Pharmacokinetics, Pharmacodynamics, Metabolism, Distribution, and Excretion of Carfilzomib in Rats

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ABSTRACT:

Carfilzomib [(2S)-N-[(S)-1-[(4S)-4-[(2R)-1-oxopentan-2-ylcarbamoyl]-2-phenylethyl]-(3-methoxy-2-[(2-methyl-1,3-thiazol-5-yl)formamido]-4-methylpentanamido)-4-methylpentanamido]-4-phenylbutanamido]-4-methylpentanamido, also known as PR-171] is a selective, irreversible proteasome inhibitor that has shown encouraging results in clinical trials in multiple myeloma. In this study, the pharmacokinetics, pharmacodynamics, metabolism, distribution, and excretion of carfilzomib in Sprague-Dawley rats were characterized. After intravenous administration, the plasma concentration of carfilzomib declined rapidly in a biphasic manner. Carfilzomib displayed high plasma clearance [195–319 ml/(min·kg)], a short-terminal half-life (5–20 min), and rapid and wide tissue distribution in rats. The exposure to carfilzomib (C_{max} and area under the curve) increased dose proportionally from 2 to 4 mg/kg but less than dose proportionally from 4 to 8 mg/kg. The high clearance was mediated predominantly by extrahepatic metabolism through peptidase cleavage and epoxide hydrolysis. Carfilzomib was excreted mainly as metabolites resulting from peptidase cleavage. Carfilzomib and its major metabolites in urine and bile accounted for approximately 26 and 31% of the total dose, respectively, for a total of 57% within 24 h postdose. Despite the high systemic clearance, potent proteasome inhibition was observed in blood and a variety of tissues. Together with rapid and irreversible target binding, the high clearance may provide an advantage in that “unnecessary” exposure to the drug is minimized and potential drug-related side effects may be reduced.

Introduction

The proteasome is a multicatalytic enzyme complex that is the primary means for protein degradation in all cells (Kisselev and Goldberg, 2001; Ciechanover, 2005). Inhibition of the proteasome leads to the accumulation of misfolded proteins and the induction of apoptosis. The proteolytic activities of the proteasome are encoded by distinct, N-terminal threonine-containing enzymes that function as chymotrypsin-like (CT-L), trypsin-like, and caspase-like proteases. Tumor cells are more susceptible than nontransformed cells to the cytotoxic effects of proteasome inhibition, making the proteasome an attractive target for the treatment of malignant diseases (Adams, 2004). The approval of bortezomib (Velcade) by the U.S. Food and Drug Administration for the treatment of multiple myeloma (MM) and mantle cell lymphoma provided clinical validation of the proteasome as a therapeutic target in oncology (Adams et al., 1998; Bross et al., 2004; Chauhan et al., 2005; Goy et al., 2005; O’Connor et al., 2005; Richardson et al., 2005). Bortezomib is a dipeptide boronic acid that reversibly inhibits the 20S proteasome by forming a tetrahedral intermediate with the side chain hydroxyl group of the N-terminal threonine (Borissonko and Groll, 2007). Despite the encouraging clinical success of bortezomib, adverse effects including painful peripheral neuropathy have been reported, and a significant fraction of patients relapse from or are refractory to treatment with bortezomib (Orlowski et al., 2002, 2007a,b; Richardson et al., 2003, 2005; Goy et al., 2005; O’Connor et al., 2005). In addition, bortezomib is metabolized primarily by cytochrome P450 3A4 (Uttamsingh et al., 2005), and coadministration of cytochrome P450 3A4 inhibitors such as ketoconazole causes a significant change in bortezomib plasma levels in humans (Venkatkarishnan et al., 2009). In the past several years, the next generation of proteasome inhibitors has been explored, with the aim to improve safety and efficacy profiles. Three small molecules [carfilzomib, (4R,5S)-4-(2-chloroethyl)-1-((1S)-cyclohex-2-eneyl(hydroxy)methyl)-5-methyl-6-oxa-2-azabicyclo[3.2.0]heptane-3,7-dione (NPI-0052), and [1R]-1-[(2S,3R)-3-hydroxy-2-[6-phenylpyridin-2-yl]carbonyl]tetrahydro-2H-benzo[d][1,3]oxaborin-3(2H)-one] have shown encouraging results in clinical trials in multiple myeloma (Adams et al., 1998; Bross et al., 2004; Chauhan et al., 2005; Goy et al., 2005; O’Connor et al., 2005). In addition, bortezomib is metabolized primarily by cytochrome P450 3A4 (Uttamsingh et al., 2005), and coadministration of cytochrome P450 3A4 inhibitors such as ketoconazole causes a significant change in bortezomib plasma levels in humans (Venkatkarishnan et al., 2009). In the past several years, the next generation of proteasome inhibitors has been explored, with the aim to improve safety and efficacy profiles. Three small molecules [carfilzomib, (4R,5S)-4-(2-chloroethyl)-1-((1S)-cyclohex-2-eneyl(hydroxy)methyl)-5-methyl-6-oxa-2-azabicyclo[3.2.0]heptane-3,7-dione (NPI-0052), and [1R]-1-[(2S,3R)-3-hydroxy-2-[6-phenylpyridin-2-yl]carbonyl]tetrahydro-2H-benzo[d][1,3]oxaborin-3(2H)-one] have shown encouraging results in clinical trials in multiple myeloma (Adams et al., 1998; Bross et al., 2004; Chauhan et al., 2005; Goy et al., 2005; O’Connor et al., 2005). In addition, bortezomib is metabolized primarily by cytochrome P450 3A4 (Uttamsingh et al., 2005), and coadministration of cytochrome P450 3A4 inhibitors such as ketoconazole causes a significant change in bortezomib plasma levels in humans (Venkatkarishnan et al., 2009).
amino]-1-oxobutyl]amino]-3-methylbutyl]boronic acid (CEP-18770) are currently in clinical development (Bennett and Kirk, 2008). Carfilzomib (Fig. 1) is a tetrapeptide epoxyketone analog of epoxomicin and eponemycin, a pair of related natural products that were discovered initially as antitumor agents in animals and later shown to inhibit the CT-L activity of the proteasome (Hanada et al., 1992; Kim et al., 1999; Meng et al., 1999a,b; Groll et al., 2000). The epoxyketone pharmacophore forms a dual covalent morpholino adduct with the N-terminal threonine. Carfilzomib shows primary selectivity for the CT-L activity and displays little activity against the trypsin-like or caspase-like activities of the proteasome (Demo et al., 2007). Upon the basis of encouraging preclinical data, including the induction of potent proteasome inhibition in blood and tissues in rodents and greater antitumor activity compared with that of bortezomib in mouse models of cancer (Demo et al., 2007; Kuhn et al., 2007), carfilzomib entered clinical testing in MM. Carfilzomib has shown encouraging results in a broad clinical trial program in MM (Alsinas et al., 2007; Jakubowiak et al., 2010a,b; Jagannath et al., 2010; Martin et al., 2010; Siegel et al., 2010; Singhal et al., 2010; Vij et al., 2010), including in patients refractory to bortezomib. Carfilzomib is currently being developed by Onyx Pharmaceuticals, Inc. for the treatment of MM and is in phase III clinical trials.

The objective of the present study was to characterize the disposition of carfilzomib in rats, a pharmacological and toxicological species used in preclinical evaluation of the compound. We show here that, after intravenous administration to Sprague-Dawley rats, carfilzomib was cleared rapidly from systemic circulation. In vitro and in vivo studies showed that carfilzomib was metabolized extensively in blood and a variety of tissues. The major metabolites detected in rat plasma, urine, and bile were from peptidase cleavage and epoxide hydrolysis. Elimination occurred mainly via biliary and renal excretion in the form of metabolites. Despite rapid systemic clearance, carfilzomib generated potent proteasome inhibition in a variety of tissues due to irreversible inhibition of its target, demonstrating the wide tissue distribution of carfilzomib upon intravenous administration. We finally show that a 30-min infusion administration resulted in a 28-fold lower maximum plasma concentration ($C_{\text{max}}$) but with comparable levels of proteasome inhibition in blood and tissues as those of an intravenous bolus.

**Materials and Methods**

**Materials.** (2S)-N-[(S)-1-[(S)-1-[(S)-1-[(S)-1-[(S)-4-Methyl-1-[[(R)-2-methylxiran-2-yl]-1-oxopentan-2-ylcarbamoyl]-2-phenylethyl]-2-[(S)-2-(2-morpholinoacetamido)-4-phenylbutanamido]-4-methylpentanamide (carfilzomib, also known as PR-171), N-[(S)-1-[(S)-1-[(S)-4-methyl-1-[(R)-2-methylxiran-2-yl]-1-oxopentan-2-ylcarbamoyl]-2-phenylethylcarbamoyl]-3-methylbutyl]-5-(morpholinomethyl)isoxazole-3-carboxamide (PR-05491), and (2S)-3-methoxy-2-[(2S)-3-methoxy-2-[(2-methyl-1,3-thiazol-5-yl)formamido]propanamido]-N-[(2S)-1-[(2R)-2-methylxiran-2-yl]-1-oxo-3-phenylpropan-2-ylpropanamide (ONX 0912), tripeptide analogs of carfilzomib shown in Fig. 1, and authentic standards of carfilzomib metabolites (M14, M15, and M16; Fig. 2 and Table 3) were synthesized at Onyx Pharmaceuticals, Inc.
Carfilzomib was formulated in 10% (w/v) sulfobutyl ether $\beta$-cyclodextrin (Captisol; Cydex Pharmaceuticals Inc., Lenexa, KS) in 10 mM citrate (pH 3.5) at a concentration of 2 mg/mL. For intravenous bolus administration, four to five animals per dose level received a single dose of carfilzomib at 2, 4, or 8 mg/kg, respectively. Blood samples (~0.4 mL) were collected before and immediately after dosing (within 10 s) and at 1, 2, 5, 15, 30, and 60 min postdose. For intravenous infusion administration, four rats received carfilzomib via a 30-min constant infusion at 8 mg/kg. Blood samples were collected before dosing, at 15 min after the start of infusion, end of infusion, and at 2, 5, 15, 30, 60, and 120 min postdose.

To assess the effects of target binding, the PK profiles of carfilzomib from two groups of male Sprague-Dawley rats (three per group) were compared. One group was pretreated with an intravenous bolus dose of 10 mg/kg ONX 0912 (also known as PR-047), a tripeptide analog of carfilzomib with potent irreversible proteasome inhibition (Zhou et al., 2009), whereas the other group was pretreated with the formulation vehicle. Thirty minutes after pretreatment, carfilzomib was administered via an intravenous bolus at 2 mg/kg. Blood samples were taken before and immediately after the carfilzomib dose and at 1, 2, 5, 15, 30, and 60 min after the carfilzomib dose.

A PK study also was conducted for M16, the diol of carfilzomib derived from epoxide hydrolysis via an intravenous bolus administration to male Sprague-Dawley rats at 5 mg/kg. M16 was formulated at 2 mg/mL in Captisol containing 10 mM citrate (pH 3.5). Blood samples were collected before dosing and at 2, 5, 10, 15, 30, 60, and 360 min postdose.

The PK of carfilzomib also was assessed in non-naïve male cynomolgus monkeys with an average weight of 3 kg. The in-life portion of the study was conducted at SNBL USA (Everett, WA). Carfilzomib was formulated in 5% hydroxypropyl-$\beta$-cyclodextrin (w/v) in 50 mM sodium citrate (pH 3.35) at a concentration of 1 mg/mL. Three animals received a single intravenous bolus dose of carfilzomib at 1 mg/kg. Blood samples were collected before dosing and at 2, 5, 15, 30, and 60 min postdose.

All of the blood samples were collected in tubes containing sodium heparin as the anticoagulant. Blood samples were kept on ice during sample collection and centrifuged at 3000g for 10 min at 4°C to obtain plasma. Plasma samples were stored in cryotubes at $-70°C$ until analysis.

**Calculation of PK Parameters.** PK parameters were calculated by non-compartmental analysis using WinNonLin 5.2 (Pharsight, Mountain View, CA). Plasma concentrations reported as below the lower limit of quantification were treated as zero for PK analysis. Area under plasma concentration-time curve (AUC) values were estimated using the linear trapezoidal method. Plasma clearance (CL) was calculated from dose/AUC extrapolated to infinity (AUC\(_{\text{inf}}\)). Terminal half-life ($t_{1/2}$) values were calculated as $\ln(2)/k_e$, where $k_e$ represents the terminal elimination rate constant obtained from the slope of the semilogarithmic plot of the concentration-time profile. Mean residence time was estimated by moment analysis. Volume of distribution at steady state ($V_{\text{ss}}$) was calculated from mean residence time $\times$ CL. $C_{\text{max}}$ was recorded as observed. The amount of M16 generated in rats after carfilzomib dosing was calculated by multiplying the AUC\(_{\text{inf}}\) of M16 by the clearance of M16 obtained in the PK study for M16.

**Pharmacodynamic Study.** Blood and tissue (adrenal, heart, liver, and lung) samples were collected from Sprague-Dawley rats receiving 8 mg/kg carfilzomib by either an intravenous bolus or a 30-min infusion administration. Blood samples were collected at predose and 0.25, 2, and 24 h postdose. For intravenous infusion, blood samples also were taken at 0.25 h after the start of infusion. Blood samples were collected at 2 and 24 h postdose. Proteasome CT-L activity was measured in lysates from cells and blood with Leu-Leu-Val-Tyr-AMC (LLVY-AMC) as the substrate as described previously (Demo et al., 2007).

**Excretion Study.** Excretion of carfilzomib was determined in male Sprague-Dawley rats with bile duct cannulation after a single intravenous bolus administration at 2 mg/kg. Bile was collected cumulatively at 0 to 4 and 4 to 8 h postdose, no bile samples were collected at 8 to 24 h postdose, and urine was collectively 0 to 4, 4 to 8, and 8 to 24 h postdose. Volumes of the bile and urine samples were measured. Acetonitrile was added to the original urine sample containers after the first thawing to achieve a final organic
content of 20% (v/v). This was done to ensure full recovery of carfilzomib from the container. Plasma samples also were collected in this study before and immediately after dosing and at 1, 2, 5, 15, 30, 60, and 120 min postdose into tubes containing sodium heparin. The urine, bile, and plasma samples were stored at −70°C until analysis.

**Blood Plasma Partitioning.** The whole blood to plasma ratio of carfilzomib was determined in vitro. Carfilzomib was spiked into prewarmed rat blood at nominal concentrations of 0.2, 2, or 10 μM. The samples were mixed gently by inverting the tubes several times and then incubated at 37°C. At 15 min, an aliquot of 100 μl of blood sample was taken and immediately quenched into 300 μl of acetonitrile containing 0.5 μM IS. The remaining blood samples were centrifuged at 3000g for 10 min at 4°C to obtain plasma.

**Metabolic Profiling in Rat Plasma, Urine, and Bile Samples.** Rat plasma samples collected in a 6-month toxicity study to support toxicokinetic evaluation of carfilzomib were used to search for circulating metabolites. Carfilzomib was given to three groups of rats at dose levels of 1, 2, or 4 mg/kg for six-28 days cycles with intravenous bolus dosing on days 1, 2, 8, 9, 15, and 16. Plasma samples were collected on the first day of cycles 1, 3, and 6 (days 1, 57, and 142, respectively) before dose and at 5, 15, 30, and 60 min postdose. Aliquots of plasma samples were pooled across animals, dose levels, collection days, and collection time points to generate one pooled sample for metabolite profiling. A pooled predose plasma sample was used as a control. The pooled samples were extracted multiple times with three volumes of acetonitrile to recover carfilzomib and metabolites. The supernatants were combined and evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted with 0.5 ml of acetonitrile/water (1:1, v/v) and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

The urine and bile samples used in metabolite profiling were obtained from the excretion study as described in the preceding section. Aliquots of bile samples or acetonitrile-treated urine samples were pooled across animals to generate one pooled bile or urine sample per time interval. The pooled urine and bile samples were subjected directly to LC-MS/MS analyses. LC-MS/MS analyses were done in positive ionization mode using precursor ion and neutral loss scans on LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) and multiple ion reaction monitoring (MRM) on an API 4000 QTrap LC/MS/MS System (AB SCIEX, Foster City, CA) using a Luna C18(2) column (150 × 4.6 mm, 3-μm particle size; Phenomenex, Torrance, CA) and gradient elution. The mobile phases were 0.4% formic acid in water adjusted to pH 3.2 with ammonium hydroxide (A) and acetonitrile (B). The flow rate was 0.6 ml/min with solvent A held at 100% for 5 min, followed by ramping solvent B from 0 to 70% in 55 min and 70 to 100% B in 2 min. The structures of the metabolites were proposed based on the molecular ion masses and fragmentation patterns generated by product ion scans. Semi-quantification was done in MRM mode by comparing the peak area of each metabolite to that of carfilzomib.

**Metabolism Study in Rat Blood and Tissue Homogenates from Adrenal, Blood, Heart, Kidney, Liver, and Lung.** The metabolism of carfilzomib was evaluated in rat blood and tissue homogenates (adrenal, heart, kidney, liver, and lung) harvested from untreated male Sprague-Dawley rats. The isolated organs were chopped into small pieces, washed several times with phosphate-buffered saline (pH 7.4), and then weighed into 2-ml Eppendorf tubes. Dulbecco’s phosphate-buffered saline (pH 7.4) then was added to the tubes to achieve a 1.5 ratio of tissue weight to Dulbecco’s phosphate-buffered saline volume. The tissues were homogenized using a TissueLyser (Retsch, Inc., Newtown, PA) at a frequency of 20 s⁻¹. Aliquots of 300 μl of rat blood or tissue homogenates were preincubated at 37°C for 5 min before the addition of 5 μl of carfilzomib working solution at a concentration of 60 μg/ml in acetonitrile. The final concentration of carfilzomib in the incubation was 1 μg/ml. The mixture was incubated at 37°C with gentle agitation. An aliquot of 40 μl of reaction mixture was taken at 2, 10, 30, 60, and 90 min and quenched with 120 μl of acetonitrile containing 0.5 μM IS. After centrifugation, the supernatants were used to measure the disappearance of carfilzomib and the formation of selected metabolites. M14, M15, and M16. The relative rates of carfilzomib metabolism in rat adrenal and liver were evaluated in a separate experiment under the same experimental conditions described above.

**Quantitative Determination of Carfilzomib and Its Metabolites in Blood, Plasma, Urine, and Bile.** Concentrations of carfilzomib and its metabolites (M14, M15, and M16) in blood, plasma, urine, and bile samples were determined by LC-MS/MS. Plasma, urine, and bile samples were stored at −70°C immediately after collection. Stability testing demonstrated that the analytes were stable under the storage condition, during the extraction process, and for at least three freeze/thaw cycles. Carfilzomib and its metabolites were quantified by comparing the peak area ratios of the analyte to IS in study samples to those of the standard calibration samples prepared in the same manner. To determine the concentration of carfilzomib in blood samples from an in vitro partitioning study, the standard calibration samples were prepared by spiking carfilzomib into blood prequenched with three volumes of acetonitrile containing 0.5 μM IS. An aliquot (40 μl) of plasma, acetonitrile-treated urine, or bile sample was mixed with 80 μl of acetonitrile containing 0.5 μM IS to extract carfilzomib and its metabolites. The extracted samples were vortexed and centrifuged. The supernatants were filtered through a 96-well filter plate (AcroPrep 96-well Filter Plate, 0.45 μm; Pall Corporation, East Hills, NY) into a 96-well microplate, and 10 μl was injected for LC-MS/MS analysis.

The LC-MS/MS system comprised either an API 3000 or an API 3200 mass spectrometer (AB SCIEX) equipped with two Shimadzu LC-10ADvp pumps and a Shimadzu SIL-HTc autosampler with controller system (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved on an ACE C18 column (2.1 × 50 mm, 3.5-μm particle size; Advanced Chromatography Technology, Aberdeen, Scotland) using gradient elution. The mobile phases were 0.1% formic acid in water (A) and methanol (B). Mass spectrometric detection was accomplished using the turbo ion spray interface in the positive ionization mode by MRM of the selective m/e transitions: 720.5 → 100 for carfilzomib, 307.3 → 100 for M14, 420.4 → 100 for M15, 738.5 → 100 for M16, and 626.3 → 199.0 for IS. The lower limit of quantification was 2.00 ng/ml with calibration ranges of 2.00 to 3000 ng/ml for blood and plasma samples and 2.00 to 1000 ng/ml for urine and bile samples.

**Results**

**Pharmacokinetics.** After a single intravenous bolus dose of 2.4, or 8 mg/kg, carfilzomib plasma concentration-time curves display a very steep initial phase (Fig. 3A). The first plasma sampling was done 5 min, an aliquot of 100 μl of sample was taken and then incubated at 37°C. At 15 min, an aliquot of 100 μl of blood sample was taken and immediately quenched into 300 μl of acetonitrile containing 0.5 μM IS. The remaining blood samples were centrifuged at 3000g for 10 min at 4°C to obtain plasma.

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<thead>
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<th>Metabolite Code</th>
<th>Proposed Structure</th>
<th>Molecular Mass</th>
<th>Retention Time</th>
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TABLE 3
Summary of carfilzomib metabolites identified in rat plasma, urine, and bile samples
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within 10 s after dosing to accurately measure the PK of carfilzomib in rats; plasma levels at 2 min were <5% of those immediately after dosing (Fig. 3A, inset). The $t_{1/2}$ value ranged from 5 to 20 min, and CL values were 195 to 296 ml/(min·kg) (Table 1). Both AUC and $C_{\text{max}}$ increased dose proportionally, and the CL values were constant from 2 to 4 mg/kg. When the dose level was increased to 8 mg/kg, however, AUC and $C_{\text{max}}$ increased less than dose proportionally, and the CL value increased. Rapid clearance and short half-life also were observed in cynomolgus monkeys after intravenous administration at 1 mg/kg (Supplemental Fig. S1).

Carfilzomib is a rapidly binding and irreversible inhibitor of the proteasome, a ubiquitously expressed target that accounts for approximately 1% of total cellular protein (Ciechanover 2005; Demo et al., 2007). To determine whether the high level of target expression affects its PK, carfilzomib was administered to two groups of rats: one pretreated with an intravenous bolus dose of the vehicle (control group) and the other pretreated with an intravenous bolus dose of ONX 0912 (formerly known as PR-047), a tripeptide analog of carfilzomib that displays potent and irreversible inhibition of the 20S proteasome (Zhou et al., 2009). A dose of 10 mg/kg was chosen for ONX 0912 to completely inhibit the primary target of carfilzomib, the CT-L activity of the proteasome, in blood and available tissues (J. Jiang and C. Kirk, unpublished observations). Thirty minutes after pretreatment, carfilzomib was administered as an intravenous bolus at 2 mg/kg to both groups of animals. No significant differences in the PK parameters between the two groups were noted, suggesting that target binding is not a major mechanism of clearance for carfilzomib (Table 2).

Pharmacodynamics. The level of proteasome inhibition could be a function of peak plasma levels or total exposure to carfilzomib. When administered as a 30-min infusion at 8 mg/kg, steady state was achieved essentially within 15 min, which is the first plasma sampling during the infusion (Fig. 3B). The steady-state concentration ($C_{\text{ss}}$) of carfilzomib is 28-fold lower than the $C_{\text{max}}$ in the bolus group at the same dose level (Table 1). The other PK parameters, including AUC, were equivalent. The level of proteasome inhibition in blood and

<table>
<thead>
<tr>
<th>Metabolite Code</th>
<th>Proposed Structure</th>
<th>Molecular Mass</th>
<th>Retention Time</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M21</td>
<td><img src="image1" alt="Structure" /></td>
<td>733</td>
<td>50.1</td>
<td>RU and RB</td>
</tr>
<tr>
<td>M22</td>
<td><img src="image2" alt="Structure" /></td>
<td>749</td>
<td>42.5</td>
<td>RB</td>
</tr>
<tr>
<td>M23</td>
<td><img src="image3" alt="Structure" /></td>
<td>753</td>
<td>39.1</td>
<td>RB</td>
</tr>
<tr>
<td>M24</td>
<td><img src="image4" alt="Structure" /></td>
<td>735</td>
<td>44.4</td>
<td>RU</td>
</tr>
</tbody>
</table>

RP, rat plasma; RU, rat urine; RB, rat bile.
* These metabolites were also observed in monkey plasma samples obtained in a 9-month toxicity study.
tissues was also equivalent between the two groups (Fig. 3C). These data demonstrate that the level of target inhibition achieved in vivo is a function of the total dose administered but not \( C_{\text{max}} \). The potent proteasome inhibition in a variety of tissues also demonstrated rapid and wide tissue distribution of carfilzomib after a single intravenous bolus or infusion administration.

**In Vitro Blood Partitioning.** The ratios of whole blood concentrations to plasma concentrations were 0.82, 0.74, and 0.63 at the nominal blood concentrations of 0.2, 2, and 10 \( \mu \text{M} \) carfilzomib, respectively. Because the calibration standards were prepared by spiking carfilzomib into prequenched whole blood, the measured whole blood levels do not represent the fraction bound to the drug target.

**Metabolism.** All of the predictable metabolites were searched in rat plasma, urine, and bile using the LC-MS/MS method described under Materials and Methods. A total of 21 metabolites were identified based on molecular ion masses and fragmentation patterns (Table 3). Although a few oxidative metabolites were observed, most metabolites were derived from peptide and epoxide hydrolysis. Semiquantification in MRM mode, assuming that carfilzomib and its metabolites exhibited similar responses under analytical conditions, has shown that morpholino-homophenylalanine (M14), morpholino-homophenylalanine-leucine (M15), and the diol of carfilzomib (M16) were the most abundant metabolites and that the oxidative metabolites were minor. Authentic standards of these three metabolites were synthesized to further confirm their structures and used for quantitative determination of their levels in plasma and excreta. Amino acids, naturally occurring dipeptides, and a low level of the dipeptide epoxycetone (M11) resulting from hydrolysis of carfilzomib also were observed. A singularly labeled carfilzomib with the label present in the aromatic ring of the phenylalanine, radioactive phenylalanine indeed was detected as a relatively abundant metabolite (J. Jiang and C. Kirk, unpublished observations). However, when nonradiolabeled carfilzomib was administered to rats as conducted in the present study, these amino acids and dipeptides could not be differentiated from endogenous ones. Therefore, they were not assessed quantitatively in this study.

As shown in Fig. 4, all three metabolites, M14, M15, and M16, formed rapidly, with measurable levels immediately postdose and peak levels within 5 min of dosing. M14 is the most abundant circulating metabolite, with a terminal half-life longer than that of the parent drug. The AUC ratios (metabolite/carfilzomib) were 2.7, 0.33, and 0.12 for M14, M15, and M16, respectively. As determined in a separate PK study, plasma clearance of M16 was rapid [110 ml/(min \( \cdot \) kg)] (Supplemental Table S1). Using this clearance value and the separate PK study, plasma clearance of M16 was rapid [110 ml/(min \( \cdot \) kg)].

In an in vitro metabolism study in rat hepatocytes using [\(^{1}H\)]carfilzomib with the label present in the aromatic ring of the phenylalanine, radioactive phenylalanine indeed was detected as a relatively abundant metabolite (J. Jiang and C. Kirk, unpublished observations). However, when nonradiolabeled carfilzomib was administered to rats as conducted in the present study, these amino acids and dipeptides could not be differentiated from endogenous ones. Therefore, they were not assessed quantitatively in this study.

As shown in Fig. 4, all three metabolites, M14, M15, and M16, formed rapidly, with measurable levels immediately postdose and peak levels within 5 min of dosing. M14 is the most abundant circulating metabolite, with a terminal half-life longer than that of the parent drug. The AUC ratios (metabolite/carfilzomib) were 2.7, 0.33, and 0.12 for M14, M15, and M16, respectively. As determined in a separate PK study, plasma clearance of M16 was rapid [110 ml/(min \( \cdot \) kg)] (Supplemental Table S1). Using this clearance value and the observed AUC\(_{\text{inf}}\) of M16 after intravenous bolus administration of carfilzomib, we estimated that approximately 6% of the carfilzomib administered to rats was biotransformed to the diol.

Carfilzomib was also metabolized rapidly in vitro after incubation with rat tissue homogenates, including adrenal, heart, kidney, liver, and lung. The half-life of carfilzomib disappearance was 4 to 39 min in blood and tissues (Fig. 5; Supplemental Fig. S2). Consistent with observations in the in vivo samples, the peptide hydrolysis products M14 and M15 were the abundant metabolites. M16 was also detected, but at a lower level. Taken together, these data suggest that carfilzomib is metabolized rapidly in rats mainly via peptidase cleavage and epoxide hydrolysis. The peptidase cleavage product M14 was the most abundant metabolite in both systemic circulation and excreta. Although M14 can be a secondary metabolite of M16 as depicted in Fig. 2, conversion of M16 to M14 is not likely the main route of M14 formation in rats because only approximately 6% of carfilzomib was converted to M16.

**Excretion.** Carfilzomib and its major metabolites were quantified in the collected urine and bile samples. Less than 1% of the intravenously dosed carfilzomib was excreted intact (Table 4). The products of peptide cleavage (M14 and M15) were the main forms of excretion. The majority of the metabolites were excreted within 4 h after dosing of carfilzomib. Renal and biliary excretion of carfilzomib and metabolites accounted for 26 and 31% of the dose, respectively, for a total of 57% recovered within 24 h postdose.

**TABLE 4**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Time</th>
<th>Percentage (%) of Dose (Urine)</th>
<th>Percentage (%) of Dose (Bile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carfilzomib</td>
<td>0–4</td>
<td>0.032 ± 0.021</td>
<td>0.017 ± 0.019</td>
</tr>
<tr>
<td></td>
<td>4–8</td>
<td>0.005 ± 0.007</td>
<td>BQL</td>
</tr>
<tr>
<td></td>
<td>8–24</td>
<td>0.003 ± 0.004</td>
<td>NA</td>
</tr>
<tr>
<td>M14</td>
<td>0–4</td>
<td>18.6 ± 10.6</td>
<td>12.9 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>4–8</td>
<td>3.86 ± 2.75</td>
<td>0.230 ± 0.108</td>
</tr>
<tr>
<td></td>
<td>8–24</td>
<td>3.29 ± 1.29</td>
<td>NA</td>
</tr>
<tr>
<td>M15</td>
<td>0–4</td>
<td>0.164 ± 0.127</td>
<td>14.7 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>4–8</td>
<td>0.027 ± 0.034</td>
<td>0.076 ± 0.056</td>
</tr>
<tr>
<td></td>
<td>8–24</td>
<td>0.045 ± 0.006</td>
<td>NA</td>
</tr>
<tr>
<td>M16</td>
<td>0–4</td>
<td>0.127 ± 0.100</td>
<td>2.64 ± 3.09</td>
</tr>
<tr>
<td></td>
<td>4–8</td>
<td>0.027 ± 0.045</td>
<td>0.013 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>8–24</td>
<td>0.010 ± 0.006</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td>0–24</td>
<td>26 ± 12</td>
<td>31 ± 5</td>
</tr>
</tbody>
</table>

NA, not applicable (bile samples were not collected from 8 to 24 h); BQL, below the quantitation limit.

---

![Fig. 4. Plasma concentration-time profiles of carfilzomib and metabolites (M14, M15, and M16). Male Sprague-Dawley rats received 2 mg/kg carfilzomib via a single intravenous bolus. Plasma samples were collected before dose, immediately after dose, and at 1, 2, 5, 15, 30, and 60 min postdose.](image-url)
Discussion

In this study, we characterized the PK, pharmacodynamics (PD), metabolism, distribution, and excretion of carfilzomib, an irreversible proteasome inhibitor, in rats, the rodent species used in pharmacology and toxicology studies of the drug candidate. The results of these studies suggest that carfilzomib displays several properties that may help to explain the favorable clinical safety profile of this agent (Vij et al., 2010).

After intravenous bolus administration, carfilzomib was cleared rapidly from systemic circulation with a $t_{1/2}$ value of <20 min. The clearance is more rapid than that reported for bortezomib in preclinical and clinical models (Papandreou et al., 2004; Hemeryck et al., 2007). Furthermore, plasma clearance at all of the dose levels was much higher than the reported hepatic blood flow for rats [55 ml/(min · kg)] (Davies and Morris, 1993), suggesting that, unlike bortezomib, carfilzomib clearance is mediated largely by extrahepatic mechanisms. We determined that the rapid clearance of carfilzomib was not due to irreversible binding of the proteasome because pre-treatment with another irreversible proteasome inhibitor (ONX 0912) did not affect the clearance of carfilzomib. Furthermore, the clearance of M16, which contains the peptide backbone of carfilzomib but lacks the ability to irreversibly bind to the proteasome, was also higher than the hepatic blood flow in rats. In addition, the high plasma clearance was not due to partitioning of carfilzomib to blood cells because the in vitro whole blood/plasma partitioning ranged from 0.64 to 0.82 across a concentration range from 0.2 to 10 μM. Because carfilzomib is an irreversible inhibitor, target inhibition is a function of proteasome turnover and persists even after the drug is cleared systemically. Indeed, rapid clearance may prevent its ability to inhibit nonproteasomal targets and thus reduce potential idiosyncratic toxicities.

The major metabolites in rat plasma, urine, and bile samples were from peptide and epoxide hydrolysis; oxidative metabolites were observed only at low levels (<0.1% of total dose in urine and bile samples). In vitro, carfilzomib was metabolized rapidly in a variety of tissues and blood also via hydrolysis. Together with the rapid (<10 s) formation of these hydrolysis metabolites in vivo, these data suggest that metabolism of carfilzomib occurs immediately upon administration and likely occurs systemically throughout the body. These metabolites lack the epoxide pharmacophore and therefore have no activity as proteasome inhibitors. Their further processing to individual amino acids also suggests that they have little toxicological impact in vivo. A minor metabolite (Phe-Leu-Epoxycetone, M11), resulting from peptidase cleavage, was detected in rat plasma, urine, and bile samples. The low abundance of M11 may be attributed to rapid secondary metabolism to phenylalanine (M7), tyrosine (M5), leucine diol (M8), and a low level of oxidized leucine epoxycetone (M2). We showed recently that dipeptide epoxycetone, morpholino-Phe-Leu-epoxycetone, is not an active proteasome inhibitor and has no activity against serum proteases in an activity-based profiling assay (Zhou et al., 2009; Arastu-Kapur et al., 2011). Therefore, the low level of M11 is unlikely to contribute to PD or induce off-target toxicity. Because peptide epoxycetones are highly selective for proteasome active sites, these findings suggest that the PD effect of carfilzomib is being driven by the parent compound (Mirabella et al., 2011). We also have noted rapid clearance and extrahepatic metabolism in the form of peptide and epoxide hydrolysis in humans, and similar to rats, the peptidase cleavage product M14 is the most abundant circulation and excretion metabolite in humans (Z. Wang, J. Yang, and C. Kirk, manuscript in preparation). Therefore, exposure to carfilzomib is unlikely to be altered in patients with hepatic impairment or taking concomitant medications that alter cytochrome P450 activity.

Carfilzomib also distributes rapidly to tissues after intravenous administration as demonstrated by potent proteasome inhibition in a variety of tissues. Potent proteasome inhibition in different tissues (except brain) after intravenous bolus administration has been described previously (Demo et al., 2007). In this study, we determined that a 30-min infusion of carfilzomib at 8 mg/kg resulted in equivalent levels of proteasome inhibition in blood and tissues despite a 28-fold lower $C_{\text{max}}$ (1.6 versus 42.9 μM) than that with intravenous bolus administration at the same dose. Therefore, we conclude that the in vivo potency of this agent is a consequence of total dose administered but not $C_{\text{max}}$. As has been described with other classes of covalent inhibitors, these data indicate that the time course of target inhibition by carfilzomib is not determined directly by its plasma exposure (Singh et al., 2011). Furthermore, we found that bolus administration of carfilzomib at 8 mg/kg resulted in a mortality rate of approximately 44% (14 of 32 animals), whereas a 30-min infusion of the same dose was well tolerated generally and did not result in mortality (0 of 24 animals; J. Jiang and C. Kirk, unpublished observations). The higher incidence of mortality with bolus administration is likely associated with the high $C_{\text{max}}$. Thus, intravenous infusion might be an alternative route of administration to bolus injection to improve the therapeutic window of carfilzomib. We have demonstrated currently that carfilzomib can be administered safely up to 56 mg/m² in MM patients with intravenous infusion, which is more than 2-fold the maximum tolerated dose for an intravenous bolus administration (Papadopoulos et al., 2010).

Despite the wide tissue distribution of carfilzomib, the apparent $V_{ss}$ values for carfilzomib were relatively small (Table 1), 0.3, 0.3, and 0.6 l/kg at 2, 4, and 8 mg/kg, respectively, when administered via an intravenous bolus. With intravenous infusion administration at 8 mg/kg, the measured $V_{ss}$ was 2 l/kg. It appears that the $V_{ss}$ value was lower at low doses (2 and 4 mg/kg). At 8 mg/kg, $V_{ss}$ obtained from intravenous infusion was higher than that from bolus administration. The specific reason for this observation was not clear. However, the apparent $V_{ss}$ values may not truly reflect the tissue distribution of an irreversible inhibitor. Moreover, as described in the preceding sections, carfilzomib is highly metabolized in a variety of tissues by peptidase and epoxide hydrolyase. This portion of carfilzomib was counted as elimination rather than as distribution.

Previously and in this study, we have noted that the liver was relatively insensitive to proteasome inhibition compared with adrenal, blood, heart, and lung (Demo et al., 2007). However, radioactivity in the liver at 0.5 h postdose is quite high (Demo et al., 2007). These disparate findings may be attributed to the competition between drug-metabolizing enzymes and binding to the proteasome within the liver. Indeed, the rate of carfilzomib disappearance in liver was more rapid relative to those in blood and other organs (adrenal, heart, and lung), as demonstrated in the in vitro study (Fig. 5; Supplemental Fig. S2). Therefore, we believe that a large portion of the radioactivity observed in rat livers represents radioactive metabolites.

Less than 1% of carfilzomib was excreted intact in urine and bile within 24 h postdose in rats. Carfilzomib is eliminated mainly in the form of metabolites, particularly peptidase cleavage products M14 and M15. In a previous report, high levels of radioactivity in bile and urine at 0.5 h postdose in the quantitative whole-body autoradiography study were noted (Demo et al., 2007), which likely represents the rapid elimination of radiolabeled metabolites. However, the radioactivity did not represent M14 or M15 because these two major metabolites do not contain the 3H-labeled phenylalanine group. The total recoveries of carfilzomib and monitored metabolites (M14, M15, and M16) in rat bile and urine and bile were 26 and 31% of the dose, respectively, for a total of 57% within 24 h postdose in the present study using nonradiolabeled carfilzomib (Table 4). The incomplete recovery of the administered carfilzomib may represent the incorporation of the amino acids from the peptide backbone of the drug into biosynthetic pathways; retention of carfilzomib bound to proteasomes in nucleated cells (erythrocytes and platelets), which cannot turnover their proteasomes; and the observed dipep-
tides, minor metabolites, or undiscovered metabolites that were not quantified in the present study. Further studies are needed to better understand the complete fate of administered carfilzomib.

In summary, carfilzomib was cleared rapidly from systemic circulation after intravenous administration to rats largely due to extrathoracic metabolism via peptide cleavage and epoxide hydrolysis. Despite rapid clearance, carfilzomib showed potent proteasome inhibition in a variety of tissues, demonstrating widespread tissue distribution. Elimination occurred mainly via biliary and renal excretion in the form of metabolites rather than intact drug. Together with the irreversible and rapid onset of target binding, the high clearance may provide an advantage in that unnecessary exposure to the drug is minimized and potential drug-related side effects may be reduced. Furthermore, the major metabolic pathways of carfilzomib result in toxicologically insignificant metabolites (modified peptides and amino acids). Carfilzomib is currently in late-stage clinical development, with encouraging safety and efficacy profiles demonstrated with intravenous bolus and infusion administrations. The studies presented here help to shed light on the disposition of this novel class of irreversible proteasome inhibitors.

Authorship Contributions

Participated in research design: Yang, Wang, Fang, Jiang, Bennett, Moineaux, and Kirk.

Conducted experiments: Yang, Wang, Fang, Jiang, Zhao, and Kirk.

Performed data analysis: Yang, Wang, Fang, Jiang, Zhao, Wong, and Kirk.

Wrote or contributed to the writing of the manuscript: Yang, Wang, Wong, and Kirk.

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