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

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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 inhibition of these three hydroxylated metabolites. Ketoconazole (5 μM), a potent 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 metabolite formation profile for K02 was observed with cDNA-expressed human CYP3A4 (Gentest). These data demonstrate that K02 is a substrate for CYP3A. Formation of 1'-hydroxymidazolam, the primary human midazolam metabolite, was markedly inhibited by K02 via competitive processes. K02 significantly inhibited the photoaffinity labeling of P-gp with azidopine and LU-49888, a photoaffinity analogue of verapamil. Transport studies with [14C]K02, using MDR1-transfected Madin-Darby canine kidney cell monolayers in the Transwell system, demonstrated that the basolateral-to-apical flux of K02 across MDR1-transfected Madin-Darby canine kidney cells was markedly greater than the apical-to-basolateral flux (ratio of 63 with 10 μM [14C]K02). This suggests that K02 is also a P-gp substrate. These studies are important for formulating strategies to increase the absorption and/or decrease the elimination of K02 and to optimize its delivery to malignant cells and parasite-infected host cells.

Cancer biology research has demonstrated that proteases are involved in all stages of cancer progression, including growth, angiogenesis, 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 or 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).

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).

1 McKerrow JH (Department of Pathology, Veterans Affairs Medical Center, San Francisco, CA; personal communication).

2 Abbreviations used are: CYP, cytochrome P450; P-gp, P-glycoprotein; MDR, multidrug resistance; MDCK, Madin-Darby canine kidney.

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midazolam (Hebert 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 cyclosporin, 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; Sparerboom 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 photofluorophore analogue of verapamil, was provided by Dr. Xiaodong Qian of BASF (Cambridge, MA). Midazolam was a gift from Hoffmann-LaRoche (Nutley, NJ). Ketoconazole was purchased from United States Pharmacopeial Convention (Rockville, MD). 7,8-Benzoflavone, β-NAPDH, quinidine, and sulfaphenazole were obtained from Sigma Chemical Co. (St. Louis, MO). 4-Dimethylamino-4’-(imidazol-1-yl)chalcone was kindly provided by Dr. George L. Kenyon, School of Pharmacy, University of California, San Francisco. Other chemicals were of reagent grade and were also purchased from Sigma. All solvents were of HPLC grade and were obtained from Fisher Scientific (Santa Clara, CA). The Bio-Rad protein assay kit with albumin protein standard was obtained from Bio-Rad (Richmond, CA). The rabbit anti-human CYP3A polyclonal antibody and preimmune IgG were kindly provided by Dr. Steven A. Wrighton, Eli Lilly and Co. (Indianapolis, IN). cDNA-expressed human CYP3A4 (M107r) was purchased from Gentest (Cambridge, MA). Azidopine (44 Ci/mmol) was obtained from Amersham (Arlington Heights, IL).

Human Liver Microsome Preparation. A human liver sample was obtained under a protocol approved by the Committee on Human Research, University of California, San Francisco. Microsomes were prepared by differential centrifugation as previously described (Bornheim and Correia, 1989). The CYP contents in microsomal suspensions were measured using carbon monoxide difference spectra (Omura and Sato, 1964), and protein concentrations were determined by the method of Lowry et al. (1951).

Incubation Procedure for K02 Metabolism by Human Liver Microsomes and cDNA-Expressed CYP3A4. General Incubation Procedure. K02 metabolism in human liver microsomes was found to be linear during 20-min incubations with microsomal protein concentrations of 1–2 mg/ml. Incubation mixtures (final volume, 0.5 ml) contained human liver microsomes (1.5 mg protein/ml) or microsomes prepared from a human B-lymphoblastoid cell line (engineered to stably express human CYP3A4 cDNA) (4 mg/ml), in 0.1 M phosphate buffer, pH 7.0. 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 13,400×g 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 described above (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 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 method of Wrighton and Ring (1994).

MS Analysis. Mass spectra and tandem mass spectra of K02 and its metabolites were acquired using a PE SCIX API III triple-quadrupole mass spectrometer (Perkin-Elmer/ABI, Foster City, CA) equipped with a heated Nebulizer inlet (Thorhill, Ontario, Canada). Positive ionization was achieved by atmospheric-pressure chemical ionization. Scan range was acquired over the range of m/z 200–700 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.

Photofluorophility Labeling of P-gp and Autoradiography. Membrane samples containing P-gp (MDR1) prepared from CEM6A cell cultures were kindly supplied by Dr. Xiaodong Wang of BASF (Cambridge, MA). CEM6A 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. Photolabeled 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 (Amerham, Arlington Heights, IL) for 30 min and dried under vacuum at 75°C. The dried gels were exposed to Hyperfilm-ECL (Amerham) for 4–5 days at
K02 concentration was 100 μM, and the experimental procedure was as described by Hunter et al. (1993). First, 2.5-ml aliquots of serum-free medium were pipetted into insert cups 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).

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, MDRI-MDCK cells were grown as epithelial layers by seeding at a density of 70,000 cells per insert. The corresponding metabolite profile of K02 in a cDNA-expressed human CYP3A4 system (with CYP3A4, exhibited concentration-dependent inhibitory effects on M12, M19, and M20 formation. At a concentration of 200 μmol/ml microsomal protein, this antibody showed 75–94% inhibitory effects on the formation of all metabolites. The formation of all metabolites was NADPH dependent, and the kinetic parameters for primary metabolites (M12, M19, and M20) of K02 in incubations with human liver microsomes and cDNA-expressed CYP3A4.

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 and the corresponding MS/MS spectra, together with that for K02, are illustrated 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. Ketocazole, 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 μmol/mg microsomal protein, this antibody showed 75–94% inhibitory effects on the formation of M12, M19, and M20 formation (fig. 3B).

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. 1B. Identical metabolite profiles were observed in HPLC chromatograms 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 metabolite profile of K02 in a cDNA-expressed human CYP3A4 system (with M12, M19, and M20). The corresponding K_M and V_max values are summarized in table 1.

Inhibition of 1’-Hydroxymidazolam Formation by K02 in Human Liver Microsomes. 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_I 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>
<thead>
<tr>
<th>Metabolite</th>
<th>K_M (μM)</th>
<th>V_max (nmol/min/mg protein)</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>
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<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>

TABLE 1

Kinetic parameters for primary metabolites (M12, M19, and M20) of K02 in incubations with human liver microsomes and cDNA-expressed CYP3A4.
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 [14C]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 peptidase 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 IC50 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. However, the \( K_M \) and \( V_{\text{max}} \) 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 membranes. 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.
Verapamil is a documented inhibitor and substrate of P-gp and has been clinically coadministered with many anticancer drugs as a P-gp inhibitor, to overcome or alleviate MDR in cancer chemotherapy (Futschek et al., 1996). Bidirectional transepithelial transport studies of K02 with MDR1-MDCK cell monolayers in the Transwell system clearly demonstrated that K02 is a substrate of P-gp.


