Snapwell™ polycarbonate filters with 0.4 μm pore diameter (Corning-Costar Corporation, Cambridge, MA), cells were harvested at 90% confluency and seeded at a density of 60,000/cm^2. All cells used for transport were cultured in parallel under identical conditions for four weeks.

Diffusion Studies

Side-by-side diffusion chambers were used (NaviCyte, Inc., Sparks, NV) in these studies. Low volume chambers were used for tissue studies and regular volume chambers were used for studies with Caco-2 cells. For low volume chambers, the exposed tissue surface area was 0.636 cm^2, and the volume of each half-chamber was 1.7 mL. For regular volume tissue culture chambers, the exposed surface area was 1.13 cm^2 and the volume of each half-chamber was 5 mL. Mixing in the chambers was controlled through a gas lift mechanism. Fasted, male Sprague-Dawley rats (250–350 g) were anesthetized by intramuscular injection of pentobarbital (30 mg/kg). Human tissue was acquired from the GI Surgical Unit at Robert Wood Johnson Hospital, New Brunswick. Caco-2 cells were cultured as described previously. All studies were performed in accordance with protocols approved by the Institutional Review Board-Use and Care of Animals at Rutgers University. Human tissue studies were performed in duplicate using multiple donors. A 1.5 to 2 cm strip of intestinal tissue (jejunum/ileum/colon) was excised, rinsed free of lumenal contents using Ringers buffer (pH 7.4) and mounted onto a diffusion half-chamber. The serosal muscle layers were stripped. The mucosal and serosal reservoirs were then filled with appropriate buffer (the mucosal buffer was pH 6.5 MES Ringer's buffer and serosal buffer was pH 7.4 Ringer's buffer, 290 mOsm/kg) and gas lifts were connected. Temperature was maintained at 37°C throughout the experiment. Samples were taken at 30, 45, 60, 75, 90 and 105 min.

Concentration Dependence

Concentration dependence studies were carried out by investigating the BL to AP transport of the three putative efflux substrates using concentrations ranging from 0.2 μM to 1000 μM. The effective permeability (cm/s) was determined using Equation 1:

$$Pe = \frac{dC/dt}{C * SA/V * 60} \quad (1)$$

where Pe is the effective permeability in cm/s, dC/dt is the slope of plot of concentration (mM) versus time (min), C is the concentration of drug in mM, SA is the surface area of the chamber in cm^2, V is the volume of each half-chamber in mL. Using nonlinear regression (Scientist v2.01, Micromath Software), kinetic values were obtained using the following equation:

$$Pe = \frac{Pc}{1 + C/Km} + Pm \quad (2)$$

where Pc is the carrier permeability (= Jmax/Km), Km is the Michaelis constant, and Pm is the nonsaturable membrane permeability, respectively. Weighted fit was performed using 1/SD as the weighting factor.

Inhibitor Studies

AP to BL and BL to AP transport of etoposide (10 μM) was investigated in the intestinal segments of rat in presence of quinidine (0.2 mM), LTC_4 (100 nM) and combination of quinidine (0.2 mM) and LTC_4 (100 nM). Quinidine has been reported to inhibit multiple efflux pathways including Pgp and MRP(16). LTC_4 has been reported to be an inhibitor of MRP(17,18) and cMOAT(6,7). The tissue was pre-incubated with the inhibitor for 20 min prior to the transport experiment. After 20 min, the inhibitor solution was removed and transport experiments commenced. During the course of the experiment, the same concentration of inhibitor(s) was present in both chambers.

Directionality Studies

For investigation of AP to BL transport, drug (spiked with a tracer amount of ^{3}H labeled drug) was placed in the mucosal side and for the investigation of BL to AP transport, drug was placed in the serosal side. The concentrations of etoposide, vinblastine and verapamil used were 10 μM, 0.1 μM, and 1 μM, respectively. Drug efflux ratios were calculated using the following equation:

$$E = \frac{Pe_1}{Pe_2} \quad (3)$$

where E = Efflux ratio, Pe_1 = BL to AP effective permeability, Pe_2 = AP to BL effective permeability.

Temperature Dependence

BL to AP transport of etoposide (10 μM), vinblastine (0.1 μM) and verapamil (1 μM) were investigated in rat ileum at the following temperatures, 3°C, 8°C, 15°C, 25°C and 37°C. Arrhenius plot of log Pe versus 1/T was constructed where T = absolute temperature (T°C + 273.16°C).

$$slope = -\frac{Ea}{2.303R} \quad (4)$$

Where Ea = Energy of activation in kcal/mole, R = gas constant = 0.001987 kcal/deg mole.

Brush Border Membrane Vesicles

Rat intestinal brush border membrane vesicles (BBMV) were prepared using a previously described method (19). A one-cycle freeze-thaw method (20) was used to preload the vesicles otherwise the experimental design is identical to that previously described (19,21). An ATP-generating solution (3 mM creatinine phosphate, 3 mM ATP, 3.6 μg/mL creatinine phosphokinase, and 5 mM $MgCl_2$) was added into vesicles loaded with 0.01 mM etoposide. The efflux buffer consisted of 150 mM sodium chloride and 20 mM Hepes, pH 7.5. For the sodium-dependence studies, 150 mM choline chloride was substituted for sodium chloride. For the proton gradient studies, the extravesicular pH was adjusted to pH 6.0 and the pH in the loading solution of the vesicles was pH 7.5. For the ATP dependence studies, the control study did not contain the ATP-generating system.

Immunoblotting

Caco-2 cells were cultured in T-75 flasks for 21–25 days. Cells were harvested by treatment with 0.05% of trypsin and 0.53 mM EDTA. The cells were rinsed twice in Ringers solution (pH 7.4) containing 25 mM glucose and centrifuged at 300×g for 10 min. Brush-border-enriched proteins were prepared with Ca^{2+} precipitation method (20). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard. Thirty five μg of protein was loaded in each lane, electrophoresed on 7% SDS-PAGE by the method of Laemmli (22) and transferred to nitrocellulose membrane. For MRP and LRP, Western blot was performed as described by Flens et al. (23). The blot was incubated for 1 hr with antibodies MRPr1 and LRP-56 at 1 μg/ml and 0.5 μg/ml, respectively. For Pgp, Western blot was performed as described by Muller et al. (24). The blot was incubated sequentially with human monoclonal antibody of C219 (8 μg/ml). Blots were incubated with either anti-mouse (for MRP and LRP) or anti-rat (for Pgp) immunoglobulin (1:10,000) for 1 hr. Pgp, MRP and LRP were detected with enhanced chemiluminescence system (Amersham, Downers Grove, IL) and exposed on Kodak film. The expression levels were quantified with a Bio Image Intelligent Quantifier (Millipore Corporation). The specificity of the C219 and LRP antibodies is very high and they do not cross react with other cellular proteins. The specificity of the MRP antibody is lower and it cross reacts with a 80 kd protein. Molecular weight markers (30 kd to 205 kd, Sigma) were used for all SDS polyacrylamide gels.

Statistical Analysis

All statistical tests were performed using Jandel Sigma stat version 2.0 (San Raphael, CA). A minimum P-value of 0.05 was used as the significance level for all tests. One way ANOVA and Tukey tests were performed on permeability data. All data are reported as the mean ± standard deviation (SD) unless otherwise noted.

RESULTS AND DISCUSSION

Investigating the intestinal secretory transport of drugs has gained considerable interest recently (11–15, 25–31). Numerous compounds such as β-lactam antibiotics, celiprolol, digoxin, DMP 728, etoposide, quinidine, ritonavir, saquinavir, verapamil, vinblastine, and others have been reported to be actively secreted by intestinal efflux systems including Pgp, the mdr-1 gene product. It has been reported that cationic compounds such as verapamil, chlorpromazine and propantheline are transported by plural Pgp-mediated efflux systems with different substrate specificities depending on the intestinal site (27). Polarized transport of digoxin, docetaxel, ritonavir, saquinavir, and vinblastine has been reported to be mediated by Pgp in human intestinal epithelial cell monolayers (11,16,30,31). Based on this body of work, it has been suggested that the intestinal secretory transport of drugs mediated by efflux pumps may act as an absorption barrier resulting in reduced oral bioavailability for certain drugs. However, to date a detailed mechanistic and kinetic investigation has not been carried out. In the present study, the intestinal efflux kinetics and regional-dependence of transport are reported using three known efflux substrates (13–15): etoposide, vinblastine and verapamil.

Concentration Dependence

The concentration dependent efflux of etoposide, vinblastine, and verapamil was investigated in rat and Caco-2 cells, passage number 26. Generally, efflux permeability was concentration dependent and saturable. However, saturation was not achieved in the ileal segment of rat for etoposide and vinblastine due to their limited solubility. Figures 1a and 1b depict the concentration dependence of etoposide efflux permeability in rat jejunum and colon, respectively. A summary of the Km, Pc (i.e., Jmax/Km) and Pm values for all the three drugs in rat tissue and for etoposide in Caco-2 cells are shown in Table 1a–c. Km values were found to be in the micromolar range varying from 4 μM to 329 μM depending on the drug and intestinal site. In Caco-2 cells, the Km value for etoposide was found to be higher than that observed in rat indicating a lower affinity for the efflux transporter(s). It appears that the efflux carrier systems in the ileum have a higher capacity and lower affinity than the colon or upper small intestine since etoposide and vinblastine efflux were not saturable up to the millimolar range. These values compare well with reported values in other systems. For example, the Km value for etoposide in membrane vesicles prepared from NIH 3T3 MRP transfectant pSRα-MRP-32 cells has been reported to be 4.4 μM (32). The Km value for vinblastine has been reported to be 18.99 μM in Caco-2 cells (14). In the present study, the Km values for vinblastine were found to be 328.9 μM and 96.8 μM in the rat jejunum and colon, respectively. These values compare quite well considering that Caco-2 cells are functionally similar to human fetal colon cells.

Directionality

BL to AP transport was found to be significantly higher ($p < 0.001$) than AP to BL transport for etoposide, vinblastine, and verapamil in rat, human intestine and Caco-2 cells. Shown in Figure 2 and Table 2 are a representative plot for etoposide and a summary of the efflux ratio results, respectively. The

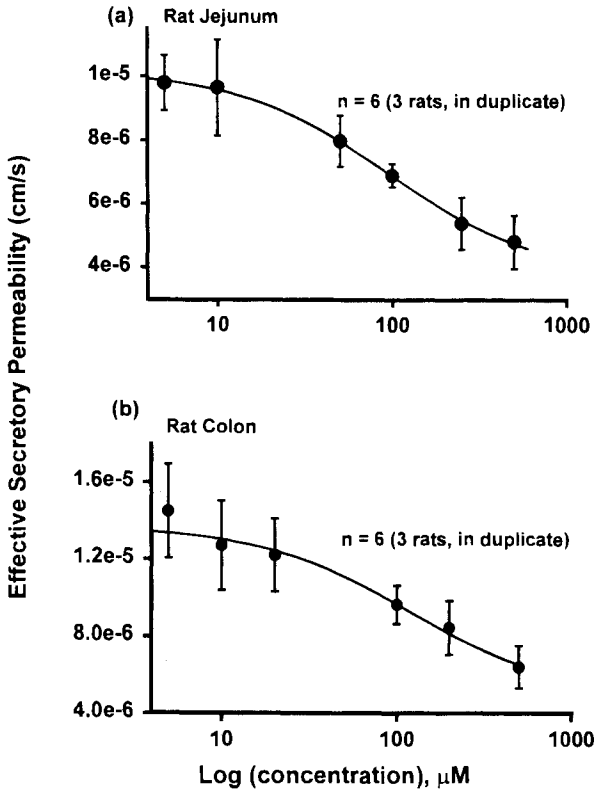

Fig. 1. Concentration dependence efflux curve. Plot of effective secretory permeability (mean ±SD) and the best fit line versus log (concentration). BL to AP transport of etoposide in rat (a) jejunum and (b) colon was investigated at various concentrations ranging from 1 μM to 500 μM. Michaelis-Menten efflux parameters were determined using weighted (1/SD) nonlinear regression and are reported in Table 1.

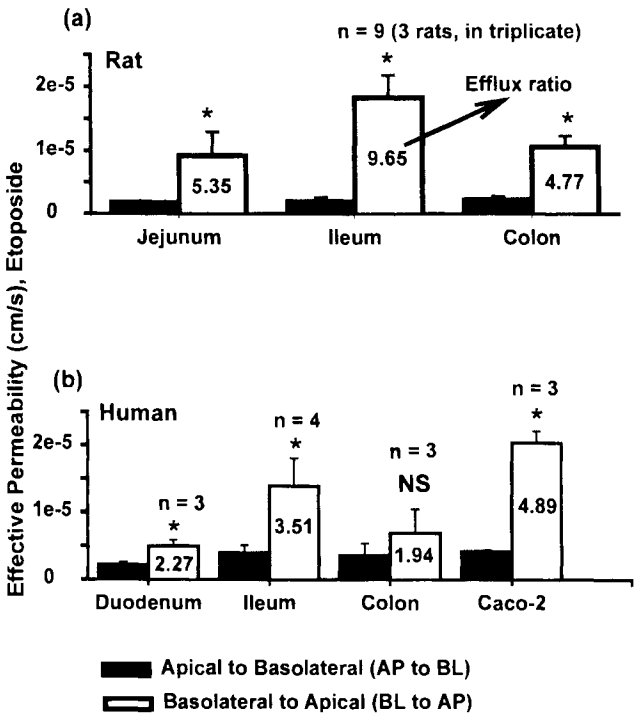

Fig. 2. Representative directionality study for etoposide. The AP to BL and BL to AP transport of etoposide in (a) rat and (b) human intestinal tissues and Caco-2 cells (passage 26) (10 μM and 5 μM, respectively) were investigated. Plot of effective permeability versus site (jejunum, ileum, colon). * Indicates that BL to AP permeability is significantly different from AP to BL permeability, $p < 0.001$. NS indicates not significantly different.

Table 1. Kinetic Parameters for the Efflux Transport of Etoposide in Rat and Caco-2 Cell (Passage No: 26), Vinblastine in Rat and Verapamil in Rat

(a) Etoposide efflux in rat and Caco-2 cell (passage no:26). Site	Km (μM)	Pc (cm/s)	Pm (cm/s)
Rat jejunum	94.16	6.49e-6	3.70e-6
Rat ileum	could not saturate	-	-
Rat colon	118.99	8.88e-6	4.83e-6
Caco-2 cells	212.96	1.28e-5	8.21e-6
(b) Vinblastine sulphate in rat. Site	Km (μM)	Pc (cm/s)	Pm (cm/s)
Rat jejunum	328.89	1.18e-6	1.06e-7
Rat ileum	could not saturate	-	-
Rat colon	96.81	2.81e-6	1.29e-6
(c) Verapamil hydrochloride in rat. Site	Km (μM)	Pc (cm/s)	Pm (cm/s)
Rat jejunum	30.82	8.83e-6	1.99e-5
Rat ileum	28.53	4.02e-5	3.00e-5
Rat colon	4.41	2.43e-5	2.05e-5

efflux permeability was significantly higher in all regions in rat and Caco-2 cells. It was observed in rat that the efflux permeability of etoposide was greater than vinblastine and verapamil. Significant efflux was observed in human duodenum and ileum, however, not in the colon. Due to the limited availability of fresh human duodenal tissue, studies were not performed with vinblastine and verapamil. In human ileum and in Caco-2 cells, the efflux permeability of vinblastine was found to be greater than etoposide and verapamil. Consistent with the concentration dependence and inhibition results, the efflux ratios in the rat and human ileal segments were significantly higher than other segments for all three drugs. These results indicate that the regional activity of efflux transporters in the ileal segment is higher than the activity in the duodenum/jejunum or colon.

Inhibition Studies

The BL to AP transport of etoposide (10 μM) in rat-jejunum, ileum and colon was investigated in presence of two well-studied mixed mechanism efflux inhibitors, quinidine and LTC_4. Quinidine (0.2 mM) is a known mixed efflux inhibitor. It inhibits Pgp, MRP and, possibly, other efflux pumps (16). LTC_4 (100 nM) is reported to be a specific inhibitor of MRP and MRP2 (cMOAT) (17,18). A combination of quinidine and LTC_4 was also used in these studies. These results are plotted in Figure 3. Inhibition was clearly demonstrated by reduction in the efflux ratio (i.e., the efflux ratios approached unity in

Table 2. Ratio of Permeability of Basolateral to Apical/Apical to Basolateral[b]

Species/Site	Etoposide[a]	Etoposide[a]+ Quinidine[a]	Etoposide[a]+ LTC_4[a]	Etoposide[a]+ Quinidine[a] + LTC_4[a]	Vinblastine sulphate[a]	Verapamil hydrochloride[a]
Human duodenum	2.27	—	—	—	—	—
Human ileum	3.52	—	—	—	19.84	2.76
Human colon	1.95	—	—	—	1.44	0.88
Caco-2	4.89	—	—	—	4.89	1.36
Rat jejunum	5.35	1.86	1.88	1.00	1.96	2.50
Rat ileum	9.66	1.00	3.95	1.55	3.27	3.61
Rat colon	4.77	1.00	1.63	1.00	2.08	3.07

[a] Concentrations: etoposide, 10 μM; quinidine, 0.2 mM; LTC4 100 nM; Vinblastine sulphate, 0.1 μM; Verapamil hydrochloride, 1 μM.
[b] All data show that BL to AP permeability is statistically significantly different form AP to BL permeability ($P < 0.001$), except human colon.

presence of inhibitors). Since an efflux ratio equal to unity is interpreted as having equal directional fluxes and there was no apparent concentration dependent absorptive flux for etoposide (not shown), these results suggest inhibition of a carrier-mediated efflux system. Efflux ratios in the presence and absence of inhibitors in the jejunal, ileal, and colonic segments of rat are depicted in Table 2. Quinidine completely inhibited etoposide efflux in the ileal and colonic segments. The efflux ratios approached unity due to an inhibition of efflux resulting in lower BL to AP permeability (Fig. 3) and higher AP to BL permeability. The rat ileum-quinidine result is apparently inconsistent with the other results and no apparent rationale can be offered. Efflux ratios were reduced significantly ($p < 0.05$) in presence of LTC_4 in rat jejunum, ileum, and colon compared to control. When the two inhibitors were used in combination, the efflux was completely inhibited in the jejunal and colonic segments. Ileal efflux was significantly inhibited but not completely inhibited consistent with the concentration dependence study results that showed that the ileal segment was not saturable. Additional work is required in identifying the function of and determining the regional differences in the expression of

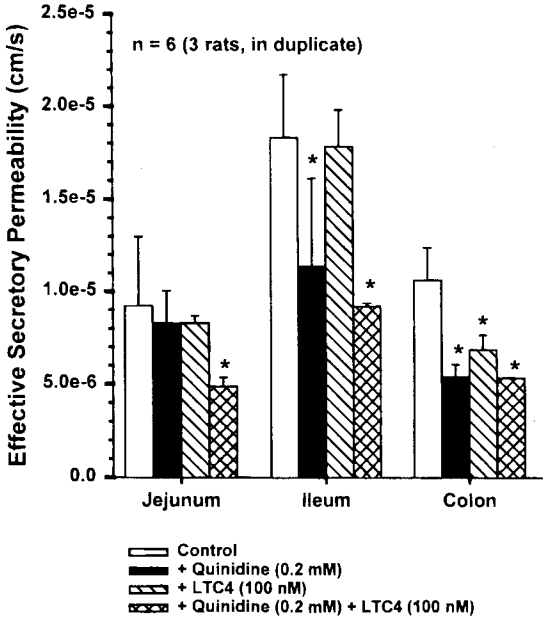

Fig. 3. Rat intestinal tissues (jejunum, ileum, colon) were pre-treated with the putative inhibitors: quinidine (0.2 mM), LTC_4 (100 nM) and combination of quinidine and LTC_4 for 20 min prior to the experiment. BL to AP transport of etoposide (10 μM) was then investigated in presence of the inhibitors. Plot of effective permeability versus intestinal site. * Indicates that BL to AP (in presence of inhibitor/s) is significantly different from BL to AP (control), $p < 0.05$.

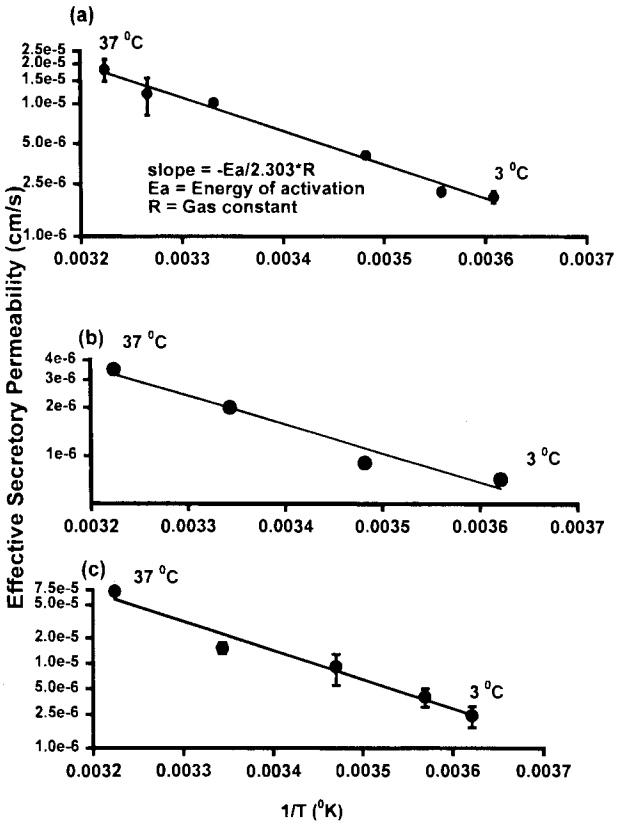

Fig. 4. Temperature dependence of (a) etoposide (10 μM) (b) vinblastine sulphate (0.1 μM) and (c) verapamil hydrochloride (1 μM) secretory permeability in rat ileum. BL to AP transport of etoposide was investigated at various temperatures ranging from 3°C to 37°C. Secretory permeability values were determined and an Arrhenius plot of log (Permeability) versus 1/T (T = absolute temperature) was constructed. Energy of activation, Ea was calculated from slope of the plot.

these efflux transporters. However, limitations in current technology (e.g., availability of truly specific inhibitors or inhibitory monoclonal antibodies) hamper these efforts.

Temperature Dependence

In order to determine the energy dependence of efflux transport, the BL to AP transport of the three drugs was investigated in rat ileum at various temperatures ranging from 3°C to 37°C. Rat ileum was selected since it had among the highest efflux transporter activity of the tissues and cells studied. Efflux was found to be temperature dependent for all three drugs. BL to AP transport decreased with decreasing temperature, indicating involvement of an active efflux transporter(s). An Arrhenius plot of log Pe versus 1/T was constructed for the three drugs (Figures 4a–c). In rat ileum, energy of activation (Ea) values were found to be 11.6, 8.3 and 15.8 kcal/mole for etoposide (10 μM), vinblastine (0.1 μM) and verapamil (1 μM) respectively. Ea values greater than 4 kcal/mole indicate active, energy dependent efflux transport (33).

Sodium, Proton and ATP Dependence

Etoposide efflux studies were also carried out using brush border membrane vesicles (BBMV) prepared from rat jejunum. Sodium-, proton-, and ATP-dependence (Figure 5a–c) of efflux was investigated. It was observed that efflux was not sodium or proton dependent, but was ATP dependent confirming the results of the temperature dependence studies. There was some difference in efflux in the presence or absence of sodium although the difference was not statistically significant. The result that efflux was not proton dependent is consistent with a previous finding (34) that intracellular pH did not affect drug extrusion by P-glycoprotein.

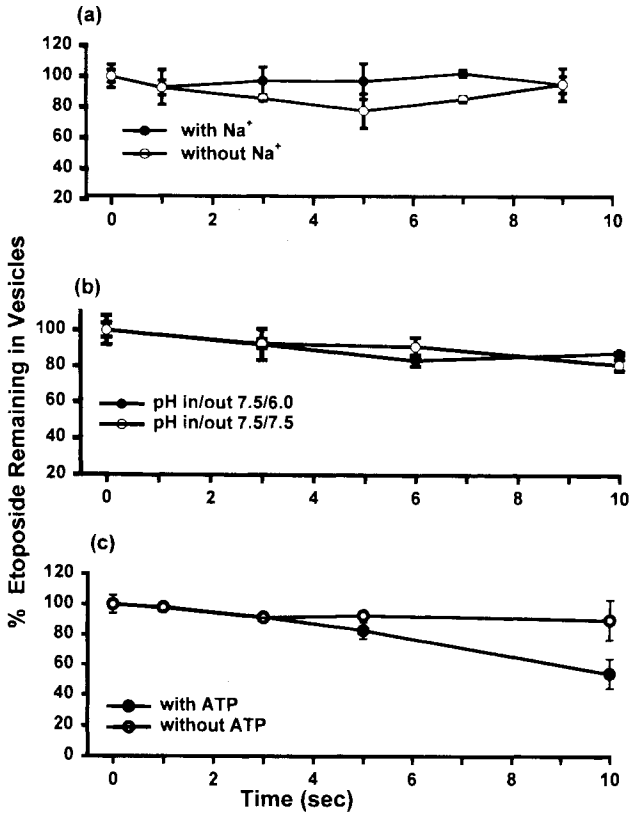

Fig. 5. (a) Sodium-, (b) proton-, and (c) ATP- dependence of etoposide (0.01 mM) efflux in rat jejunal BBMV. See text for experimental details.

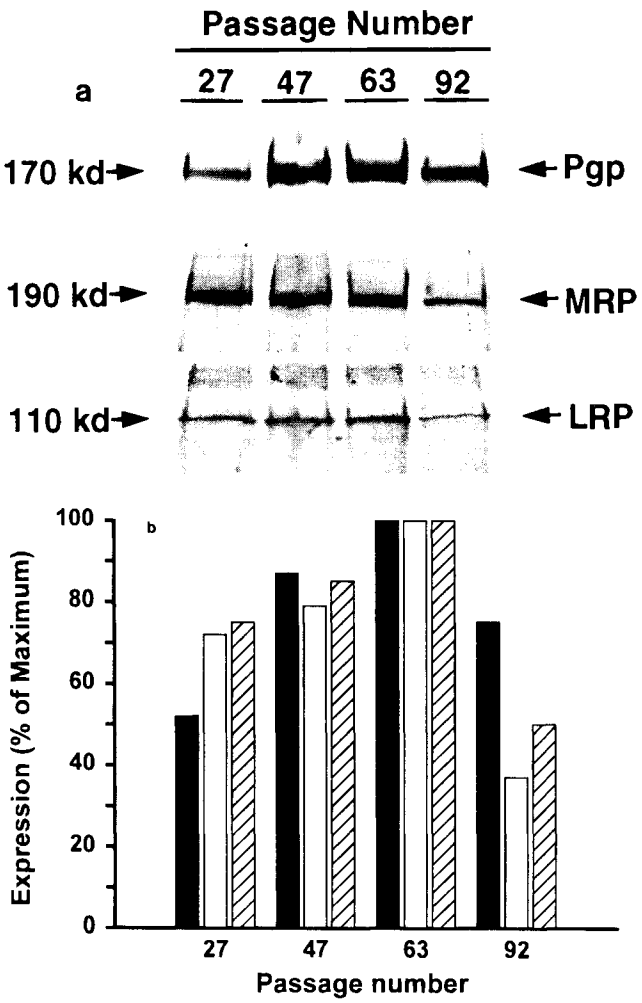

Fig. 6. (a) Western blot analysis of Pgp, MRP and LRP in Caco-2 cells (passage numbers 27, 47, 63 and 92). (b) Plot of Relative expression (% of Maximum) versus passage number.

Immunoblotting

The presence of Pgp, MRP and LRP was confirmed in Caco-2 cells using Western blot analysis. The expression of the transporters varied depending on the passage number (Figure 6a). The expression level of transporters increased up to passage number 63 and after passage 63, the expression was found to decline (Figure 6b). It has been previously reported that expression and activity of Pgp varied with some culturing conditions in Caco-2 cell monolayers (35). It is difficult to compare the functional expression of the individual transporters with the immunoquantitation results given that specific inhibitors are not well characterized or readily available.

CONCLUSIONS

In order to determine if efflux transporters truly limit the oral bioavailability of drugs, a kinetic investigation is required as a starting point. The results of our studies indicate that active and saturable efflux of drugs does take place in rat and human intestine and in Caco-2 cells. A mechanistic and kinetic investigation of efflux revealed that the efflux process is carrier-mediated and energy-, species-, substrate-, and site-dependent. Efflux appears to be mediated by Pgp, MRP and possibly other efflux pumps, however, the specific roles cannot be determined given the limitations of current technology.

Most recent reports have implicated intestinal drug efflux in the reduced oral BA of HIV protease inhibitors, digoxin, and numerous other drugs based only on directional flux studies (16,36). However, key kinetic parameters that give an indication of saturability (Km), capacity (Pc or Jmax) and regional dependence are required to make an assessment of the importance of efflux as it relates to oral bioavailability. The current results begin to clarify the role of efflux in determining oral drug bioavailability, however, critical questions remain unanswered. On the one hand, with the exception of the ileal segment, it appears that efflux may be saturable at typical drug dosages suggesting that efflux may not play an important role. On the other hand, the efflux carriers appear to have activity along the entire length of the intestinal tract unlike "absorptive" carriers which are generally limited to the small intestine. This suggests that there is a much higher probability that an efflux event may occur. Therefore, the importance of these efflux transporters in reducing oral drug bioavailability is still not clear. There is some indication, however, that efflux transporters may affect the *in vivo* disposition of drugs (9,10,13,37,38). For example, Leu and Huang demonstrated that the serum levels of intestinally administered etoposide in rats increased in the presence of intravenously administered quinidine (13). Also, certain drugs originally intended to treat peripheral symptoms owing to their inability to enter the brain, were found to regain CNS activity when the Pgp barrier was removed or reduced (37,38). Therefore, the role of efflux in the *in vivo* disposition of drugs requires further investigation.

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