SELECTIVE INHIBITION OF HUMAN CYTOCHROME P450 3A4 BY N-[2(R)-HYDROXY-1(S)-INDANYL]-5-[2(S)-(1,1-DIMETHYLETHYLAMINOCARBONYL)-4-[[FURO[2,3-B]PYRIDIN-5-YL]METHYL]PIPERAZIN-1-YL]-4(S)-HYDROXY-2(R)-PHENYLMETHYL-PENTANAMIDE AND P-GLYCOPROTEIN BY VALSPODAR IN GENE TRANSFECTANT SYSTEMS

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ABSTRACT:
Our previous report showed that L754.394 and valsodar (PSC833) are potent inhibitors of midazolam hydroxylation in human jejunum microsomes and vectorial transport of vinblastine in Caco-2 cells, respectively. In the present study, to directly examine the interactions of these compounds as well as other substrates with CYP3A4 and P-glycoprotein (P-gp), we performed in vitro inhibition studies using recombinant CYP3A4-expressed microsomes and an MDR1-transfected cell line, LLC-MDR1, respectively. In CYP3A4-expressed microsomes, both L754.394 and ketoconazole, at a concentration less than 0.5 μM, are the most potent inhibitors of the formation of 1’-hydroxymidazolam, a major metabolite of midazolam formed by CYP3A4. The greatest inhibitory effect on the transcellular transport of digoxin in LLC-MDR1 cells was observed in the presence of valsodar (<0.1 μM), followed by verapamil. From a comparison of the IC50 values, it was shown that L754.394 and valsodar exhibited the highest selectivity for CYP3A4 and P-gp, respectively. To demonstrate such specificity, both midazolam hydroxylation and digoxin transport were observed in CYP3A4-transfected Caco-2 cells, which coexpress both P-gp and CYP3A4, in the presence or absence of L754.394 (0.5 μM) and valsodar (1.0 μM). L754.394 almost completely inhibited midazolam hydroxylation, but not digoxin transport, whereas almost complete inhibition of digoxin transport was observed in the presence of valsodar, but inhibition of the hydroxylation was minimal. Thus, the present study has demonstrated that L754.394 has a specific inhibitory effect on CYP3A4, whereas valsodar is specific for P-gp.

Intestinal first-pass metabolism catalyzed by CYP3A4 has been recognized to be clinically important for several drugs, such as cyclosporin A, tacrolimus, and midazolam (Kolars et al., 1991; Thummel et al., 1996; Floren et al., 1997). CYP3A4 is the most abundant cytochrome P450 (CYP)1 isoform in the liver and small intestinal epithelial cells (Watkins et al., 1987; de Waziers et al., 1990; Paine et al., 1997). It accounts for about 30 and 70% of the total CYP in the liver and small intestine, respectively (Watkins et al., 1987). More than half of all drugs administered to humans are metabolized by CYP3A4 (Li et al., 1997), leading us to believe that, depending on their physicochemical properties, certain types of these drugs may undergo metabolism in the small intestine during gastrointestinal absorption. On the other hand, P-glycoprotein (P-gp) is located at the brush border membranes of the small intestine and acts as an efflux transporter for amphipathic compounds (Hunter et al., 1993; Saitoh and Aungst, 1995; Terao et al., 1996). It is currently believed that intestinal P-gp affects the absorption of drugs after oral administration (Sparreboom et al., 1997b). Wacher et al. (1995) reported that the substrate specificity for CYP3A4 overlaps that for P-gp, such that many substrates of CYP3A4 are also substrates or inhibitors of P-gp. Lown et al. (1997b) and Zhang et al. (1998) have proposed that these proteins act synergistically in the small intestine as an absorption barrier, and this may result in a significant first-pass effect on drugs in the small intestine following their oral administration. Ito et al. (1999) have developed a pharmacokinetic model for drug absorption, including metabolism by CYP3A4 inside the epithelial cells, P-gp-mediated efflux into the lumen, intracellular diffusion from the luminal to the basal side, and subsequent permeation through the basal membrane. This mathematical analysis can simulate the synergistic increase in drug absorption by simultaneously inhibiting both CYP3A4 and P-gp (Ito et al., 1999). However, there is no evidence yet on the contribution of CYP3A4 and P-gp to the intestinal first-pass removal of poorly absorbed drugs. To answer this question, we need to investigate specific inhibitors that can recognize these proteins separately. For such a purpose, in the present study, different inhibition studies of CYP3A4-mediated metabolism (midazolam hydroxy-
lution) and P-gp-mediated vectorial transport (transcellular transport of digoxin) were performed in the presence of substrates and/or modulators of these proteins. In addition, to demonstrate the possible application of these inhibitors in gastrointestinal absorption studies, we examined their specific inhibition in CYP3A4-transfected Caco-2 (CYP3A4-Caco-2) cells, which simultaneously express both CYP3A4 and P-gp.

**Experimental Procedures**

**Materials.** [3H]Digoxin and [14C]mannitol were purchased from Amersham International (Buckinghamshire, UK). L754.394 was kindly supplied by Merck Research Laboratories (West Point, PA). Valspodar (PSC833) and cyclosporin A were kindly donated by Novartis Pharma AG (Basel, Switzerland). Midazolam and 1-hydroxymidazolam were supplied by Nippon Roche (Tokyo, Japan). Verapamil was obtained from Wako Pure Chemicals (Osaka, Japan). Ketoconazole was purchased from Funakoshi (Tokyo, Japan). All other chemicals were of reagent grade and were obtained commercially.

**Metabolism Study in the Recombinant CYP3A4-Expression System.** Microsomes from B lymphoblastoid cells expressing recombinant human CYP3A4 were used. Midazolam (2 μM) was incubated with a reaction mixture consisting of 0.2 mg/ml microsomal protein and an NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 0.1 U/ml glucose-6-phosphate dehydrogenase, 6 mM MgCl2) in 100 mM potassium phosphate buffer (pH 7.4). After a 5 min preincubination, with or without different concentrations of inhibitors (except for L754.394, which was preincubated for 20 min) at 37°C in a shaking water bath, the enzyme reaction was initiated by addition of midazolam, followed by incubation for 20 min. The reaction was terminated by adding 4 volumes of methanol/acetonitrile (1:1), and then the reaction mixture was centrifuged at 10,000 g for 5 min. An aliquot of supernatant was evaporated to dryness. The residues were reconstituted in mobile phase for analysis by HPLC.

**Cell Culture.** All cultures were maintained in a humidified 37°C incubator in 5% CO2 in air. An MDR1-transfected cell line (LLC-MDR1 cells) was kindly donated by Dr. P. Borst in the Netherlands Cancer Institute, Amsterdam (Smith et al., 1998). LLC-MDR1 cells were maintained in M199 medium supplemented with 10% fetal bovine serum, 10 mM Hepes, and 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and amphotericin B. CYP3A4-Caco-2 cells, which were originally developed by Crespi et al. (1996), were maintained under almost identical conditions to the Caco-2 cells except that 15% fetal bovine serum was added to the medium, and vector-bearing cells were selected by resistance to 100 μg/ml hygromycin B. For transport experiments, LLC-MDR1 cells were grown on a polycarbonate terephthalate (PET) insert (3.0-μm pore size, 6.4-mm diameter, Falcon, Franklin Lakes, NJ) at a density of 1.5 × 105 cells/well. The cells were cultured for 4 days with one medium replacement. Caco-2 cells were grown on a PET insert (1.0-μm pore size, 10.5-mm diameter, Falcon) at about 1.0 × 106 cells/well and cultured for 3 weeks. CYP3A4-Caco-2 cells were grown on a PET insert (1.0-μm pore size, Falcon) at about 1.2 × 106 cells/cm2 and cultured for 14 days. Twenty-four hours before the start of the experiment, cells were preincubated with 100 nM p-nitrophenol 12-nitrate 13-acetate to increase CYP3A4 expression.

**Transport Study in Cell Monolayers.** The confluent cells were washed with Hanks’ balanced salt solutions (HBSS), followed by the addition of HBSS, with or without an appropriate concentration of inhibitors, to both the apical (AP) and basal (BL) sides of the cell monolayer. After preincubation for 20 min, the experiment was started by replacing the HBSS at either the AP or BL side of the cell monolayer with HBSS containing 100 nM [3H]digoxin (at 0.25 μCi/ml), [14C]mannitol (at 0.04 μCi/ml), and the corresponding inhibitor. The cells were incubated at 37°C, and an aliquot of medium was withdrawn from each compartment at specified times. The appearance of radioactivity in the acceptor compartment was monitored. In addition, paracellular flux was monitored as the appearance of [14C]mannitol in the acceptor compartment. For the determination of the cellular protein, the monolayer cells were first solubilized with 1 M of NaOH at 37°C for 2 h, followed by neutralization with 1 M HCl. The protein concentration in the solubilized cells was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

**Metabolism Study in CYP3A4-Transfected Caco-2 Monolayers.** After midazolam (1 μM) was added to the AP side, experiments proceeded for 4 h at 37°C. An aliquot of medium was withdrawn from both the AP and BL sides at 4 h. For the analysis of 1-hydroxymidazolam, 1 ml of 50 mM NaCO3 (pH 12.5) containing 25 ng of etozolam as an internal standard was added to the samples (0.5–1.5 ml), followed by extraction with 7 ml of ethyl acetate. The organic phase was evaporated to dryness under nitrogen, and the residue was reconstituted in mobile phase for analysis by HPLC.

**Analytical Methods.** To determine the radiolabeled compounds ([3H]digoxin and [14C]mannitol), samples were transferred to counting vials, mixed with scintillation cocktail (Goldscint; National Nuclear, Tokyo, Japan), and placed in a liquid scintillation counter (LS 6000SE; Beckman Instruments, Fullerton, CA). Midazolam and 1-hydroxymidazolam were measured using an HPLC system (Hitachi L-6300 pump, Hitachi AS-4000 autosampler, and Hitachi L-4250 UV spectrophotometric detector) equipped with a reverse-phase column (Tosoh TSK Gel ODS-80Ts, 250 × 4.5-mm inner diameter, with a TSK guard column). The mobile phase consisted of methanol/acetonitrile/10 mM phosphate buffer (pH 7.4) (5:5:10, v/v/v). The flow rate was 1 ml/min, and elution from the column was monitored at 220 nm.

**Data Analysis.** The permeability of [3H]digoxin (cleared volume, μl/mg of protein) was calculated by dividing the amount transported by the initial concentration of [3H]digoxin in the donor compartment. The permeation clearance (μl/min/mg of protein) was obtained by linear regression analysis of the slope of the permeation profile against time. For the determination of the IC50 for midazolam hydroxylation, the ratio (R) of the appearance of the metabolite in the presence of inhibitor to that in its absence was fitted to the following equation:

\[
R = \frac{IC_{50}}{IC_{50} + I}
\]

For the determination of the IC50 for digoxin transport, we assumed that the maximum inhibition was observed in the presence of 3 μM valsopdar. The permeation clearances of digoxin in the absence of inhibitors (A), in the presence of 3 μM valsopdar (B), and in the presence of each inhibitor (C) were used to determine the R value at each inhibitor condition as \((C - B)/(A - B)\). The IC50 was obtained by the same fitting procedure as in the case of midazolam hydroxylation. Fitting was carried out by an iterative nonlinear least-squares method, the input data being weighted as the reciprocal of the square of the observed values. Statistical analysis was performed by Student’s t test to identify significant differences between various treatment groups.

**Results**

**Effects of Different Inhibitors on Midazolam Hydroxylation Activity in the CYP3A4-Expression System.** The metabolic study of midazolam was performed in CYP3A4-recombinant microsomes to identify the most potent inhibitor of CYP3A4 among the four study compounds (ketoconazole, L754.394, verapamil, and valsopdar) examined. Figure 1 shows the inhibition profile for CYP3A4-mediated metabolism. L754.394 and ketoconazole reduced the formation of 1-hydroxymidazolam, the main metabolite of midazolam, at relatively low concentrations, whereas the effect of verapamil and valsopdar was minimal (Fig. 1). The IC50 values of L754.394 and ketoconazole were <0.05 μM, whereas those of verapamil and valsopdar were >7 μM (Table 1). In this experiment, microsomes were preincubated in the presence of NADPH for 20 min for L754.394 and 5 min for the other three compounds. When the microsomes were preincubated with each of the compounds for 20 min in the presence of NADPH and the reaction was initiated by the addition of midazolam, the obtained IC50 for ketoconazole and verapamil were 0.0628 and 7.55 μM, respectively, whereas the metabolic activity in the presence of 10 μM valsopdar was 85.6% of control. On the other hand, when the microsomes were preincubated for 20 min with midazolam, but without NADPH, and the reaction was initiated by the addition of NADPH, the obtained IC50 for ketoconazole and vera-
experiments.

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bition by L754.394 was observed. The metabolic activity in such case 
cells (Fig. 3). Verapamil also inhibited the digoxin flux above 10 
dar was a potent inhibitor of the BL to AP flux of digoxin across both 
verapamil in a dose-dependent manner (Fig. 3). In particular, valspo-
BL to AP transport of digoxin in Caco-2 and LLC-MDR1 cells. In 
Figure 3 shows the inhibition profiles for the 
transport of digoxin was found in the parent LLC-PK1 cells.

consequence of P-gp expression on the AP domain. No vectorial 
transport was inhibited in these cells, whereas the AP to BL transport 
was increased (Fig. 2). This vectorial transport property is the direct 
result of the presence of both CYP3A4 and P-gp in Caco-2 cells.

The ratio of the IC50 for CYP3A4 and P-gp (i.e., IC50 for CYP3A4/ 
IC50 for P-gp) indicated that L754.394 and valsapar exhibit a 100-
fold higher selectivity for CYP3A4 and P-gp inhibition, respectively 
(Table 1).

Effects of L754.394 and Valsapar on Digoxin Transport and 
Midazolam Hydroxylation in CYP3A4-Transfected Caco-2 Cells.

Because Caco-2 cells have minimal CYP3A4 activity, CYP3A4-
Caco-2 cells that express both CYP3A4 and P-gp were used to 
simultaneously evaluate the specific effect of the inhibitors (Fig. 4). In 
the presence of L754.394 (0.5 μM), formation of 1'-hydroxymidazo-
amol was almost completely inhibited (Fig. 4B), whereas the transport 
of the parent compound from the AP to BL side was only minimally 
affected (Fig. 4A). The BL to AP transport of digoxin in the presence 
of L754.394 (0.5 μM) was the same as in the control experiment (Fig. 
4C), whereas valsapar (1 μM) inhibited the transport of digoxin, the 
AP to BL transport being almost comparable with the BL to AP 
transport in its presence (Fig. 4C). Approximately 29 and 23% inhibi-
tion of the formation of 1'-hydroxymidazolam on the AP and BL 
side, respectively, was observed in the presence of valsapar (Fig. 
4B). Thus, L754.394 and valsapar are able to selectively inhibit 
mamidol metabolism and digoxin transport, respectively. The per-
meation clearance of [14C]mannitol in the absence of each inhibitor 
was 0.696 ± 0.048 μl/min/mg of protein in the CYP3A4-transfected 
Caco-2 cells and 98 to 120% of control in the presence of 1.0 μM 
valsapar or 0.5 μM L754.394.

**Discussion**

It is generally accepted that metabolism by CYP3A4 and efflux by 
P-gp in gut epithelial cells limit the oral bioavailability of several 
medications (Wu et al., 1995; Saitoh and August, 1995). To evaluate 
their role in intestinal absorption, it is necessary to identify specific 
inhibitors for each protein. We previously reported that L754.394 and 
valsapar are potent inhibitors of midazolam hydroxylation in human 
jejunum and liver microsomes, and vectorial transport of vinlbastine 
in Caco-2 cells, respectively (Achira et al., 1999). Although there are 
abundant quantities of CYP3A4 in human liver and small intestine 
and P-gp is constitutively expressed in Caco-2 cells, caution should 
be exercised in demonstrating their direct inhibitory action on CYP3A4 
and P-gp. In the present study, to directly demonstrate their inhibitory 
effect on human CYP3A4 and P-gp, we carried out in vitro inhibition 
studies of midazolam hydroxylation and vectorial transport of digoxin 
in recombinant CYP3A4-expressed microsomes and LLC-MDR1 
cells, respectively.

The Ki of L754.394 and ketoconazole for midazolam hydroxylation 
was lower than that of valsapar and verapamil in recombinant 
CYP3A4-microsomes (Fig. 1). L754.394 is reported to be a potent 
inactivator of CYP3A4 (Chiba et al., 1995). In a similarly designed 
experiment, von Molke et al. (1996) determined the Ki values (0.0037 
μM) of ketoconazole for the formation of 1'-hydroxymidazolam in 
human liver microsomes, and this was consistent with the present
result (Table 1). The IC\textsubscript{50} values of the four compounds obtained in the present study using recombinant CYP3A4-microsomes (Table 1) were also comparable with the IC\textsubscript{50} values using human jejunum microsomes (0.0076, 0.03, 4.03, and 29.9 μM for L754.394, ketoconazole, valspodar, and verapamil, respectively, Achira et al., 1999). Based on these results, it can be concluded that L754.394 and ketoconazole are more potent inhibitors of CYP3A4, compared with the other two compounds.

The inhibition profile of each compound toward vectorial digoxin transport showed a similar tendency in both Caco-2 and LLC-MDR1 cell monolayers (Fig. 3), the IC\textsubscript{50} value of valsopodar being the lowest (Table 1). Valsopodar is a nonimmunosuppressive cyclosporin A derivative and is now under clinical investigation as a modulator of multidrug resistance because it is a potent inhibitor of P-gp function (Keller et al., 1992; Watanabe et al., 1995). The \( K_e \) values of valsopodar for the transport of daunorubicin mediated by P-gp into rat canalicular membrane vesicles was estimated to be 0.3 μM (Böhme et al., 1993), which is consistent with our results. Thus, of the four compounds examined, valsopodar is the most potent inhibitor of P-gp. By comparing the IC\textsubscript{50} ratios between CYP3A4 and P-gp (Table 1), L754.394 and valsopodar proved to be 100 times more selective toward CYP3A4 and P-gp, respectively.

Crespi et al. (1996) reported that CYP3A4-transfected Caco-2 cells showed a 100-fold increase in testosterone 6β-hydroxylation activity, compared with the parental cell lines. Therefore, it is possible to simultaneously evaluate the specific inhibition by L754.394 and valsopodar. L754.394 and valsopodar specifically inhibited midazolam hydroxylation and digoxin transport, respectively (Fig. 4), indicating that these compounds are also specific inhibitors of CYP3A4 and P-gp, respectively, in an intact cell monolayer. However, we should also note the weak effect of L754.394 on the transport of midazolam (Fig. 4A). This can be explained if we consider that the metabolism in CYP3A4 transfect Caco-2 monolayers is still low and not enough to affect the net transport of parent compound. Actually, hydroxylation
of midazolam accounted for, at most, 10% of its transport in the control (Fig. 4, A and B). Thummel et al. (1996) reported that the small intestine is a major site for the presystemic CYP3A-mediated metabolism of midazolam after oral administration. The inhibition of CYP3A-mediated metabolism both in the small intestine and the liver has been suggested to contribute to the pharmacokinetic interaction between midazolam and clarithromycin (Gorski et al., 1998). Therefore, our present findings suggest that the metabolism taking place in the CYP3A4-transfected Caco-2 cell monolayers is not still extensive compared with that in vivo. Thus, a more cautious interpretation is required when the transcellular transport in this cell line is compared with in vivo gastrointestinal absorption. To monitor the oral absorption of new drugs during the initial stages of their development, we should establish optimum conditions to observe the higher CYP3A4-mediated metabolism, which will have a potential effect on the transcellular transport of the parent compound.

Controversial results have been obtained regarding the cooperative activity of CYP3A4 and P-gp as a barrier to gastrointestinal drug absorption. Lown et al. (1997b) reported that the Cmax and oral clearance of cyclosporin A appeared to correlate with both the liver CYP3A4 activity, as measured by the erythromycin breath test, and the intestinal P-gp concentration, but did not correlate with the intestinal CYP3A4 levels. Gomez et al. (1995) found that ketoconazole increased the gastrointestinal absorption of cyclosporin A, although ketoconazole had no effect on its systemic clearance. Assuming that ketoconazole does not influence the hepatic blood flow rate or the fraction of dose absorbed, this result may be accounted for by inhibition of intestinal CYP3A4. In contrast, as discussed above, the inhibition of P-gp by ketoconazole may also be possible. Lown et al. (1997a) found that the area under the curve of felodipine after giving it orally with grapefruit juice were 370% of the control. The intestinal CYP3A4 content after administration of grapefruit juice was reduced to 38% of the control, although the liver CYP3A4 activity and the intestinal P-gp content were unaffected, leading us to believe that the increase in the oral area under the curve of felodipine may be due to the inhibition of intestinal CYP3A4. However, other constituents of grapefruit juice may also inhibit the function of P-gp. To determine the role of CYP3A4 and P-gp as a barrier to absorption, it is important to administer a specific inhibitor that can selectively recognize each protein.

In the present study, the inhibitors were added to both the AP and BL side of the monolayer cells. If the specific inhibitors identified in the present study are orally administered in vivo, such inhibitors will be exposed first from the apical side (gut lumen). Therefore, it would still be possible, in in vivo studies, to observe reduced (or increased) selectivity between valsodar and L754,394 and a difference in the relative potency of the series of inhibitors studied. Further studies are needed to fully characterize the selectivity of these compounds found in vivo.

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References


Fioren LC, Bekersky I, Benet LZ, Mekki Q, Dressler D, Lee JW, Roberts JP and Hebert MF

![Fig. 4. Effects of L754,394 and valsodar on the transcellular transport of midazolam (A), midazolam hydroxylation (B), and transcellular transport of digoxin (C) in CYP3A4-transfected Caco-2 cell monolayers.](image-url)


