Evaluation of Capravirine as a CYP3A Probe Substrate: In Vitro and in Vivo Metabolism of Capravirine in Rats and Dogs

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ABSTRACT:

Metabolism of [14C]capravirine was studied via both in vitro and in vivo means in rats and dogs. Mass balance was achieved in rats and dogs, with mean total recovery of radioactivity >86% for each species. Capravirine was well absorbed in rats but only moderately so in dogs. The very low levels of recovered unchanged capravirine and the large number of metabolites observed in rats and dogs indicate that capravirine was eliminated predominantly by metabolism in both species. Capravirine underwent extensive metabolism via oxygenation reactions (predominant pathways in both species), depicolylation and carboxylation in rats, and decarbamation in dogs. The major circulating metabolites of capravirine were two depicolyated products in rats and three decarbamated products in dogs. However, none of the five metabolites was observed in humans, indicating significant species differences in terms of identities and relative abundances of circulating capravirine metabolites. Because the majority of in vivo oxygenated metabolites of capravirine were observed in liver microsomal incubations, the in vitro models provided good insight into the in vivo oxygenation pathways. In conclusion, the diversity (i.e., hydroxylation, sulfoxi-
dation, sulfone formation, and N-oxidation), multiplicity (i.e., mono-, di-, tri-, and tetraoxygenations), and high enzymatic specificity (>90% contribution by CYP3A4 in humans, CYP3A1/2 in rats, and CYP3A12 in dogs) of the capravirine oxygenation reactions observed in humans, rats, and dogs in vivo and in vitro suggest that capravirine can be a useful CYP3A substrate for probing catalytic mechanisms and kinetics of CYP3A enzymes in humans and animal species.

Capravirine represents a novel non-nucleoside reverse transcriptase inhibitor that had been developed for the treatment of human immunodeficiency virus type 1 (Fujiwara et al., 1998, 1999; Ohkawa et al., 1998; Ren et al., 2000; Bu et al., 2004, 2005, 2006, 2007). In humans, capravirine is predominantly metabolized to a variety of mono-, di-, tri-, and tetraoxygenated metabolites via individual or combined oxygenation reactions of N-oxidation, sulfonation, sulfone formation, and hydroxylation (Bu et al., 2004).

Because several possible oxygenation reactions may be involved in the formation and/or sequential metabolism of a single metabolite, it is not possible to determine definitive pathways and their relative contributions to the overall metabolism of capravirine using conventional approaches. For this reason, a human liver microsome-based sequential incubation method has been developed to deconvolute the complicated sequential metabolism of capravirine in humans (Bu et al., 2005). Briefly, this method includes three fundamental steps: 1) a primary incubation (for a time of $t_1$) of [14C]capravirine in human liver microsomes, 2) isolation of [14C]-metabolites from the primary incubation, and 3) a sequential incubation (for another time of $t_2$) of each isolated [14C]-metabolite that is supplemented with an ongoing human liver microsomal incubation (same content as the primary incubation except the use of nonlabeled capravirine). A critical idea of this sequential incubation method is that the overall process keeps the amounts of [14C]-metabolites relevant, and mass balance is maintained, which allows us to assign definitive pathways and estimate their relative contributions based on the extent of both the disappearance of the isolated precursor [14C]-metabolites and the formation of sequential [14C]-metabolites. An advantage of this system is that the sequential incubation of each isolated [14C]-metabolite is conducted under conditions mimicking the “ongoing” incubation of the supplemental mixture, and the sequential metabolism of the [14C]-metabolite is monitored selectively by radioactivity in the presence of all relevant nonlabeled metabolic components. The sequential incubation method should be applicable to mechanistic studies of other compounds involving complicated sequential metabolism when radiolabeled materials are available. We were able to develop the new method largely due to the biotransformation features of capravirine that allow the study of metabolic diversity (i.e., hydroxylation, sulfonation, sulfone formation, and N-oxidation) as well as multiplicity (i.e., mono-, di-, tri-, and tetraoxygenations).

In a further study, P450 enzymes responsible for the primary and sequential oxygenation reactions of capravirine in human liver microsomes have been identified at the specific pathway level (Bu et al., 2006). The total oxygenation of capravirine is mediated predominantly (>90%) by CYP3A4 and marginally (<10%) by CYP2C8, 2C9, and 2C19. However, all sequential oxygenation reactions are mediated exclusively by CYP3A4.

Based on the aforementioned diversity, multiplicity, and high en-

ABBREVIATIONS: P450, cytochrome P450; LC, liquid chromatography; RAM, radioactivity monitoring; MS, mass spectrometry; BDC, bile duct-cannulated; MS", multistage ion-trap mass spectrometry; amu, atomic mass unit(s).
zymatic specificity of the capravirine oxygenation reactions observed in humans, capravirine appears to be a useful CYP3A4 probe substrate. To further assess the applicability of capravirine as a suitable P450 probe substrate, it is necessary to characterize the metabolism of capravirine in preclinical species. Therefore, the purpose of the current study was to investigate both the in vitro and in vivo metabolism of capravirine in rats and dogs. Qualitative and quantitative metabolic profiles of capravirine in the two species in vitro and in vivo were obtained using liquid chromatography coupled with radioactivity monitoring and mass spectrometry (LC-RAM-MS) and are compared with those in humans.

Materials and Methods

Materials. For animal studies, capravirine was synthesized by Sumika Fine Chemicals (Chou-ku, Osaka, Japan) and [14C]capravirine (>99% radiochemical purity) was synthesized by the Developmental Research Laboratories of Shionogi and Co. (Toyonaka, Osaka, Japan). For liver microsomal incubations, rat (St. Louis, MO). Ritonavir was extracted from commercially available capsules (Norvir). Human, rat, and dog liver microsomes were prepared at Pfizer (Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog CYP3A12 (all with reductase and cytochrome (Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog liver microsomes were prepared at Pfizer (Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog CYP3A12 (all with reductase and cytochrome P450s, Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog CYP3A12 (all with reductase and cytochrome P450s, Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog CYP3A12 (all with reductase and cytochrome P450s, Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog CYP3A12 (all with reductase and cytochrome P450s, Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog CYP3A12 (all with reductase and cytochrome P450s, Groton, CT). Recombinant rat CYP3A1 and CYP3A2, recombinant dog CYP3A12 (all with reductase and cytochrome P450s, Groton, CT).

Animal Studies. All procedures performed on animals were in accordance with established guidelines and were reviewed and approved by an independent institutional review board. The jugular vein-cannulated male Sprague-Dawley rats were conditioned for 5 days and the bile duct-cannulated (BDC) rats were held in conditioning for 24 h before treatment. [14C]Capravirine was administered as a single oral dose of 200 mg/kg (150 μCi/kg) in 0.5% methylcellulose. Blood was collected for metabolic profiling and structure determination institutional review board. The jugular vein-cannulated male Sprague-Dawley rats were conditioned for 5 days and the bile duct-cannulated (BDC) rats were held in conditioning for 24 h before treatment. [14C]Capravirine was administered as a single oral dose of 200 mg/kg (150 μCi/kg) in 0.5% methylcellulose. Blood was collected for metabolic profiling and structure determination. For control incubations, blank rabbit sera (0.2 mg/ml) were first preincubated for 5 min. Reactions were started by the addition of NADPH and terminated by the addition of 2 ml of ice-cold acetonitrile. Samples were vortexed and then centrifuged for 5 min. The supernatant from each incubation tube was transferred into an appropriate polypropylene tube for evaporation to dryness under N2 at 40°C. The dried residues were reconstituted in 110 μl of 30:70 (v/v) methanol-20 mM ammonium acetate (pH 4.0), and an aliquot (100 μl) of each reconstituted solution was injected for LC-RAM-MS analysis.

Ritonavir Inhibition. [14C]Capravirine (2 μM) was coincubated with ritonavir at concentrations ranging from 0 to 2 μM in rat or dog liver microsomes for 10 min at 37°C. All other incubation conditions and sample preparation procedures were the same as those described above under Microsomal Metabolism. The inhibitor concentrations were chosen on the basis of their ability to inhibit CYP3A4 with relative specificity (von Moltke et al., 1998).

Recombinant P450 Metabolism. [14C]Capravirine (2 μM) was incubated with each of the three P450 Supersomes (rat CYP3A1 and CYP3A2 and dog CYP3A12) at 20 nM for 0 or 20 min at 37°C. All other incubation conditions and sample preparation procedures were the same as those described above under Microsomal Metabolism.

Antibody Inhibition. The anti-rat CYP3A2 antisera (0.2 mg/ml) were first incubated with rat liver microsomes (0.2 mg/ml) or anti-dog CYP3A12 antisera (0.2 mg/ml) with dog liver microsomes (0.2 mg/ml) for 30 min at room temperature. For control incubations, blank rabbit sera (0.2 mg/ml) were first preincubated with rat or dog liver microsomes (0.2 mg/ml) for 30 min at room temperature. [14C]Capravirine (2 μM) was then added, and the mixture was preincubated for 5 min at 37°C. Reactions were started by the addition of NADPH and continued for 10 min at 37°C. All other incubation conditions and sample preparation procedures were the same as those described above under Microsomal Metabolism.

LC-RAM-MS. Methods used for metabolite profiling and identification in the present study were the same as those reported in a previous study (Bu et al., 2004). Briefly, an Agilent 1100 high-performance liquid chromatography system (Agilent Technologies, Wilmington, DE) was coupled with a model 3 β-RAM radiodetector (IN/US Systems, Inc. Tampa, FL) and a Finnigan LCQ-Deca XP ion-trap mass spectrometer (Thermo Electron Corporation, San Jose, CA). Separation was performed through an Aqua C18 column (150 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA) with a gradient mobile phase at a flow rate of 1.0 ml/min. Ion-trap scans (MS+, n = 1–4) were conducted under electrospray ionization conditions. Laura (version 3.0; IN/US Systems, Inc.) and Xcalibur V1.2 (Thermo Electron Corporation) software systems were used to control the radiodetector and the LC-MS system, respectively, for data acquisition and processing.

Results

Excretion. In BDC rats after oral administration of [14C]capravirine, radioactivity was eliminated predominantly by biliary excretion and secondarily by urinary and fecal excretion (Table 1). The low
fetal recovery of radioactivity (<10%) suggested that capravirine was well absorbed in rats. In BDC dogs after oral administration of [14C]capravirine, approximately half of the radioactivity underwent biliary excretion (Table 1). The combined recovery of urinary and biliary radioactivity suggested that capravirine was fairly well absorbed (approximately 60% of the dose) in dogs. After oral administration of [14C]capravirine, approximately half of the radioactivity underwent biliary radioactivity suggested that capravirine was fairly well absorbed in rats. In BDC dogs after oral administration of [14C]capravirine, approximately half of the radioactivity underwent biliary excretion (Table 1). The combined recovery of urinary and biliary radioactivity suggested that capravirine was fairly well absorbed (approximately 60% of the dose) in dogs. After oral administration of [14C]capravirine, approximately half of the radioactivity underwent biliary excretion (Table 1). The combined recovery of urinary and biliary radioactivity suggested that capravirine was fairly well absorbed (approximately 60% of the dose) in dogs.

In Vivo Metabolism. In rat plasma, three metabolites (C17, C23, and C24) were radiochemically quantified (Fig. 1A); unchanged drug and several trace metabolites were observed by LC-MS only (data not shown). In the urine of BDC rats, five metabolites (C4, C11, C12, C15, and C22) were radiochemically quantified, with C4 and C15 being the two most abundant urinary metabolites (Table 2). In the bile of rats, 10 metabolites (C3, C4, C8, C10, C11, C12, C15, C22, C25, and C26) were radiochemically quantified, with C11 and C15 being the two most significant biliary metabolites (Table 2). As with plasma, unchanged parent and a number of trace metabolites in either urine or bile were observed by LC-MS only (Table 2). In the feces of BDC rats, unchanged capravirine and two coeluting metabolites (C19 and C20) were radiochemically quantifiable, and a variety of other trace fecal metabolites were observed by LC-MS only (Table 2).

In dog plasma, unchanged parent represented the most abundant metabolic component, and six metabolites were radiochemically quantifiable, including C9, C9a, C23, C25a, C26, and C27 (Fig. 1B). In addition, a number of trace circulating metabolites were observed by LC-MS only (data not shown). In the urine of dogs, eight metabolites (C3, C4, C9, C11, C15, C22, C23, and C26) were radiochemically quantified, with C9 being the most abundant urinary metabolite (Table 2). In the bile of dogs, seven metabolites (C3, C4, C9, C11, C15, C22, and C26) were radiochemically quantified, with C9 and C11 being the two most significant biliary metabolites (Table 2). In the feces of dogs, five metabolites (C3, C5, C9, C18, and C23) were radiochemically quantified, with C3 and C9 being the two major metabolites (Table 2). In all dog excreta, unchanged capravirine and a variety of trace metabolites were observed by LC-MS only (Table 2).

**Metabolite Identification.** A total of 22 capravirine metabolites were observed in rats and dogs (Fig. 1; Table 2), of which 14 metabolites (C3, C4, C9, C10, C11, C12, C15, C18, C19, C20, C22, C23, C25b, and C26) had been identified in previous studies in humans (Bu et al., 2004, 2005). In the preclinical study, structure elucidation was performed only for the 8 new capravirine metabolites (C5, C8, C9a, C17, C24, C25, C25a, and C27).

The regiochemistry for sulfoxidation, sulfone formation, N-oxidation, and hydroxylation of capravirine was generally determined on the basis of the characteristic mass spectrometric fragmentation patterns of each metabolite (Bu et al., 2004). Under LC-MSn (n = 2–4) conditions, neutral losses of 48 amu (SO) and 64 amu (SO2) were suggestive of sulfonation and sulfone formation, respectively. The presence of the product ion at m/z 169 under LC-MS2 scanning was indicative of N-oxidation. The primary hydroxylation was distinguished from the tertiary hydroxylation at the isopropyl group of capravirine in terms of relative abundance in the loss of H2O under LC-MSn (n = 2–4) conditions, with the former losing H2O as a minor

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**TABLE 1**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Excretion (0–168 h)</th>
<th>Total Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urinary</td>
<td>Biliary</td>
</tr>
<tr>
<td>Rats (BDC, n = 4)</td>
<td>10.9</td>
<td>67.4</td>
</tr>
<tr>
<td>Dogs (BDC, n = 2a)</td>
<td>15.5</td>
<td>45.4</td>
</tr>
<tr>
<td>Dogs (intact, n = 4)</td>
<td>18.6</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., not applicable.

*a* For this group, all samples were collected through 48 h postdosing.

**TABLE 2**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Rat (BDC)</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Bile</td>
</tr>
<tr>
<td></td>
<td>(0–24 h)</td>
<td>(12–48 h)</td>
</tr>
<tr>
<td>CPV</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C3</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C4</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C5</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>C8</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C9</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C10</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C11</td>
<td>1.2</td>
<td>6.6</td>
</tr>
<tr>
<td>C12</td>
<td>0.40</td>
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<tr>
<td>C15</td>
<td>1.9</td>
<td>9.4</td>
</tr>
<tr>
<td>C18</td>
<td>✓</td>
<td>X</td>
</tr>
<tr>
<td>C19</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C20</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C22</td>
<td>0.25</td>
<td>4.6</td>
</tr>
<tr>
<td>C23</td>
<td>✓</td>
<td>X</td>
</tr>
<tr>
<td>C25</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>C25b</td>
<td>✓</td>
<td>X</td>
</tr>
<tr>
<td>C26</td>
<td>3.4</td>
<td>0.73</td>
</tr>
</tbody>
</table>

CPV, capravirine; X, not detectable by either LC-MS or LC-RAM; ✓, detectable by LC-MS only.

*a* C19 and C20 coeluted.
fragmentation pathway and the latter losing H₂O as a predominant fragmentation pathway.

Metabolite C5 had a protonated molecular ion [M + H]⁺ at m/z 499 (451 + 16 + 30 CH₃ → COOH), consistent with a mono-oxygenated and carboxylated product of capravirine (Table 3). The characteristic neutral loss of 48 amu (m/z 456→408 and 438→390) was indicative of a sulfoxide. The formation of the fragment ion at m/z 169 supported an N-oxide. The loss of H₂O as a minor fragmentation pathway together with the presence of the fragment ion at m/z 408 suggested hydroxylation at a primary carbon atom of the isopropyl group of capravirine.

Metabolite C8 showed [M + H]⁺ at m/z 497 (451 + 16 + 30 CH₃ → COOH), consistent with a mono-oxygenated and carboxylated product of capravirine (Table 3; Fig. 2). The absence of both the fragment ion at m/z 169 and the neutral loss of 48 amu suggested that the mono-oxygenation was at the isopropyl group of capravirine. Because the loss of H₂O was minor (Fig. 2), the hydroxylation was most likely at a primary carbon atom of the isopropyl group. Thus, the carboxylation was proposed to be at the primary carbon atom of the isopropyl group (Fig. 2).

Metabolite C9a (present exclusively in dog plasma) exhibited [M + H]⁺ at m/z 440 (451 + 2 × 16 - 43), consistent with a dioxygenated and decarbamated product of capravirine (Table 3). However, an insufficient sample quantity made further identification of C9a not possible. Metabolite C17 showed [M + H]⁺ at m/z 408 (451 + 3 × 16 - 91), consistent with a trioxigenated and decarbamated product of capravirine (Table 3). The characteristic neutral loss of 64 amu (m/z 329→265) was suggestive of a sulfone, and the predominant loss of H₂O (m/z 408→390 and 347→329) suggested that hydroxylation was at the tertiary carbon atom of the isopropyl group of capravirine. Metabolite C24 had [M + H]⁺ at m/z 392 (451 + 2 × 16 - 91), consistent with a dioxygenated and decarbamated product of capravirine (Table 3). The characteristic neutral loss of 64 amu (m/z 331→267) was indicative of a sulfone. The structure of C24 was confirmed by chromatographic and mass spectral comparisons to its authentic standard (data not shown).

Metabolite C25 showed [M + H]⁺ at m/z 481 (451 + 30 CH₃ → COOH), consistent with a carboxylated product of capravirine (Table 3). Metabolite C25a exhibited [M + H]⁺ at m/z 424 (451 + 16 - 43), consistent with a mono-oxygenated and decarbamated metabolite of capravirine (Table 3). The absence of both the fragment ion at m/z 169 and the neutral loss of 48 amu (Table 3) suggested that the mono-oxygenation was at the isopropyl group of capravirine. Based on relative abundance in the loss of H₂O (data not shown), a primary hydroxylation at the isopropyl group was proposed. Metabolite C27 had [M + H]⁺ at m/z 408 (451 - 43), consistent with a decarbamated product of capravirine (Table 3). The structure of C27 was confirmed by chromatographic and mass spectral comparisons to its authentic standard (data not shown).

On the basis of the preclinical metabolite profiling and structure elucidation, a combined metabolic scheme of capravirine in rats and dogs can be proposed (Fig. 3).

**Microsomal Metabolism.** Capravirine underwent extensive oxygenation reactions to a variety of primary and sequential metabolites in either rat (Fig. 4) or dog (Fig. 5) liver microsomes. Note that the radiochemically quantifiable in vitro metabolites (C3, C4, C9, C11, C15, C18, C19, C20, C22, C23, C25b, and C26) were all observed in the aforementioned in vivo studies. In addition, all of the in vitro metabolites observed in the preclinical species were observed in human liver microsomal incubations (Bu et al., 2005).

In rat liver microsomal incubations, the disappearance of capravirine and the formation of all metabolites showed linear kinetics only up to 5 min (Fig. 6), and were in contrast to those observed in the incubation of capravirine in human liver microsomes in which all reactions were linear over the entire incubation period of 60 min (Bu et al., 2005). After the initial 5-min incubation in rat liver microsomes, abundances of some metabolites (C9, C18, C19, C20, C23, C25b, and C26) increased to a maximum and then declined, whereas abundances of other metabolites (C3, C4, C11, C15, and C22) continued to increase over the entire incubation time of 60 min (Fig. 6). As a strategy, this kind of sequential metabolism-time profiles under the nonlinear conditions helped us to assign oxygenation pathways and their relative contributions to the overall metabolism of capravirine in rat liver microsomes. Metabolites C23 (the sulfoxide) and C25b (the sulfone) represented the two most significant metabolites within the
linear range (the first 5 min), whereas C15 (the N-oxide of C23) and C22 (the N-oxide of C25b) became the two most abundant metabolites after 40 min. Such characteristic sequential metabolism-time profiles of the four major metabolites clearly suggested that C23 was primarily metabolized to C15 and C25b, followed by the sequential oxygenation of C25b predominantly to C22 (Fig. 6). After 10 min of incubation, the consumption of C23 (by sequential metabolism) exceeded its formation from capravirine (<20% parent remaining at 10 min). This led to the decrease in the abundance of C23 with time, which sequentially led to the observed decrease in the abundance of C25b. Cumulative profiles of C15 and C22 indicated that the two N-oxidation products of C23 and C25b did not undergo significant sequential metabolism, suggesting an inactivation effect of the pyridinyl N-oxidation on the sequential metabolism of capravirine metabolites in rat liver microsomes. This effect is consistent with that observed in human liver microsomes (Bu et al., 2005). The same inactivation effect appeared to be true for the formation-time profiles of metabolites C4 and C11 (Fig. 6). In contrast to C23 (the sulfoxide), C26 (the N-oxide, a minor metabolite of capravirine in rat liver microsomes) might provide a secondary contribution to the formation of C15. In addition, both structural features and sequential metabolism-time profiles of C9 and C18 suggested that C9 was sequentially metabolized to C18, which, in turn, was further metabolized to C11 (Fig. 6). Finally, abundances of the two coeluted metabolites C19 and C20 were too low to provide a significant effect on any of their possible sequential metabolites. On the basis of all results described above, sequential oxygenation pathways of capravirine in rat liver microsomes under linear conditions (within the first 5 min of incubation) were proposed (Fig. 7). The metabolic scheme of capravirine in rat liver microsomes is qualitatively similar to that of capravirine in human liver microsomes (Bu et al., 2005).

In dog liver microsomal incubations, the disappearance of capravirine and the formation of some metabolites (C9, C15, C19, C20, C23, C25b, and C26) exhibited linear kinetics up to 10 min of
incubation (Fig. 8). The formation of other metabolites (C3, C4, C11, C18, and C22) became radiochemically quantifiable only after 10 min and showed linear kinetics from 10 to 60 min of incubation (Fig. 8). At 10 min, C23 (the sulfoxide) accounted for more than 60% of total radioactivity and the capravirine remaining was less than 20%, suggesting that the metabolism after 10 min represented mainly the sequential oxygenation reactions of the sulfoxide (Fig. 8). Taking the similar data interpretation strategies used for the aforementioned rat liver microsomal incubations, we propose sequential oxygenation pathways of capravirine in dog liver microsomes under linear conditions (within the first 10 min of incubation) (Fig. 9).

**Ritonavir Inhibition.** The metabolism of capravirine in human liver microsomes is inhibited by ritonavir in a typical concentration-dependent manner (Bu et al., 2006). Similarly, the effect of ritonavir on the metabolism of capravirine in rat and dog liver microsomes was investigated in the present study. At different ritonavir concentrations (0–2 μM), the percentage inhibition of either the overall metabolism of capravirine or the formation of each metabolite by ritonavir can be estimated using the following equation: percentage inhibition = 100 × (A0 − A1)/A0, where A0 represents the amount percentage of total radioactivity) of a metabolite or the amount (100% – percentage

![Fig. 4. A representative radiochromatogram generated from an incubation of [14C]capravirine (CPV) (2 μM) in rat liver microsomes (0.2 mg/ml) for 10 min, recombinant rat CYP3A1 (20 nM) for 20 min, or recombinant rat CYP3A2 (20 nM) for 20 min.](image1)

![Fig. 5. A representative radiochromatogram generated from an incubation of [14C]capravirine (CPV) (2 μM) in dog liver microsomes (0.2 mg/ml) for 10 min or recombinant dog CYP3A12 (20 nM) for 20 min.)](image2)

![Fig. 6. Sequential metabolism-time courses of [14C]capravirine (CPV) (2 μM) in rat liver microsomes (0.2 mg/ml).](image3)
of total radioactivity) of capravirine metabolized in the absence of ritonavir and $A_{HI}$ is the amount of the metabolite or the amount of capravirine metabolized in the presence of ritonavir at a concentration of [I]. Ritonavir is a specific and potent inhibitor of CYP3A4 in humans (von Moltke et al., 1998), CYP3A1/2 in rats (Debri et al., 1995; Solon et al., 2002; Kageyama et al., 2005), and CYP3A12 in dogs (Fraser et al., 1997; Lu et al., 2005). In addition, ritonavir at 2 μM can achieve the maximal inhibition of CYP3A4 activity in human liver microsomes (Bu et al., 2006).

In rat liver microsomes, the metabolism of capravirine was inhibited by ritonavir in a concentration-dependent manner (Fig. 10A). Because the formation of most (C3, C4, C9, C11, C15, C18, C23, and C25b) of the metabolites in the rat liver microsomal incubation was completely inhibited by ritonavir at 2 μM (Fig. 10A), ritonavir at 2 μM also appeared to achieve the maximal inhibition of CYP3A1/2 activity in rat liver microsomes. Under the maximal inhibition conditions, the 90% inhibition of the overall metabolism of capravirine (Fig. 10A) suggested that CYP3A1 and CYP3A2 were predominantly responsible for the metabolism of capravirine in rat liver microsomes. Specifically, the formation of C3, C4, C9, C11, C15, C18, C23, and C25b appeared to be mediated exclusively, C22 primarily (~90%), and C19/C20 and C26 moderately (~40% for C19/C20 and ~60% for C26), by CYP3A1/2 (Fig. 10A). The partial mediation of the formation of C19/C20 and C26 by CYP3A1/2 and CYP3A12 in rat liver microsomes is consistent with that observed in human liver microsomes (Bu et al., 2006). Because of the minor contribution of both C19/C20 and C26 to the overall metabolic profile of capravirine in rat liver microsomes (Fig. 6), no further attempts were made to identify other enzymes responsible for the formation of the three minor metabolites.

In dog liver microsomes, the metabolism of capravirine was also inhibited by ritonavir in a concentration-dependent manner (Fig. 10B). The overall metabolism of capravirine and the formation of all metabolites (except C19/C20) observed in the dog liver microsomal incubation appeared to be completely inhibited by ritonavir at 2 μM (Fig. 10B), suggesting that all oxygenation reactions of capravirine were mediated almost exclusively by CYP3A12 in dog liver microsomes.

**Recombinant P450 Metabolism.** To further confirm the enzymes primarily responsible for the metabolism of capravirine in rat and dog liver microsomes, incubations of capravirine were performed using...
commercially available recombinant rat CYP3A1 and CYP3A2 (Fig. 4) and recombinant dog CYP3A12 (Fig. 5). All capravirine metabolites formed in the rat liver microsomal incubation were observed in the incubations of either recombinant CYP3A1 or CYP3A2 (Fig. 4). However, the much higher similarity in relative abundances of the metabolites between the microsomal and recombinant CYP3A2 incubations compared with the observed microsomal and recombinant CYP3A1 incubations suggested that CYP3A2 might play a more important role in the metabolism of capravirine in rat liver microsomes. In addition, the high similarity in metabolic profiles between the dog liver microsomal and CYP3A12 incubations (Fig. 5) suggested that CYP3A12 indeed plays a dominant role in the metabolism of capravirine in dog liver microsomes.

**Antibody Inhibition.** The enzymatic specificity of capravirine metabolism in rat and dog liver microsomes was also assessed by specific antibody inhibition. In the presence of the anti-rat CYP3A2 antisera (0.2 mg/ml) in the rat liver microsomal incubation, the total metabolism of capravirine was inhibited at 89%, and the inhibition levels of the metabolite formations were similar to those observed in the rat liver microsomal incubations of capravirine in the presence of 0.5 μM ritonavir (Fig. 10A). Likewise, in the presence of the anti-dog CYP3A12 antisera (0.2 mg/ml) in the dog liver microsomal incubation, the inhibition of the total metabolism of capravirine was achieved at 93%, and the inhibition levels of the metabolite formations were similar to those observed in the dog liver microsomal incubations of capravirine in the presence of 1.0 μM ritonavir (Fig. 10B).

**Discussion**

Mass balance was achieved in both rats and dogs, with mean total recovery of radioactivity > 86% for each species. Capravirine was well absorbed in rats, consistent with its absorption in humans (Bu et al., 2004), whereas lower absorption of capravirine was observed in dogs. Because only trace levels of unchanged capravirine were detected in dog feces, capravirine most likely underwent significant gastrointestinal metabolism by gut microflorae and/or enterocytes (Wacher et al., 1996; Andrieux et al., 2002, Doherty and Charman, 2002) in dogs. In addition, the overall low (in rats) or trace (in dogs) levels of unchanged capravirine as well as the large number of metabolites observed in rats and dogs indicated that capravirine was

![Diagram of proposed sequential oxygenation pathways of capravirine](image-url)
eliminated predominantly by metabolism in both species, consistent with capravirine metabolism observed in humans (Bu et al., 2004).

Three major circulating metabolites (C19, C20, and C26) of capravirine in humans (Bu et al., 2004) were all observed in rat and dog plasma, suggesting that both preclinical species were suitable for toxicity assessment of capravirine. Two depicolylated metabolites (C17 and C24) were the major plasma components in rats, and three decarbamated metabolites (C9a, C25a, and C27) represented the major plasma components in dogs. However, none of these five metabolites was observed in humans, indicating significant species differences in terms of identities and relative abundances of circulating capravirine metabolites.

In general, N-oxides and sulfoxides may undergo significant gastrointestinal degradation or reduction to their parents or precursor metabolites by gut microflora (Andrieux et al., 2002, Doherty and Charman, 2002), meaning that these oxidative metabolites may not be observed in feces at significant levels. On the basis of the identities of the metabolites radiochemically quantified in fecal samples, this appeared to be true for capravirine metabolites involving N-oxidation in humans (Bu et al., 2004), rats, and dogs (Table 2). However, this appeared only to be true for capravirine metabolites involving sulfoxidation in rats (Table 2) but not in dogs (Table 2) and humans (Bu et al., 2004), suggesting a species dependence of the gut degradation of the capravirine sulfoxides.

Oxidation reactions (mono-, di-, tri-, and tetra-) represent the major metabolic pathways of capravirine in rats and dogs, with depicolylolation and carboxylation as the minor pathways in rats and decarbamamation as the minor reaction in dogs. On the basis of all capravirine metabolites identified in the present study, oxidation sites are selective to sulfoxidation/sulfone formation, pyridinyl N-oxidation, and hydroxylation at the isopropyl group of capravirine (Fig. 3), which is consistent with the oxygenation reactions of capravirine in humans (Bu et al., 2004). In contrast with the metabolism of capravirine in humans in whom several glucuronides and a sulfate were observed (Bu et al., 2004), none of such conjugation products was found in the preclinical species.

Because the majority of the in vivo oxygenated metabolites were also observed in liver microsomal incubations, the in vitro models provided very useful information for understanding the in vivo oxygenation pathways. The in vitro study of capravirine in rat liver microsomes suggested that the oxygenated metabolites without N-oxidation (i.e., C9, C18, C23, and C25b) underwent extensive sequential oxygenation reactions, whereas the oxygenated metabolites with N-oxidation (i.e., C4, C11, C15, C22, and C26) underwent little or no sequential oxygenation reactions (Figs. 6 and 7). The in vitro finding was in agreement with the rat in vivo finding for which all oxygenated metabolites with N-oxidation (i.e., C4, C10, C11, C12, C15, C22, and C26) represented the major/minor metabolites (not undergoing significant sequential metabolism) in bile, whereas all oxygenated metabolites without N-oxidation (i.e., C9, C18, C23, and C25b) were presented in small amounts or not detected (undergoing significant sequential metabolism) in bile (Table 2; Fig. 3). Likewise, the same conclusion can be drawn for the in vitro (Figs. 8 and 9) and in vivo (Table 2; Fig. 3) studies of capravirine in dogs. Note that an exception was present for C9 in dogs. In the dog liver microsomal incubation, C9 did not undergo significant sequential oxygenation (Fig. 8), which was in contrast to that observed in rats and humans. However, the metabolic profile of C9 was consistent between the in vitro and in vivo studies of capravirine in dogs (Fig. 8 versus Table 2).

In conclusion, the diversity (i.e., hydroxylation, sulfoxidation, sulfone formation, and N-oxidation), multiplicity (i.e., mono-, di-, tri-, and tetra-oxygenations), and high enzymatic specificity (>90% contribution for CYP3A4 in humans, CYP3A1/2 in rats, and CYP3A12 in dogs) of the capravirine oxygenation reactions observed in humans, rats, and dogs both in vivo and in vitro suggested that capravirine can be a useful CYP3A substrate for probing catalytic mechanisms and kinetics of CYP3A enzymes in humans and animal species.

References


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