Rhinoviruses are the primary causative agent implicated in human upper respiratory tract infections and are responsible for a large percentage of common cold incidents each year (Couch, 1990; Rueckert 1990; Gwaltney and Rueckert, 1997). The production of mature virion particles requires a functional rhinovirus 3C protease, which is responsible for post-translational cleavage of viral polyprotein precursor into active enzymatic proteins (Krausslich and Wimmer, 1988; Rueckert, 1990). The importance of this enzyme in viral replication has led to its identification as a novel therapeutic target (e.g., Kaldor et al., 1995; Webber et al., 1996; Dragovich et al., 1998a,b; Kong et al., 1999a,b; Harr et al., 1999). The current study describes and evaluates the metabolism of AG7088, a potent and irreversible peptidomimetic inhibitor of rhinovirus 3C protease.

In vitro metabolism of AG7088, which comprises four main building blocks denoted P1/P1', P2, P3, and P4, was studied in liver microsomes isolated from mice, rats, rabbits, dogs, monkeys, and humans. The structures of the metabolites were characterized by liquid chromatography (LC)-tandem mass spectrometry and LC-NMR methods. Hydrolysis of the ethyl ester to produce metabolite M4 (AG7185) is the predominant pathway in all species, with the greatest activity observed in rodents and rabbits, followed by monkeys, dogs, and humans. Several hydroxylation products were identified as minor metabolites, including diastereomers M1 and M2, with a hydroxy group at the P1-lactam moiety, and M3, with a hydroxy group at the methyl position of the methylisoxazole ring. Rodent and rabbit liver microsomes formed almost exclusively the acid metabolite M4 (AG7185), with very little hydroxylated metabolites, whereas monkey liver microsomes formed more secondary metabolites (i.e., acid analogs of the hydroxylated metabolites). The overall metabolic profile of AG7088 formed in dog liver microsomes closely resembled that of human liver microsomes; therefore, this species may be the most appropriate animal model relative to humans for exposure to AG7088 and its metabolites.

ABSTRACT:
In vitro metabolism of AG7088 [trans-(4S,2'R,5'S,3'S)-4-{2'-4-(4-fluorobenzyl)-6'-methyl-5'-(5'-methylisoxazole-3'-carbonylamino)-4-oxoheptanoylamino)-5-(2'-oxopyrrolidin-3''-yl)pent-2-enolic acid ethyl ester] was studied in liver microsomes isolated from mice, rats, rabbits, dogs, monkeys, and humans. The structures of the metabolites were characterized by liquid chromatography (LC)-tandem mass spectrometry and LC-NMR methods. Hydrolysis of the ethyl ester to produce metabolite M4 (AG7185) is the predominant pathway in all species, with the greatest activity observed in rodents and rabbits, followed by monkeys, dogs, and humans. Several hydroxylation products were identified as minor metabolites, including diastereomers M1 and M2, with a hydroxy group at the P1-lactam moiety, and M3, with a hydroxy group at the methyl position of the methylisoxazole ring. Rodent and rabbit liver microsomes formed almost exclusively the acid metabolite M4 (AG7185), with very little hydroxylated metabolites, whereas monkey liver microsomes formed more secondary metabolites (i.e., acid analogs of the hydroxylated metabolites). The overall metabolic profile of AG7088 formed in dog liver microsomes closely resembled that of human liver microsomes; therefore, this species may be the most appropriate animal model relative to humans for exposure to AG7088 and its metabolites.

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Liver microsomes (1 mg of protein/ml) were incubated with AG7088 (25 mM (1 mM) in 100 mM potassium phosphate buffer, pH 7.4, at 37°C in a shaking microsomes (1 mg/ml) were preincubated with NADPH-regenerating solution microsomes (male beagle, 20 mg of protein/ml, 0.57 nmol of P450/mg). The above with some exceptions. Incubations were performed using dog liver 60 with gentle vortexing. The final incubation volume was 5 ml, and it contained under a gentle stream of nitrogen using a Dri-Block sample concentrator was transferred to another set of test tubes, and the solvent was evaporated which the reaction was terminated by the addition of acetonitrile (2 ml). All samples were vortexed for 2 min on an SP Multi-tube Vortexer (Baxter, Marietta, OH). Water-acetonitrile 8:2 (400 m from Cambridge Isotopes. Preparation of AG7088 Stock solutions for Microsomal Incubations. A 50 mM solution of AG7088 in dimethyl sulfoxide was initially prepared and subsequently diluted with acetonitrile to achieve a 1 mM solution.

AG7088 Incubations in Various Animal and Human Liver Microsomes. Liver microsomes (1 mg of protein/ml) were incubated with AG7088 (25 μM) in 100 mM potassium phosphate buffer, pH 7.4, at 37°C in a shaking water bath for 5 min before initiating the reaction with NADPH (2 mM). The final volume of each incubation was 0.5 ml. After the addition of NADPH, a 200-μl aliquot was removed and placed into a test tube containing 2 ml of acetonitrile to serve as a time 0 sample. The remainder of the incubate proceeded for 30 min, after which the reaction was terminated by the addition of acetonitrile (2 ml). All samples were vortexed for 2 min on an SP Multi-tube Vortexer (Baxter, McGaw Park, IL) and then centrifuged at 2500g for 20 min. The organic layer was transferred to another set of test tubes, and the solvent was evaporated under a gentle stream of nitrogen using a Dri-Block sample concentrator (Techne, Princeton, NJ) at 50°C. The sample residues were stored at −20°C until analyzed by LC-MS/MS.

Microsomal incubations were performed on a larger scale to generate samples for LC-NMR analysis. The protocol was similar to that described above with some exceptions. Incubations were performed using dog liver microsomes (male beagle, 20 mg of protein/ml, 0.57 nmol of P450/mg). The microsomes (1 mg/ml) were preincubated with NADPH-regenerating solution (1 mM) in 100 mM potassium phosphate buffer, pH 7.4, at 37°C in a shaking water bath for 10 min before initiating the reaction with AG7088 (20 μM), with gentle vortexing. The final incubation volume was 5 ml, and it contained 60 μg of AG7088. The incubation was allowed to proceed for 2 h, after which the reaction was terminated by the addition of ice-cold acetonitrile (15 ml). The sample was then centrifuged at 4000 rpm for 20 min, and the supernatant was concentrated on a Speed-Vac (Savant Instruments, Forma Scientific, Inc., Marietta, OH). Water-acetonitrile 8:2 (400 μl) was added to the concentrated microsomal extract, and solid-phase extraction was performed as follows. After washing the solid-phase extraction cartridge (Varian BondElut LRC, 40-μm, 200-mg C18) with methanol, the cartridge was equilibrated with water-acetonitrile (8:2). The microsomal extract was then passed through the cartridge followed by another 1 ml of the equilibration solvent (fraction 1). Thereafter, the column was washed with water-acetonitrile (1:1, 2 ml; fraction 2) and finally with acetonitrile (2 ml; fraction 3). Analytical HPLC analysis of the fractions indicated that early eluting, proteinaceous material was effectively concentrated into the first fraction, while metabolites and unmetabolized parent drug were contained in fraction 2. Fraction 2 was concentrated and stored at −20°C until analyzed by LC-NMR.

LC-MS/MS. AG7088 metabolites produced by small-scale incubates were analyzed by LC-MS/MS. Interpretation of the product ion mass spectra (MS/ MS) of each metabolite was conducted in reference to the parent drug. The LC-MS/MS system included a Waters 2690 Separation Module (Waters, Milford, MA) and a Quattro II triple stage quadrupole mass spectrometer controlled by Masslynx 3.0 software (Micromass, Beverly, MA). The chromatography was performed on a reversed phase column (Waters Symmetry C18, 2.1 × 150 mm, 5 μm) using a gradient elution method at a flow rate of 0.2 ml/min. The mobile phase contained 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). Gradient elution was programmed linearly from 20 to 80% B over 20 min. The mass spectrometer was operated under the following conditions: electrospray in positive ion mode using a Crossflow counter electrode, capillary voltage = 3.2 kV, cone voltage = 30 V, source temperature = 140°C, collision energy = 22 eV, and collision gas cell pressure = 1.4 × 10⁻³ mBar. Data were collected by MS1 under full scan mode (m/z 150–800 at a scan speed of 1.30 s), and daughter ion scan mode to obtain structural information.

LC-NMR. AG7088 and metabolites M1, M2, M3, and M4 were analyzed by LC-NMR on a Bruker Avance 500-MHz spectrometer (Bruker Instruments, Fremont, CA). The system was configured with a 4-mm H/H¹ inverse-geometry LC (LC SEI) probe equipped with x,y,z-gradients, an HP1100 analytical HPLC with binary pump and variable wavelength UV detector, and a Bruker 12-loop peak sampling unit (BPSU-12). The LC-NMR software interface was HyStar NT Version 1.2 (Bruker Instruments). Chromatography was performed on a reversed phase column (Ckapcell C18, 4.6 × 250 mm, 5 μm) using a gradient elution method at a flow rate of 1 ml/min. Mobile Phase A contained 0.1% TFA in deuterium oxide (99.9% D), while mobile phase B contained 0.1% TFA in acetonitrile. The solvent gradient was increased linearly from 20% B to 50% B over 30 min. The UV absorbance was monitored at 220 nm. LC-NMR was performed in stop-flow mode. AG7185 (M4) eluted at 41% mobile phase B with a retention time (tR) of 21.6 min; M3 at 45% B with tR = 25.1 min, M1/M2 at 47% B with tR = 27.3 min, and AG7088 at 50% B with tR = 30.4 min. Solvent suppression was performed using either double presaturation of the residual solvent resonances, or WET (Ogg et al., 1994; Smallcombe et al., 1995) solvent suppression with ¹H decoupling. Spectra were acquired using 64k data points and a spectral width of 10,000 Hz. Typically, data were acquired overnight (7K–20K transients). In addition, reference LC-NMR spectra were acquired on synthetic standards of AG7185 and AG7088 (50-μg quantities with retention times of 21.5 and 30.0 min, respectively), and one-dimensional spectra were acquired with 1K transients. To obtain ¹H resonance assignments for AG7088 under the LC solvent conditions, magic angle gradient double quantum-filtered correlation spectroscopy (van Zijl et al., 1995) was performed on AG7088 synthetic standard (200 μg of AG7088, tR = 30.3 min). The two-dimensional data were collected in phase-sensitive mode using TPPL and gradient pulses were employed for coherence transfer selection. For each of the 256 t₁ increments, 704 transients were acquired using 2K data points over a spectral width of 7000 Hz, with the carrier centered on the acetonitrile resonance. All spectral data were referenced to acetonitrile at 2.0 ppm.

Results

HPLC Profiles of AG7088 Metabolites. The HPLC chromatographic profiles of AG7088 and metabolites produced by hepatic microsomes from the mouse, rat, rabbit, dog, monkey, and human are illustrated in Fig. 2, a to f, respectively. The metabolites are designate M1 to M6 on the basis of increasing polarity or decreasing retention time on a reversed phase HPLC column. M4 (AG7185) was the predominant metabolite in all six species studied, especially in rodents and rabbits. Metabolites M1 to M3 were more prevalent in human and dog liver microsomes, whereas M5 and M6 were more abundant in monkey liver microsomes. The abundance of each metabolite was graded from 1+ to 5+ (Table 1) based on its peak height relative to AG7088 (Fig. 2, a–f). Results for M1 and M2 were pooled (vide infra).

Structural Elucidation by LC-MS/MS and LC-NMR. Parent drug. AG7088 (Fig. 1) comprises four main building blocks: P1, a

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3 Abbreviations used are: LC-MS/MS, liquid chromatography–tandem mass spectrometry; LC-NMR, liquid chromatography-NMR spectroscopy; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.
AG7088 was accomplished using LC-MS/MS and LC-NMR and was facilitated by comparative analysis with the parent compound. The product ion mass spectrum of AG7088 (MH\(^+\) = 599; Fig. 3a) provided structural information mainly from the fragmentation at the P1 to P2 linkage (m/z 210, 227, 345, and 373). In addition, fragmentation at the P3 to P4 linkage produced an ion at m/z 455. In the LC-NMR spectrum of AG7088, key functional groups were clearly represented. These included the fluorophenyl (δ 7.15, dd, 2H; δ 6.97, dd, 2H) and methyl isoxazole (δ 6.45, s, 1H; δ 2.44 s, 3H) rings, the trans-α,β-unsaturated ethyl ester (δ 6.53, dd, 1H; δ 5.23, d, 1H; δ 4.14, q; δ 1.25, t, 3H), the valine methyl and Cα protons (δ 0.94, d, 3H; δ 0.82, d, 3H; δ 4.55, d, 1H), and the P1 lactam protons adjacent to the nitrogen (δ 3.25, 3.18, m), as well as several aliphatic resonances associated with P2 (δ 2.61–3.10).

Metabolites M1 and M2. The MS1 full scans of metabolites M1 and M2 showed that both peaks contained the ion at m/z 615 as the most abundant ion, consistent with monohydroxy metabolites of AG7088. Their MS/MS spectra were almost identical, in which the protonated molecule and most of the fragment ions containing the P1/P1' moiety readily lost a water molecule (e.g., m/z 208, 225, 453, and 597; Fig. 3b), presumably to form thermodynamically more stable or fully conjugated products. In contrast, the fragment ions that did not contain the P1/P1' moiety remained unchanged (e.g., m/z 345 and 373). This mass spectral information localized the hydroxy groups of M1 and M2 to the P1/P1' moiety. However, it was not possible to definitively assign the structures from these data alone, and M1 and M2 were further characterized by LC-NMR.

LC-NMR was performed on a metabolite-enriched fraction obtained from a larger-scale microsomal incubation and solid-phase extraction procedure. Chromatography of this fraction resulted in coelution of M1 and M2 as a broad peak; thus, the LC-NMR data were recorded on a mixture of M1 and M2. The resulting spectrum was deceptively simple and more consistent with that of a single compound than a mixture. We ultimately recognized that these data represented a pair of diastereomers. The spectrum was characterized by a single set of well resolved resonances in the downfield region (Fig. 4). The aromatic protons of the fluorophenyl ring gave rise to two well defined triplets (δ 7.15, 2H; 6.98, 2H), identical to those observed in AG7088. The sharp methine proton of the methyl isoxazole ring was present at 6.46 ppm. One new resonance, not present in AG7088, appeared at 5.19 ppm (t, 1H), adjacent to an olefinic proton (δ 5.5 Hz, 1H), and the Val Cα proton (δ 4.56, 1H) gave rise to a broad doublet of doublets. Although these patterns were unique from those of AG7088 (δ 6.52, 1H) appeared as a doublet of triplets, while the Val Cα proton (δ 4.56, 1H) gave rise to a broad doublet of doublets. Although these patterns were unique from those of AG7088 (δ 6.52, 1H), adjacent to an olefinic doublet (δ 5.23 ppm (1H)). The only other notable differences in this region of the spectrum were associated with the apparent multiplet structures of two of the signals. One of the olefinic protons (δ 6.52, 1H) appeared as a doublet of triplets, while the Val Cα proton (δ 4.56, 1H) gave rise to a broad doublet of doublets. Although these patterns were unique from those of AG7088 (δ 6.52, 1H), modification of the structure to accommodate additional couplings in two remote portions of the molecule seemed unlikely. A more plausible interpretation was that each of these signals represented a pair of resonances (2 × 0.5H) arising from two diastereomers in the M1/M2 mixture. The slight chemical shift offset between resonances gave rise to what appeared to be a change in multiplet structure when, in fact, the spin-spin couplings for each pair of resonances remained unchanged. The presence of two compounds of identical mass and mass spectral fragmentation pattern, similar HPLC retention time, and two sets of nearly degenerate NMR signals strongly suggested that M1 and M2 were a pair of diastereomers.

Despite the mixture of two diastereomers, it was possible to characterize the structures of M1 and M2 from the LC-NMR spectrum. Perhaps the most informative piece of data came from the chemical shift of the new proton at 5.09 ppm, which was consistent with a

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Fig. 2. HPLC chromatograms (analog UV signal at 220 nm) of AG7088 metabolites formed by liver microsomes in various species (mouse (a), rat (b), rabbit (c), dog (d), monkey (e), and human (f)).

Glu-derived γ-lactam containing an ethyl propenoate Michael-acceptor moiety (P1'); P2, 4-fluoro-Phe; P3, Val, which is joined to P2 via a ketomethylene isostere; and P4, a methyl isoxazole (Dragovich et al., 1998a,b, 1999a,b). Identification of the microsomal metabolites of
hemiaminal (oxidation of the P1-lactam at the γ-position). This assignment provided the best fit for all possible P1 oxidation products when compared with model compounds (Baker and Sifniades, 1979; Farina et al., 1984; Williams et al., 1991; Butler and Capon, 1992; Potts et al., 1992). Moreover, the appearance of this signal coincided with the disappearance of the two methylene protons adjacent to the lactam nitrogen of AG7088 (δ 3.25, 3.18; data not shown), which had been assigned from the AG7088 magic angle double quantum-filtered correlation spectroscopy data. The presence of the hemiaminal is also consistent with the observed dehydration in the mass spectrometer, a fragmentation pattern that has been observed for other hemiaminals or acetals (Scheuer and de Silva, 1980; R. Bannwart, B. Potts, and M. Ouellette, unpublished observations). Although this functional group could give rise to two diastereomers in dynamic equilibrium, the presence of two unique sets of resonances indicated that the rate of interconversion was slow on the NMR time scale, and while variable temperature NMR (up to 55°C) resulted in modest sharpening of some resonances, complete coalescence between species was never achieved. Nevertheless, the collective data for M1/M2 obtained from LC-NMR and LC-MS are entirely consistent with a pair of diastereomers with a hydroxy group at the γ-position of the P1-lactam moiety.

**Metabolite M3.** The MS1 full scan spectrum of M3 also showed the ion at m/z 615 as the most abundant ion, consistent with a monohydroxy metabolite of AG7088. In contrast to M1 and M2, M3 retained all fragment ions that included the P1/P1’ moiety (e.g., m/z 210, 227, and 455), but shifted +16 Da on ions that contained the P4 moiety (e.g., m/z 143, 361, and 389). These data (Fig. 3c) localized the M3 hydroxy group to the methylisoxazole ring. LC-NMR definitively established the site of oxidation as the P4 methyl group. The AG7088 methyl singlet at δ 2.44 was replaced by a sharp methylene singlet at δ 4.70, consistent with substitution with oxygen, while the neighboring methine proton of the isoxazole ring was modestly downfield shifted from δ 6.45 to δ 6.66. No other changes were apparent in the NMR spectrum.

**Metabolite M4.** The MS1 full scan spectrum of M4 showed the ion at m/z 571 as the most abundant ion, consistent with a monohydroxy metabolite of AG7088. In contrast to M1 and M2, M3 retained all fragment ions that included the P1/P1’ moiety (e.g., m/z 210, 227, and 455), but shifted +16 Da on ions that contained the P4 moiety (e.g., m/z 143, 361, and 389). These data (Fig. 3c) localized the M3 hydroxy group to the methylisoxazole ring. LC-NMR definitively established the site of oxidation as the P4 methyl group. The AG7088 methyl singlet at δ 2.44 was replaced by a sharp methylene singlet at δ 4.70, consistent with substitution with oxygen, while the neighboring methine proton of the isoxazole ring was modestly downfield shifted from δ 6.45 to δ 6.66. No other changes were apparent in the NMR spectrum.

**Metabolite M5.** The MS1 full scan spectrum of M5 indicated that it is the carboxylic acid analog of M1/M2, and must therefore represent a pair of diastereomers. Finally, the MS1 full scan and product ion (Fig. 3f) mass spectra of M6 indicated that it is the carboxylic acid analog of M3. These compounds were not assessed by LC-NMR.

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**TABLE 1**

Metabolite profiles of AG7088 in hepatic microsomes from five animal species and humans

<table>
<thead>
<tr>
<th>Species</th>
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<th>M4</th>
<th>M5</th>
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**FIG. 3.** Product ion mass spectra of AG7088 (a) and its metabolites M1/M2 (b); M3 (c); M4 (d); M5 (e); and M6 (f), and assignment of fragment ions.
Discussion

While the sensitivity of LC-MS/MS provides rapid metabolite information during drug discovery or development, it often lacks the accuracy of pinpointing the precise location of metabolism. LC-NMR, on the other hand, offers detailed structural information that is complementary to the LC-MS/MS data. In this case, the complementary results obtained from LC-MS/MS and stop-flow LC-NMR were critical in providing definitive structural information for these metabolites. The overall metabolic pathway for AG7088 as proposed from these data is summarized in Fig. 5. The LC-MS/MS data were suffi-

Fig. 4. LC-NMR spectra of AG7088 (top; R = H) and metabolites M1/M2 (bottom; R = OH), expanded about the region from 4.45 to 7.40 ppm.

Fig. 5. Metabolic pathway for AG7088 in vitro, as deduced from LC-MS/MS and LC-NMR analysis.
cient to determine the structure of hydrolysis product M4, while LC-NMR provided confirmation of its identity. LC-MS/MS also provided a highly plausible structure for M3 and localized the site of oxidation for M1 and M2 to the P1 moiety, while LC-NMR allowed the specific sites of oxidation to be identified. Clearly, the combination of hyphenated techniques provided a powerful means of elucidating the structures of all of the metabolites with very limited quantities of material.

The metabolic pathway of AG7088 was evaluated across species, including mouse, rat, rabbit, monkey, dog, and human. Hydrolysis of the ethyl ester to produce M4 is the predominant pathway in all species, with rodents and rabbits being the most active, followed by monkeys. Hydrolysis occurred in dog and human liver microsomes at a more moderate rate. Hydroxylation on the methyl position of the methylisoxazole ring to give M3 also appeared to be a prominent pathway in monkey, dog, and human liver microsomes. Two additional metabolites, M1 and M2, were identified as a pair of diastereomers with a hydroxy group at the γ-position of the P1-lactam moiety. Rabbit, dog, and human liver microsomes formed more M1/M2 when compared with mouse, rat, and monkey liver microsomes. M1/M2 were subject to further hydrolysis to form secondary metabolite M5, which represents a putative mixture of diastereomers.

The metabolic profile of AG7088 in dog liver microsomes resembled that of human liver microsomes, indicating that the dog may be the most appropriate animal model relative to humans for exposure to AG7088 and its metabolites. Although AG7088 appeared to be stable that of human liver microsomes, indicating that the dog may be the most appropriate animal model relative to humans for exposure to AG7088 and its metabolites. Although AG7088 appeared to be stable that of human liver microsomes, indicating that the dog may be the most appropriate animal model relative to humans for exposure to AG7088 and its metabolites. Although AG7088 appeared to be stable.

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References


