OVERLAPPING SUBSTRATE SPECIFICITIES OF CYTOCHROME P450 3A AND P-GLYCOPROTEIN FOR A NOVEL CYSTEINE PROTEASE INHIBITOR

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(Received May 12, 1997; accepted December 18, 1997)

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

ABSTRACT:

K02 (morpholine-urea-Phe-Hphe-vinylsulfone), a newly developed peptidomimetic, acts as a potent cysteine protease inhibitor, especially of cathepsins B and L (which are associated with cancer progression) and cruzain (a cysteine protease of Trypanosoma cruzi, which is responsible for Chagas’ disease). Here we investigated the effects of the disposition of K02 using in vitro systems, characterizing the interaction of the drug with human cytochrome P450 (CYP) 3A and P-glycoprotein (P-gp), a mediator of multidrug resistance (MDR) to cancer chemotherapy and a countertransporter in the intestine that limits oral drug bioavailability. P-gp functions as an ATP-dependent drug efflux pump to reduce intracellular cytotoxic concentrations. An HPLC assay was developed to analyze K02 and its metabolites formed in human liver microsomes. Three major primary metabolites were determined by LC/MS/MS to be hydroxylated products of the parent compound. A rabbit anti-CYP3A polyclonal antibody (200 μl antibody/mg microsomal protein) produced 75–94% inhibition of the formation of these three hydroxylated metabolites. Ketoconazole (5 μM), a selective CYP3A inhibitor, produced up to 75% inhibition, whereas other CYP-specific inhibitors, i.e. quinidine (CYP2D6), 7,8-benzo-flavone (CYP1A2), and sulfaphenazole (CYP2C9), showed no significant effects. An identical metabolism formation profile for K02 was observed with cDNA-expressed human CYP3A4 (Genentest). These data demonstrate that K02 is a substrate for CYP3A. Formation of 1'-hydroxymidazolam, the primary human midazolam metabolite, was markedly inhibited by K02

Chemotaxis and metastasis (Elliott and Sloane, 1996). A key event necessary for tumor growth and invasion of tumor cells into normal tissues is the removal of extracellular matrix by matrix-degrading proteases, which can be broadly classified into three major groups, i.e. serine proteases, cysteine proteases (e.g. cathepsins B and L), and metalloproteinases (Chen, 1992). Recently, these proteases have become targets for the development of inhibitors, which may serve as potential therapeutic agents (DeClerk and Imren, 1994; Clawson, 1996).

Trypanosoma cruzi, a protozoan parasite, is the etiologic agent of American trypanosomiasis, which is responsible for Chagas’ disease. The major proteolytic activity of T. cruzi is a cathepsin L-like cysteine protease, termed cruzain, that is expressed in all life stages of the parasite (Scharfstein et al., 1986; Bontempi et al., 1989; North et al., 1990). Cruzain has been shown to be a promising therapeutic target for antiparasite drug design (McKerrow et al., 1995; McGrath et al., 1995).

K02 (morpholine-urea-Phe-Hphe-vinylsulfone) is one of a class of newly developed peptidomimetics that act as potent cysteine protease inhibitors (Palmer et al., 1995). K02 is an irreversible inhibitor of cruzain, that is expressed in all life stages of the parasite (Scharfstein et al., 1986; Bontempi et al., 1989; North et al., 1990). Cruzain has been shown to be a promising therapeutic target for antiparasite drug design (McKerrow et al., 1995; McGrath et al., 1995).

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tease inhibitors all appear to be substrates for CYP3A (Prueksaritanont et al., 1994; Kumar et al., 1996; Chiba et al., 1996). Recently, CYP3A has also been found to be expressed in some human neoplasms (Fritz et al., 1993; Kivisto et al., 1995; Murray et al., 1994, 1995a,b), which indicates that CYP3A may deactivate cancer drugs within the tumor cells if the drugs are substrates for CYP3A (Wacher et al., 1995). The contribution of intestinal CYP3A-mediated metabolism to poor oral drug bioavailability has been shown to be clinically important for several drugs, such as cyclosporine, tacrolimus, and midazolam (Hebert et al., 1992; Wu et al., 1995; Thummel et al., 1996).

A major impediment to effective cancer chemotherapy is MDR, a process by which tumor cells rid themselves of anticancer drugs (Patel and Rothenberg, 1994). One important mechanism for MDR is known to involve the overexpression of P-gp in tumor cells in response to drug exposure (Endicott and Ling, 1989; Ling, 1995). P-gp is a member of a superfamily of highly conserved ATP-binding cassette transport proteins (Gottesman and Pastan, 1993). P-gp functions as an ATP-dependent drug efflux pump to reduce intracellular cytotoxic concentrations. In humans, there are two MDR genes, MDR1 and MDR2, with MDR1 conferring drug resistance. MDR1 is expressed on the apical surfaces of many epithelial cells in a number of tissues; for example, it is expressed at high levels in columnar epithelial cells of the jejunum (Thiebaut et al., 1987). Although its exact physiological roles are still under investigation, P-gp is generally considered to play an important detoxification role via countertransport. Several pharmacokinetic studies using P-gp-knockout mice demonstrated the importance of P-gp in limiting oral drug bioavailability when drugs are P-gp substrates (Schinkel et al., 1995, 1997; Sparreboom et al., 1997; van Asperen et al., 1996).

A striking overlap of the substrates for CYP3A and P-gp, including a wide variety of hydrophobic therapeutic agents (and many anticancer drugs), and their tissue distributions has been observed (Wacher et al., 1995). These observations led us to suggest that CYP3A and P-gp might play complementary roles in drug disposition by biotransformation (phase I) and antitransport (phase III), especially in the villi of the small intestine, where CYP3A and P-gp act synergistically as an oral drug bioavailability barrier. Several pharmacokinetic studies using CYP3A and/or P-gp inhibitors illustrated the importance of CYP3A and P-gp in drug absorption and disposition (Floren et al., 1997; Gomez et al., 1995; van Asperen et al., 1997). For the present work, we studied the interaction of K02, a promising prototypical cysteine protease inhibitor, with CYP3A and P-gp systems in vitro.

Materials and Methods

Chemicals and Biochemicals. K02 and [14C]K02 (27.6 Ci/mmol) were kindly supplied by Arris Pharmaceutical Corp. (South San Francisco, CA); LU-49888 (28 Ci/mmol), a ‘H-labeled photofinity analogue of verapamil, was provided by Dr. Xiaodong Qian of BASF (Cambridge, MA). CEM 5k is a clonal antibody or preimmune rabbit IgG was incubated with microsomes for 30 min at room temperature before the K02 incubation, as described above (engineered to stably express human CYP3A4 cDNA) (4 mg/ml), in 0.1 M phosphate buffer, plus K02 (10–200 μM) in methanol (≤1%, v/v). After a 5-min preincubation at 37°C, the reaction was initiated by addition of NADPH (1 mM), and the mixture was incubated at 37°C for 10 min. The reaction was stopped by addition of 0.5 ml of ice-cold precipitation reagent (62.5% methanol/37.5% acetonitrile). The internal standard, i.e. 4-dimethylamino-4’-(imidazol-1-yl)chalcone in methanol, was added. The resultant mixture was vortex-mixed and centrifuged at 13,400g for 12 min, to precipitate protein. An aliquot of the supernatant (100 μl) was subjected to HPLC analysis.

**Chemical Inhibition Study.** The reversible inhibitors for CYPs used in this study, i.e. ketoconazole (CYP3A, 7,8-benzoflavone (CYP1A2), quinidine (CYP2D6), and sulfaphenazole (CYP2C9), were added to the incubation mixture at the same time as K02 (50 μM). The incubation protocol was the same as that described above (see General Incubation Procedure).

**HPLC Assay for K02 and Metabolites.** A Shimadzu LC-600 HPLC system and a Waters 710B WISP autosampler were used with a Shimadzu SPD-10A UV spectrophotometric detector operated at 220 nm. A Du Pont Zorbax SB-C18 column (3.0 × 250 mm; MAC-MOD Analytical, Inc., Chadds Ford, PA) 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. Under these conditions, K02 and the internal standard eluted at 29 and 35 min, respectively. All K02 metabolites eluted between 8 and 24 min. Chromatographic data were collected with a Hewlett Packard 3392A integrator.

**Inhibition of Midazolam Oxidation by K02 in Human Liver Microsomes.** The procedure for incubation of midazolam, in the presence or absence of K02, with human liver microsomes was the same as described above. HPLC analysis of midazolam, 4-hydroxymidazolam, and 1’-hydroxymidazolam was carried out following the methods of Wrighton and Ring (1994).

**MS Analysis.** Mass spectra and tandem mass spectra of K02 and its metabolites were acquired using a PE SCIEX API III triple-quadrupole mass spectrometer (Perkin-Elmer/ABI, Foster City, CA) equipped with a heated Nebulizer inlet (Thornhill, Ontario, Canada). Positive ionization was achieved by atmospheric-pressure chemical ionization. Scans were acquired over the range of m/z 250–700 for MS and m/z 50–600 for MS/MS. Collisional activations were accomplished for MS/MS using 90% argon/10% nitrogen as the collision gas, at an energy of 25 eV.

**Photoaffinity Labeling of P-gp and Autoradiography.** Membrane samples containing P-gp (MDR1) prepared from CEM4 cells were kindly supplied by Dr. Xiaodong Wang of BASF (Cambridge, MA). CEM4 is a derived drug-resistant cell line of human leukemic lymphoblasts selected using vinblastine (Beck et al., 1986). Membrane protein (200 μg) was preincubated with 100 nM [3H]LU-49888 (about 2 μCi) (Qian and Beck, 1990) or 50 nM azidopine (2 μCi), in the presence or absence of K02, in a buffer containing 250 mM sucrose and 10 mM Tris-HCl, pH 7.4, at room temperature for 30 min and then was irradiated with UV light (254 nm), at a distance of 10 cm, for 10 min at 4°C. Photoabeled proteins were diluted (1:1) with Laemmli sample buffer and separated by one-dimensional 4–15% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad). After staining with Coomassie blue and destaining, the gels were soaked in Amplify solution (Amer- sham, Arlington Heights, IL) for 30 min and dried under vacuum at 75°C. The dried gels were exposed to Hyperfilm-ECL (Amer sham) for 4–5 days at
K02 concentration was 100 μM. The experimental procedure was as described by Hunter et al. (1993). 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 them onto permeable filter matrices of Transwell inserts (4.7-cm² growth area; Corning Costar Corp., Cambridge, MA), in six-well cluster plates, for 4–5 days. The integrity of the cell monolayers was assessed by transepithelial electrical resistance measurements with a Millipore Millicell-ERS resistance system (Millipore Corp., Bedford, MA).

**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 of Transwell inserts (4.7-cm² growth area; Corning Costar Corp., Cambridge, MA), in six-well cluster plates, for 4–5 days. The integrity of the cell monolayers was assessed by transepithelial electrical resistance measurements with a Millipore Millicell-ERS resistance system (Millipore Corp., Bedford, MA).

Measurement of Bidirectional Transepithelial Fluxes of [14C]K02. The experimental procedure was as described by Hunter et al. (1993). First, 2.5-ml and 1.5-ml aliquots of serum-free medium 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. Fifty microliters of solution were taken from the accumulation side every 30 min and subjected to liquid scintillation counting (Beckman LS1801 scintillation counter; Beckman Instruments, Palo Alto, CA).

**Results**

Biotransformation of K02 in Human Liver Microsomes. Fig. 1A illustrates a typical HPLC metabolite profile for K02 in a human liver microsomal incubation. Three major metabolites, i.e., M12, M19, and M20 (numbered with reference to retention times), were chosen for further study to identify the major CYPs involved in biotransformation. The formation of all metabolites was NADPH dependent, and the formation of these three metabolites appeared to follow Michaelis-Menten-type kinetics. The corresponding K_M and V_max values are summarized in Table 1.

**Determination of Metabolite Structure by Tandem MS.** M12, M19, and M20 were determined by LC/MS to be single-hydroxylated products of the parent compound K02. Analysis of tandem mass spectra of these three major primary metabolites revealed the possible hydroxylation sites. The deduced structures of M12, M19, and M20 (fig. 4) are shown in Fig. 2. Other K02 metabolites shown in Fig. 1A were determined to be isomers of M12, M19, and M20, in terms of the corresponding hydroxylation regions, and to be sequentially hydroxylated products (data not shown).

**CYP3A4, a Primary Enzyme Responsible for K02 Biotransformation in Human Liver Microsomes.** Chemical Inhibition Study. Ketocozazole, a reversible inhibitor of CYP3A4, strongly inhibited the formation of M12, M19, and M20, with IC_50 values in the range of 1–4 μM (fig. 3A). Quinidine, 7,8-benzoflavone, and sulfaphenazole, selective chemical inhibitors of CYP2D6, CYP1A2, and CYP2C9, respectively, at their recommended inhibitor concentrations (Halpert et al., 1994) had no significant effects on the formation of M12, M19, and M20 (fig. 4).

**Immunoinhibition Study.** Rabbit anti-CYP3A polyclonal antibody (Gorski et al., 1994), which is known to cross-react with human CYP3A4, exhibited concentration-dependent inhibitory effects on M12, M19, and M20 formation. At a concentration of 200 μM of microsomal protein, this antibody showed 75–94% inhibitory effects on the formation of M12, M19, and M20 (fig. 4).

**Metabolism by cDNA-Expressed CYP3A4.** The HPLC metabolite profile of K02 in a cDNA-expressed human CYP3A4 system (with CYP reductase coexpression) is shown in Fig. 3B. Identical metabolite profiles were observed in HPLC chromatographs obtained for K02 incubations with human liver microsomes and cDNA-expressed human CYP3A4 (fig. 1). The formation of M12, M19, and M20 appeared to follow Michaelis-Menten kinetics with the corresponding IC_50 values. The Lineweaver-Burk plot shown in Fig. 5 demonstrates that K02 competitively inhibited formation of 1'-hydroxymidazolam, a major metabolite formed by CYP3A4 in humans. The concentration range of midazolam was 12.5–150 μM. The K_M for 1'-hydroxymidazolam was determined to be 11.9 ± 0.6 μM and the K_M for K02 was found to be 12.1 ± 0.2 μM by nonlinear regression analysis using PCNONLIN version 4.0 (Statistical Consultants Inc., Lexington, KY), based on a simple competitive-inhibition Michaelis-Menten model.

### Table 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>K_M (μM)</th>
<th>V_max (nmol/mg/min)</th>
<th>Incubation System</th>
</tr>
</thead>
<tbody>
<tr>
<td>M12</td>
<td>37.6 ± 3.4</td>
<td>1.51 ± 0.01</td>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>M12</td>
<td>10.9 ± 2.9</td>
<td>0.53 ± 0.04</td>
<td>cDNA-expressed CYP3A4</td>
</tr>
<tr>
<td>M19</td>
<td>52.4 ± 3.5</td>
<td>0.30 ± 0.02</td>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>M19</td>
<td>18.0 ± 3.2</td>
<td>0.45 ± 0.03</td>
<td>cDNA-expressed CYP3A4</td>
</tr>
<tr>
<td>M20</td>
<td>33.3 ± 8.4</td>
<td>1.19 ± 0.06</td>
<td>Human liver microsomes</td>
</tr>
<tr>
<td>M20</td>
<td>31.2 ± 0.1</td>
<td>0.65 ± 0.01</td>
<td>cDNA-expressed CYP3A4</td>
</tr>
</tbody>
</table>
Inhibition by K02 of Photoaffinity Labeling of P-gp. K02 significantly inhibited the photoaffinity labeling of P-gp with azidopine and LU-49888, a photoaffinity analogue of verapamil, as depicted in the autoradiograph shown in fig. 6. Densitometric readings for labeled P-gp bands in the autoradiographs, in the absence and presence of K02 (10 μM), yielded 80 and 74% (fig. 6, lanes 2 and 6) inhibition of LU-49888 and azidopine labeling, respectively, compared with controls (0 μM K02) (fig. 6, lanes 1 and 5).

Bidirectional Transepithelial Transport Studies Using MDR1-MDCK Cell Monolayers in the Transwell System. As depicted in fig. 7, the basolateral-to-apical flux of 10 μM \[^{14}C\]K02 across MDR1-MDCK cell monolayers was markedly greater than the apical-to-basolateral flux (ratio, 63). This specific basolateral-to-apical transport was significantly inhibited by vinblastine, an avid P-gp substrate (48% inhibition at 25 μM).

**Discussion**

K02 is the first member of a newly developed class of vinylsulfone peptidomimetics, which act as potent mechanism-based cysteine protease inhibitors, to be subjected to biotransformation studies using human liver microsomal preparations and transport studies using MDR1-MDCK cell monolayers in the Transwell system. K02 and other compounds in its class act as potent inhibitors of cathepsins B and L (two cancer-associated cysteine proteases) and cruzain (the major cysteine protease present in *T. cruzi*). The study described here demonstrates that K02 is a CYP3A substrate and CYP3A plays a primary role in the oxidative biotransformation of K02 in human liver microsomal preparations. These conclusions are based on the results of an integrated *in vitro* approach using 1) selective CYP chemical inhibitors, 2) immunoinhibition with an anti-CYP3A antibody, and 3) cDNA-expressed human CYP3A4. Both ketoconazole, a CYP3A-selective inhibitor, and rabbit anti-CYP3A antibody demonstrated potent and concentration-dependent inhibitory effects on the formation of three major primary hydroxylated metabolites of K02, designated M12, M19, and M20. The IC\(_{50}\) values for ketoconazole were in the range of 1–4 μM, which are typical values for this potent reversible CYP3A inhibitor. Representative chemical inhibitors of other major CYP enzymes responsible for human drug metabolism, *i.e.*
quinidine (CYP2D6), 7,8-benzoflavone (CYP2C9), and sulfa-
phenazole (CYP1A2), at their recommended concentrations showed
no significant effects on K02 metabolite formation. Incubation of K02
with cDNA-expressed human CYP3A4 produced a metabolite profile
almost identical to that obtained with human liver microsomes. How-
ever, the $K_M$ and $V_{max}$ values obtained in these two incubation
systems were not exactly comparable, with differences of up to 4-fold.
We speculate that the CYP conformations and/or the interactions of
NADPH-CYP reductase with CYP are not exactly the same in these
two systems, because of differing conditions of the microsomal mem-
branes. K02 also competitively inhibited the formation of 1'-hydroxymidazolam, which is the major midazolam metabolite in humans

and is produced by CYP3A4 (Thummel et al., 1994a,b). This suggests
the potential for drug-drug interactions of K02 with other CYP3A
substrates when the drugs are administered concomitantly.

K02 strongly inhibited the photoaffinity labeling of P-gp with
azidopine and LU-49888, a photoaffinity analogue of verapamil.
Ketoconazole on the intestinal metabolism, transport, and oral bioavailability of cyclosporine A (CyA) and its metabolite, cyclosporine GL, is that coadministration of ketoconazole with CyA results in reduced oral bioavailability of CyA. Our pharmacokinetic studies conducted in male Sprague-Dawley rats demonstrate that ketoconazole is a substrate of P-gp, as evidenced by the reduction in oral bioavailability of CyA following coadministration with ketoconazole. These data clearly support the hypothesis that there is substrate specificity overlap of CYP3A and P-gp.
retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proc Natl Acad Sci USA* **85**:4486–4490.


