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Transport of a New Erectogenic Udenafil in Caco-2 Cells

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P-glycoprotein, an ATP-dependent efflux pump, is a membrane transporter that influences the absorption and excretion of drugs. There is a striking overlap between the substrates for CYP3A4 and P-glycoprotein. This study was designed to assess whether udenafil, a substrate of CYP3A4, is also a P-glycoprotein substrate. Udenafil stimulated P-glycoprotein ATPase activity, a putative measure of P-glycoprotein affinity, although with lower affinity than a proven substrate, verapamil. Bidirectional transport studies of udenafil using Caco-2 cell monolayers showed that its efflux (15.9-22.8 $\times$ 10^{-6} cm/s) was significantly higher than its influx (3.7-9.1 $\times$ 10^{-6} cm/s). P-glycoprotein inhibitors such as cyclosporine, tariquidar and verapamil significantly increased the influx of udenafil and decreased the efflux of udenafil. These results indicate that udenafil is a substrate for P-glycoprotein. The low bioavailability, variable absorption and drug-drug interactions of udenafil may be related to the variability of CYP3A4 and P-glycoprotein expression and to possible CYP3A4 and P-glycoprotein interactions.

Key words: Udenafil, P-glycoprotein, Caco-2, Transport

INTRODUCTION

Udenafil, 5-[2-propoxy-5-(1-methyl-2-pyrrollydinylethyl-amidosulfonyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazollo(4,3-d)pyrimidin-7-one (DA-8159, Fig. 1), is a potent and selective inhibitor of cGMP-specific phosphodiesterase type 5 (PDE5) (Ahn *et al.*, 2003; Kang *et al.*, 2005; Oh *et al.*, 2000). Udenafil has been used as an oral therapy for erectile dysfunction in Korea since December, 2005. Udenafil is biotransformed to hydroxy-udenafil, *N*-desmethyl-udenafil and DA-8164 (Choi *et al.*, 2002). CYP3A4 was identified as the major enzyme responsible for udenafil *N*-dealkylation to its major metabolite, DA-8164, which has a 50% lower PDE5 inhibitory potency than udenafil (Ji *et al.*, 2004). In rats, udenafil showed dose-dependent pharmacokinetics after oral administration of 10-100 mg/kg doses (Shim *et al.*, 2003). After oral administration of 30 mg/kg udenafil to rats, the extent of absolute bioavailability was approximately 38.0%, 0.67% of oral dose was not absorbed, the hepatic first-pass effect was approximately 9.6% of the oral dose, and the intestinal first-pass effect was 58.0% of the oral dose (Shim *et al.*, 2003).

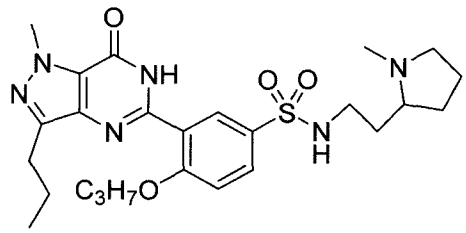

Fig. 1. Chemical structure of udenafil

P-glycoprotein is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of membrane transport proteins, which evidently act as ATP-driven pumps to extrude xenobiotics from the interior of cells (Hunter *et al.*, 1993). Expression of P-glycoprotein in normal human tissues, particularly within the cellular membranes of the kidney, adrenal gland, brain capillary endothelial cells, liver, gastrointestinal tract and placenta, suggests that it plays a role in the absorption and excretion of P-glycoprotein substrates (Ambudkar *et al.*, 1999; Williams and Sinko, 1999). Futhermore, absorption of a drug through the intestines may vary in individuals

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due to P-glycoprotein polymorphism (Hoffmeyer *et al.*, 2000). CYP3A4 and P-glycoprotein also play important roles in limiting oral drug delivery by their joint presence in small intestinal enterocytes, the significant overlap in their substrate specificities and the poor oral bioavailability of drugs that are substrates for both P-glycoprotein and CYP3A (Benet and Cummins, 2001; Kim *et al.*, 2006; Wacher *et al.*, 1995, 2001; Williams and Sinko, 1999).

The intestinal first-pass effect is reported to significantly contribute to poor oral bioavailability of udenafil, a substrate for CYP3A4 (Shim *et al.*, 2003). P-glycoprotein-mediated efflux may also limit net absorption of udenafil and contribute to dose-dependent pharmacokinetics for udenafil. The purposes of this study were to characterize the affinity of udenafil for P-glycoprotein through P-glycoprotein substrate-induced ATPase activity in recombinant human P-glycoprotein (Sarkardi *et al.*, 1992) and to evaluate the possible enterocytic transport of udenafil using human colonic cell line Caco-2, a well-established intestinal model for assessing drug absorption and P-glycoprotein-mediated transport (Hidalgo, 2001; Meunier *et al.*, 1995).

MATERIALS AND METHODS

Materials

Udenafil was a gift from Dong-A Pharmaceutical Co. (Yongin, Korea). Lucifer yellow, verapamil hydrochloride, cyclosporine, indomethacin, genestein and other cell culture medium components were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hanks buffered salt solution (HBSS), non-essential amino acids (NEAA), fetal bovine serum (FBS), Minimal Essential Medium (MEM) and high glucose Dulbecco's Modified Eagle Medium (DMEM) were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Human P-glycoprotein membranes prepared from baculovirus-infected insect cells were obtained from Gentest (Woburn, MA, U.S.A.).

Effect of udenafil on P-glycoprotein-dependent ATPase activity

Membrane fractions were diluted in Tris-MES buffer (pH 6.8, containing 2 mM dithiothreitol, 50 mM KCl and 5 mM sodium azide) and incubated for 5 min at 37°C with various concentrations of udenafil or a positive control, verapamil (2, 10, 20, 40, 60, 80, 120 and 180 μM). The reactions were incubated for 20 min at 37°C after the addition of 4 mM Mg-ATP and stopped by the addition of 10% ice-cold sodium dodecylsulfate (SDS). For each reaction, an identical incubation containing 100 μM sodium *ortho*-vanadate, an inorganic phosphate analogue and P-glycoprotein-specific inhibitor, served as a control for baseline ATPase activity due to non-P-glycoprotein ATPases in the membrane preparation and spontaneous non-enzymatic ATP hydrolysis. An eight-point standard curve of 0-150 nM inorganic phosphate was incubated in duplicate in each plate prior to incubation. Covered mixtures were incubated at 37°C for 5 min, and incubations were terminated by the addition of 30 μL of ice-cold SDS including 0.1% Antifoam A. The amount of released inorganic phosphate was determined by the method of Drueckes *et al.* (1995): 200 μL of a color solution (35 mM ammonium molybdate in 15 mM zinc acetate and 10% ascorbic acid, 1:4 at pH 5.0) was added to each well, and the mixture was incubated for 20 min at 37°C. The absorbance of the inorganic phosphate-molybdate complex was read at 750 nm using a microplate reader, and the inorganic phosphate concentration was calculated from the standard curve. Non-linear regression curves for the relationship between concentration and activity were estimated on the basis of the Michaelis-Menten model for enzyme kinetics. K_m and V_{max} values were derived using Enzyme Kinetics software (version 1.1, SPSS Science Inc., Richmond, CA, U.S.A.).

Cell culture

Caco-2 cells were cultured at 37°C in MEM, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/mL penicillin and 0.1 mg/mL streptomycin, in a humidified 37°C incubator with 5% carbon dioxide. Caco-2 cells (passage number 35~36) were seeded at a density of 60,000 cells/cm^2 onto Transwell™ permeable polycarbonate inserts (12-mm id, 0.4-mm pore size, BD, Cambridge, MA, U.S.A.). Medium was changed every other day after seeding. The cell monolayers were used approximately 21 days post-seeding. To ensure cell monolayer integrity, the transepithelial electrical resistance (TEER) was measured using an EVOM Epithelial Tissue Voltammeter and an Endohm-12 electrode (World Precision Instruments, Sarasota, FL, U.S.A.). Caco-2 cells with TEER values ≥300 Ω·cm^2 were used for transport experiments.

Measurement of bidirectional udenafil transport in Caco-2 cell monolayers

Caco-2 cell monolayers were incubated in transport buffer (HBSS with 10 mM glucose and 25 mM HEPES adjusted to pH 7.4) for 30 min at 37°C. Udenafil solutions in transport buffer (1, 2, 5, 10 and 20 μM) were added to the apical side (for apical-to-basolateral permeability measurements) or basolateral side (for basolateral-to-apical permeability measurements) of the inserts. For apical-to-basolateral transport, the inserts were moved to wells with fresh transport buffer at various times over a 1.5-h period,

and aliquots were removed from each well. For basolateral-to-apical transport, the transport media in the inserts were removed and replaced with 0.5 mL of fresh transport buffer at various times over a 1.5 h period. The aliquots were stored at -20°C until analysis. Upon completion of all transport experiments, TEER was measured to ensure that cell monolayer integrity and viability had not been adversely affected by the experimental conditions.

Udenafil flux was expressed as a rate (pmol/h/cm^2). The observed basolateral-to-apical flux would be the result of passive diffusion plus active transport, and the apical-to-basolateral flux would be the result of passive diffusion minus active transport. The active transport rate of udenafil was calculated as (basolateral-to-apical flux - apical-to-basolateral flux)/2 (Walle and Walle, 1998). The Hill equation model, $V = V_{max} \cdot [S]^n/(K_m^n + [S]^n)$, best described the kinetics of udenafil active transport in Caco-2 cells. V is the active transport rate at substrate concentration [S]; V_{max} the maximum velocity; K_m, the substrate concentration at which the active transport rate is 50% of V_{max}.

The apparent permeability coefficients (P_{app}, cm/s) were calculated using $P_{app} = (dQ/dt)/(A \cdot C_D)$ where Q is the amount of udenafil transported over time t of the experiment, A is the surface area of the porous membrane in cm^2, and C_D is the initial concentration of udenafil added to the donor compartment.

In studies where udenafil transport was performed in conjunction with inhibition of P-glycoprotein or multidrug resistance-associated protein (MRP), cells were preincubated with transport media containing the inhibitors for 30 min before initiation of the study. The inhibitors included 50 μM verapamil, 5 μM cyclosporine, 5 μM tariquidar, 75 μM indomethacin and 50 μM genestein (Martin *et al.*, 1999; Silverman, 2000). The udenafil concentration used in these experiments was 5 μM. During the transport study, these modifying agents were also present both in donor chamber with udenafil and in the receiver chamber at the same concentrations listed above.

LC-MS/MS analysis of udenafil

The concentrations of udenafil in the cell incubates were analyzed by slight modification of our previous LC-MS/MS method (Kim *et al.*, 2003). To 40 μL of the incubates, 10 μL of internal standard (sildenafil) working solution and 800 μL of dichloromethane were added and vortex-mixed for 5 min. The organic layer was colleted after centrifuging at 5000 × g for 5 min and evaporated to dryness. The residues were dissolved in mobile phase and the aliquot was analyzed by LC-MS/MS. The separation was performed on a Luna-phenylhexyl column (3 μm, 2.0 mm i.d. × 100 mm, Phenomenex, Torrance, CA, U.S.A.) using a mixture of acetonitrile and 5 mM ammonium formate (55:45, v/v) at a flow rate of 0.2 mL/min. The column and autosampler tray were maintained at 30°C and 4°C, respectively. The eluent was introduced directly into the tandem quadrupole mass spectrometer (Micromass UK Ltd., Manchester, UK) through the positive ionization electrospray interface. The ion source and desolvation temperature were held at 120°C and 350°C, respectively. The optimum cone voltage for ionization of udenafil and sildenafil was 40 V. The molecular ions of udenafil and sildenafil were fragmented at collision energies of 30 and 13 eV, respectively, using argon as the collision gas. Multiple reaction monitoring (MRM) mode using specific precursor/product ion transitions was employed for the quantification: the transitions of *m/z* 517→*m/z* 283 for udenafil and *m/z* 475→*m/z* 100 for sildenafil. Peak areas for all components were automatically integrated using MassLynx 3.5 software (Micromass UK Ltd). The amount of udenafil in the incubates was determined by interpolation from standard curves constructed in transport media from 1 to 100 pmol udenafil.

Data analysis

Differences between treatments were evaluated using Student's unpaired *t*-test, with a statistical significance level of $p < 0.05$.

RESULTS AND DISCUSSION

In general, most drugs that stimulate P-glycoprotein ATPase activity are transported by P-glycoprotein (Ambudkar *et al.*, 1999). Udenafil stimulated P-glycoprotein ATPase activity, as seen in Fig. 2, with a K_m value of 24.8 μM and a V_{max} value of 36.4 nmol phosphate released/mg P-glycoprotein/min. The prototype P-glycoprotein substrate, verapamil, had a K_m value of 8.8 μM and a V_{max} value of

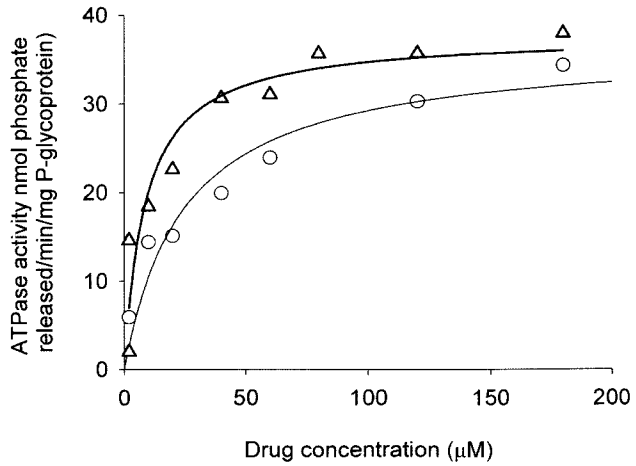

Fig. 2. Michaelis-Menten plot of *ortho*-vanadate-sensitive substrate-induced P-glycoprotein ATPase activity as measured by inorganic phosphate released by udenafil (○) and verapamil (△). Data are the mean of duplicate incubations.

37.7 nmol phosphate released/mg P-glycoprotein/min. The V_{max}/K_m ratio amounted to 1.5 and 4.3 for udenafil and verapamil, respectively. Udenafil had a lower affinity than the archetypal substrate verapamil (K_m, 24.8 vs. 8.7 μM), but had as high capacity as verapamil (V_{max}, 36.4 vs. 37.7 nmol phosphate released/mg P-glycoprotein/min) (Fig. 2), suggesting that udenafil may be transported by P-glycoprotein.

Both the apical-to-basolateral and basolateral-to-apical fluxes of udenafil across Caco-2 cell layers were linear with time up to 1.5 h (Fig. 3) under all conditions. The transport of udenafil through Caco-2 monolayers occurred in both apical-to-basolateral and basolateral-to-apical directions (Fig. 3). The apical-to-basolateral P_{app} value in Caco-2 cells of $>2\times10^{-6}$ cm/s should, in general, be associated with efficient intestinal absorption in humans (Gres *et al.*, 1998). The apical-to-basolateral P_{app} values for 1 to 20 μM udenafil were 2.9-9.6 $\times10^{-6}$ cm/sec (Table I), suggesting that udenafil may be effectively absorbed in the intestinal tract. These data are consistent with the previous report that the low oral bioavailability of udenafil in rats was more dependent on intestinal and hepatic first-pass effect than on lack of absorption (Shim *et al.*, 2003).

The basolateral-to-apical flux was 2.5 to 5.5-fold greater than the apical-to-basolateral flux at concentrations of 1-20 μM. P_{app} values for basolateral-to-apical transport were significantly higher than those for apical-to-basolateral transport at each udenafil concentration (Table I), indicating the presence of an efflux pump to remove udenafil from within cell membranes. P_{app} values for basolateral-to-apical transport were fairly consistent (15.9-22.8 $\times$ 10^{-6} cm/s) at concentrations from 1 to 20 μM. As P-glycoprotein becomes saturated at higher drug concentrations, the apical-to-basolateral transport, *i.e.* absorption, of udenafil would significantly increase at 20 μM (from 2.9-6.6 $\times$ 10^{-6} cm/s at 1-10 μM to 9.1 $\times$ 10^{-6} cm/s) (Table I). As udenafil concentrations increased in the range of 1-20 μM, the absorptive apical-to-basolateral flux displayed nonlinear increases that correlated with saturation of the active transport system. The active transport rate of udenafil across Caco-2 cell monolayers was found to have a sigmoidal saturation curve that fit the Hill equation (Fig. 4). The apparent K_m and V_{max} values for P-glycoprotein-mediated efflux of udenafil in Caco-2 cell monolayers were 14.2 μM and 720.5 pmol/hr/cm^2, respectively, and were comparable to those previously reported for the P-glycoprotein substrates, vinblastine (19.0 μM and 643 pmol/h/cm^2) (Hunter *et al.*, 1993) and paclitaxel (16.5 μM, 1050 pmol/hr/cm^2) (Walle and Walle, 1998).

P-glycoprotein or MRP inhibitors offer an alternate way to evaluate the active transporter involved in udenail efflux. P-glycoprotein inhibitors, such as 5 μM cyclosporine, 50 μM verapamil and 5 μM tariquidar, significantly increased apical-to-basolateral transport from 3.4 $\times$ 10^{-6} to 11.8-15.6

Table I. Apparent permeability coefficients (P_{app}) of udenafil through Caco-2 cell monolayers in apical-to-basolateral (A→B) and basolateral-to-apical (B→A) directions from cells grown on filter inserts suspended in culture

Concentration (μM)	P_{app} (A → B) ($\times 10^{-6}$ cm/s)	P_{app} (B → A) ($\times 10^{-6}$ cm/s)
1	3.7 ± 0.6	15.9 ± 2.8^{a,**}
2	2.9 ± 0.5	15.9 ± 2.6^{a,***}
5	4.5 ± 0.8	21.6 ± 2.8^{a,***}
10	6.6 ± 1.3	22.8 ± 2.4^{a,***}
20	9.1 ± 4.4^{b,*}	21.3 ± 1.6^{a,***}

Data are mean ± S.E. (n = 6).
Significant differences between transport directions [(a)] and udenafil concentration in a given direction [(b)] are shown. *: p<0.05; **: p<0.01; ***: p<0.001

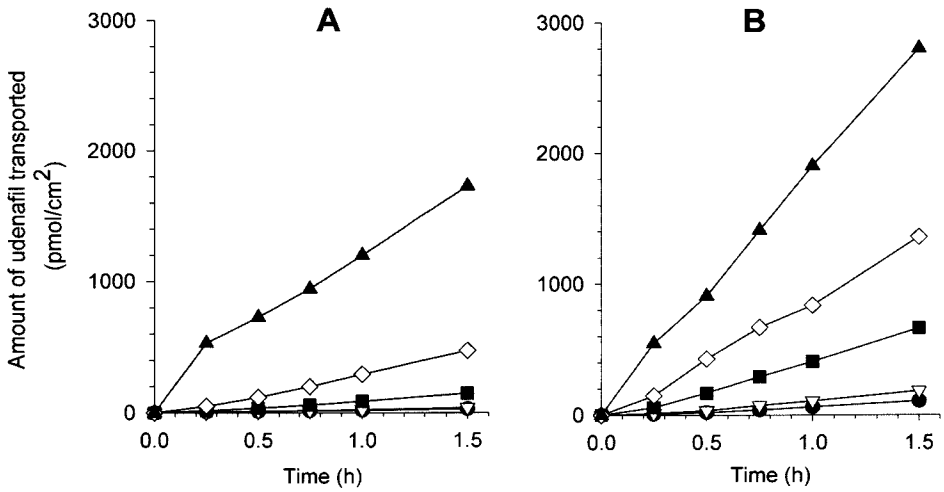

Fig. 3. (A) Apical-to-basolateral and (B) basolateral-to-apical transepithelial flux of various concentrations of udenafil across Caco-2 cell monolayers. Data represent means of six determinations. ●: 1 μM; ▽: 2 μM; ■: 5 μM; ◇: 10 μM; ▲: 20 μM.

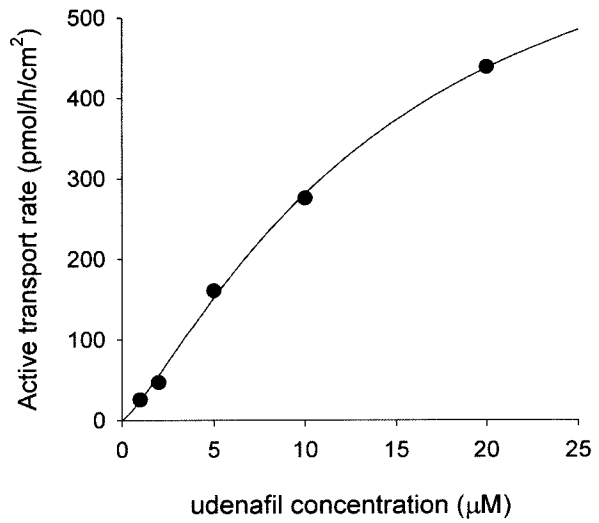

Fig. 4. Michaelis-Menten plot of udenafil active transport. The active transport rate was calculated as (basolateral-to-apical flux – apical-to-basolateral flux)/2. Data are the mean of six determinations.

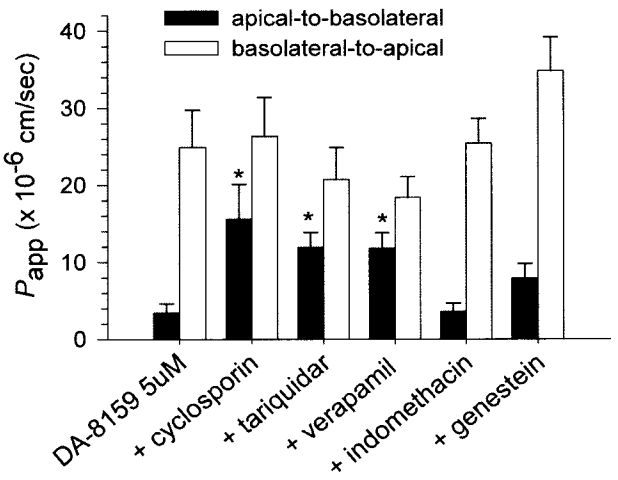

Fig. 5. Effects of P-glycoprotein inhibitors and MRP inhibitors, indomethacin and genestein, on udenafil transport in Caco-2 monolayers. Udenafil concentration was 5 μM. Cyclosporine, 5 μM; tariquidar, 5 μM; verapamil, 50 μM; indomethacin, 75 μM; genestein, 50 μM. Each data is the mean ± S.E. of four experiments. Significant differences between modifier addition and udenafil itself in a given direction, * $p<0.05$.

$\times 10^{-6}$ cm/s (Fig. 5). MRP inhibitors, including 75 μM indomethacin and 50 μM genestein, did not affect P_{app} values for either apical-to-basolateral or basolateral-to-apical transport (Fig. 5). These data indicate that udenafil is a P-glycoprotein substrate.

Although udenafil is metabolized by CYP3A4, the major CYP isoform in human intestine, the metabolites of udenafil were not detected in Caco-2 cells expressing low levels of CYP3A4 (Gan *et al.*, 1996; Schmiedlin-Ren *et al.*, 1997).

The simultaneous expression and cooperative nature of CYP3A4 and P-glycoprotein in the intestine may uniquely affect substrate absorption and cause the potential drug interactions (Silverman, 2000; Wacher *et al.*, 2001). This study demonstrated that udenafil, a CYP3A4 substrate, is also a substrate for the P-glycoprotein efflux pump. P-glycoprotein-mediated efflux of udenafil can contribute to the dose-dependent pharmacokinetics of udenafil in rats (Shim *et al.*, 2003). As a result, the inter-individual variability in the pharmacokinetics and the therapeutic effectiveness of udenafil may be related to variability in P-glycoprotein and CYP3A4 expression.

Furthermore, any co-administered drugs that inhibit or induce P-glycoprotein and/or CYP3A4 may alter the bioavailability of udenafil and potentially lead to changes in the pharmacokinetics of udenafil in patients.

In conclusion, this study demonstrates that udenafil, a CYP3A4 substrate, is also a substrate for P-glycoprotein, suggesting that P-glycoprotein-mediated efflux and CYP3A4-catalyzed metabolism account for the heterogeneity in udenafil bioavailability. Co-administration of inhibitors and inducers of P-glycoprotein and/or CYP3A4 could also induce the changes in the plasma concentrations of udenafil.

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