CYP1A-MEDIATED METABOLISM OF THE JANUS KINASE-3 INHIBITOR 4-(4′-HYDROXYPHENYL)-AMINO-6,7-DIMETHOXYPYRAZINE: STRUCTURAL BASIS FOR INACTIVATION BY REGIOSELECTIVE O-DEMETHYLATION

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ABSTRACT:

Here we report the phase I metabolism of the rationally designed Janus kinase-3 (JAK) inhibitor 4-(4′-hydroxyphenyl)-amino-6,7-dimethoxypyrazine (WHI-P131; JANEX-1). JANEX-1 was metabolized by the cytochrome P450 enzymes CYP1A1 and CYP1A2 in a regioselective fashion to form the biologically inactive 7-O-demethylation product 4-(4′-hydroxyphenyl)-amino-6-methoxy-7-hydroxypyrazine (JANEX-1-M). Our molecular modeling studies indicated that the CYP1A family enzymes bind and demethylate JANEX-1 at the C-7 position of the quinazoline ring since the alternative binding conformation with demethylation at the C-6 position would result in a severe steric clash with the binding site residues. The metabolism of JANEX-1 to JANEX-1-M in pooled human liver microsomes followed Michaelis-Menten kinetics with \( V_{\text{max}} \) and \( K_m \) values (mean \pm S.D.) of 34.6 \pm 9.8 pmol/min/mg and 107.3 \pm 66.3 \mu M, respectively. \( \alpha \)-Naphthoflavone and furafurylline, which both inhibit CYP1A2, significantly inhibited the formation of JANEX-1-M in human liver microsomes. There was a direct correlation between CYP1A activities and the magnitude of JANEX-1-M formation in the liver microsomes from different animal species. A significantly increased metabolic rate for JANEX-1 was observed in Aroclor 1254-, \( \beta \)-naphthoflavone-, and 3-methylcholanthrene-induced microsomes but not in clofibrate-, dexamethasone-, iso- niad-, and phenobarbital-induced microsomes. The formation of JANEX-1-M in the presence of baculovirus-expressed CYP1A1 and 1A2 was consistent with Michaelis-Menten kinetics. The systemic clearance of JANEX-1-M was much greater than that of JANEX-1 (5525.1 \pm 1926.2 ml/h/kg versus 1458.0 \pm 258.6 ml/h/kg). Consequently, the area under the curve for JANEX-1-M was much smaller than that for JANEX-1 (27.5 \pm 8.0 versus 94.8 \pm 18.4 \mu M \cdot h; P < 0.001).

Acute allergic reactions, also known as immediate (type I) hypersensitivity reactions, including anaphylaxis with a potentially fatal outcome, are triggered by three major classes of proinflammatory mediators, namely preformed granule-associated bioactive amines (e.g., histamine, serotonin) and acid hydrolases (e.g., prostaglandin D2, and platelet activating factor), and newly synthesized arachidonic acid metabolites (e.g., leukotriene C4, prostaglandin D2, and platelet activating factor), which both inhibit CYP1A2, significantly inhibited the formation of JANEX-1-M in human liver microsomes. There was a direct correlation between CYP1A activities and the magnitude of JANEX-1-M formation in the liver microsomes from different animal species. A significantly increased metabolic rate for JANEX-1 was observed in Aroclor 1254-, \( \beta \)-naphthoflavone-, and 3-methylcholanthrene-induced microsomes but not in clofibrate-, dexamethasone-, iso- niad-, and phenobarbital-induced microsomes. The formation of JANEX-1-M in the presence of baculovirus-expressed CYP1A1 and 1A2 was consistent with Michaelis-Menten kinetics. The systemic clearance of JANEX-1-M was much greater than that of JANEX-1 (5525.1 \pm 1926.2 ml/h/kg versus 1458.0 \pm 258.6 ml/h/kg). Consequently, the area under the curve for JANEX-1-M was much smaller than that for JANEX-1 (27.5 \pm 8.0 versus 94.8 \pm 18.4 \mu M \cdot h; P < 0.001).

1 Abbreviations used are: TNF, tumor necrosis factor; PTK, protein tyrosine kinases; JAK3, Janus kinase-3; JANEX-1, 4-(4′-hydroxyphenyl)-amino-6,7-dimethoxypyrazine; HPLC, high-performance liquid chromatography; JANEX-1-M, 4-(4′-hydroxyphenyl)-amino-6-methoxy-7-hydroxypyrazine; P450, cytochrome P450; FMO, flavin-containing monooxygenase; ARO, Aroclor 1254; BNF, \( \beta \)-naphthoflavone; CFB, clofibrate; DEX, dexamethasone; ISO, isoniazid; 3-MC, 3-methylcholanthrene; PHEN, phenobarbital; DMSO, dimethyl sulfoxide; MS, mass spectrometry; PBS, phosphate-buffered saline; LC, liquid chromatography; DNP, dinitrophenyl; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); BSA, bovine serum albumin; TEA, triethylammonium; CL, clearance; \( R_h \), retention time; VDW, van der Waals; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

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The microsomes from ICR/CD-1 mice, Sprague-Dawley rats, Dunkin-Hartley guinea pigs, New Zealand white rabbits, beagle dogs, and cynomolgus monkeys were purchased from In Vitro Technologies, Inc. (Baltimore, MD). Induced rat liver microsomes from treatment of Sprague-Dawley rats with Aroclor 1254 (ARO) (single dose of 100 mg/kg i.p.), β-naphthoflavone (BNF) (100 mg/kg/day i.p. for 4 days), clofibrate (CFB) (200 mg/kg/day i.p. for 4 days), dexamethasone (DEX) (50 mg/kg/day i.p. for 4 days), isoniazid (ISO) (200 mg/kg/day i.p. for 4 days), 3-methylcholanthrene (3-MC) (54 mg/kg/day i.p. for 4 days), and phenobarbital (PHE) (80 mg/kg/day i.p. for 4 days) were also purchased from In Vitro Technologies, Inc. The protein concentrations and specific activities of each P450 isoform were provided in the data sheets by the manufacturer.

JANEX-1 was synthesized as previously described (Narla et al., 1998). Its structure (Fig. 3A) and physicochemical properties were previously reported (Narla et al., 1998; Sudbeck et al., 2000).

The microsomes containing baculovirus-expressed P450 enzymes, including CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP4A11, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and recombinant human flavin-containing monoxygenase (FMO)-3, were purchased from GENTEST. All enzymes were coexpressed with oxidoreductase. CYP2A6, 2B6, 2C8, 2C9, 2C19, and 2E1 were also coexpressed with cytochrome b5.

The purpose of the present study was to investigate the phase I metabolism of JANEX-1. Here, we show that JANEX-1 is metabolized by the cytochrome P450 enzymes CYP1A1 and CYP1A2 in a regioselective fashion to form the biologically inactive 7-O-demethylation product 4′-(4′-hydroxyphenyl)-amino-6-methoxy-7-hydroxyquinazoline (JANEX-1-M). Our molecular modeling studies indicated that the cytochrome P450A1A family enzymes bind and demethylate JANEX-1 at the C-7 position of the quinazoline ring since the alternative binding conformation with demethylation at the C-6 position would result in a severe steric clash with the binding site residues.

Materials and Methods

**Chemicals.** Acetonitrile and aceton were purchased from Fisher Chemicals (Fair Lawn, NJ). Furafylline and (S)-(+)-mephenytoin were purchased from Ultrafine Chemicals (Manchester, UK). Ketocazolazone was from ICN Biomedicals, Inc. (Aurora, OH), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The reagents for synthesis were purchased from Aldrich (Milwaukee, WI). The human liver microsomes (individuals: H003, H006, H023, H030, H042, H043, H056, H066, H070, H089, H093, and H112; pooled: H161) were purchased from GENTEST (Woburn, MA). The protein concentrations and specific activities of each P450 isoform in the human microsomes were provided in the data sheets by the manufacturer.

The microsomes containing baculovirus-expressed P450 enzymes, including CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP4A11, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and recombinant human flavin-containing monoxygenase (FMO)-3, were purchased from GENTEST. All human liver microsomes (individ-
and human liver cytosol were studied using incubations conducted as described above.

Inhibition Experiments. The effects of various cytochrome P450 substrates/inhibitors on the formation of JANEX-1-M in human liver microsomes (catalog no. H161) were studied by measuring JANEX-1-M formation after preincubation with the following compounds: α-naphthoflavone, furafylline, coumarin, diethyldithiocarbamic acid, orphenadrine, sulfaphenazole, (S)-methylnaphtoin, quindine, p-nitrophenol, ketonazole, troleandomycin, and lauric acid. All inhibitors were dissolved in DMSO and added to the incubations at a final DMSO concentration of 0.5% (v/v). Control incubations (without inhibitors) also contained 0.5% DMSO. The final concentration of JANEX-1 was 50 μM, and final concentrations of P450 substrates/inhibitors were those recommended in previous publications (Kajita et al., 2000; Wynafulda, 2000).

Incubation of JANEX-1 with Microsomes Containing Baculovirus-Expressed Enzymes. The metabolism of JANEX-1 by specific cytochrome P450 isoforms was studied using incubations conducted as described above, except that baculovirus-expressed P450 enzymes were used at a concentration of 50 pmol/ml for the incubation. JANEX-1 was used at a final concentration of 50 μM in these experiments.

Identification of Metabolite(s) by LC-MS. Mass spectrum analysis was carried out using atmospheric pressure ionization-electrospray and a high-energy-dynode electron multiplier (Hewlett Packard, Palo Alto, CA), which is connected to the LC system (Chen et al., 1999b). The conditions for mass spectrum analysis were set at a fragmentor of 75 V, a drying gas flow of 10 l/min, a nebulizer pressure of 25 psig, and a drying gas temperature of 350°C.

Pharmacokinetic Studies in Mice. Female CD-1 mice (6–8 weeks old) from Charles River Laboratories, Inc. (Wilmington, MA) were housed in a USDA-accredited animal care facility under standard environmental conditions. All rodents were housed in microisolator cages (Lab Products, Inc., Maywood, NJ) containing autoclaved bedding. Mice were allowed free access to autoclaved pellet food and tap water throughout the study. All animal studies were approved by the Parker Hughes Institute Animal Care and Use Committee, and all animal care procedures conformed to the principles outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC).

A 50-μl solution of JANEX-1 or JANEX-1-M (40 mg/kg) dissolved in DMSO was administered intravenously via the tail vein. This volume of DMSO is well tolerated by mice when administrated by rapid i.v. or i.p. injection. Four mice per time point were used for pharmacokinetic studies. Blood samples (~500 μl) were obtained from the ocular venous plexus by retro-orbital venipuncture at 0, 2, 5, 10, 15, 30, 45, 60, 120, 240, and 360 min after i.v. injection.

For studying the in vivo metabolism of JANEX-1, 10 mice received a 100 mg/kg i.v. bolus dose of JANEX-1, and then the mice were collectively placed in the Nalgene metabolic cage system (Nalgene Company, Rochester, NY). Urine was collected at 0 to 6, 6 to 24, 24 to 30, 30 to 48, and 48 to 72 h.

In Vitro Mast Cell Biology Experiments. RBL-2H3 cells (a rat origin mucosal mast cell line kindly provided by Dr. R. P. Siraganian (NIH, Bethesda, MD)) in 48-well tissue culture plates were sensitized with a monoclonal anti-dinitrophenyl (DNP)-IgE antibody (0.24 mg/ml) by overnight incubation at 4°C. Monoclonal antibody (mAb) 2B3 with the JANEX-1 or JANEX-1-M (20 mg/kg i.p.) twice at 1-h intervals before the antigen challenge. Control mice were treated with an equal volume of vehicle. Thirty minutes after the last dose of JANEX-1 or JANEX-1-M or vehicle, mice were injected intradermally with 20 mg of DNP-IgE (left ears) or PBS (right ears) in a 20-μl volume using a 30-gauge needle, as previously described (Miyajima et al., 1997). After 20 h, mice were treated with JANEX-1 or

**Fig. 1. Detection of a metabolite in urine of JANEX-1-treated mice.**

A, representative chromatograms from blank mice urine; B, urine from mice administered i.v. with JANEX-1; C, on-line MS spectrum. HPLC column, Zorbax SB-phenyl; mobile phase, acetonitrile/water containing 0.1% TFA (28:72, v/v); flow rate, 0.6 ml/min; detected with UV at 340 nm and selected ion monitoring at m/z of 284 and 298 (positive ion).
challenged with 100 µg of antigen (DNP-BSA) in 200 µl of 2% Evans blue dye intravenously. Mice were sacrificed by cervical dislocation 30 min after the antigen challenge. For quantitation of Evans blue dye extravasation, as a measure of anaphylaxis-associated vascular hyperpermeability, 8-mm skin specimens were removed from the ears of mice, minced in 2 ml of formamide, and incubated at 80°C for 2 h in a water bath to extract the dye. The absorbance was read at 590 nm.

**HPLC Determination of JANEX-1 and Its Metabolites.** The levels of JANEX-1 and its metabolite in the microsome systems and plasma were determined using a quantitative HPLC method (Chen et al., 1999a). In brief, 800 µl of acetone was added to the microsome system to terminate the reaction. Following centrifugation (300g; 5 min), the supernatant was transferred to a clean tube and dried under a slow, steady stream of nitrogen gas. The residue was reconstituted in 50 µl of methanol/water (9:1, v/v). A 35-µl aliquot of the reconstituted solution was injected for HPLC analysis using a Hewlett Packard series 1100 instrument equipped with a quaternary pump, an autosampler, an auto electronic degasser, an automatic thermostatic column compartment, a diode array detector, and a computer with Chemstation software. For pharmacokinetic modeling, an appropriate model was chosen on the basis of the lowest sum of weighted squared residuals, the lowest standard error of the fitted parameters, and the dispersion of the residuals. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile. The systemic clearance (CL) was determined by dividing the dose by the area under the curve.

**Homology Model of Human CYP1A1 and Docking of JANEX-1-M.** A homology model of human CYP1A1 was constructed based on the crystal structure of CYP2C5 (PDB accession number: 1d6) using homology and docking modules with INSIGHT II software (Molecular Simulations, Inc., San Diego, CA). The homology modeling of CYP1A1 was carried out in two steps summarized as follows: 1) the most reasonable sequence alignment between the CYP1A1 and a coordinate template was determined; and 2) new coordinates were assigned to the CYP1A1 residues according to the template coordinates (based on the sequence alignment), followed by the determination of loop coordinates and an energy minimization of the entire structure. The homology model of human CYP1A1 was used to generate a crystal-like simulation system and plasma, except that a 250 µl of acetone was added to the microsome system to terminate the reaction. Following centrifugation (300g; 5 min), the supernatant was transferred to a clean tube and dried under a slow, steady stream of nitrogen gas. The residue was reconstituted in 50 µl of methanol/water (9:1, v/v). A 35-µl aliquot of the reconstituted solution was injected for HPLC analysis using a Hewlett Packard series 1100 instrument equipped with a quaternary pump, an autosampler, an auto electronic degasser, an automatic thermostatic column compartment, a diode array detector, and a computer with Chemstation software for data analysis. A 250 × 4-mm Lichrospher 100 RP-18 (5 µm) analytical column and a 4 × 4-mm Lichrospher 100 RP-18 (5 µm) guard column were obtained from Hewlett Packard. The mobile phase contained acetonitrile/water (0.1% of TFA and 0.1% TEA) (28:72, v/v), which was degassed automatically by the electronic degasser system. The column was equilibrated and eluted under isocratic conditions using a flow rate of 0.6 ml/min at ambient temperature. The wavelength of detection was set at 340 nm for JANEX-1 and its metabolite.

The levels of JANEX-1 and its metabolite in the urine of mice treated with JANEX-1 was determined using the above-described procedures for the microsome system and plasma, except that a 250 × 4.6-mm Zorbax SB-phenyl analytical column (5 µm) and a mobile phase containing acetonitrile/water (0.1% of TFA) (28:72, v/v) at flow rate of 0.6 ml/min were used. On-line MS detection was set up with a selected ion model at both 284 and 298 and a fragmentor voltage of 75 V.

**Enzyme Kinetic Analysis and Pharmacokinetic Analysis.** Enzymatic kinetic analysis and pharmacokinetic analysis were performed using WinNonlin program V 3.0 (Pharsight, Mountain View, CA) (Chen et al., 1999b, 2001; Uckun et al., 1999a,b). Kinetic parameters such as V_{max} (maximum reaction velocity) and K_{m} (the substrate concentration that corresponds to 50% V_{max}) were estimated by fitting the data to the Michaelis-Menten model \( V = \frac{V_{max} \cdot C}{K_m + C} \), where C is the drug concentration and r is the Hill coefficient. Metabolic clearance was estimated as (CL_{met} = \frac{V_{max}}{K_m}) and Eadie-Hofstee plots were constructed to determine whether the kinetics was monoo- or biphasic. The relationship between the formation of JANEX-1-M and various cytochrome P450 activities was examined by calculating the nonparametric Spearman rank correlation coefficient using Instat computer software, version 3.0 (GraphPad Software, Inc., San Diego, CA).

For pharmacokinetic modeling, an appropriate model was chosen on the basis of the lowest sum of weighted squared residuals, the lowest Schwartz criterion, the lowest Akaike information criterion value, the lowest standard errors of the fitted parameters, and the dispersion of the residuals. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration-time profile. The systemic clearance (CL) was determined by dividing the dose by the area under the curve.
interactions with nearby residues were avoided. Otherwise, an even sampling of various initial positions was used within the general binding region that is known from crystal structures of P450 enzyme complexes. Subsequently, an automatic docking procedure was followed and evaluated using the Ludi score function (Bohm, 1992) for maximum binding affinity. Finally, the docked position of the molecule was inspected and compared with known compounds in superimposed homologous protein complexes using templates with PDB access codes 1BVV, 1F4T, 1E9X, and 1EA1.

Results

Metabolism of JANEX-1 in Mice and Human Microsomes. We used analytical HPLC to examine mouse urine samples collected from a group of 10 mice between 0 to 6, 6 to 24, 24 to 30, 30 to 48, and 48 to 72 h after i.v. injection of a 100 mg/kg bolus dose of JANEX-1 lactobionate for the presence of JANEX-1 and potential JANEX-1 metabolites. The parent compound JANEX-1 was detected at a retention time (RT) of 10.2 min in urine samples collected between 0 to 6 and 6 to 24 h but not at later time points (Fig. 1, A and B). A potential metabolite peak with a retention time of 7.9 min was also detected in the same urine samples (Fig. 1B). The on-line LC-MS yielded m/z values of 298 for the parent compound and 284 for the potential metabolite (Fig. 1C), prompting the hypothesis that the putative metabolite is a demethylated form (M-14) of JANEX-1.

To test the hypothesis that JANEX-1 is metabolized by demethylation, we next examined the in vitro metabolism of WHI-P31 in human liver microsomes. The representative metabolite profile of JANEX-1 following incubation with human hepatic microsomes is presented in Fig. 2A. The parent compound is shown with a R_T of 7.3 min, whereas the major metabolite of JANEX-1 in the microsome system had a R_T of 5.9 min (JANEX-1-M). Notably, the on-line LC-MS yielded for JANEX-1-M an m/z value of 284 (Fig. 2B), which is the same as the m/z value of the putative in vivo metabolite of JANEX-1 detected in mouse urine samples (see Fig. 1C).

Notably, the formation of JANEX-1-M was significantly decreased when NADP was omitted from the system (6.1 versus 47.1 pmol/min/mg, P < 0.0001; Fig. 2C). No metabolite was formed in denatured microsomes or in the absence of microsomes (Fig. 2C). Furthermore, the P450 inhibitor proadifen (SKF-525A) abolished the microsomal metabolism of JANEX-1 (Fig. 2C). Human liver cytosol and FMO did not participate in the in vitro metabolism of JANEX-1 (Fig. 2C). Taken together, these results indicate that JANEX-1 is metabolized by cytochrome P450-mediated enzymatic demethylation.

Identification of 4-(4’-Hydroxyphenyl)-amino-6-methoxy-7-hydroxyquinazoline as the Major Metabolite of JANEX-1. We next sought to determine the structural identity of JANEX-1-M, the major
metabolite of JANEX-1. Since demethylation could occur on either one of the two methoxy groups attached to the quinazoline ring of JANEX-1, it was important to determine whether the 6-O-demethylated or the 7-O-demethylated form of JANEX-1 was the actual metabolite (Fig. 3A). To this end, we first used sodium ethanethiolate to demethylate the methoxy groups of JANEX-1. The demethylation reaction yielded two structural isomers, which were separated by flash chromatography on silica and then further purified by recrystallization from methanol. The electrospray ionization-MS yielded a molecular mass of 284 for both synthetic isomers.

The retention times of the two synthesized demethylation products (compound 1 and compound 2) were different, but the retention time of compound 1 was identical to the retention time of JANEX-1-M formed in the microsome system (Fig. 3B). Furthermore, compound 1 had the same m/z (i.e., 284) as the urine-derived or microsome-derived JANEX-1-M by LC-MS (Fig. 3C). Therefore, compound 1 was further characterized to elucidate the structural features of the metabolite of JANEX-1.

Compound 1 was crystallized and analyzed using X-ray crystallography to determine the molecular structure of JANEX-1-M. The crystal structure of compound 1 showed that it is the 7-O-demethylation product of JANEX-1. The refined small molecule X-ray crystal structure of JANEX-1-M is shown in Fig. 3D; data statistics are listed in Table 1, and atomic coordinates are listed in Table 2. All nonhydrogen atoms were refined using anisotropic displacement parameters. Hydrogen atoms were placed at ideal positions (taking into consideration hydrogen bonding interactions for H-3 and H-4; Fig. 3D) and refined as riding atoms with relative isotropic displacement parameters. Hydrogen atoms were placed at ideal positions and refined as riding atoms with relative isotropic displacement parameters. Goodness of fit on \( R = 0.073, \omega R^2 = 0.073 \), and \( w = 1/ \sigma^2 \) for the structure refinement.

### Table 1

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### Activity and Pharmacokinetic Features of JANEX-1-M, 4-(4′-Hydroxyphenyl)-amino-6-methoxy-7-hydroxyquinazoline

Targeting JAK3 in mast cells with JANEX-1 inhibits IgE receptor-mediated mast cell responses (Malaviya et al., 1999). To examine the effect of JANEX-1-M on IgE receptor/FeceRI-mediated mast cell degranulation and cytokine release, IgE-sensitized RBL-2H3 mast cells were preincubated with increasing concentrations of JANEX-1-M, JANEX-1, or vehicle for 1 h before challenge with antigen (DNP-BSA). Notably, JANEX-1 prevented mast cell degranulation and release of preformed granule-associated β-hexosaminidase (Fig. 4A) and release of the proinflammatory cytokine TNF-α (Fig. 4B) in a concentration-dependent fashion, with near to complete inhibition at \( \approx 30 \mu M \). Unlike JANEX-1, its 7-O-demethylated metabolite JANEX-1-M did not inhibit mast cell degranulation or TNF-α release after IgE receptor/FeceRI cross-linking (Fig. 4, A and B). These results demonstrate that JANEX-1-M is \( >10 \)-fold less potent than JANEX-1 in inhibiting IgE receptor-mediated mast cell responses.

Increased vascular permeability induced by mast cell mediators, such as histamine and leukotrienes, is a hallmark of anaphylaxis (Oettgen et al., 1994). Therefore, we next compared the effects of JANEX-1 and JANEX-1-M on vascular permeability in a well characterized murine model of passive cutaneous anaphylaxis (Miyajima et al., 1997). JANEX-1, but not JANEX-1-M, significantly inhibited the IgE/antigen induced plasma exudation, as measured by extravasation of systemically administered Evans blue dye, in mice that had been presensitized with antigen-specific IgE (Fig. 4C).

We next examined the pharmacokinetics of JANEX-1-M in mice in side-by-side comparison with the parent compound JANEX-1. Following i.v. injection of JANEX-1-M at a dose level of 40 mg/kg, the plasma concentration of JANEX-1-M as a function of time can best be described by using a two-compartment model (Fig. 4D). Both JANEX-1-M and JANEX-1 had moderate volumes of distribution at steady state (JANEX-1-M: \( V_d = 811.6 \pm 336.0 \) ml/kg, \( n = 4 \); JANEX-1: \( V_d = 857.7 \pm 50.8 \) ml/kg), which is slightly larger than the volume of water in the body (Davies and Morris, 1993) (Table 3). JANEX-1-M had a larger volume of distribution at the central compartment (745.7 ± 40.7 versus 429.4 ± 206.1 ml/kg, \( P < 0.02 \)) and a shorter elimination half-life (33.0 ± 20.1 versus 77.1 ± 10.2 min, \( P < 0.01 \)) than the parent compound JANEX-1 (Table 3). The CL of JANEX-1-M was much faster than that of JANEX-1 (5525.1 ± 1926.2 versus 1458.0 ± 258.6 ml/kg, \( P < 0.01 \)), which is higher than the blood flow to either the kidney or the liver (Davies and Morris, 1993) (Fig. 4D; Table 3). Consequently, the area under the curve value for JANEX-1-M was much smaller than that for JANEX-1 (27.5 ± 8.0 versus 94.8 ± 18.4 µM·h, \( P < 0.001 \)) (Table 3).

### Kinetics of JANEX-1 Metabolism in Liver Microsomes and Its Relationship to CYP1A2 Activity

The metabolism of JANEX-1 to JANEX-1-M in pooled human liver microsomes followed Michaelis-Menten kinetics with \( V_{max} \) and \( K_m \) values (mean ± S.D.) of 34.6 ± 9.8 pmol/min/mg and 107.3 ± 66.3 µM, respectively (Fig. 5A). The corresponding rate of metabolic clearance for JANEX-1 was 0.3 µl/min/mg.

An Eadie-Hofstee plot of the formation of JANEX-1-M (\( V \)) versus \( V/S \) in human liver microsomes was monophasic (Fig. 5B), suggesting that a single enzymatic pathway was the main contributor to the metabolism of JANEX-1. Notably, at a final concentration of 50 µM, the rate of JANEX-1-M formation from JANEX-1 in human liver microsomes (\( n = 12 \)) correlated with the microsomal CYP1A2 (phenacetin O-deethylase) activity (\( r = 0.95, P < 0.0001 \)) (Fig. 5C) but not with total P450 content, cytochrome C reductase activity, cytochrome \( b_5 \) activity, or the activities of other cytochrome P450 enzymes, including CYP2A6 (cumarin 7-hydroxylase), CYP2B6 ([S]-mephenytoin N-demethylase), CYP2C8 (paclitaxel 6α-hydroxylase),
CYP2C9 (diclofenac 4'-hydroxylase), CYP2C19 [(S)-mephénytoin 4'-hydroxylase], CYP2D6 (bufuralol 1'-hydroxylase), CYP2E1 (chlorzoxazone 6-hydroxylase), CYP3A4 (testosterone 6α-hydroxylase), CYP4A (lauric acid 12-hydroxylase), and FMO (methyl p-tolyl sulfide oxidase) (data not shown). These results prompted the hypothesis that JANEX-1 metabolism is mediated primarily by CYP1A2.

The effects of 12 cytochrome P450 substrates/inhibitors on the formation of JANEX-1-M in human liver microsomes are presented in Table 4. In all 12 compounds tested, only α-naphthoflavone and furafylline, which both inhibit CYP1A2, significantly inhibited the formation of JANEX-1-M in human liver microsomes, which supports the hypothesis that JANEX-1 metabolism is mediated primarily by CYP1A2.

We next studied the metabolism of JANEX-1 in induced Sprague-Dawley rat liver microsomes. ARO, BNF, 3-MC are the inducers for the CYP1A subfamily (Waxman, 1999) (Fig. 6A). CYP4A is induced by CFB (Waxman, 1999), CPR3A by DEX (Grange et al., 1994; Waxman, 1999), CYP2E by ISO (Grange et al., 1994), and CYP2B by PHEN (Waxman, 1999). A significantly increased metabolic rate for JANEX-1 was observed in ARO-, BNF-, and 3-MC-induced microsomes but not in CFB-, DEX-, ISO-, and PHEN-induced microsomes (Fig. 6B). These results further support the importance of CYP1A in the metabolism of JANEX-1 to form JANEX-1-M. The results also exclude a significant role for CYP4A, CPR3A, CYP2E, and CYP2B in the formation of JANEX-1-M.

We also examined the interspecies differences in JANEX-1 metabolism in relationship to the CYP1A content of the microsomes. There was a direct correlation between CYP1A activity and the magnitude of JANEX-1-M formation in the liver microsomes from different animal species (Fig. 6C). The metabolic rate was the highest in rabbit liver microsomes, which had the highest CYP1A activity, and lowest in canine microsomes, which had the lowest CYP1A activity (Fig. 6C).

Metabolism of JANEX-1 by Recombinant Human CYP1A1 and CYP1A2. We next sought to determine the identity of the cytochrome P450 isoform, which is responsible for the metabolism of JANEX-1 by using recombinant cytochrome P450 isoforms (Crespi, 1995). The results presented in Fig. 7A indicate that metabolism of JANEX-1 to form JANEX-1-M is mainly mediated by cytochrome P450 1A1 and 1A2.

The metabolic formation of JANEX-1-M in baculovirus-expressed CYP1A1 and 1A2 was consistent with Michaelis-Menten kinetics (Fig. 7B). The estimated $V_{\text{max}}$ and $K_{\text{m}}$ values for JANEX-1-M were $1842.7 \pm 268.8 \text{ pmol/min/nmol}$ and $1.5 \pm 0.6 \mu \text{M}$ in the presence of CYP1A1 and $9292.4 \pm 307.9 \text{ pmol/min/nmol}$ and $8.5 \pm 0.8 \mu \text{M}$ in the presence of CYP1A2, respectively. Therefore, the average meta-
bolic clearance of JANEX-1 was 1228.5 μl/min/nmol in the presence of CYP1A1 and 1093.2 μl/min/nmol in the presence of CYP1A2.

Structural Basis for the Regioselective O-Demethylation of JANEX-1 by CYP1A1/CYP1A2. We next set out to elucidate the structural basis for the regioselective demethylation of JANEX-1 by enzymes of the CYP1A family. To this end, we constructed a series of three-dimensional models of human cytochrome P450 enzymes based on their amino acid sequence similarity with several cytochrome P450
proteins with known crystal structures, including rabbit CYP2C5 (see Materials and Methods). A homology model of CYP1A1 was established using the HOMOLOGY module within the INSIGHT II program (Molecular Simulations, Inc.) (see Materials and Methods). The binding mode of JANEX-1 was determined by a docking procedure using the AFFINITY module within INSIGHT II, with a fixed distance between the O-7 atom and the heme iron atom. Next, the homology model of CYP1A1 was superimposed with crystal structures of a number of P450 enzymes and their complexes with small molecules (PDB access codes: 1DT6, 2C17A, 1FAG, and 2BMH) (Hasemann et al., 1994; Hishiki et al., 2000).

The crystal structures of the ligated P450 enzyme complexes revealed a general orientation of ligand binding in which a ligand is typically 2 Å away from the heme iron stacking against the I helix and extending toward the BC loop. The comparison showed that the docked position of JANEX-1 was consistent with the general orientation. In the docked model, the quinazoline group of JANEX-1 is situated on top of the heme group of CYP1A1 (see Fig. 8A), with the O-7 atom close to the iron in the heme center. On one side, the quinazoline is stacked against the I helix, containing residues 317 to 321; the side chain of A317 has VDW contact with the quinazoline ring. On the same side, the 4-hydroxyphenyl group is stacked against the F helix, containing residue G225, and is sandwiched from the other side by residue F123. Mostly perpendicular to the aromatic ring plane of the ligand, T111 from the BC loop forms a hydrogen bond with the 4-hydroxyl group. Residue V228 is near the hydroxyphenyl ring and V382 is in nonbonded contact with the 6-methoxyl group. This model provides an opportunity to examine what residues might be involved in the binding of JANEX-1 and what might be the reasons for regioselective demethylation. Based on the model, it is clear that an alternative binding conformation, with O-6 close to the iron center, is unlikely (explained in Fig. 8B). This model of CYP1A1 and crystal structures of other P450 enzymes reveal that the binding site is asymmetric, with the heme iron at the center of a bottom plateau of a “valley-shaped” region. The alternative conformation (with demethylation at the C-6 position) would result in a severe steric clash with the residues that are associated with the heme (Fig. 8B).

Finally, we examined which residue(s) might be responsible for JANEX-1 being favorably catalyzed by CYP1A enzymes. Our model indicated that T111 in CYP1A1 forms a hydrogen bond with JANEX-1. An ideal hydrogen bond could result in a nearly 8-fold improvement in the binding constant based on a LUDI score function (Bohm, 1992). T111 is conserved in the 1A family but is not found in other P450 enzymes that we examined, except CYP2A6. It is also noted that a small residue at 225 (a glycine in CYP1A1 and a valine in CYP1A2) has close VDW contact with the 4-hydroxyphenyl ring. This would be less feasible when 225 is a larger residue, as is the case

**TABLE 4**

<table>
<thead>
<tr>
<th>Inhibitors (μM)</th>
<th>Specificity</th>
<th>% Control for JANEX-1-M Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthoflavone (50)</td>
<td>CYP1A2</td>
<td>9.8 ± 1.8</td>
</tr>
<tr>
<td>Furafylline (50)</td>
<td>CYP1A2</td>
<td>17.8 ± 4.0</td>
</tr>
<tr>
<td>Coumarin (50)</td>
<td>CYP2A6</td>
<td>89.0 ± 3.4</td>
</tr>
<tr>
<td>Diethylthiocarbamic acid (50)</td>
<td>CYP2A6</td>
<td>89.1 ± 2.7</td>
</tr>
<tr>
<td>Orphenadrine (50)</td>
<td>CYP2B6</td>
<td>89.7 ± 1.2</td>
</tr>
<tr>
<td>Sulfinpyrazone (10)</td>
<td>CYP2C9</td>
<td>78.5 ± 2.5</td>
</tr>
<tr>
<td>(S)-Mephenytoin (250)</td>
<td>CYP2C19</td>
<td>94.1 ± 8.0</td>
</tr>
<tr>
<td>Quinidine (5)</td>
<td>CYP2D6</td>
<td>89.0 ± 4.8</td>
</tr>
<tr>
<td>p-Nitrophenol (100)</td>
<td>CYP2E1</td>
<td>91.8 ± 2.9</td>
</tr>
<tr>
<td>Ketoconazole (5)</td>
<td>CYP3A4</td>
<td>86.4 ± 2.9</td>
</tr>
<tr>
<td>Troleandomycin (50)</td>
<td>CYP3A4</td>
<td>88.7 ± 1.8</td>
</tr>
<tr>
<td>Lauric acid (50)</td>
<td>CYP4A11</td>
<td>90.0 ± 11.2</td>
</tr>
</tbody>
</table>

FIG. 5. A, kinetics of formation of JANEX-1-M in human liver microsomes; B, Eadie-Hofstee plot for the O-demethylation by human liver microsomes; and C, correlation of the JANEX-1-M formation with CYP1A2 activities in individual human liver microsomes.

JANEX-1 was used at a final concentration of 50 μM in these experiments.
for other enzymes. Considering the tight fit in this region, a larger residue may be sufficient to impair the binding of JANEX-1.

Discussion

Our experimental data presented here provides unprecedented evidence that the JAK3 inhibitor JANEX-1 is metabolized by cytochrome P450 enzymes CYP1A1 and CYP1A2 in a regioselective fashion to form the biologically inactive 7-O-demethylation product 4-(4′-hydroxyphenyl)-amino-6-methoxy-7-hydroxyquinazoline. Our molecular modeling studies indicated that the cytochrome 1A family enzymes must bind and demethylate the molecule at the C-7 position of the quinazoline ring since the alternative binding conformation with demethylation at the C-6 position would result in a severe steric clash with the residues that are associated with the heme iron of the enzyme at the center of a bottom plateau of a valley-shaped binding region. Our model further indicated that the combination of the hydrogen bond with T111 and the presence of a small residue at 225 makes the cytochrome 1A family most suitable to bind JANEX-1 and demethylate the molecule at the C-7 position of the quinazoline ring.

O-Demethylation has been reported for other 6,7-dimethoxy-quinazoline compounds, including prazosin (Taylor et al., 1977), doxazosin (Kaye et al., 1986), and DDQ (Yamato et al., 1982). The metabolic pathway of JANEX-1 is similar to that of DDQ, which was biotransformed in vivo to form only the 7-demethylation metabolite, but it is different from the metabolism of prazosin and doxazosin, which were metabolized in vivo to form both 6- and 7-demethylation metabolites. However, the detailed metabolic pathways of prazosin, doxazosin, and DDQ have not been reported.

CYP3A is the most abundant cytochrome P450 enzyme subfamily in human liver, which is abundantly expressed in the intestinal mucosa (Kronbach et al., 1989; Crespi, 1995). CYP3A did not appear to be
involved in the demethylation of JANEX-1. This may account for the good oral bioavailability of JANEX-1 in animals (Uckun et al., 1999b). In human tissues from nonsmokers, the CYP1A1 gene is expressed at very low levels (Crespi, 1995). Due to the low abundance of CYP1A1 in human tissues, CYP1A1 would be unlikely to control the metabolism of JANEX-1 in patients. CYP1A2 activity varies considerably from individual to individual and appears to be modulated by environmental factors (Crespi, 1995). Therefore, the O-demethylation of JANEX-1 is likely to show a significant patient-to-patient variation in clinical settings. It is also likely that the metabolism of JANEX-1 in patients will be affected by other drugs that are metabolized by or induce CYP1A2 subfamily members. Finally, further characterization of the other routes of excretion and metabolism pathways is required to better understand the metabolite pharmacokinetics of JANEX-1-M.

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References


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