Effects of Ketoconazole on the Intestinal Metabolism, Transport and Oral Bioavailability of K02, a Novel Vinylsulfone Peptidomimetic Cysteine Protease Inhibitor and a P450 3A, P-Glycoprotein Dual Substrate, in Male Sprague-Dawley Rats1

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ABSTRACT
We investigated the effects of ketoconazole on the oral bioavailability of morpholine-urea-phenylalanine-homophenylalanine-vinylsulfone-phenyl (K02), a vinylsulfone peptidomimetic cysteine protease inhibitor, and a P450 3A (CYP3A) and P-glycoprotein dual substrate, in male Sprague-Dawley rats, so as to evaluate the roles of CYP3A and P-gp in K02 disposition. Male Sprague-Dawley rats (8–10 wk old, n = 3–6) were administered a single dose of K02 (10 mg/kg) i.v. or (30 mg/kg) p.o. with or without a concomitant oral dose of ketoconazole (20 mg/kg). Blood samples were collected from 2 min to 8 h after administration through a implanted jugular vein cannula. K02 plasma concentrations were determined by liquid chromatography/mass spectrometer/mass spectrometer analysis. Ketoconazole markedly raised the area under the curve of orally administered K02 from 9.4 ± 4.4 to 102 ± 24 mg ⋅ min/liter and decreased K02 oral plasma clearance from 3810 ± 1620 to 306 ± 60 ml/min/kg. With concomitant ketoconazole dosing, the changes of AUC of i.v. administered K02 (from 94 ± 17 to 107 ± 14 mg ⋅ min/liter) and clearance (from 110 ± 22 to 95 ± 13 ml/min/kg) were not significant, although K02 oral bioavailability increased from 2.9 ± 1.4 to 31.0 ± 7.5% (P < .001). In summary, ketoconazole, a dual inhibitor of CYP3A and P-glycoprotein, can effectively increase K02 oral bioavailability by inhibiting the CYP3A/P-gp absorption barrier in the small intestine.

K02 (aka K11002) (Arris Pharmaceutical Corporation, South San Francisco, CA) is a member of a newly developed class of vinylsulfone peptidomimetic cysteine protease inhibitors (Palmer et al., 1995). K02 is an irreversible inhibitor to many cysteine proteases, such as cathepsins B, K, L, S and cruzain, exhibiting nanomolar Ki values in vitro. Thus, K02 is a promising lead compound for anticancer and antiparasite drug development (McGrath et al., 1995; McKerrow et al., 1995). Previous in vitro metabolism and transport studies demonstrated that K02 is a substrate of cytochrome P450 3A (CYP3A) as well as a substrate P-gp (Zhang et al., 1998). CYP3A is the major subfamily of hepatic and intestinal CYP enzymes leading to oxidative biotransformation both in humans and rats (Kolars et al., 1992a, 1994; McKinnon et al., 1995). CYP3A is the primary CYP subfamily responsible for phase I metabolism of more than 50% of drugs administered to humans which are metabolized by CYPs (Benet et al., 1996). CYP3A comprises 30 and 70% of total CYPs in liver and intestine, respectively (Kolars et al., 1992b; Shimada et al., 1994; Watkins et al., 1987). Among the CYP3A subfamily, CYP3A4 is the most abundant and most important enzyme in human drug metabolism. Immunohistochemical studies suggest that the major CYP3A protein present in liver, jejunum, colon and pancreas is CYP3A4. CYP3A4 has a very broad substrate specificity. It is known that CYP3A4 catalyzes the metabolism of a large, growing number of structurally diverse and clinically important drugs, covering a wide therapeutic range, e.g., antiarrhythmics, antifungals, calcium-channel blockers, cancer chemotherapeutic agents (e.g., etoposide, paclitaxel, vinblastine and vincristine), hormones, immunosuppressants (cyclosporine, tacrolimus and rapamycin) (Wacher et al., 1995) and HIV protease inhibitors (e.g., saquinavir, indinavir, ritonavir and nelfinavir) (Chiba et al., 1997; Fitzsimmons and Collins, 1997; Kumar et al., 1996; Li et al., 1995; Perry and Benfield, 1997). Intestinal first-pass

ABBREVIATIONS: K02, morpholine-urea-phenylalanine-homophenylalanine-vinylsulfone-phenyl; CYP, cytochrome P450; P-gp, P-glycoprotein; MDR, multidrug resistance; ABC, ATP-binding cassette; IC50, 50% inhibitory concentration; MDCK, Madin-Darby canine kidney cells; AUC, area under curve; CL, clearance; F, oral drug bioavailability; MAT, mean absorption time; MRT, mean resident time; Tmax, peak time; Vss, volume of distribution at steady state; HPLC, high-performance liquid chromatography.
metabolism mediated by CYP3A has been recently recognized to be clinically important for several drugs, such as cyclosporine, tacrolimus and midazolam (Floren et al., 1997; Hebert et al., 1992; Thummel et al., 1996).

P-gp is a plasma membrane glycoprotein belonging to the superfamily of ABC transporters (Gottesman and Pastan, 1993). It functions as an ATP-dependent drug efflux pump to reduce intracellular concentrations. P-gp is expressed on the apical surfaces of many epithelial cells in a number of tissues, e.g., at high levels in columnar epithelial cells of the jejunum and colon, renal proximal tubule epithelium, the luminal surface of biliary hepatocytes (Thiebaut et al., 1987). Studies on knockout mice with disruption of mdr1a or mdr1b, of P-gp from the intestine. For example, the oral bioavailability of paclitaxel is three-fold higher in mdr1a (-/-) mice compared with mdr1a (+/+ ) mice (Sparreboom et al., 1997).

A striking overlap of substrate specificity and tissue distribution for CYP3A and P-gp have been observed (Wacher et al., 1995). This suggests that CYP3A and P-gp may play complementary roles in drug absorption, distribution, metabolism and excretion by biotransformation and countertransport, especially in the villi of the small intestine. Our hypothesis is that CYP3A and P-gp act synergistically in the small intestine as a barrier to oral drug absorption.

Our purpose was to quantitate the effect of ketoconazole, a potent CYP3A inhibitor and a reported P-gp reversal agent in P-gp over-expressed multidrug resistant cell lines (Siegmund et al., 1994), on the oral bioavailability of K02, a CYP3A and P-gp substrate.

**Materials and Methods**

**Materials**

K02 (Mu-Phe-Hph-YS-Phe, aka K11002) and [14C]-K02 (27.6 Ci/mol), with the label on the carbonyl carbon of the morpholine urea moiety, radiopurity > 95%, were kindly supplied by Dr. James Palmer of Atris Pharmaceutical Corporation. Ketoconazole was purchased from USPC (Rockville, MD). 7,8-Benzoflavone, β-NADPH, quinidine and sulfaphenazole were obtained from Sigma Chemical Co. (St. Louis, MO). 4-Dimethylamino-4’-imidazolyl)chalcone was kindly provided by Dean George L. Kenyon, School of Pharmacy, UCSF. Other chemicals were of reagent grade and also purchased from Sigma. All solvents were of HPLC grade and obtained from Fisher Scientific (Santa Clara, CA). Anti-rat CYP3A2 polyclonal antibody and preimmune IgG were purchased from Gentest (Woburn, MA).

**Rat Intestinal Microsome Preparations**

The animal protocol for obtaining male SD rat intestine was approved by the Committee on Animal Research, UCSF. Microsomes were prepared by differential centrifugation (Bornheim and Correia, 1989). The CYP content in microsomal suspensions was measured using the carbon monoxide difference spectrum (Omura and Sato, 1964), and protein concentration was determined using a commercial procedure (Protein Assay Kit, BioRad, Richmond, CA) with human albumin as a standard.

**Incubation Procedure for K02 Metabolism in Rat Intestinal Microsomes and HPLC Assay.**

The same incubation procedure and HPLC assay were used as described previously (Zhang et al., 1998). Briefly, incubation mixtures (final volumes of 0.5 ml) contained rat intestinal microsomes (1.5 mg protein/ml) in 0.1 M phosphate buffer, and K02 (50 μM) in methanol (not more than 1% v/v). After a 5-min preincubation at 37°C, the reaction was initiated by adding NADPH (1 mM) and incubated at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of ice-cold precipitation reagent (62.5% methanol, 37.5% acetonitrile). The internal standard, 4-dimethylamino-4’-imidazolyl)chalcone in methanol, was added. The resultant mixture was vortexed and centrifuged at 13,400 × g for 12 min to precipitate protein. An aliquot of the supernatant (100 μl) was subjected to HPLC analysis. For the chemical inhibition experiments, the CYP reversible inhibitors (Halpert et al., 1994), ketoconazole (CYP3A), 7,8-benzoflavone (CYP1A2), quinidine (CYP2D6) and sulfaphenazole (CYP2C9) were added to the incubation mixture at the same time with K02 (50 μM). The Km of M20 formation is 33 μM. For the immunoinhibition study, a designated amount of polyclonal anti-rat CYP3A2 rabbit antibody or preimmune rabbit IgG was incubated with microsomes (200 μg microsomal protein, 1 mg/ml) for 30 min at room temperature before the K02 incubation (50 μM K02). For the HPLC assay, a Shimadzu LC-600 HPLC system (Kyoto, Japan) and a Waters 710B WISP autosampler (Milford, MA) were used with a Shimadzu SPD-10A UV spectrophotometric detector operated at 220 nm. A Du Pont Zorbax SB-C18 column (MAC-Mod Analytical, Inc., Chadds Ford, PA), 3.0 × 250 mm was used with a precolumn 0.2 mm frit. The isocratic mobile phase of 10 mM potassium phosphate, pH 6.0: methanol: acetonitrile (44:35:21; v/v/v) was delivered at 0.5 ml/min. Chromatographic data were collected through a Hewlett Packard 3392A integrator (Santa Clara, CA).

**Cell Culture and Transepithelial Transport in the Transwell System**

**Cell culture.** The MDR1-MDCK cell line was kindly provided by Dr. Ira Pastan, National Institutes of Health (Pastan et al., 1988). MDR1-MDCK cells were maintained in culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 80 ng/ml colchicine. For transport experiments, MDR1-MDCK cells were grown as epithelial layers by seeding onto permeable filter matrices (final volumes of 0.5 ml) in six-well cluster plates for 4 to 5 days. The integrity of the cell monolayers were measured by transepithelial electrical resistance using a Millipore Millicell-ERS resistance system (Millipore Corporation, Bedford, MA).

**Measurement of bidirectional transepithelial fluxes of [14C]-K02.** The detailed experimental procedure used was that described by Hunter et al. (1993). Serum-free medium, 1.5 and 2.5 ml, were pipetted into insert cups (apical solution) and six-well plates (basolateral solution), respectively. The medium on either the apical or basolateral side of the monolayers contained 10 μM [14C]-K02. The monolayers were then incubated at 37°C for up to 3 hr. Aliquots (50 μl) of solution were taken from the receiving side every 30 min and subjected to liquid scintillation counting (Beckman LS1801 scintillation counter, Beckman Instruments, Inc., Palo Alto, CA). For the inhibition study, ketoconazole was added to the basolateral solution at various concentrations.

**In Vivo Pharmacokinetic Studies**

Male SD rats (B & K, Fremont, CA, 8–10 wk old) were studied. All rats had an indwelling cannula (0.020 in. ID/0.037 in. OD Silastic laboratory tubing, Dow Corning, Midland, MI) implanted in the right jugular vein for blood sampling. The surgery was performed under ketamine/xylazine/acepromazine (22/2.5/0.75 mg/kg, i.p.) anesthesia. For the rats receiving a K02 i.v. dose, a separate cannula was implanted in the left jugular vein for K02 administration. The sur-
gery was performed one day before the study. Each group of rats ($n = 3–6$) were administered a single dose of K02 (10 mg/kg) i.v. or (30 mg/kg) p.o. by gavage with or without a concomitant p.o. dose of ketoconazole (20 mg/kg). K02 and ketoconazole were administered as solutions in DMSO:0.17% sodium phosphate, 0.08% citric acid anhydrous:propylene glycol (10:20:70, v:v:v). Blood samples (100–200 μl) were collected from 2 min to 8 hr after administration through the right jugular vein cannula.

**Determination of K02 Plasma Protein Binding and Blood to Plasma Ratio**

The rat plasma protein binding of [14C]-K02 was determined by ultrafiltration (Lin et al., 1996). Briefly, [14C]-K02 was incubated with rat plasma for 30 min at 37°C in a concentration range of 0.1 to 10 μg/ml. The plasma samples were transferred to Centrifree tubes (Amicon Co., Danvers, MA) and centrifuged at 1500 × g for 15 min. Aliquots of both original plasma and ultrafiltrate samples were subject to scintillation counting. The unbound fraction of K02 was estimated from the ratio of count of ultrafiltrate to that of original plasma sample. The binding of [14C]-K02 to the Centrifree tube was found to be negligible (<0.05%). K02 blood to plasma ratio was measured by the following procedure: briefly, [14C]-K02 was incubated with rat fresh blood for 60 min at 37°C in a concentration range of 0.1 to 10 μg/ml. Blood samples were centrifuged at 4000 rpm for 4 min. Aliquots of plasma and original blood sample were subject to scintillation counting. K02 blood to plasma ratio was calculated directly from ratio of count of blood sample to that of plasma sample.

**LC/MS/MS Determination of K02 Plasma Concentrations**

A 25-μl aliquot of rat plasma was subjected to protein precipitation with 125 μl of acetonitrile containing 0.02 ng/μl of internal standard (Lovastatin). After vortexing and centrifugation, the supernatant was transferred to vial and 10 μl were injected into LC/MS/MS system. The chromatographic separation was achieved with a C18 column (5 μm, 4.6 × 50 mm) using a mobile phase of 20/80% water/acetonitrile containing 0.06% trifluoroacetic acid at a flow rate of 0.8 ml/min. The retention times of K02 and internal standard are 0.6 and 1.1 min, respectively. The mass spectrometer was an API III PE-Sciex (Thornhill, Canada) equipped with an atmospheric pressure chemical ionization interface. The positive ions monitored with MRM for the quantification of K02 at m/z 562 → 233 and at m/z 405 → 199 for internal standard. The accuracy and precision of the assay for the spiked standard rat plasma at the K02 concentration range of 1 ng/ml to 4 μg/ml were within 12 and 10%, respectively.

**Pharmacokinetic Analysis**

Noncompartmental methods (systems analysis) were used to calculate the K02 pharmacokinetic parameters. MAT are calculated as the ratio of the area under the first moment curve divided by AUC for the p.o. dose minus the same parameter calculated for the i.v. dose (MRT).

**Results**

CYP3A2 is the principle cytochrome P450 for K02 oxidation in rat intestinal microsomes. Similar biotransformation profiles were observed for HPLC chromatographs obtained following K02 incubations in human liver, rat liver and intestinal microsomes, although the catalytic activity for K02 oxidation in rat intestinal microsomes is about 10% of that in rat liver microsomes (fig. 1). M20 (numbered with reference to retention time), an hydroxylated product of K02 (Zhang et al., 1998) and a major metabolite formed in rat microsomal incubation systems, was chosen for the identification of the major CYPs involved in K02 biotransformation in the rat intestinal microsomes. The M20 formation activi-
IC$_{50}$ value of about 1 µM (fig. 3). Quinidine, 7,8-benzoflavone and sulfaphenazole, selective chemical inhibitors of CYP2D6, CYP1A2 and CYP2C9 at their recommended inhibitor concentrations respectively, had no significant effects on the formation of M20 (fig. 2). Rabbit anti-rat CYP3A2 polyclonal antibody significantly inhibited M20 formation (fig. 2), although no effect was found for control preimmune antibody.

Ketoconazole effectively inhibits the P-glycoprotein mediated transport of K02 across MDR1-MDCK cell monolayers. Ketoconazole inhibited K02 basolateral to apical (B-A) flux. As depicted in figure 4, the B-A flux of 10 µM $^{14}$C-K02 across MDR1-MDCK cell monolayers was markedly inhibited by ketoconazole (100 µM in basolateral compartment), although K02 apical to basolateral (A-B) flux was increased. The concentration-dependent inhibition of K02 B-A flux by ketoconazole is demonstrated in figure 5. The IC$_{50}$ of ketoconazole was 120 µM obtained by nonlinear regression.

Pharmacokinetics of K02 in male SD rats in the absence and presence of ketoconazole. K02 plasma concentration versus time curves for i.v. (10 mg/kg) and p.o. (30 mg/kg) administration in the absence and presence of a concomitant ketoconazole p.o. dose (20 mg/kg) are depicted in figures 6 and 7. Pharmacokinetic parameters of K02 calculated by noncompartmental methods are listed in table 1. The K02 protein binding and blood to plasma concentration ratio were measured over a wide concentration range, and found to be concentration independent with $f_u = 0.045 \pm 0.008$ and $C_P/C_U = 0.85 \pm 0.02$. Here a concomitant ketoconazole oral dose (20 mg/kg) raised the AUC of orally administered K02 from 9.4 ± 4.4 to 102 ± 24 mg · min/liter and decreased K02 CL/F from 3810 ± 1620 to 306 ± 60 ml/min/
kg. For i.v. administered K02 with concomitant oral ketoconazole changes vs. control for AUC (from 94 ± 17 to 107 ± 14 mg · min/liter), CL (from 110 ± 22 to 95 ± 13 ml/min/kg), volume of distribution at steady-state ($V_{ss}$) (from 1.9 ± 0.3 l/kg to 2.0 ± 0.6 l/kg) and MRT (from 17.4 ± 2.0 to 21.4 ± 8.3 min) were not significant. However, the concomitant ketoconazole oral dose (20 mg/kg) markedly increases K02 oral bioavailability from 2.9 ± 1.4 to 31.0 ± 7.5% (P < .001) (table 1).

**Discussion**

Our previous in vitro metabolism study demonstrated that K02 is a substrate of CYP3A and that CYP3A is the principle CYP for K02 oxidation in human liver microsomes (Zhang et al., 1998). We confirmed that CYP3A is also a major CYP enzyme for K02 oxidation in rat liver and intestinal microsomes (fig. 2). Ketoconazole, a potent CYP3A inhibitor, can significantly inhibit K02 biotransformation in rat intestinal microsomes with an IC$_{50}$ value of approximately 1 μM (fig. 3). In vitro microsomal incubations demonstrated that the catalytic activity of rat intestinal microsomes is much less than that of rat liver microsomes. However, because of the difficulty of enterosomes preparation in vitro, the quantitative significance of in vivo intestinal metabolism may be more than suggested by in vitro intestinal microsomal activity.

Our previous transport studies (Zhang et al., 1998) demonstrated that K02 is a substrate of P-gp. As shown in figure 4, K02 exhibits marked differences in bidirectional transepithelial transport across MDR1-MDCK cell monolayers. Ketoconazole can effectively inhibit P-gp mediated transport of K02 by decreasing K02 B-A flux and increasing K02 A-B flux (fig. 4). The IC$_{50}$ value for ketoconazole inhibition of K02 transport in MDR1-MDCK cells is approximately 120 μM (fig. 5). This is the first reported direct evidence for ketoconazole inhibition of P-gp mediated transport in Transwell systems.

These in vitro results provided the basis for studying the influence of CYP3A and P-gp in K02 oral bioavailability. Several pharmacokinetic studies using ketoconazole have demonstrated the importance of CYP3A and/or P-gp in drug absorption, distribution, elimination and toxicity (Floren et al., 1997; Gomez et al., 1996; Spina et al., 1997; Yuan et al., 1997).

Oral ketoconazole dosing (20 mg/kg) had little effect on the pharmacokinetics of i.v. K02 as seen in figure 6 and in the calculated pharmacokinetic parameters using noncompartmental methods (table 1). It is very obvious that the primary influence of the concomitant oral dosing of ketoconazole (20 mg/kg) on K02 oral data is explained by effects on the absorption process ($F$ increases more than 10-fold, table 1), although ketoconazole causes minimal changes in $T_{max}$ and MAT (table 1).
The primary goal of this study was to support the hypothesis that CYP3A-mediated intestinal metabolism and P-gp-mediated countertransport process in the gut create a bioavailability barrier for drugs which are CYP3A and/or P-gp substrates, and to demonstrate that this CYP3A/P-gp oral bioavailability barrier can be overcome by oral coadministration of CYP3A and/or P-gp inhibitors, e.g., ketoconazole. The most important evidence in support of this hypothesis is the 10-fold increase of K02 oral bioavailability with coadministration of an oral dose of ketoconazole (table 1) without significant changes in other pharmacokinetic parameters, e.g., CL. These results demonstrate that ketoconazole inhibits the CYP3A-mediated metabolism and P-gp mediated countertransport of K02 in the gut, thus effectively blunting the intestinal first-pass effect. Due to the combined effect of CYP3A and P-gp in the gut, it is not possible to quantitatively assess the relative contributions of CYP3A and P-gp on K02 bioavailability. Due to the continual cycling of drug between the gut lumen and enterocytes, resulting from passive absorption processes and P-gp countertransport, we hypothesize that inhibiting P-gp will reduce the access of substrates to intestinal CYP3A. However, on the basis of our in vitro studies showing that ketoconazole is a much more potent inhibitor of CYP3A mediated metabolism of K02 (IC50 = 1 μM) than of P-gp-mediated transport (IC50 = 120 μM), and because concomitant ketoconazole dosing caused no significant changes in T1/2 and the MAT (table 1), it is likely that ketoconazole mainly contributes to the inhibition of CYP3A-mediated gut metabolism of K02. Additional studies are required to substantiate this possibility.

In conclusion, this study confirms that the oral bioavailability of CYP3A and P-gp substrates, such as K02, can be dramatically altered after a concomitant oral dose of a CYP3A and P-gp inhibitor, with no effect on other pharmacokinetic parameters, such as plasma clearance.

### References


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