IN VITRO AND IN VIVO EVALUATIONS OF INTESTINAL BARRIERS FOR THE ZWITTERION L-767,679 AND ITS CARBOXYL ESTER PRODRUG L-775,318

Roles of Efflux and Metabolism

THOMAYANT PRUEKSAINTANONT, POLLY DELUNA, LYNN M. GORHAM, BENNETT MA, DOSINDA COHN, JIANMEI PANG, XIN XU, KWAN LEUNG, AND JIUNN H. LIN

Department of Drug Metabolism, Merck Research Laboratories

(Received October 16, 1997; accepted February 4, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:
The barriers to oral delivery of the hydrophilic zwitterion L-767,679 (I) and its carboxyl ester prodrug L-775,318 (II) were examined. In the Caco-2 cell model, transport of II, but not I, was strongly oriented in the secretory direction. The basal-to-apical transport of II displayed saturable kinetics and was markedly inhibited by verapamil and quinidine, known P-glycoprotein inhibitors. In Caco-2 cells, metabolism of I was not observed, whereas hydrolysis of II was modest (<20%). In the in situ rat intestinal loop model, verapamil did not affect the absorption of I but significantly increased the absorption of II. I was resistant to intestinal metabolism, whereas II underwent hydrolysis partially in rat lumen but more extensively in rat intestinal tissue and blood. In vitro metabolism studies indicated that verapamil also inhibited the hydrolysis of II in rats. The inhibition was relatively specific for the intestinal and not the luminal esterases. These results suggested that the intestinal absorption of I was limited not by intestinal efflux or metabolism but more likely by the low lipophilicity of I. However, an efflux system, likely mediated by P-glycoprotein, played an important role in limiting the absorption of II. In rats, metabolism served as an additional barrier to the absorption of II. Verapamil increased the intestinal absorption of the prodrug by inhibiting the efflux system in the two models studied, as well as possibly inhibiting metabolism in rats. For the first time, secretory transport was identified as a cause of the failure to increase the absorption of a lipophilic and cationic prodrug developed to overcome the absorption problem.

Hydrophilic and ionic compounds generally possess low membrane permeability and therefore are poorly absorbed after oral administration. For some drugs, metabolism has also been identified as an important intestinal barrier (Krishma and Klotz, 1994; Friedman and Amidon, 1991; Prueksaritanont et al., 1996a). More recently, it has been increasingly recognized that the intestinal absorption of a compound could also be limited in part by intestinal efflux systems (Hunter et al., 1993a; Karlsson et al., 1993; Terao et al., 1996; Fricker et al., 1996; Cavet et al., 1996; Lang et al., 1997). P-glycoprotein is a well-recognized secretory transporter localized on epithelia of animal and human tissues, such as intestine, kidney, liver, and adrenal glands, as well as the blood-brain barrier (Thiebaut et al., 1987; Croop et al., 1989; Hsing et al., 1992). Features common to most P-glycoprotein substrates recognized to date include hydrophobicity and an amino group (Ford and Hait, 1990). Active secretory transport has also been implicated for some polar compounds, including anions and small peptides (Saitoh et al., 1996; Burton et al., 1993; Langguth et al., 1997).

L-767,679 (I), a potent fibrinogen receptor antagonist, is a highly polar (log P < -3) and zwitterionic compound containing peptide linkages (fig. 1). In animal models, the low oral bioavailability of I was attributed to poor absorption and not to first-pass metabolism (Prueksaritanont et al., 1997). Although the low intestinal permeation of I was likely the result of low lipophilicity, the possible involvement of intestinal efflux could not be completely ruled out. Nevertheless, a prodrug approach, aiming to improve intestinal permeability by increasing lipophilicity, was undertaken (Prueksaritanont et al., 1997; Hutchinson et al., 1996). For several prodrugs in this structural series, extensive hepatic/intestinal first-pass metabolism to metabolites other
than the corresponding active drugs, and not poor absorption of the prodrugs, was demonstrated to be primarily responsible for the low oral bioavailability of both the prodrugs and their active drugs after administration of the prodrugs to animals (Prueksaritanont et al., 1996a, 1997). However, our preliminary studies in Caco-2 cells indicated that absorption of some of the prodrugs also was limited. Structurally, the cationic and lipophilic prodrugs are potential substrates for P-glycoprotein.

In view of the above discussion, it was desirable to gain some insight into intestinal barriers for the active drug I, as well as its prodrug. The benzyl ester prodrug L-775,318 (II) (fig. 1), a relatively lipophilic prodrug (log P = 0.7), was chosen for the study. In the present study, the intestinal transport mechanism for and metabolism of I and II were examined using an in vitro Caco-2 cell model, in conjunction with known transport modifiers (verapamil and quinidine) and an esterase inhibitor (PMSF) (Ford and Hait, 1990; Wacher et al., 1995; Morgan et al., 1994). The Caco-2 cell system offers considerable advantages, including its human origin and a simple epithelial monolayer structure that enables directional transport studies. This human intestinal cell line also expresses functional transporters, such as dipeptide carriers and P-glycoprotein, and some drug-metabolizing enzymes, which are all known to be present in human small intestines (Fricker et al., 1996; Prueksaritanont et al., 1996b; Hunter et al., 1993b; Adibi, 1997). Although good agreement was observed between Caco-2 cell and in vivo findings, with respect to P-glycoprotein involvement, in several studies (Terao et al., 1996; Fricker et al., 1996; Leu and Huang, 1995), recent results showed that Caco-2 cells underestimated the absorption of P-glycoprotein substrates, compared with in vivo observations (Yee, 1997). Apparently, there are some discrepancies between in vitro and in vivo conditions that are relevant to the contribution of P-glycoprotein to the transport of compounds. Therefore, in this study, the intestinal absorption of I and II was also investigated using an in situ rat intestinal loop technique. Because the intestine is known to contain drug-metabolizing enzymes, including esterases (Friedman and Amidon, 1991; Prueksaritanont et al., 1996a; Kaminsky and Fasco, 1992; Heymann and Mentlein, 1988), the in vitro metabolism of I and II was also examined using rat intestinal S9 preparations, in the absence and presence of verapamil and PMSF. Because of the presence of esterases in the rat lumen (Friedman and Amidon, 1991; Prueksaritanont et al., 1996a; Campbell et al., 1987), similar in vitro metabolism studies were also conducted for II using rat intestinal lumen washes.

### Materials and Methods

**Chemicals.** I \[N-{[7-(piperazin-1-yl)-3,4-dihydro-1(1H)-isoquinolinonine-2-yl(acetyl)-3-(S)-ethylamino]-β-alanine} and its ester prodrug II (fig. 1) were synthesized at Merck Research Laboratories (West Point, PA), as described (Hutchinson et al., 1996). PMSF, verapamil, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO), whereas quinidine was obtained from Aldrich (St. Louis, MO). Solvents used for analysis were of analytical or HPLC grade (Fisher Scientific, Pittsburgh, PA). The human intestinal cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in Opti-MEM medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum, nonessential amino acids, and L-glutamine and were used at passages 20–50.

**In Vitro Caco-2 Cell Studies.** Caco-2 cells, grown on 12-mm Millicel polycarbonate filter inserts (Millipore, Bedford, MA) at 100,000 cells/cm² for 3 weeks, were used throughout. The integrity of the monolayer was monitored by determining transepithelial electrical resistance (320–490 Ω cm²) and lucifer yellow permeation. In addition, the transport of caffeine, a compound known to be well transported transcellularly and well absorbed in vivo, was also monitored. The transport studies with I and II were carried out in triplicate or quadruplicate at 37°C using 1–1000 µM I or II, with an apical compartment pH of 7.4 [10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer] or 5.5 [10 mM 2-(N-morpholino)ethanesulfonic acid buffer] and a basal compartment pH of 7.4 [10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer]. Samples were taken from the recipient compartment at designated times and were replaced with an equal volume of fresh buffer. Calculation of cumulative transport included correction for dilution effects. For studies of the inhibitory effects of verapamil, PMSF, and quinidine, the incubations were performed at pH 7.4 for both donor and recipient compartments, with inhibitor concentrations of 200 µM verapamil, 250 µM quinidine, or 500 µM PMSF. Solutions from the two compartments were analyzed for I and II by the HPLC method described below. Both compounds were adsorbed negligibly to the wells or filters as judged by virtually 100% recovery.

### In Situ Rat Intestinal Loop Studies

The studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (230–320 g) were prepared for the intestinal absorption study according to the procedure of Barr and Riegelman (1970). In brief, a segment of proximal jejunum (about 20 cm long) was isolated and tied off at both ends to form a closed loop. The mesenteric vein that collected blood from the ligated segment of the intestine was cannulated for blood collection. The animal was placed under a heating lamp to help maintain the body temperature at 37°C. Dosing solution (0.5 mg of I or II/2 ml of normal saline), with or without verapamil (500 µM), was injected directly into the lumen of the intestinal loop. Blood samples were collected at appropriate times, and the blood lost from the mesenteric vein was continuously replaced with an equal volume of heparinized fresh rat blood, through the cannulated jejunal vein. At the end of the experiment (1 hr), the remaining contents in the intestinal loop were collected. The loop was rinsed with 2 x 3 ml of saline, and the washes were combined. An aliquot of the intestinal wash was immediately mixed with 3 ml of aceton to stop the hydrolytic reaction. The intestine was homogenized using 4 volumes of saline, and an aliquot was taken and immediately extracted with 3 ml of aceton to terminate the hydrolytic reaction. Blood samples (25–75 µl) were also extracted using 3 ml of aceton. The aceton extracts were evaporated, reconstituted with the mobile phase, and analyzed for I, II, and verapamil by the HPLC methods described below.

### In Vitro Metabolism Studies

Subcellular fractions of rat intestine (N = 3) were prepared as described previously (Prueksaritanont et al., 1996b). Studies of the inhibition of S9 protein, 0.2 µmol of NADPH, 2.5–100 nmol of I or II, 2 µmol of MgCl₂, and 20 µmol of phosphate buffer, pH 7.4. The reaction was terminated, after incubation at 37°C for various times up to 60 min, by the addition of 0.2 ml of acetonitrile. After centrifugation, the supernatant was analyzed by HPLC. The inhibitory effects of verapamil, PMSF, and quinidine on the metabolism of II were investigated using similar conditions but in the presence of verapamil (25–500 µM), PMSF (100–1500 µM), or quinidine (100–2500 µM) and with an incubation time of 10 min. A preliminary study indicated that the hydrolytic reaction was linear during the 10-min incubation.

The rat lumen wash was collected after rinsing of the small intestine (~50 cm long, N = 2) with 2 x 3 ml of normal saline and was used on the same day. Studies on the hydrolysis of II (10–250 µM) in rat intestinal lumen or blood were performed using 0.5 ml of intestinal lumen wash or whole blood, which was also obtained from rats (N = 2) on the day of the experiment. At various times during incubation at 37°C, the reaction was stopped by the addition of 5 ml of aceton. The aceton extracts were evaporated, reconstituted with the mobile phase, and analyzed by HPLC. The effects of verapamil on hydrolysis by the lumen wash were also investigated at a substrate concentration of 250 µM and inhibitor concentrations of 100 and 500 µM.

### Analytical Procedures

A HPLC method for simultaneous determination of I and its prodrug II (Prueksaritanont et al., 1997) was used with minor modifications. The system consisted of a Waters 600E multisolute delivery system, a Waters 717 Plus autosampler, and a Jasco 821-FP fluorescence detector. The sample analysis of I and II was conducted with a Spherisorb SCX column (4.6 x 250 mm, 5 µm), with the mobile phase (solvent A, acetonitrile; solvent B, 0.04 M phosphate buffer, pH 4.2) being delivered at a flow rate of 1 ml/min (maintenance at 27% solvent A for 5 min, gradient to 60% solvent A in 1 min, and maintenance at 60% solvent A for 6 min). The
effluent was monitored at excitation and emission wavelengths of 245 nm and 440 nm, respectively.

Analysis of verapamil concentrations in the intestinal lumen, gut, and blood was also conducted using an HPLC method reported earlier (Busse et al., 1995), with some modifications. The chromatographic system consisted of a C18 Novapak column (4.6 × 150 mm, 5 µm; Waters) and a mobile phase of 50% acetonitrile in 0.05 M phosphoric acid solution containing 1.7% (v/v) triethanolamine. The effluent was monitored using a fluorescence detector (Jasco 821-FP) set at excitation and emission wavelengths of 280 nm and 310 nm, respectively.

Data Analysis. Apparent \( K_{m} \) and \( V_{max} \) values were estimated using a nonlinear regression program (Enzfitter; Biosoft, Ferguson, MO). The intrinsic clearance was estimated by dividing \( V_{max} \) by \( K_{m} \). Determination of the type of inhibition was based on visual inspection of 1) double-reciprocal plots of the data and 2) the patterns of changes in \( K_{m} \) and \( V_{max} \) values in the presence and absence of inhibitors. \( K_{i} \) values for competitive inhibition were then estimated by fitting nontransformed data to the following equation, using a nonlinear regression program (PCnonlin; Scientific Consulting, Cary, NC): \( V = (V_{max} \times S)/(S + K_{m}(1 + [I]/K_{i})) \), where \( S \) and \( I \) represent substrate (\( I \)) and inhibitor (verapamil, PMSF, or quinidine) concentrations, respectively.

Statistical analysis was performed using analysis of variance (Statview; Abacus Concepts, Berkeley, CA). A \( p \) value of \(<0.05 \) was considered statistically significant.

Results

**In Vitro Caco-2 Cell Studies.** For all Caco-2 cell monolayers used, the apical-to-basal transport of caffeine, a positive control for transcellular transport, was \( >18\% \)/hr. In most cases, the apical-to-basal or basal-to-apical transport of lucifer yellow, a compound known to be transported paracellularly, was \( <1\% \)/hr. Transport of \( I \) across Caco-2 cell monolayers was comparable at the apical pH values of 5.5 and 7.4 (data not shown), and therefore subsequent studies were conducted only at pH 7.4. Over the concentration range of 10–100 \( \mu \)M, the transport of \( I \) in both the apical-to-basal and basal-to-apical directions was independent of the initial concentration (fig. 2A). The apical-to-basal permeation was slightly but not statistically greater than the permeation in the opposite direction (fig. 2A). There appeared to be a correlation between the transport of \( I \) and that of lucifer yellow (fig. 2B), although the correlation was higher for the apical-to-basal direction (\( r^2 = 0.998 \)) than for the basal-to-apical direction (\( r^2 = 0.74 \)). Verapamil, a known P-glycoprotein inhibitor, did not affect the transport of \( I \) in either direction (fig. 2C). No metabolism of \( I \) was observed in experiments with Caco-2 cells.

Transport of \( II \) also was not affected by pH (data not shown), but it was strongly oriented in the secretory direction. Over the concentration range studied, the apical-to-basal transport was much lower (2–4-fold) than the basal-to-apical transport (fig. 3A). For the apical-to-basal direction, there was a correlation (\( r^2 = 0.82 \)) between the transport of \( II \) and that of lucifer yellow (fig. 3B), suggesting a possible involvement of the paracellular pathway in the net transport of \( II \) in this direction. In the basal-to-apical direction, such a correlation was not observed (fig. 3B), indicating that mechanisms other than the paracellular process were primarily involved. The basal-to-apical transport displayed saturable kinetics, with an apparent \( K_{m} \) of 440 \( \mu \)M and a \( V_{max} \) of 15 pmol/hr/10^6 cells.

During the incubation of \( II \) with Caco-2 cells, \( I \) was the only metabolite observed. Interestingly, the metabolism was variable (2–20% of the transported drug), and \( I \) was detected only in the basal compartment (not in the apical compartment) and only when the drug was initially added to the apical chamber (data not shown). Under similar conditions, hydrolysis was not observed in the absence of the monolayer. Similar observations were reported for other ester prodrugs (Prueksaritanont et al., 1996a; Hovgaard et al., 1995). Considering that \( I \) was poorly transported, these results suggested that the hydrolytic reaction occurred during the transport of \( II \) by intracellular esterases. Because the metabolism of \( II \) was relatively minor, overall hydrolysis of the transport of \( II \) that were calculated using either only \( II \) or both \( II \) and \( I \) were similar.

In the presence of verapamil or quinidine, the apical-to-basal transport of \( II \) was increased significantly (>5-fold) (fig. 4A), whereas the basal-to-apical transport was inhibited markedly (~5-fold) (fig. 4B). PMSF did not affect the transport of \( II \) in either direction (fig. 4). In these experiments, however, the hydrolysis of \( II \) by Caco-2 cells was found to be minimal (<10%). In all cases, the transport of lucifer yellow was comparable in the absence and presence of the inhibitors, suggesting that the paracellular transport of \( II \) was not affected by the inhibitors.

**In Situ Rat Intestinal Loop Studies.** The intestinal absorption of \( I \) was limited, in both the absence and presence of verapamil. At the end of the experiment (60 min), the total dose absorbed, as reflected by the sum of the amounts of \( I \) recovered in blood and intestine, was 3.9 ± 2.0% in the absence of verapamil and 3.2 ± 0.7% in the presence of verapamil (table 1). In blood, the amount of \( I \) recovered over the experimental time course was essentially the same with or without verapamil (fig. 5A). Verapamil also did not appear to affect the total recovery of \( I \) in the intestinal tissue or intestinal lumen (table 1). Total recovery of \( I \) in all sections (blood, intestine, and lumen) was >85% (table 1), suggesting minimal metabolism of \( I \) in rats.

The intestinal absorption of \( II \) was also limited and was comparable to that of \( I \) in the absence of verapamil. Based on the total recovery in blood and intestinal tissue, the absorption of \( II \) was increased significantly from 4.8 ± 2.2% without verapamil to 9.3 ± 2.5% with verapamil (table 2). Only \( I \) (not \( II \)) was detected in blood, consistent with a preliminary study that showed very rapid hydrolysis of the prodrug in rat blood (virtually complete within a few minutes). In the presence of verapamil, \( I \) also was the only species detected in blood at all sampling times, suggesting that verapamil did not appreciably affect the rate of hydrolysis of \( II \). In blood, the recovery of \( II \) as \( I \) during the 60-min collection period was significantly higher in the presence of verapamil (fig. 5B). At the end of the experiment, the total recovery of \( I \) in blood was increased ~3-fold by verapamil (table 2). Verapamil also increased (~2-fold), although not statistically significantly, total recovery (\( II \) plus \( I \)) in intestinal tissue (table 2). In intestinal tissue, \( II \) was only a minor fraction (<1%) recovered in either the presence or absence of verapamil (table 2). The results suggested that rat intestinal esterases hydrolyzed the prodrug efficiently. Because the intestinal recoveries were determined only at 60 min, which is potentially far too long a time for such a rapid hydrolytic reaction, it was unclear whether the rate of hydrolysis of \( II \) in the intestine was affected by verapamil. Interestingly, the recoveries of \( I \) in the tissue after administration of \( II \), either with or without verapamil (table 2), were ~2-fold higher than the corresponding values after administration of \( I \) (table 1).

The majority of the dose administered (>80%) was found in the rat lumen. Of the dose recovered in the lumen at the end (60 min) of either experiment (with or without verapamil), >65% of \( II \) remained unchanged (table 2). These results indicated that \( II \) was not metabolized extensively in the rat lumen and, consequently, this metabolism was not the major factor contributing to the poor absorption of \( II \). In addition, the luminal metabolism of \( II \) appeared not to be affected by verapamil. In these studies, virtually complete recovery of the administered dose as \( I \) and \( II \) was observed (table 2), indicating that \( I \) was the primary metabolite of \( II \) after intestinal administration of \( II \) in rats.

In the \textit{in situ} studies, verapamil concentrations were also measured. Verapamil was absorbed substantially from the intestinal loop. The total recoveries of verapamil in blood and intestinal tissue were
comparable in the presence of II (33.6 ± 10.6%) or I (28.4 ± 4%). Although blood concentrations of verapamil appeared to be higher when verapamil was coadministered with II, compared with I (fig. 6A), the differences were not statistically significant. In both cases, ~20% of the dose administered was recovered in the intestinal tissue and ~40–55% was detected in the intestinal lumen (fig. 6B), with the resulting total recovery of >70%. The results suggested that the differential effects of verapamil on the absorption of I and II were not the result of differences in verapamil concentrations in blood and intestine.

**In Vitro Metabolism Studies. Intestinal Metabolism.** I was resistant to metabolism, whereas the prodrug II was metabolized extensively by rat intestinal S9 fractions, in agreement with the *in situ* observations. The metabolism of II, both in the presence and in the absence of NADPH, yielded exclusively I, indicating the primary involvement of esterases. The hydrolysis of II displayed monophasic kinetics, with an apparent $K_M$ of 720 μM and a $V_{max}$ of 36 nmol/min/mg S9 protein. *In vitro* metabolism was also assessed, to examine a potential inhibitory effect of verapamil on the hydrolysis of II. Verapamil appeared to be a competitive inhibitor of a II-hydrolyzing enzyme system (fig. 7A), with an apparent $K_i$ value of 90 μM. Under similar conditions, PMSF competitively inhibited hydrolysis, with a $K_i$ value of 20 μM (fig. 7B). These results suggested that verapamil was a relatively potent inhibitor of the intestinal hydrolysis of II.

**Intestinal Luminal Metabolism.** Hydrolysis of II was also examined using the intestinal lumen wash obtained from untreated rats. II was hydrolyzed considerably (fig. 8), presumably because of the presence of esterases in the rat lumen; hydrolysis was minimal in the absence of the lumen wash. Verapamil, in the concentration range examined, did not alter the rate of hydrolysis of II (fig. 8), in agreement with the *in situ* observations. The findings also suggested that the hydrolysis of II in the intestine and that produced by the lumen wash resulted from different hydrolytic enzymes and that verapamil was a relatively specific inhibitor of the intracellular intestinal esterases.

**Discussion**

This study used both *in vitro* Caco-2 cell and *in situ* rat intestinal loop models to characterize the absorption barriers for I and its prodrug II. The results with Caco-2 cells suggested that the low levels of absorption of I were not the result of polarized efflux systems. The primary pathway for the transport of I appeared to be paracellular. These conclusions were based on the findings that the transport of I in both the apical-to-basal and basal-to-apical directions was concentration independent and was correlated with that of lucifer yellow. In addition, the transport of I was not inhibited by verapamil. Considering that the apical-to-basal permeation was slightly greater than the basal-to-apical permeation and that I was detected on the basal side when II was initially placed in the apical chamber, I could also be transported (albeit to a much lesser extent) transeellularly. In contrast, the prodrug II was transported predominantly by the transeellular pathway and modestly by the paracellular route. The net transeellular transport of II was attenuated by polarized and saturable efflux systems. These conclusions were made based on the following findings: 1) basal-to-apical transport in Caco-2 cells was up to 5-fold greater than apical-to-basal permeation, 2) the transport of II only in the apical-to-basal (and not the basal-to-apical) direction was correlated with that of lucifer yellow in the corresponding directions, 3) verapamil and quinidine increased the apical-to-basal transport and decreased the basal-to-apical transport of II, and 4) the secretory transport displayed saturable kinetics. The apparent $K_M$ value for II was ~400 μM, which is considerably higher than values reported for cyclosporine and vinblastine, known P-glycoprotein substrates.

---

**Fig. 2.** A. Bidirectional transport of I as a function of concentration; B. correlation between the transport of I and that of lucifer yellow; C. effects of verapamil on the apical-to-basal (A-TO-B) and basal-to-apical (B-TO-A) transport of I.

Results are means ± SD (*N* = 3). For A and B, all incubations were performed at 37°C for 60 min, using pH 7.4 for both compartments. For C, all incubations were performed at 37°C, using 10 μM I and pH 7.4 for both compartments, in the absence or presence of verapamil (200 μM).
Fricker et al., 1996; Hunter et al., 1993b), but comparable to those noted for digoxin and pristinamycin, also P-glycoprotein substrates (Cavet et al., 1996; Phung-Ba et al., 1995). The results suggested that the polarized efflux of II might be moderate and would be less favorable in the presence of a compound with much higher affinity.

To examine the in vivo contribution of the intestinal efflux system, the transport of I and II was further investigated using the in situ rat intestinal loop model. Qualitatively, results obtained from the in situ experiment, for both I and II, agreed well with those from Caco-2 cells. Quantitatively, however, there appeared to be a discrepancy. The apparently greater effects of verapamil on the transport of II in Caco-2 cells, compared with those in rats, might be a result of species differences in the amount and/or function of the responsible secretory transporter expressed in the two models. In Caco-2 cells, P-glycoprotein could be expressed at very high and variable levels, depending on several factors, including the passage number and culture medium (Burton et al., 1997). In the present study, the jejunum section of rat intestine, which is known to contain P-glycoprotein and possibly other secretory transporters (Hsing et al., 1992; Saitoh and Aungst, 1995), was used. It is not known whether the transporters present in the Caco-2 cells and rat intestine exhibited different affinities for the substrate II ($K_M$) or the inhibitor verapamil ($K_i$). The disparity observed could have resulted from differences in the concentrations of II and verapamil in these two systems, relative to their respective $K_i/K_M$ values. Further studies on the quantitative contribution of the intestinal efflux system in vivo appear warranted.

Considering that verapamil and quinidine are P-glycoprotein inhibitors (Ford and Hait, 1990; Wacher et al., 1995), that verapamil is not a potent inhibitor of the multidrug resistance-associated protein (another efflux carrier) (Lautier et al., 1996; Flens et al., 1996), and that P-glycoprotein is expressed substantially in Caco-2 cells and rat intestine (Hsing et al., 1992; Hunter et al., 1993b), it is likely that II was a substrate for P-glycoprotein. Because P-glycoprotein is also expressed in the human gastrointestinal tract (Thiebaut et al., 1987), it is conceivable that P-glycoprotein also would play a role in the intestinal absorption of II in humans. Interestingly, at physiological pH, II is a cation with reasonable lipophilicity, in common with most P-glycoprotein substrates (Ford and Hait, 1990). To our knowledge, the involvement of P-glycoprotein has not been demonstrated previ-
ously to be an absorption barrier for an ester prodrug, although similar findings were noted for peptides with a masked carboxyl terminus (Lang et al., 1997). Also, not all cationic prodrugs screened in our laboratory exhibited preferential secretory transport in Caco-2 cells. Apparently, more studies are needed for a better understanding of the substrate specificity of intestinal efflux systems.

The present study also indicated that metabolism was not a barrier to intestinal absorption of I in Caco-2 cells or rats. A similar conclusion is anticipated for humans, based on an earlier metabolism study of I using human intestinal S9 fractions (Prueksaritanont et al., 1997). In the case of II, intestinal metabolism also was not a major barrier in Caco-2 cells, because metabolism was not extensive. In rats, however, metabolism by esterases in the intestinal lumen served as an additional, although not principal, barrier to the absorption of II. Based on the available data, it is unclear whether esterases in the intestinal tissue also played an important role in limiting the absorption of II in rats. If II were absorbed substantially before hydrolysis, this metabolism would not pose a major barrier to the intestinal availability of I after administration of II, because I was not a substrate for an efflux system. However, the systemic availability of I after administration of II could still be low, because I, once formed inside the cells, is not expected to be readily transported across the basal membrane into the bloodstream. In fact, this is consistent with the greater accumulation of I observed in intestinal tissue after administration of II (table 2), compared with administration of I (table 1). In view of the low recoveries of I plus II in intestinal tissue and blood and the high levels of unchanged II in the rat lumen in the presence of verapamil (table 2), mechanisms other than intestinal efflux and metabolism might also

![Graph A](image1.png)

**Fig. 5.** Recovery of I in rat blood as a function of sampling time after administration of I (A) or II (B).

Results are means ± SD (N = 4) obtained after administration of I or II (0.5 mg/2 ml) in the rat intestinal loop, in the absence (control) or presence of verapamil (500 μM). *, Statistically significant differences from control.

### TABLE 2

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Blood, I</th>
<th>Intestine</th>
<th>Lumen</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of dose</td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Without verapamil</td>
<td>0.3 ± 0.1</td>
<td>4.3 ± 2.1</td>
<td>0.1 ± 0.1</td>
<td>18.2 ± 5.7</td>
</tr>
<tr>
<td>With verapamil</td>
<td>1.4 ± 0.8*</td>
<td>7.6 ± 3.1</td>
<td>0.3 ± 0.4</td>
<td>22.9 ± 4.9</td>
</tr>
</tbody>
</table>

Results (means ± SD, N = 4) were obtained at the end of a 60-min experiment after administration of II (0.5 mg/2 ml) in rat intestinal loops, with or without verapamil (500 μM). *Statistically significant difference (p < 0.05) from control (without verapamil).

![Graph B](image2.png)

**Fig. 6.** Recovery of verapamil in rat blood, as a function of sampling time (A), and in gut and lumen (B).

Results are means ± SD (N = 4) obtained after administration of verapamil (500 μM) in the rat intestinal loop, with I or II (0.5 mg/2 ml).
very weak inhibitor of esterases (study demonstrated, for the first time, that verapamil is also a potent inhibition of both efflux and esterase activity was responsible for the increased absorption of...and toxicity. Thus, increasing the hydrophobicity of compounds using a prodrug approach may not always result in increased absorption. On the contrary, as was demonstrated in this study, poor absorption resulting from active intestinal secretion of prodrugs is also possible. The potential involvement of intestinal efflux should be examined in the evaluation of cationic prodrugs.

Acknowledgments. The authors thank Drs. K. C. Kwan and J. Hochman for critical review of the manuscript and Dr. J. H. Hutchinson and M. J. Breslin for synthesis of L-767,679 and L-775,318.

References


