IN VITRO EVALUATION OF THE DISPOSITION OF A NOVEL CYSTEINE PROTEASE INHIBITOR

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ABSTRACT:

K11777 (N-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl) is a potent, irreversible cysteine protease inhibitor. Its therapeutic targets are cruzain, a cysteine protease of the protozoan parasite Trypanosoma cruzi, and cathepsins B and L, which are associated with cancer progression. We evaluated the metabolism of K11777 by human liver microsomes, isolated cytochrome P450 (CYP) enzymes, and flavin-containing monooxygenase 3 (FMO3) in vitro. K11777 was metabolized by human liver microsomes to three major metabolites: N-oxide K11777 (Km = 14.0 ± 4.5 μM and apparent Vmax = 3460 ± 3190 pmol·mg⁻¹·min⁻¹, n = 4), β-hydroxy-homoPhe K11777 (Km = 16.8 ± 3.5 μM and Vmax = 1260 ± 1090 pmol·mg⁻¹·min⁻¹, n = 4), and N-desmethyl K11777 (Km = 18.3 ± 7.0 μM and Vmax = 2070 ± 1830 pmol·mg⁻¹·min⁻¹, n = 4). All three K11777 metabolites were formed by isolated CYP3A and their formation by human liver microsomes was inhibited by the CYP3A inhibitor cyclosporine (50 μM, 54–62% inhibition) and antibodies against human CYP3A4/5 (100 μg of antibodies/100 μg microsomal protein, 55–68% inhibition). CYP2D6 metabolized K11777 to its N-desmethyl metabolite with an apparent Km (9.2 ± 1.4 μM) lower than for CYP3A4 (25.0 ± 4.0 μM) and human liver microsomes. The apparent Km for N-oxide K11777 formation by cDNA-expressed FMO3 was 109 ± 11 μM. Based on the intrinsic formation clearances and the results of inhibition experiments (CYP2D6, 50 μM bufuralol; FMO3 mediated, 100 mM methionine) using human liver microsomes, it was estimated that CYP3A contributes to >80% of K11777 metabolite formation. K11777 was a potent (IC50 = 0.06 μM) and efficacious (maximum inhibition 85%) NADPH-dependent inhibitor of human CYP3A4 mediated 6′-β-hydroxy lovastatin formation, suggesting that K11777 is not only a substrate but also a mechanism-based inhibitor of CYP3A4.

K11777 (N-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl, Fig. 1) is a recently developed peptidomimetic that is a potent, irreversible inhibitor of cysteine proteases such as cathepsin B (E.C. 3.4.22.1) and L (E.C. 3.4.22.15) as well as cruzain. Cruzain is a cysteine protease of Trypanosoma cruzi, a protozoan parasite and the etiological agent of American trypanosomiasis or Chagas’ disease. Chagas’ disease is the leading cause of heart disease in Latin America (Filardi and Brener, 1987; Libow et al., 1991; Parada et al., 1997). The currently available therapeutic options for Chagas’ disease are generally considered unsatisfactory. Due to significant toxicity, chemotherapy with nifurtimox or benznidazole must be carried out under close medical control (Gorla et al., 1988). Although both drugs significantly shorten the acute phase and decrease mortality of Chagas’ disease, they fail to eradicate the parasite in 40% of the patients and are not used during the chronic phase of the disease (Kirchhoff, 1993).

Recent chemotherapeutic approaches have focused on developing specific inhibitors of cruzain (Cazzullo et al., 1997). Inhibition of cruzain prevented growth and differentiation of T. cruzi in cell culture models (Harth et al., 1993), showing that cruzain is a promising therapeutic target. In addition, K11777 inhibits the cysteine proteases cathepsins B and L, which are significantly involved in all stages of cancer progression, including growth, angiogenesis, invasion, and metastasis (Chen, 1992; Elliott and Sloane, 1996).

K11777 is the most recent development in a line of peptidomimetic cysteine protease inhibitors (personal communication, Arris Pharmaceutical Corp., South San Francisco, CA). In comparison to its predecessor K11002, for which the oral bioavailability in Sprague-Dawley rats was 3% (Zhang et al., 1998b), K11777 possesses an N-methyl-piperazine ring instead of a morpholine-urea ring, increasing solubility in intestinal fluids and thereby oral bioavailability. Indeed, oral bioavailability of K11777 (mean of 19.9%, unpublished data) proved to be significantly greater than that of K11002.

It was our goal to evaluate the oxidative metabolism of K11777 by liver monooxygenases and to assess the influence of the N-methyl-piperazine ring on the in vitro metabolite pattern and monooxygenases involved with K11777 in comparison to K11002 (Zhang et al., 1998a).

Materials and Methods

Chemicals. K11777 and K11002 (Arris Pharmaceutical Corp.), lovastatin (Merck, Sharp & Dohme, Rahway, NJ), iraconazole (Janssen Pharmaceutical Inc., Titusville, NJ), and cyclosporine (Novartis Pharma AG, Basel, Switzerland) were kind gifts of the manufacturers.

(±)-Bufuralol was purchased from UFC Ltd. (Manchester, UK) and NADP, isocitric acid, and isocitrate dehydrogenase for the NADPH-generating system were purchased from Sigma-Aldrich (St. Louis, MO). Physical and chemical properties of chemicals were controlled according to the manufacturer’s specifications.

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MgCl₂, 2.5 mM NADP, 45 mM isocitric acid, and 1.75 U isocitrate dehydrogenase in 0.1 M NaCl80°C. Protein concentrations were determined using the bicinchoninic acid method.

SOLVENTS AND BUFFER COMPONENTS. All solvents were HPLC grade and were from Fisher Scientific (Santa Clara, CA). CYP and FMO3 enzymes as well as monoclonal antibodies against human CYP3A and human liver microsomes were from Gentest (Woburn, MA). All solvents were HPLC grade and were from Fisher Scientific (Santa Clara, CA). All other chemicals were reagent grade and were purchased from Sigma.

Isolation of Microsomes. The collection of human liver tissue samples for in vitro drug metabolism studies was approved by the Committee on Human Subjects Research, University of California, San Francisco. Microsomes were isolated by differential centrifugation as described by Guengerich (1982) with the following modifications: instead of Tris buffer, 0.1 M NaCl, pH 7.4, was used. After ultracentrifugation, the supernatant was discarded and the residue reconstituted in 4 times its volume of a buffer solution containing 0.1 mM NaCl/KCl phosphate buffer/glycerol (4:1, v/v) and stored at −80°C. Protein concentrations were determined using the bicinchoninic acid method described by Smith et al. (1985). Bovine serum albumin was used as the standard. CYP concentrations were determined using the method of Omura and Sato (1964) following the protocol of Estabrook and Werringloer (1978).

Metabolism by Human Liver Microsomes. Microsomal protein (0.05–0.2 mg), buffer, and K11777 (in acetonitrile/water, 9:1 v/v, final concentration 5–150 μM) were preincubated for 5 min. The reaction was started by adding 0.4 ml of an NADPH-generating system, containing 5 mM EDTA, 25 mM MgCl₂, 2.5 mM NADPH, 45 mM isocitric acid, and 1.75 U isocitrate dehydrogenase in 0.1 M NaCl/KCl phosphate buffer (pH 7.4). Inhibition studies with methionine to assess FMO3 activity (vide infra), because FMO3 is inactivated by preincubation without NADPH, were carried out as follows: the substrate, inhibitor (if applicable), NADPH-generating system, and buffer were preincubated at 37°C for 4 min. The reaction was started by addition of FMO3 (5 μl). All assays were incubated for 10 min.

Extraction and Quantification of K11777 and Its Metabolites. After protein precipitation by addition of 0.4 ml of acetonitrile to the reaction mixtures, samples were vortexed for 10 s and centrifuged at 4°C and 10,900g for 5 min. The protein precipitation reagent acetonitrile also contained the internal standard K11002 (250 ng/ml). Supernatants were transferred into 1.8-ml HPLC vials (Hewlett-Packard, Palo Alto, CA).

K11777 and its metabolites were quantified by HPLC/electrospray-MS in combination with an on-line switching extraction step using a Hewlett-Packard series 1100 liquid chromatograph that consisted of a G1313A autosampler, G1312A binary pump, G1322A degasser, and G1316A column thermostat, connected to a 5989B mass spectrometer (all Hewlett-Packard) equipped with an Iris hexapole ion guide (Analytica of Branford, Branford, CT) by a 5987A electro spray interface (Hewlett-Packard). Solvent for the on-line extraction column was delivered by an additional binary HPLC pump (Perkin-Elmer, Norwalk, CT), controlled via the external contacts of the HP1100 HPLC system. The HPLC/MS system was controlled and data were processed using ChemStation software, revision A04.02 (Hewlett-Packard). The column switching HPLC/MS system was set up as described in detail by Kirschner et al. (1999). In brief, 100-μl samples were injected onto a 10 × 2-mm extraction column filled with Hypersil ODS-1 of 10-μm particle size (Shandon, Chadwick, UK). Samples were washed with a mobile phase of 2 mM NH₄-acetate/methanol (8:2, v/v). The flow was 6 ml/min. After 1 min, the switching valve was activated and the analytes were eluted in the backflush mode from the extraction column onto a 35 × 4.6-mm analytical column (Phenomenex, Torrance, CA) filled with Capcell Pak UG-120A CN material, 5-μm particle size (Shiseido, Tokyo, Japan). The mobile phase consisted of 2 mM ammonium acetate and methanol. The following gradient was run: 0 min, 60% methanol; 1 min, 60% methanol; 10 min, 88% methanol. For the following 2.5 min, the column was washed with 95% methanol and was reequilibrated to the starting conditions for 3 min. The flow was 0.4 ml/min and the column temperature 40°C. The electrospray interface was adjusted to the following parameters (nomenclature according to the ChemStation software): nebulizer gas: nitrogen of grade 5.0 purity, 80 psi; drying gas: nitrogen 5.0, flow, 40 (arbitrary units), 330°C; Vcap, ~5300 V; Vend, ~2600 V; Vcy1, ~5000 V, capillary exit voltage, 88 V. The following parameters were used for mass spectrometry analysis: quadrupole temperature, 120°C; multiplier voltage, 2.275 V; X-ray, 10,000 V. Positive ions [M+H]⁺ of K11777 and its metabolites gave the best signal-to-noise ratio and were recorded in the single ion mode: m/z = 575.2 for K11777 (retention time 8.7 min); m/z = 591.2 for N-oxide K11777 (retention time 7.4 min) and β-hydroxy-homoPhe K11777 (retention time 8.2 min); and m/z = 561.2 for N-desethyl K11777 (retention time 11.8 min). The dwell time for each ion was 100 ms.

The assay had the following specifications for K11777: lower limit of detection (signal/noise = 3:1), 0.5 ng/ml; lower limit of quantitation, 1.25 ng/ml; linearity, 1.25–500 ng/ml (r² = 0.996); and interassay variability (3 days, n = 15), 6.2% for 20 ng/ml and 5.3% for 160 ng/ml. The specifications for the metabolites, as checked by spiking inactivated reaction mixtures with isolated metabolites, did not differ from those of K11777. The recovery of K11777 and its metabolites was >80%. Within-batch stability of extracted samples was established for at least 24 h and all samples were analyzed within this time period.

Determination of the Apparent Michaelis-Menten Constants Kₘ and Vₘₐₓ. Liver microsomes (n = 4 for each concentration), CYP (n = 3), or FMO3 (n = 3) enzyme preparations were incubated with the following K11777 concentrations: 5, 10, 15, 20, 30, 40, 60, 80, and 100 μM (for FMO3 additionally 150 μM) as described above, for 10 min. Higher K11777 concentrations could not be tested due to limited solubility above 150 μM.

Microsomes from baculovirus-infected insect cells expressing the following human monoxygenases were used: CYP1A1 (lot 10), 1A2 (lot 12), 2B6 (lot 2), 2C8 (lot 2), 2C9 (Arg146) (lot 5), 2C19 (lot 3), 2D6 (lot 14), 2E1 (lot 5), 3A4 (lot 15/20), 3A5 (lot 8), and FM03 (lot 8) (all from Gentest). CYP enzymes, except 3A5, 1A1, 1A2, and 2D6, were coexpressed with NADPH-CYP reductase and cytochrome b₅. The metabolism assays (final volume of 1 ml) were carried out as described for the human liver microsomes using 100 μM K11777, except when FM03-mediated metabolism was assessed: The assays containing the substrate, inhibitor (if applicable), NADPH-generating system, and buffer were preincubated at 37°C for 4 min. The reaction was started by addition of FM03 (5 μl). All assays were incubated for 10 min.

FIG. 1. Chemical structures of K11777 in comparison to K11002.

The arrow marks structural differences.

as well as NADPH were purchased from Sigma (St. Louis, MO). Recombinant CYP and FMO3 enzymes as well as monoclonal antibodies against human CYP3A and human liver microsomes were from Gentest (Woburn, MA). All solvents were HPLC grade and were from Fisher Scientific (Santa Clara, CA). All other chemicals were reagent grade and were purchased from Sigma.

Isolation of Microsomes. The collection of human liver tissue samples for in vitro drug metabolism studies was approved by the Committee on Human Research, University of California, San Francisco. Microsomes were isolated by differential centrifugation as described by Guengerich (1982) with the following modifications: instead of Tris buffer, 0.1 M NaCl, pH 7.4, was used. After ultracentrifugation, the supernatant was discarded and the residue reconstituted in 4 times its volume of a buffer solution containing 0.1 mM NaCl/KCl phosphate buffer/glycerol (4:1, v/v) and stored at −80°C. Protein concentrations were determined using the bicinchoninic acid method described by Smith et al. (1985). Bovine serum albumin was used as the standard. CYP concentrations were determined using the method of Omura and Sato (1964) following the protocol of Estabrook and Werringloer (1978).

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Metabolism by cDNA-Expressed, Isolated CYP Enzymes and FMO3.
Heat-denatured microsomes, control microsomes from insect cells (Gentest), and incubation mixtures without NADPH were used as negative controls. Microsomal preparations were inactivated by incubation at 95°C for 5 min. Heat-denatured microsomal preparations were also used to establish stability of the study drug during incubation and as matrix for quality control samples during study sample analysis, but were not used to assess the relative contributions of FMO3 and CYP to K11777 metabolism. Therefore, conditions were chosen that inactivated all drug-metabolizing enzymes. After heat inactivation, microsomes or isolated enzymes did not show any detectable activity as assayed using standard substrates (CYP3A,Lovastatin; CYP2D6, (±)-bufuralol; FMO3, methionine). \( k_{\text{m}} \) and \( V_{\text{m}} \) were determined after data fitting using Hanes-Woolf plots. Data were fitted using the SigmaPlot software (version 5.0; SPSS Inc., San Rafael, CA).

**Structural Evaluation of K11777 Metabolites by MS/MS.** Metabolites were isolated after incubation of K11777 with human liver microsomes and the NADPH-generating system using semipreparative HPLC. Extraction and setup of the HPLC system were as described for HPLC/MS analysis of K11777, except that an injection volume of 400 \( \mu l \) and that in place of the mass spectrometer a G1314A variable wavelength UV monitor (Hewlett-Packard, Palo Alto, CA) were used. Chromatograms were recorded at a UV wavelength of 210 nm. Metabolite fractions were collected manually and purity was established by HPLC/UV and HPLC/MS analysis. Structures of the isolated metabolites were evaluated by electrospray-MS/MS in combination with collision-induced dissociation and analysis of the fragmentation patterns using a PE SCIEX API III triple-quadrupole mass spectrometer (Perkin-Elmer/Applied Biosystems, Foster City, CA) equipped with a heated nebulizer inlet (Thornhill, Ontario, Canada). The isolated metabolite fractions were introduced into the electrospray interface by continuous infusion (5 \( \mu l/min \)) via a syringe pump (Harvard Apparatus, Holliston, MA). Positive ions were recorded. Scans were acquired over a mass range of \( m/z \) 70 to 600. For collision-induced dissociation, the collision energy was set to 60 eV and the collision gas was used as the collision gas.

**Identification of the Monooxygenases Involved in K11777 Metabolism.** The monooxygenases involved were identified by metabolism of K11777 by isolated, recombinant CYP and FMO enzymes (vide supra). In addition, K11777 metabolism was inhibited by specific chemical inhibitors and antibody bodies. The effects of the specific CYP3A inhibitors iraconazole and cyclosporine (Guengerich, 1995) on K11777 metabolism were assessed. Cyclosporine was dissolved in methanol and iraconazole in acetone/dimethyl sulfoxide (2:1, v/v). Inhibitor solution (10 \( \mu l \)) resulting in final concentrations of 0.1, 1, 5, and 20 \( \mu M \) for iraconazole and 0.1, 1, 10, and 50 \( \mu M \) for cyclosporine was added to the liver microsomal preparations containing 10 \( \mu M \) K11777. Microsomes to which 10 \( \mu l \) of the vehicle was added were used as controls. The mixtures were incubated and analyzed as described above.

To exclude that iraconazole, which like K11777 possesses a piperazine ring, interacted with FMO3 and iraconazole and cyclosporine (Guengerich, 1995) on K11777 metabolism was assessed. Cyclorsporine was dissolved in methanol and iraconazole in acetone/dimethyl sulfoxide (2:1, v/v). Inhibitor solution (10 \( \mu l \)) resulting in final concentrations of 0.1, 1, 5, and 20 \( \mu M \) for iraconazole and 0.1, 1, 10, and 50 \( \mu M \) for cyclosporine was added to the liver microsomal preparations containing 10 \( \mu M \) K11777. Microsomes to which 10 \( \mu l \) of the vehicle was added were used as controls. The mixtures were incubated and analyzed as described above.

For inhibition of K11777 metabolism by specific CYP antibodies, 100 \( \mu g \) of microsomal protein isolated from human liver (pool of four) in 20 \( \mu l \) of 0.1 M Tris buffer (pH 7.4) was preincubated on ice with 0, 1, 2, 5, or 10 \( \mu l \) of CYP3A4/5 antibody solution (1 \( \mu l = 10 \mu g \) of protein) (Gentest) for 25 min. Then buffer (final volume 1 ml), K11777 (final concentration 50 \( \mu M \)), and the NADPH-generating system were added. Samples were incubated and extracted as described above. To exclude a non-specific effect of high protein concentrations, control samples were incubated with rabbit preimmune serum (Gentest) at the same concentration as the highest antibody concentration used (\( n = 4 \)).

To estimate the relative contributions of CYP2D6 and FMO3 to the overall metabolism of K11777, K11777 metabolism by human liver microsomes was inhibited by addition of 50 \( \mu M \) (±)-bufuralol (CYP2D6 inhibitor, Gut et al., 1984) or 100 mM methionine (FMO3 substrate, Ripp et al., 1999). As controls, K11777 metabolism catalyzed by isolated CYP3A, CYP2D6, or FMO3 enzymes was inhibited using 50 \( \mu M \) (±)-bufuralol or 100 mM methionine. The effect of the inhibitors and antibodies on K11777 metabolite formation was statistically evaluated by analysis of variance (GLM procedure, SAS, version 6.12; SAS Institute, Cary, NC).

**Inhibition of the CYP3A-Mediated Lovastatin-6β-hydroxylation by K11777 and K11002.** To assess and compare inhibition of CYP3A-mediated metabolism by K11777 and K11002, their effect on the CYP3A-dependent 6β-hydroxylation of lovastatin (Wang et al., 1991) was evaluated. K11777 and K11002 (0, 0.012, 0.06, 0.12, 1.2, 12, and 120 \( \mu M \)) were incubated with 25 \( \mu M \) of human microsomal CYP3A4 protein with or without (control) an NADPH-generating system at 37°C. After a preincubation period of 30 min, the reaction was started by addition of 1 mmol of NADPH and lovastatin (final concentration in the assay 100 \( \mu M \)). For comparison, lovastatin was added together with K11777 or K11002 and the NADPH-generating system without a preincubation period. Lovastatin and its metabolite 6β-hydroxy lovastatin were quantified by HPLC as described previously (Jacobsen et al., 1999).

**Inhibition of the CYP2D6-Mediated (±)-Bufuralol-1’-hydroxylation by K11777.** To evaluate the effect of K11777 on the CYP2D6-mediated metabolism of (±)-bufuralol (Gut et al., 1984), K11777 (0, 0.012, 0.06, 0.12, 1.2, and 120 \( \mu M \)) was incubated with 25 \( \mu M \) of isolated human CYP 2D6, and with NADPH-generating system at 37°C. After a preincubation period of 30 min, (±)-bufuralol (final concentration 50 \( \mu M \)) and 1 mmol of NADPH were added. As controls, the inhibition assay was carried out with a 30-min preincubation period without NADPH (\( n = 4 \)) and without preincubation (\( n = 4 \)). (±)-Bufuralol and its 1’-hydroxy metabolite were quantified by HPLC/MS as described for K11777 with the following gradient: 0 min 70% methanol, 6 min 95% methanol. The column was washed with 95% methanol for 2 min and reequilibrated to the starting conditions within 4.5 min. Bufuralol and 1’-hydroxy bufuralol ([M+H]⁺ were recorded in the single ion mode: \( m/z = 262.2 \), bufuralol (retention time 8.4 min); \( m/z = 278.2 \), 1’-hydroxylbufuralol (retention time 7.0 min). The dwell time for each ion was 500 ms.

**Results**

**In Vitro Metabolism of K11777 by Human Liver Microsomes.** After incubation of K11777 with human liver microsomes, three metabolites were detected by HPLC/UV as well as HPLC/MS in the scan and single ion mode (Fig. 2). These peaks were neither detected in control incubations containing heat-inactivated microsomes nor during incubations with microsomes without NADPH. Two metabolites with a molecular ion [M+H]⁺ of \( m/z = 591.2 \) were detected, which is a mass difference of +16 amu compared with K11777. Their HPLC retention times of 7.4 and 8.2 min (Fig. 2) were different, indicating oxidation of K11777 at two different positions. A third metabolite had a retention time longer than K11777 (11.8 versus 8.7 min). Its molecular ion of \( m/z = 561 \) indicated demethylation in one position. Additional metabolites were not detected (limit of detection 0.5 ng/ml). The formation of all three metabolites was linear for incubation periods up to 15 min and microsomal protein concentrations from 0.025 to 0.2 mg/ml. Apparent Km, apparent Vmax, and intrinsic formation clearances (ClFmax, Vmax/Km) for the three K11777 metabolites are shown in Table 1. The ClFmax values indicated that formation of the N-oxide of K11777 (structural identification, vide infra) was the main metabolism reaction with a mean ClFmax of 3.7-fold higher than for 6β-hydroxy homoPhe K11777 and 2.5-fold higher than for N-desmethyl K11777.

**Structural Evaluation of K11777 Metabolites by MS/MS.** The mass spectra and proposed fragmentation pathways of K11777 and its isolated metabolites are shown in Fig. 3. Corresponding fragments of metabolites and K11777 are compared in Table 2. Analysis of the fragmentation patterns and comparison with K11777 suggested the following structures for the three metabolites.

\( \text{N-oxide K11777 (retention time 7.4 min).} \) In comparison to K11777 (Fig. 3A), no fragment with \( m/z = 101 \), which comprises the N-methyl-
piperazine ring, but two new fragments with \( m/z = 99 \) and \( m/z = 117 \) were detected (Table 2). The fragment \( m/z = 117 \) appeared to represent hydroxylation at the \( N \)-methyl-piperazine ring. This was confirmed by the other new fragment at \( m/z = 99 \), which was most likely \( m/z = 117 - H_2O \). All fragments not containing the \( N \)-methyl-piperazine ring were identical with those of K11777, whereas all fragments containing the \( N \)-methyl-piperazine ring had \( m/z \) values 16 amu greater than the corresponding K11777 fragments (Table 2). Further structural identification based solely upon MS/MS analysis was not possible.

**β-Hydroxy homoPhe K11777 (retention time 8.2 min)** (Fig. 3B). The molecular ion \([M+H]^+\) of 591.2 indicated hydroxylation in one position. The fragment of \( m/z = 274 \) showed that the \( N \)-methyl-piperazine Phe- part of the molecule was unchanged (Table 2) and hydroxylation occurred at the homoPhe-vinylsulfone-phenyl part of K11777. In comparison to K11777, two additional fragments at \( m/z = 331 \) \([M+H]^+\) and \( m/z = 368 \) \([M+K]^+\) were present (Fig. 3, A and B). These could be explained by loss of the homoPhe group from fragment \( m/z = 434 \), suggesting that the hydroxylation had most likely occurred at the first C atom of the homoPhe residue of K11777. This fragment was probably caused by the loss of homoPhe (105 \( m/z \)) from fragment \( m/z = 434 \) (\( N \)-methyl-piperazine-Phe-homoPhe-vinyl-residue). The formation of this additional fragment could be explained by \( \alpha \)-cleavage of the hydroxy-homoPhe residue due to hydroxylation at the \( \beta \)-C-atom of this residue.

**N-desmethyl K11777 (retention time 11.8 min)**. All fragments not containing the \( N \)-methyl-piperazine ring were identical with those of K11777, whereas all fragments containing the \( N \)-methyl-piperazine ring had \( m/z \) values 14 amu lower than the corresponding K11777 fragments (Table 2). Fragment 101 \( m/z \) (\( N \)-methyl-piperazine) was shifted to 87 \( m/z \), indicating that the structure of this metabolite was most likely N-desmethyl K11777.

**Identification of the Monoxygenases Involved in K11777 Metabolism.** K11777 metabolism by isolated CYP3A4 and 2D6 was linear during incubation periods up to 10 min; metabolism by FMO3 for 15 min. Reaction kinetics was linear for protein concentrations from 0.025 to 0.2 mg/ml. The K11777 metabolite profiles after incubation with human liver microsomes and cDNA-expressed human CYP3A4 were identical, as confirmed by mass spectrometry. In
addition, in vitro incubations indicated that CYP2D6 and FMO3 might constitute major pathways for the formation of \(N\)-desmethyl and \(N\)-oxide K11777, respectively. The apparent \(K_m\) and apparent \(V_{\text{max}}\) values are shown in Table 3. It must be taken into account that the cDNA-expressed isolated CYP and FMO preparations varied significantly in enzyme activities, which also did not reflect the

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>(N)-Methyl-piperazine</th>
<th>(N)-Methyl-piperazine-Phe</th>
<th>Phe-homoPhe-vinylsulfone-phenyl</th>
<th>New Fragments</th>
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<tbody>
<tr>
<td>K11777</td>
<td>101</td>
<td>274</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N)-Oxide K11777</td>
<td>117</td>
<td>274</td>
<td></td>
<td></td>
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<tr>
<td>(N)-Oxide K11777, (117-H_2O = 99)</td>
<td>101</td>
<td>274</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\beta)-Hydroxy homoPhe-K11777</td>
<td>101</td>
<td>274</td>
<td>473</td>
<td>331</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(473 + O = 489)</td>
<td>369 [331 + K]+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(489-H_2O = 471)</td>
<td></td>
</tr>
<tr>
<td>(N)-Desmethyl K11777</td>
<td>87</td>
<td>274-CH(_3) = 260</td>
<td></td>
<td>475</td>
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</table>

**Fig. 3.** Tandem mass spectra of K11777 (A) and its metabolite \(\beta\)-hydroxy homoPhe K11777 (B).
formation was 97.1%. The addition of 1 mM homoPhe, and the inhibitor cyclosporine reduced the formation of N\textsubscript{-}oxide, \textbeta\textsubscript{-}hydroxy-homoPhe, and N\textsubscript{-}desmethyl metabolites (Table 4).

In addition to CYP3A, CYP2D6, and FMO3, incubation of all other CYP enzymes with K11777 metabolites by human liver microsomes with an IC\textsubscript{50} of 50\textmu M inhibited K11777 metabolite formation rates by human cDNA-expressed CYP enzymes and cDNA-expressed FMO3.

Data are presented as mean ± standard deviation (n = 3).

### TABLE 3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>N\textsubscript{-}Oxide K11777</th>
<th>\textbeta\textsubscript{-}Hydroxy-homoPhe K11777</th>
<th>N\textsubscript{-}Desmethyl K11777</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K\textsubscript{m} [\textmu mol \cdot \textit{L}^{-1}]</td>
<td>V\textsubscript{max} [\textmu mol \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{protein}]</td>
<td>LOD [pmol \cdot \text{mg}^{-1} \text{protein}]</td>
</tr>
<tr>
<td>CYP1A4</td>
<td>23.2 ± 3.1 (13.2 ± 0.3)</td>
<td>2695 ± 97 (13.2 ± 0.5)</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>22.8 ± 3.5 (12.1 ± 0.1)</td>
<td>212 ± 12 (12.1 ± 0.1)</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>FMO3</td>
<td>109.3 ± 10.8 (18.8 ± 0.6)</td>
<td>8565 ± 68 (18.8 ± 0.6)</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

LOD, concentration below lower limit of detection (0.5 ng/ml).

### TABLE 4

K11777 metabolite formation rates by human cDNA-expressed CYP enzymes and cDNA-expressed FMO3.

K11777 (final concentration: 100\textmu M) was incubated for 15 min with 25 pmol of CYP enzymes isolated from baculovirus-infected insect cells expressing human CYP 1A1, 1A2, 2B6, 2C8, 2C9-Arg, 2C19, 2D6, 2E1, 3A4, or 3A5 and 0.125 mg of microsomal protein isolated from baculovirus-infected insect cells expressing human FMO3.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>N\textsubscript{-}Oxide K11777</th>
<th>\textbeta\textsubscript{-}Hydroxy-homoPhe K11777</th>
<th>N\textsubscript{-}Desmethyl K11777</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mmol CYP \cdot \textit{min} [pmol/mg protein \cdot \textit{min}]</td>
<td>pmol/mmol CYP \cdot \textit{min} [pmol/mg protein \cdot \textit{min}]</td>
<td>pmol/mmol CYP \cdot \textit{min} [pmol/mg protein \cdot \textit{min}]</td>
</tr>
<tr>
<td>1A1</td>
<td>0.091 ± 0.009 (12.8 ± 1.2)</td>
<td>0.015 ± 0.002 (2.0 ± 0.3)</td>
<td>0.145 ± 0.017 (20.5 ± 2.4)</td>
</tr>
<tr>
<td>1A2</td>
<td>0.079 ± 0.002 (21.0 ± 0.6)</td>
<td>0.028 ± 0.001 (7.3 ± 0.3)</td>
<td>0.153 ± 0.002 (40.3 ± 0.7)</td>
</tr>
<tr>
<td>2B6</td>
<td>0.046 ± 0.001 (5.9 ± 0.2)</td>
<td>&lt;LOD</td>
<td>0.070 ± 0.005 (9.0 ± 0.6)</td>
</tr>
<tr>
<td>2C8</td>
<td>0.164 ± 0.006 (82.0 ± 2.8)</td>
<td>0.038 ± 0.003 (18.8 ± 1.4)</td>
<td>0.192 ± 0.006 (95.7 ± 3.0)</td>
</tr>
<tr>
<td>2C9-Arg</td>
<td>0.044 ± 0.002 (21.1 ± 1.0)</td>
<td>&lt;LOD</td>
<td>0.045 ± 0.001 (82.0 ± 1.0)</td>
</tr>
<tr>
<td>2C19</td>
<td>0.057 ± 0.005 (20.1 ± 1.8)</td>
<td>&lt;LOD</td>
<td>0.046 ± 0.001 (16.6 ± 0.1)</td>
</tr>
<tr>
<td>2D6</td>
<td>0.168 ± 0.014 (27.5 ± 2.3)</td>
<td>&lt;LOD</td>
<td>4.37 ± 10.6 (717 ± 58.3)</td>
</tr>
<tr>
<td>2E1</td>
<td>0.023 ± 0.001 (13.4 ± 0.5)</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>3A4</td>
<td>0.877 ± 0.105 (87.0 ± 10.4)</td>
<td>0.320 ± 0.047 (31.7 ± 4.7)</td>
<td>0.550 ± 0.037 (54.7 ± 3.7)</td>
</tr>
<tr>
<td>3A5</td>
<td>0.166 ± 0.003 (124 ± 2.1)</td>
<td>0.024 ± 0.001 (17.8 ± 0.4)</td>
<td>0.124 ± 0.001 (93.0 ± 0.6)</td>
</tr>
<tr>
<td>FMO3</td>
<td>[1800 ± 97.7]</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

LOD, concentration below lower limit of detection (0.5 ng/ml).

Enzymes’ relative in vivo activities. Therefore, apparent V\textsubscript{max} data in Table 3 and formation rates in Table 4 only allow comparison of the formation of different metabolites by the same enzyme but not comparison of the formation of metabolites by different enzymes. In addition to CYP3A, CYP2D6, and FMO3, incubation of all other CYP enzymes with K11777 resulted in detectable amounts of one or more K11777 metabolites (Table 4).

Antibodies against human CYP3A enzymes inhibited K11777 metabolism by human liver microsomes with an IC\textsubscript{50} of 50 \textmu g of antibody/100 \textmu g human liver microsomal protein (Fig. 4). Compared with uninhibited controls, 100 \textmu g of antibody/100 \textmu g human liver microsomal protein reduced N\textsubscript{-}oxide K11777, \textbeta\textsubscript{-}hydroxy-homoPhe K11777, and N\textsubscript{-}desmethyl K11777 formation by 55 ± 10, 68 ± 8, and 61 ± 11%, respectively (Fig. 4). In the presence of rabbit preimmune serum at the same protein concentration as the highest antibody concentration used, N\textsubscript{-}oxide K11777 formation was 91.8 ± 1.4% and N\textsubscript{-}desmethyl K11777 formation was 97.1 ± 4.2% of controls without serum.

The addition of 50 \textmu M of the specific CYP3A substrate and inhibitor cyclosporine reduced the formation of N\textsubscript{-}oxide, \textbeta\textsubscript{-}hydroxy-homoPhe, and N\textsubscript{-}desmethyl K11777 by 54 ± 8, 61 ± 9, and 62 ± 9%, and the addition of 1 \textmu M itraconazole by 79 ± 4, 83 ± 4, and 82 ± 7%, respectively (mean ± standard deviation, n = 4).

(±)-Bufuralol (50 \textmu M) inhibited N\textsubscript{-}demethylation of K11777 by...
Human liver microsomes metabolized K11777 to three metabolites. The metabolic pathways proposed on the basis of our study results are shown in Fig. 6. For two metabolites, N-desmethyl K11777 and \(-\beta\)-hydroxy-homoPhe K11777, structures could be proposed based on the MS/MS fragmentation patterns. For the third metabolite (HPLC retention time 7.4 min), the fragmentation pattern suggested hydroxylation at the N-methyl-piperazine ring. This metabolite was generated by incubation of K11777 with FMO3. FMOs are microsomal enzymes that catalyze the NADPH- and \(O_2\)-dependent oxidation of several nitrogen-, sulfur-, selenium- and phosphorus-containing compounds (Ziegler, 1993). Currently, five FMO enzymes, FMO1 to 5 have been identified, each exhibiting its own unique species- and tissue-dependent expression (Hines et al., 1994). The predominant FMO enzyme in adult human liver is FMO3, which was the reason we used it in our study. It is known that FMO-mediated \(O\)-oxidation occurs preferentially at the \(N\)-methyl nitrogen atom of \(N\)-methyl-\(N'\)-substituted pipерazines, e.g., \(N\)-oxidation of clozapine (Tugnait et al., 1997) and the \(N\)-methyl-piperazine-substituted phenothiazine drug trifluoperazine (Sofer and Ziegler, 1978). Based on these data, the most likely structure of the K11777 metabolite hydroxylated at the \(N\)-methyl-piperazine ring is \(N\)-oxide K11777.

Although the pH optimum of FMO3 is between 9 and 9.5 (Lang et al., 1998), these conditions are not relevant in vivo in humans and we adjusted our assays assessing FMO3 activity to pH 7.4. It must also be noted that only apparent enzyme kinetic parameters could be reported for assays based on liver microsomal preparations. Microsomes do not represent an isolated enzyme but a mixture of different enzymes and, as demonstrated by our study, more than one CYP enzyme is involved in the metabolism of K11777.

Finding that K11777 was metabolized by isolated CYP3A enzymes, and that the specific CYP3A substrate cyclosporine and anti-bodies against human CYP3A4/5 were effective inhibitors of K11777 metabolite formation in human liver microsomes, led us to conclude that CYP3A is primarily involved in K11777 metabolism. Although all other tested cDNA-expressed CYP enzymes were able to generate at least one of the three K11777 metabolites, the inhibition experiments with bufuralol and methionine indicated that CYP3A4 is responsible for at least 66% of the formation of \(N\)-desmethyl K11777, whereas FMO3 does not seem to be a relevant metabolic pathway.

The choice of effective inhibitor concentrations was confirmed by the inhibition experiments with isolated CYP2D6 and FMO3, showing \(>80\%\) inhibition of the isolated enzymes. Based on this information and the intrinsic formation clearances (CL_{int}, Table 1) in human liver microsomes, it can be estimated that CYP3A is responsible for more than \(80\%\) of the total metabolism of K11777.

Nevertheless, it is interesting that K11777 is a substrate for such a wide variety of CYP enzymes. It is well established that \(N\)-demethylation reactions are catalyzed by many different CYP enzymes, such as 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 (Coutts et al., 1994). For example, the \(N\)-demethylation of clozapine, a drug also containing an
N-methyl-piperazine ring, is catalyzed by CYP enzymes 1A2, 2C9, 2C19, 2D6, and 3A4 (Linnet and Olesen, 1997). In our study, we used a sensitive HPLC/MS assay that enabled us to detect metabolite concentrations as low as 0.5 ng/ml.

In addition to being a substrate, K11777 was a potent inhibitor of CYP3A with an inhibition constant in the nanomolar range. Inhibition of CYP3A by K11777 was most potent and efficient after a preincubation period of 30 min in the presence of NADPH, indicating that K11777 is a mechanism-based inhibitor. In contrast, K11777 did not inhibit the catalytic activity of CYP2D6, the other major CYP enzyme involved in K11777 metabolism. The structurally related protease inhibitor K11002 was also a mechanism-based inactivator of CYP3A, indicating that the phenylalanine-homo-phenylalanine-vinylsulfone-phenyl part of the molecule rather than the morpholine-urea ring of K11002 or the N-methyl-piperazine ring of K11777 was involved.

As mentioned above, the structural difference between K11777 and K11002 is the replacement of the morpholine-urea ring of K11002 by an N-methyl-piperazine ring. This modification resulted in significant differences of the in vitro drug metabolism of K11777 in comparison to K11002. Zhang et al. (1998a) identified the three major metabolites of K11002 as hydroxy metabolites. The hydroxylation positions were at the phenylalanine, homo-phenylalanine, and the phenyl residue. The morpholine-urea ring was not involved. Introduction of the N-piperazine ring shifted the main target of metabolism reactions from the phenylalanine-homo-phenylalanine-vinylsulfone-phenyl part of the molecule to the N-methyl-piperazine ring. In contrast to K11002, two of the three K11777 metabolism sites were at the N-methyl-piperazine ring. Also, the N-methyl-piperazine ring allowed for additional metabolic pathways, e.g., via FMO3. In comparison to K11002, K11777 exhibited smaller $K_m$ values for its metabolism by human liver microsomes. Those described for K11002 ranged between 38 to 52 $\mu$M (Zhang et al., 1998a) and were more than 2-fold higher than those found for K11777 here.

In conclusion, the N-methyl-piperazine ring is the major metabolism site of K11777, and N-oxygenation, N-demethylation, and hydroxylation by CYP3A4 constitute the major metabolic pathways (Fig. 6). Intestinal CYP3A enzymes have been shown to play an important role in the low and variable oral bioavailability of drugs that are CYP3A substrates (Benet et al., 1996), and may be one of the factors responsible for the low oral bioavailability of K11777. In addition, many clinically important drugs that are CYP3A substrates, inhibitors, and/or inducers (Rendic and DiCarlo, 1997) may interact with K11777 pharmacokinetics. Vice versa, we have shown that K11777 is a potent and effective mechanism-based inhibitor of

Fig. 6. Proposed metabolic pathways of K11777 in human liver microsomes.
CYP3A and thus can be expected to affect the pharmacokinetics of coadministered drugs, if they are CYP3A substrates.

References


