Characterization of the Major Metabolites of Verapamil as Substrates and Inhibitors of P-glycoprotein

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ABSTRACT

Verapamil is subject to extensive oxidative metabolism mediated by cytochrome P450 enzymes with less than 5% of an oral dose being excreted unchanged in urine. Furthermore, verapamil is known to be a potent inhibitor of P-glycoprotein function. There is evidence from in vivo investigations that some verapamil metabolites might be actively transported. The aim of the present study was to investigate P-glycoprotein-mediated transport and inhibition properties of verapamil and its metabolites norverapamil, D-620, D-617, and D-703. Polarized transport of these compounds was assessed in P-glycoprotein-expressing Caco-2 and L-MDR1 cells ( LLC-PK1 cells stably transfected with human MDR1-P-glycoprotein). Inhibition of P-glycoprotein-mediated transport by these compounds was determined using digoxin as P-glycoprotein substrate. At concentrations of 5 μM, significant differences between basal-to-apical and apical-to-basal apparent permeability coefficients were observed for D-617 and D-620 in all P-glycoprotein-expressing cell monolayers, indicating that both are P-glycoprotein substrates. In contrast, no P-glycoprotein-dependent transport was found for verapamil, norverapamil, and D-703 in Caco-2 cells and for D-703 in L-MDR1 cells. Moreover, verapamil, norverapamil, and D-703 inhibited P-glycoprotein-mediated digoxin transport with IC50 values of 1.1, 0.3, and 1.6 μM, respectively, whereas D-617 and D-620 did not (at concentrations up to 100 μM). We conclude that verapamil phase I metabolites exhibit different P-glycoprotein substrate and inhibition characteristics, with the N-dealkylated metabolites D-617 and D-620 being P-glycoprotein substrates and norverapamil and D-703 being inhibitors of P-glycoprotein function, which may influence P-glycoprotein-dependent drug disposition and elimination.

Recent studies indicate that the MDR1 gene product P-glycoprotein has considerable impact on bioavailability, tissue concentrations, and pharmacodynamic effects of drugs (Schinkel et al., 1995; Kim et al., 1998; Fromm et al., 1999; Greiner et al., 1999). P-glycoprotein is an ATP-dependent efflux transporter with wide substrate specificity. Substrates of P-glycoprotein are structurally unrelated compounds, such as some anticancer drugs, immunosuppressive agents, HIV-protease inhibitors, central nervous system active drugs, and cardiovascular drugs (Hunter et al., 1993; Schinkel et al., 1995; Cavet et al., 1996; Terao et al., 1996; Kim et al., 1998). P-glycoprotein is located in the apical (luminal) membrane of epithelial cells of different tissues (i.e., brush border membrane of proximal tubule cells in kidneys, brush border membrane of enterocytes in intestine, canicular membrane of hepatocytes, capillary endothelial cells of the brain). It functions as an efflux pump, thereby limiting intracellular accumulation of xenobiotics (Gottesman and Pastan, 1993; Saitoh and Aungst, 1995; Kim et al., 1999).

In the past, research has focused on the consequences of cytochrome P450-mediated drug metabolism for the bioavailability and pharmacokinetics of drugs (Guengerich, 1995). There is, however, increasing knowledge on the role of P-glycoprotein-mediated transport for drug disposition. Furthermore, modification of P-glycoprotein-mediated drug transport is involved in certain drug interactions. For example, it is now recognized that drug interactions resulting in increased serum levels of digoxin are due to the inhibition of P-glycoprotein-mediated transport (Fromm et al., 1999). Due to its location in tissues with excretory function, the inhibition of P-glycoprotein activity results in a reduced drug elimination via the bile or the urine. Moreover, the inhibition of intestinal P-glycoprotein leads to a decreased secretion of drugs out of the enterocytes back into the intestinal lumen, thereby increasing bioavailability (Mayer et al., 1996). On the other hand, the induction of intestinal P-glycoprotein expression by rifampicin has been shown to determine the

ABBREVIATION: Papp, apparent permeability coefficient.
decrease of digoxin plasma concentration after oral administration (Greiner et al., 1999).

However, until now, little data are available on whether phase I drug metabolites also interact with P-glycoprotein. The calcium channel blocker verapamil is widely used for the treatment of supraventricular arrhythmias, coronary heart disease, and arterial hypertension. Verapamil is subject to extensive oxidative metabolism mediated by cytochrome P450 enzymes, with less than 5% of a dose being excreted unchanged in urine after oral administration (Eichelbaum et al., 1979; Mikus et al., 1990). The complex pattern of verapamil oxidative metabolism and the contribution of the different cytochrome P450 enzymes involved (e.g., CYP3A4, CYP2C9, CYP2C8, CYP1A2) has extensively been studied (Kroemer et al., 1993; Busse et al., 1995). Major metabolic steps are the formation of D-617, norverapamil, D-620, and D-703 (Fig. 1). Exogenous factors such as diet and comedication have been identified as factors that modify the disposition of verapamil (Fromm et al., 1996; Darbar et al., 1998).

Verapamil is known to inhibit P-glycoprotein-mediated transport in a dose-dependent manner (Tsuruo et al., 1982). Furthermore, verapamil and norverapamil increase the sensitivity of resistant cells to cytotoxic drugs through inhibition of P-glycoprotein-mediated transport (Häußerman et al., 1991), and verapamil has been used as a multidrug-resistance-modifying agent with cancer chemotherapy (Eichelbaum et al., 1993; Schumacher et al., 1993). The following lines of evidence indicate the possibility of the existence of an active transport for verapamil metabolites in humans. First, in addition to glomerular filtration, renal secretion of the verapamil metabolites D-617 and D-620 was observed in healthy volunteers (Mikus et al., 1990). Second, using an intestinal perfusion catheter, we observed the accumulation of D-617, D-620, and, to a lesser extent, norverapamil in an isolated intestinal segment after the i.v. administration of verapamil to healthy volunteers (von Richter et al., 1999), supporting the notion that some verapamil metabolites might be subject to an active basal-to-apical transport from the circulation via the enterocytes into the intestinal lumen. Finally, it is not known so far whether verapamil metabolites other than norverapamil are P-glycoprotein inhibitors, thereby possibly contributing to drug interactions.

Using P-glycoprotein-expressing cell lines (Caco-2, L-MDR1) we tested the hypothesis that phase I drug metabolites can be substrates or inhibitors of P-glycoprotein, thereby providing further insights into an understanding of the elimination of verapamil metabolites and their potential role for drug interactions.

**Experimental Procedures**

**Materials**

$[{}^{3}H]$Digoxin (19 Ci/mmol) and $[{}^{3}H]$inulin (3.3 mg/mCi) were supplied by New England Nuclear Research Products (Boston, MA).

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**Fig. 1.** Chemical structure of verapamil and its major metabolites. Percent values indicate the amount of a given oral dose excreted in the urine over a 48-h interval (Eichelbaum et al., 1979).
Verapamil, norverapamil, D-617, D-620, and D-703 were obtained from Knoll AG (Ludwigshafen, Germany). Unlabeled digoxin was purchased from Sigma Chemie (Deisenhofen, Germany).

Transport in Cultured LLC-PK1, L-MDR1, and Caco-2 Cells

Transport Studies. Transport was studied using Caco-2 (passage number 21–50), L-MDR1, and LLC-PK1 cells (passage numbers 7–23 and 7–27, respectively). Caco-2 cells are a human colon carcinoma cell line, LLC-PK1 cells are porcine kidney epithelial cells, and L-MDR1 cells are LLC-PK1 cells stably transfected with human MDR1 cDNA. When grown as a monolayer on semipermeable filters, these cells become polarized, and P-glycoprotein is expressed in Caco-2 and L-MDR1 cells on their apical surface, allowing the study of vectorial transcellular transport (i.e., basal-to-apical and apical-to-basal transport; Schinkel et al., 1995; Kim et al., 1998; Fromm et al., 1999). L-MDR1 and LLC-PK1 cells were kindly provided by Dr. A. H. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands), and Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were plated onto Transwell filters (Costar, Cambridge, MA) and grown under identical conditions as previously described (Kim et al., 1998; Fromm et al., 1999). Transport experiments were performed on day 7 after plating. About 1 h before the start of the transport experiment, the medium in each compartment was replaced by serum-free medium (OptiMEM; Life Technologies, Grand Island, NY). For transport experiments, the medium in each compartment was then replaced with 80 μl of serum-free medium with the addition of the drug (0.5 and 5 μM for D-703, 0.1 to 100 μM for D-617, and D-620) on the basal or the apical side of the monolayer. The amount of drug appearing in the opposite compartment (basal or apical) after 1, 2, 3, and 4 h was measured in 50-μl aliquots, and drug transport was calculated as a percent of the amount added. Moreover, the apparent permeability coefficients (P_ap) from the initial basal-to-apical and apical-to-basal transport rates were determined according to the following:

\[ P_{\text{app}} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \text{cm/s} \]

where \( dQ/dt \) (micromoles per second) is the initial transport rate, \( C_0 \) (micromoles per cubic centimeter) is the initial concentration in the donor chamber, and \( A \) (square centimeters) is the surface area of the monolayer (Artursson and Karlsson, 1991).

Inhibition Experiments. Inhibition of P-glycoprotein-mediated transport across confluent Caco-2 cell monolayers was determined in a similar manner after the addition of the putative inhibitor to both the apical and the basal compartments, using radiolabeled [3H]digoxin (5 μM) as prototypical P-glycoprotein substrate. Concentrations ranging from 0.01 to 100 μM were chosen for verapamil, norverapamil, and D-703, and concentrations ranged from 0.1 to 100 μM for D-617 and D-620. Complete inhibition of P-glycoprotein-mediated transport would be expected to result in the loss of the basal-to-apical versus apical-to-basal transport difference for digoxin. Net basal-to-apical transport was calculated after 4 h by subtracting the apical-to-basal from the basal-to-apical transport rate. The corresponding IC_{50} values for verapamil, norverapamil, and D-703 were calculated with GraFit 4.0 (Erithacus Software Ltd., Staines, UK).

Digoxin transport was determined for each plate (12 wells) in the absence of any inhibitor as positive control (2 wells/plate). Moreover, integrity of the monolayer was assessed by measuring transepithelial translocation of [3H]inulin (2 wells/plate; Hidalgo et al., 1989). Only plates with a transepithelial translocation of inulin of less than 5% within 4 h were used for further studies. To exclude an influence of verapamil and verapamil metabolites on monolayer integrity, we performed additional control experiments to confirm cell monolayers integrity by [3H]inulin in the presence of maximal substrate and inhibitor concentrations.

Drug Analyses

Drug concentrations of verapamil, norverapamil, D-617, D-620, and D-703 were determined by a modification of an HPLC-electrospray mass spectrometry assay developed by von Richter et al. (in press). To each 50-μl aliquot, 75 μl d\textsubscript{6}H\textsubscript{2}O and 25 μl of internal standard (containing D-832 and 3H\textsubscript{3}-norverapamil) were added, reaching a final probe volume of 150 μl. Separation of the substances was achieved on a LUNA C8 analytical column (150 × 2 mm i.d., 5 μm particle size) with 5 mM ammonium acetate-acetonitril (70:30) as the mobile phase. With a gradient pump, assay run time was 15 min. With the mass spectrometer operated in the selected-ion monitoring, the limits of quantification were 1 pmol/150 μl for verapamil, norverapamil, D-617, D-620, and D-703.

Aliquots (50 μl) containing radiolabeled digoxin and inulin were analyzed by liquid scintillation counting (Beckmann, Unterschlesheim, Germany) after the addition of 5 ml of Aqua Safe 300 Plus (Zinsser Analytic, Frankfurt am Main, Germany).

Statistical Analysis

All data are presented as mean ± S.D. Mean values were calculated from at least three experiments conducted on different days. Differences in P_{app} values calculated from initial basal-to-apical and apical-to-basal transport rates were tested for significance by paired t tests (Instat, 1997; GraphPad Software, San Diego, CA). A value of P < .05 was required for statistical significance.

Results

Transport Studies. At concentrations of 5 μM, there was no significant difference between the P_{app} values for basal-to-apical and apical-to-basal in Caco-2 cells for verapamil, norverapamil, and D-703 (Table 1 and Fig. 2). However, significant differences in P_{app} values were detectable for verapamil (basal-to-apical, 27.8 ± 3.1; apical-to-basal, 18.7 ± 0.7; P < 0.05) and norverapamil (basal-to-apical, 18.9 ± 4.8; apical-to-basal, 2.2 ± 0.4; P < .05) in P-glycoprotein-overexpressing L-MDR1 cells. These differences were even more pronounced at concentrations of 0.5 μM (verapamil: basal-to-apical, 28.9 ± 2.4; apical-to-basal, 6.7 ± 6.0; norverapamil:

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>P_{app} (basal-apical)</th>
<th>P_{app} (apical-basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>11.9 ± 1.3</td>
<td>12.9 ± 6.4</td>
</tr>
<tr>
<td>Norverapamil</td>
<td>9.4 ± 1.8</td>
<td>6.3 ± 7.1</td>
</tr>
<tr>
<td>D-703</td>
<td>3.6 ± 1.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>D-617</td>
<td>15.5 ± 4.5</td>
<td>1.6 ± 0.6*</td>
</tr>
<tr>
<td>D-620</td>
<td>17.7 ± 6.0</td>
<td>2.6 ± 0.8*</td>
</tr>
</tbody>
</table>

* P < .05, P_{app} (basal-apical) versus P_{app} (apical-basal)

** P < .01, P_{app} (basal-apical) versus P_{app} (apical-basal)
Fig. 2. Transepithelial transport of verapamil, norverapamil, D-703, D-617, and D-620 across Caco-2, L-MDR1, and LLC-PK1 monolayers. ■, translocation from the basal to the apical compartment. △, translocation from the apical to the basal compartment. Values are mean ± 1 S.D. from three or more experiments.
basal-to-apical, 23.9 ± 4.0; apical-to-basal, 2.5 ± 4.3; \( P < .05 \).

\( P_{\text{app}} \) values for D-620 and D-617 were significantly greater from basal-to-apical than from apical-to-basal in Caco-2 and L-MDR1 cells (Table 1 and Fig. 2).

In LLC-PK1 cells, apical-to-basal and basal-to-apical transport differences were absent for all compounds (Table 1 and Fig. 2).

**Inhibition Experiments.** For verapamil, norverapamil, and D-703, we found an inhibition of P-glycoprotein-mediated digoxin transport in Caco-2 cells with \( IC_{50} \) values of 1.1 \( \mu \)M for verapamil, 0.3 \( \mu \)M for norverapamil, and 1.6 \( \mu \)M for D-703 (Fig. 3). Inhibition of digoxin transport was absent with D-617 and D-620 even at high concentrations (100 \( \mu \)M, data not shown).

**Discussion**

Verapamil phase I metabolites D-617 and D-620 showed markedly greater initial \( P_{\text{app}} \) values from the basal to the apical compartment than from the apical to the basal compartment of P-glycoprotein-expressing cell monolayers, indicating that both compounds are P-glycoprotein substrates. These in vitro data are in accordance with in vivo observations suggesting active transport of D-617 and D-620 in gut wall mucosa and kidneys. Using a multiluminal perfusion catheter, concentrations of verapamil and metabolites were determined after the i.v. administration of the calcium channel blocker to healthy volunteers in an isolated jejunal segment. D-617, D-620, and norverapamil were found in considerably higher concentrations in the intestinal lumen compared with in plasma, suggesting the existence of active intestinal secretion mechanisms (von Richter et al., 1999). Moreover, data indicate that active renal secretion of D-617 and D-620 occurs in addition to glomerular filtration in humans (Mikus et al., 1990). Because P-glycoprotein is known to be expressed in the apical membrane of enterocytes and proximal tubule cells of kidneys, it can be concluded that P-glycoprotein-mediated transport of D-617 and D-620 contributes at least in part to the intestinal and renal secretion of phase I metabolites of verapamil in humans.

Moreover, our data provide evidence that at concentrations of 5 \( \mu \)M, neither verapamil and norverapamil nor D-703 is subject to a relevant P-glycoprotein-dependent transport in that we found no difference between the basal-to-apical and the apical-to-basal \( P_{\text{app}} \) values across confluent monolayers of Caco-2 cells. However, significant transport of verapamil and norverapamil was detectable in P-glycoprotein-overexpressing L-MDR1 cells at 5 \( \mu \)M, and this difference was even more pronounced at lower concentrations.

Concentration-dependent verapamil transport has been reported by other authors. Saitoh and Aungst (1995) observed a decrease of verapamil efflux in rat jejunum with increasing verapamil concentrations. Similarly, an increased permeability of verapamil was found in rat jejunum using increasing luminal verapamil concentrations, which has been interpreted as saturation of an efflux mechanism (Sandström et al., 1998). It was also concluded from rat studies that a decreased permeability of verapamil enantiomers during the coadministration of rifampicin could be due to the induction of P-glycoprotein (Sandström and Lennernäs, 1999). Although in these experiments polarized verapamil transport was observed even at concentrations higher than those we used in our experiments, differences may be explained by the expression of transporters other than P-glycoprotein in rat intestine in comparison with our cell lines, which might also be involved in verapamil secretion. Central nervous system accumulation of verapamil occurred in \textit{mdr1a} knockout mice in comparison with control animals, indicating a P-glycoprotein-mediated efflux of verapamil at the blood-brain barrier. In accordance with our findings, these observations were made at low verapamil plasma concentrations (Hendrikse et al., 1998).

Experiments with LLC-PK1 cells indicate that passive dif-
fuson of verapamil and its metabolites is different due to alterations in physicochemical properties. Because transcellular translocation was compared between LLC-PK1 cells and P-glycoprotein-expressing L-MDR1 cells for each compound, observed polarized basal-apical translocation in L-MDR1 cells in the absence of such a polarized transport in LLC-PK1 cells can be attributed to P-glycoprotein function.

Inhibition studies indicate that verapamil, norverapamil, and D-703 are potent inhibitors of P-glycoprotein-mediated transport of digoxin in Caco-2 monolayers. The inhibitory potency of norverapamil (IC50 = 0.3µM) exceeded that of verapamil (IC50 = 1.1 µM) and D-703 (IC50 = 1.6 µM). It should be noted, however, that D-703 is present in plasma predominantly as glucuronide. Even at high concentrations, neither D-617 nor D-620 exerted a relevant inhibition of digoxin transport. Inhibition of P-glycoprotein-mediated transport by verapamil is well documented in various in vitro and in vivo settings. Häußermann et al. (1991) found increased sensitivity of resistant human lymphoma cell lines to the P-glycoprotein substrate vincristine after the administration of verapamil and norverapamil, whereas D-617 remained without effect on cell sensitivity. In accordance with these findings, P-glycoprotein-inhibiting properties of verapamil were used to reverse multidrug resistance in chemotherapy and to increase plasma concentrations of P-glycoprotein substrates (Eichelbaum et al., 1993; Schumacher et al., 1993).

The question arises of why verapamil and metabolites have different properties as P-glycoprotein substrates and inhibitors. These differences in substrate and inhibitor specificities could be due to differences in spatial arrangement of the electron donor pattern, as suggested by Seelig (1998). Our results are in agreement with the model in which compounds with more functional units (verapamil, norverapamil, and D-703) bind more strongly to P-glycoprotein than do compounds with only one functional unit (D-617 and D-620). This would explain why verapamil, norverapamil, and D-703 potentially inhibit P-glycoprotein function, whereas the N-dealkylated metabolites D-617 and D-620 are good substrates of P-glycoprotein (Seelig, 1998). For verapamil and norverapamil, it can be hypothesized that at higher substrate concentrations, these compounds inhibit their own transport via P-glycoprotein. The involvement of different P-glycoprotein binding sites for verapamil and its metabolites is likely to be a mechanism to explain differences in substrate specificity and inhibition characteristics of these compounds (Shapiro et al., 1999). Similar to our results with verapamil and its phase I metabolites, different P-glycoprotein transport and inhibition characteristics of the parent drug and its metabolites have been reported for cyclosporine. Gan et al. (1995) noted that the major primary metabolites of cyclosporine A generated via metabolism by cytochrome P450 3A4 are subject to P-glycoprotein-dependent transport, whereas the parent drug inhibits P-glycoprotein function. It has therefore been hypothesized that cytochrome P450 3A4-dependent metabolites might be better substrates for P-glycoprotein than are the parent compounds.

Taken together, we draw the following conclusions. First, phase I metabolites can be substrates of P-glycoprotein (e.g., D-617, D-620), thereby contributing to overall drug elimination. Second, phase I metabolites can also inhibit P-glycoprotein function (e.g., norverapamil, D-703), thereby possibly modifying intracellular concentration of P-glycoprotein substrates and contributing to drug interactions. This is particularly important for gut wall mucosa, which serves due to colocalization of cytochrome P450 3A4 and P-glycoprotein as a protective barrier and limits oral bioavailability of xenobiotics. Finally, gut wall and liver, which express both drug-metabolizing enzymes and P-glycoprotein, are likely to be the major sites of the complex interaction between transport and drug metabolism of verapamil and of its phase I metabolites.

In general, metabolism and transport are both part of a complex cellular detoxification mechanism, interacting in a synergistic way to limit toxicity of xenobiotics. Metabolism by different enzymes of the cytochrome P450 family leads to metabolites being in part good P-glycoprotein substrates, limiting bioavailability of a drug and contributing to its renal, biliary, and intestinal excretion. As a possible general mechanism, elimination of these metabolites via P-glycoprotein transport may in some cases decrease the extent of product inhibition of cytochrome P450 enzymes, which promotes generation of new metabolites (Watkins, 1997).

In summary, our data indicate that verapamil and its phase I metabolites exhibit different P-glycoprotein substrate and inhibition characteristics, with N-dealkylated D-617 and D-620 being P-glycoprotein substrates and norverapamil and D-703 being potent P-glycoprotein inhibitors, which are likely to influence P-glycoprotein-dependent drug disposition and elimination.

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References


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