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

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Running Title Page

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AUC	area under plasma concentration over time curve
AUC_{last}	area under plasma concentration over time curve calculated to the last measurable concentration
AUC_{inf}	area under plasma concentration over time curve extrapolated to infinity
BQL	below the quantitation limit
C_{max}	maximum concentration
C_{ss}	steady state concentration

CL	plasma clearance
C-L	caspase-like
CT-L	chymotrypsin-like
DPBS	dulbecco's phosphate buffer saline
IS	internal standard
i.v.	intravenous
LC-MS/MS	liquid chromatography tandem mass spectrometry
LLOQ	lower limit of quantification
MRT	mean residence time
PK	pharmacokinetics
PD	pharmacodynamics
QWBA	quantitative whole body autoradiography
RB	rat bile
RP	rat plasma
RU	rat urine
$t_{1/2}$	terminal half life
T-L	trypsin-like
V_{ss}	volume of distribution at steady state

Abstract

Carfilzomib (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. Following intravenous administration, the plasma concentration of carfilzomib declined rapidly in a biphasic manner. Carfilzomib displayed high plasma clearance (195 to 319 mL/min/kg), a short terminal half life (5-20 minutes) and rapid and wide tissue distribution in rats. The exposure to carfilzomib (C_{max} and AUC) increased dose proportionally from 2 to 4 mg/kg but less than dose proportionally from 4 to 8 mg/kg. The high clearance was predominantly mediated 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 about 26% and 31% of the total dose, respectively, for a total of 57% within 24 hours post-dose. 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.

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Introduction

The proteasome is a multicatalytic enzyme complex that is the primary means for protein degradation in all cells [Ciechanover, 2005; Kisselev and Goldberg, 2001]. 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 which function as chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L) proteases. Tumor cells are more susceptible than non-transformed cells to the cytotoxic effect 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 treatment of multiple myeloma (MM) and mantle cell lymphoma provided clinical validation of the proteasome as a therapeutic target in oncology [Bross et al., 2004; Adams et al., 1998; Chauhan et al., 2005; Richardson et al., 2005; Goy et al., 2005; O'Connor 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 [Borissenko 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 and 2007a and 2007b; Richardson et al., 2003 and 2005; O'Connor et al., 2005; Goy et al., 2005]. In addition, bortezomib is primarily metabolized by cytochrome P450 3A4 (CYP3A4) [Uttamsingh et al., 2005] and co-administration of CYP3A4 inhibitors such as ketoconazole causes a significant change of bortezomib plasma levels in humans [Venkatakrishnan et al., 2009].

In the past years, the next generation of proteasome inhibitors has been explored aiming at improving safety and efficacy profiles. Three small molecules (carfilzomib, NPI-0052 and CEP-18770) are currently in clinical development [Bennett and Kirk, 2009].

Carfilzomib (**Figure 1**) is a tetrapeptide epoxyketone analog of epoxomicin and eponemycin, a pair of related natural products that were initially discovered as anti-tumor agents in animals and later shown to inhibit the CT-L activity of the proteasome [Hanada et al., 1992; Meng et al., 1999a and 1999b; Kim et al., 1999; 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 T-L or C-L activities of the proteasome [Demo et al., 2007]. Based upon encouraging preclinical data, including the induction of potent proteasome inhibition in blood and tissues in rodents and greater anti-tumor activity as compared to 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 [Alsina et al., 2007; Siegel et al., 2010; Vij et al., 2010; Jakubowiak et al., 2010a; Jakubowiak et al., 2010b; Martin et al., 2010; Jagannath et al., 2010 and Singhal et al., 2010], including patients refractory to bortezomib. Currently, carfilzomib is being developed by Onyx Pharmaceuticals for the treatment of MM 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 following intravenous (i.v.) administration to Sprague Dawley rats, carfilzomib was rapidly cleared from systemic

circulation. In vitro and in vivo studies showed that carfilzomib was extensively metabolized 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 to its target, demonstrating wide tissue distribution of carfilzomib upon i.v. administration. Finally, we show that a 30-minute infusion administration resulted in a 28-fold lower maximum concentration (C_{max}) but with comparable levels of proteasome inhibition in blood and tissues as an i.v. bolus.

Material and Methods

Materials. Carfilzomib [(2S)-N-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylcarbamoyl)-2-phenylethyl)-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)-4-methylpentanamide], PR-054591 [N-((S)-1-((S)-1-((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-ylcarbamoyl)-2-phenylethylcarbamoyl)-3-methylbutyl)-5-(morpholinomethyl)isoxazole-3-carboxamide] and ONX 0912 [(2S)-3-methoxy-2-[(2S)-3-methoxy-2-[(2-methyl-1,3-thiazol-5-yl)formamido] propanamido]-N-[(2S)-1-[(2R)-2-methyloxiran-2-yl]-1-oxo-3-phenylpropan-2-yl]propanamide], tripeptide analogues of carfilzomib shown in **Figure 1**, and authentic standards of carfilzomib metabolites (M14, M15 and M16; **Figure 2 and Table 3**), were synthesized at Onyx Pharmaceuticals, Inc. PR-054591 was used as the internal standard (IS) for all quantitative analyses. Rat whole blood and rat and monkey plasma in sodium heparin were from Bioreclamation (East Meadow, NY). Tissue homogenates were prepared in

house. All other reagents were obtained from commercial sources with an appropriate quality.

Pharmacokinetic (PK) studies. All animal studies were conducted in accordance with the guide for the Care and Use of Laboratory Animals. Male Sprague Dawley rats, weighing ~ 250-350 g and containing cannulas in the jugular vein and carotid artery, were obtained from Charles River Laboratories (Hollister, CA) and used for all PK studies. Animals were allowed to acclimate for a minimum of three days prior to experimentation and allowed food and water *ad libitum.*

Carfilzomib was formulated in 10% (w/v) sulfobutylether betacyclodextrin (Captisol^{TM}) in 10 mM citrate (pH 3.5) at a concentration of 2 mg/mL. For i.v. bolus administration, four to five animals per dose level received a single dose of carfilzomib at 2, 4 or 8 mg/kg, respectively. Blood (~0.4 mL) samples were collected prior to and immediately after dosing (within 10 seconds) and at 1, 2, 5, 15, 30 and 60 minutes post-dose. For i.v. infusion administration, four rats received carfilzomib via a 30-minute constant infusion at 8 mg/kg. Blood samples were collected prior to dosing, at 15 minutes post start of infusion (PSI), end of infusion and at 2, 5, 15, 30, 60 and 120 minutes post-dose.

To assess the effect of target binding, the PK profiles of carfilzomib from two groups of male Sprague Dawley rats (3/group) were compared. One group was pre-treated with an i.v. 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], while the other group was pre-treated with formulation vehicle. Thirty minutes after pre-treatment, carfilzomib was administered via i.v. bolus at 2 mg/kg.

Blood samples were taken prior to and immediately after the carfilzomib dose and at 1, 2, 5, 15, 30 and 60 minutes post carfilzomib dose.

A PK study was also conducted for M16, the diol of carfilzomib derived from epoxide hydrolysis via i.v. bolus administration to male Sprague Dawley rats at 5 mg/kg. M16 was formulated at 2 mg/mL in Captisol^{TM} containing 10 mM citrate (pH 3.5). Blood samples were collected prior to dosing and at 2, 5, 10, 15, 30, 60, 120 and 360 minutes post-dose.

PK of carfilzomib was also assessed in non-näive 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-β-cyclodextrin (HPBCD; w/v) in 50 mM sodium citrate (pH 3.35) at a concentration of 1 mg/mL. Three animals received a single i.v. bolus dosing of carfilzomib at 1 mg/kg. Blood samples were collected prior to dosing and at 2, 5, 15, 30 and 60 minutes post-dose.

All blood samples were collected in tubes containing sodium heparin as the anti-coagulant. Blood samples were kept on ice during sample collection and centrifuged at 3000 g for 10 minutes 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 lower limit of quantification (LLOQ) were treated as zero for PK analysis. Area under the concentration-time curve (AUC) values were estimated using linear trapezoidal method. Plasma clearance (CL) was calculated from Dose/AUC_{inf}. Terminal half life ($t_{1/2}$) values were calculated as ln(2)/k, where k

represents the terminal elimination rate constant obtained from the slope of the semi-logarithmic plot of the concentration-time profile. Mean residence time (MRT) was estimated by moment analysis. Volume of distribution at steady state (V_{ss}) was calculated from MRT × CL. Maximum concentration (C_{max}) was recorded as observed. The amount of M16 generated in rats following carfilzomib dosing was calculated by multiplying the AUC_{inf} of M16 by the clearance of M16 obtained in the PK study for M16.

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

Excretion Study. Excretion of carfilzomib was determined in male Sprague Dawley rats with bile duct cannulation following a single i.v. bolus administration at 2 mg/kg. Bile was collected cumulatively at 0–4 and 4–8 hours post-dose, no bile samples were collected at 8-24 hours post-dose; urine was collected 0–4, 4–8 and 8–24 hours post-dose. Volumes of the bile and urine samples were measured. Acetonitrile was added to the original urine sample containers following 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 were also collected in this study prior to and

immediately after dosing and at 1, 2, 5, 15, 30, 60 and 120 minutes post-dose into tubes containing sodium heparin. The urine, bile and plasma samples were stored at -70°C until analysis.

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

Metabolite 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 the dose levels of 1, 2, or 4 mg/kg for six 28-day cycles with i.v. 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) prior to dose and at 5, 15, 30, and 60 minutes post-dose. 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 pre-dose 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

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 directly subjected to LC MS/MS analyses.

LC MS/MS analyses were done in positive ionization mode using precursor ion and neutral loss scans on an LTQ™ mass spectrometer (Thermo Scientific, San Jose, CA) 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 minutes, followed by ramping solvent B from 0–70% in 55 minutes and 70–100% B in 2 minutes. The structures of metabolites were proposed based on the molecular ion mass and fragmentation pattern generated by product ion scans. Semi-quantitation was done in MRM mode by comparing the peak area of each metabolite to carfilzomib.

Metabolism Study in Rat Blood and Tissue Homogenates from Liver, Kidney, Lung, Heart and Adrenal. The metabolism of carfilzomib was evaluated in rat blood and tissue homogenates (liver, kidney, lung, heart and adrenal) harvested from untreated male Sprague Dawley rats. The isolated organs were chopped into small pieces, washed with phosphate buffer saline (pH 7.4) for several times and then weighed

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into 2 mL Eppendorf tubes. Dulbecco's phosphate buffer saline (DPBS, pH 7.4) was then added to the tubes to achieve a 1:5 ratio of tissue weight: DPBS volume. The tissues were homogenized using a Tissuelyser (Retsch, Inc) at a frequency of 20 s^{-1}. Aliquots of 300 µL rat blood or tissue homogenates were pre-incubated at 37ºC for 5 minutes prior to the addition of 5 µL of carfilzomib working solution at 60 µg/mL in acetonitrile. The final concentration of carfilzomib in the incubation was 1 µg/mL. The mixture was incubated at 37ºC with gentle agitation. An aliquot of 40 µL reaction mixture was taken at 2, 10, 20, 30, 60 and 90 minutes, quenched with 120 µL 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 rate of carfilzomib metabolism in rat liver and adrenal gland was 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 extraction process, and for at least three freeze/thaw cycles. Carfilzomib and metabolites were quantified by comparing the peak area ratios of the analyte to IS in study samples to the standard calibration samples prepared in the same manner. To determine the concentration of carfilzomib in blood samples from in vitro partitioning study, the standard calibration samples were prepared by spiking carfilzomib into blood pre-quenched with three volumes of acetonitrile containing 0.5 µM IS. An aliquot (40

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μL) of plasma, bile or acetonitrile-treated urine sample was mixed with 80 μL of acetonitrile containing 0.5 μM IS to extract carfilzomib and metabolites. The extracted samples were vortexed and centrifuged. The supernatants were filtered through a 96-well filter plate (AcroPrep p6 Filter Plate, 0.45 μm, Pall Corporation, Ann Arbor, MI) 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 (ABSCIEX, Foster City, CA) equipped with two Shimadzu LC- 10ADvp pumps and a Shimadzu SIL-HTc autosampler with controller system (Shimadzu, Columbia, MD). 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 ionspray interface in the positive ionization mode by MRM of the selective *m/z* 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 LLOQ was 2.00 ng/mL with a calibration range of 2.00–3000 ng/mL for blood and plasma samples and 2.00–1000 ng/mL for urine and bile samples.

Results

PK. Following a single i.v. bolus dose of 2, 4 or 8 mg/kg, carfilzomib plasma concentration over time curves display a very steep initial phase (**Figure 3A**). First plasma sampling was done within 10 seconds after dosing in order to accurately measure the PK of carfilzomib in rats; plasma levels at 2 minutes were <5% of those immediately

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after dosing (**Figure 3A insert**). The terminal half life ($t_{1/2}$) ranged from 5 to 20 minutes and CL values were 195 to 296 mL/min/kg (**Table 1**). Both AUC and C_{max} increased dose proportionally and the CL values was constant from 2 to 4 mg/kg. When the dose level was increased to 8 mg/kg, however, AUC and C_{max} increased less than dose proportionally and the CL value increased. Rapid clearance and short half life were also observed in cynomolgus monkeys following i.v. administration at 1 mg/kg [**Supplemental Figure S1**].

Carfilzomib is a rapidly binding and irreversible inhibitor of the proteasome, a ubiquitously expressed target that accounts for ~1% of total cellular protein (Demo et al., 2007; Ciechanover 2005). In order to determine if the high level of target expression affects its PK, carfilzomib was administered to two groups of rats: one pre-treated with an i.v. bolus dose of the vehicle (control group) and the other pre-treated with an i.v. 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 in order to completely inhibit the primary target of carfilzomib, the CT-L activity of the proteasome, in blood and available tissues (Data not shown). Thirty minutes after pretreatment, carfilzomib was administered as an i.v. 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**).

PD. The level of proteasome inhibition could be a function of peak plasma levels or total exposure to carfilzomib. When administered as a 30-minute infusion at 8 mg/kg, steady state was essentially achieved within 15 minutes, which is the first plasma

sampling during the infusion (**Figure 3B**). The steady state concentration (C_{ss}) of carfilzomib is 28-fold lower than the C_{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 tissues was also equivalent between the two groups (**Figure 3C**). These data demonstrate that the level of target inhibition achieved in vivo is a function of the total dose administered but not C_{max}. The potent proteasome inhibition in a variety of tissues also demonstrated rapid and wide tissue distribution of carfilzomib following a single i.v. bolus or infusion administration.

In Vitro Blood Partitioning. The ratio of whole blood concentration to plasma concentration was 0.82, 0.74 and 0.63 at the nominal blood concentrations of 0.2, 2 and 10 μM of carfilzomib, respectively. Since the calibration standards were prepared by spiking carfilzomib into pre-quenched whole blood, the measured whole blood levels do not represent the fraction bound to drug target.

Metabolism. All predictable metabolites were searched in rat plasma, urine and bile using the LC/MS/MS method described in the "Materials and Methods" section. A total of 21 metabolites were identified based on molecular ion mass and fragmentation pattern (**Table 3**). Although a few oxidative metabolites were observed, most metabolites were derived from peptide and epoxide hydrolysis. Semi-quantitation in MRM mode, assuming that carfilzomib and its metabolites exhibited similar response under the analytical conditions, has shown that, morpholino-homophenylalanine (M14), morpholino-homophenylalanine-leucine (M15) and diol of carfilzomib (M16) were the most abundant metabolites and 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 low level of the dipeptide epoxyketone (M11) resulting from hydrolysis of carfilzomib, were also observed. In an in vitro metabolism study in rat hepatocytes using ^{3}H-carfilzomib with the label present in the aromatic ring of the phenylalanine, radioactive phenylalanine was indeed detected as a relatively abundant metabolite (data not shown). However, when non-radiolabeled carfilzomib was administrated to rats as conducted in the present study, these amino acids and dipeptides could not be differentiated from endogenous ones. Therefore, they were not quantitatively assessed in this study.

As shown in **Figure 4**, all three metabolites, M14, M15, and M16, formed rapidly with measurable levels immediately post-dose and peak levels within 5 minutes of dosing. M14 is the most abundant circulating metabolites with a terminal half life longer than 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/kg) (**Supplemental data Table S1**). Using this clearance value and the observed AUC_{inf} of M16 following i.v. bolus administration of carfilzomib, we estimated that about 6% of the carfilzomib administered to rats was biotransformed to the diol.

Carfilzomib was also rapidly metabolized in vitro following incubation with rat tissue homogenates, including liver, kidney, lung, heart and adrenal gland. The half-life of carfilzomib disappearance was 4–39 minutes in blood and tissues (**Figure 5 and Supplemental Figure 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 rapidly metabolized in rats mainly via peptidase cleavage and epoxide hydrolysis. 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 **Figure 2**, conversion of M16 to M14 is not likely the main route of M14 formation in rats since only about 6% of carfilzomib was converted to M16.

Excretion. Carfilzomib and its major metabolites were quantified in the collected bile and urine 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. Majority of the metabolites were excreted within 4 hours 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 hour post-dose.

Discussion

In this study, we characterized the PK, 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 carfilzomib displays several properties that may help explain the favorable clinical safety profile of this agent (Vij et al., 2010).

Following i.v. bolus administration, carfilzomib was rapidly cleared from systemic circulation with $t_{1/2}$ of less than 20 minutes. The clearance is more rapid than what has been reported for bortezomib in preclinical and clinical models (Papandreou et

al., 2004 and Hemeryck et al., 2007). Furthermore, plasma clearance at all dose levels was much higher than reported hepatic blood flow for rats (55 mL/min/kg) [Davies and Morris, 1993], suggesting that, unlike bortezomib, carfilzomib clearance is mediated by extrahepatic mechanisms. We determined that the rapid clearance of carfilzomib was not due to irreversible binding of the proteasome as pretreatment 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. Finally, the high plasma clearance was not due to partitioning of carfilzomib to blood cells as the in vitro whole blood/plasma partitioning ranged from 0.64 to 0.82 across a concentration range of 0.2 to 10 μM. Since carfilzomib is an irreversible inhibitor, target inhibition is a function of proteasome turnover and persists even after drug is systemically cleared. Indeed, rapid clearance may prevent its ability to inhibit non-proteasomal targets and thus reduce potential idiosyncratic toxicities.

The major metabolites in rat plasma, bile and urine 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 rapidly metabolized in a variety of tissues and blood also via hydrolysis. Together with the rapid (<10 seconds) 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 epoxyketone 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

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minor metabolite (Phe-Leu-Epoxyketone, 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 low level of oxidated leucine epoxyketone (M2). Recently we showed that dipeptide epoxyketone, Morpholino-Phe-Leu-Epoxyketone, is not an active proteasome inhibitor and has no activity against serine proteases in an activity based profiling assay (Zhou et al., 2009; Kapur et al., 2011). Therefore, the low level of M11 is unlikely to contribute to PD or induce off-target toxicity. Since peptide epoxyketones 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 have also 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 (Wang et al., Manuscript in Preparation). Therefore, exposure to carfilzomib is unlikely to be altered in patients with hepatic impairment or taking concomitant medications that alter CYP activity.

Finally, carfilzomib rapidly distributes to tissues following i.v. administration as demonstrated by the potent proteasome inhibition in a variety of tissues. Potent proteasome inhibition in different tissues (except brain) following i.v. bolus administration was previously described [Demo et al., 2007]. In this study, we determined that a 30-minute infusion of carfilzomib at 8 mg/kg resulted in equivalent levels of proteasome inhibition in blood and tissues despite a 28-fold lower C_{max} (1.6 vs. 42.9 μM) than with i.v. bolus administration at the same dose. Therefore, we conclude

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the in vivo potency of this agent is a consequence of total dose administered but not $C_{max.}$ As has been described with other classes of covalent inhibitors, these data indicate that the time course of target inhibition by carfilzomib is not directly determined 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 ~44% (14 out of 32 animals) while a 30-minute infusion of the same dose was generally well tolerated and did not result in mortality (0 of 24 animals; data not shown). The higher incidence of mortality with bolus administration is likely associated to the high C_{max}. Thus, i.v. infusion might be an alternative route of administration to bolus injection to improve therapeutic window of carfilzomib. Currently, we have demonstrated that carfilzomib can be safely administered up to 56 mg/m^2 in MM patients with i.v. infusion, which is more than 2-fold the maximum tolerated dose for an i.v. bolus administration [Papadopoulos et al., 2010].

Despite the wide tissue distribution of carfilzomib, the apparent steady-state volume of distribution (V_{ss}) for carfilzomib was relatively small (**Table 1**): 0.3, 0.3 and 0.6 L/kg at 2, 4 and 8 mg/kg, respectively, when administered via i.v. bolus. With i.v. infusion administration at 8 mg/kg, the measured V_{ss} was 2 L/kg. It appears the V_{ss} value was lower at low dose levels (2 and 4 mg/kg). At 8 mg/kg, V_{ss} obtained from i.v. infusion was higher than 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 hydrolase. This portion of carfilzomib was accounted as elimination rather than as distribution.

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Previously and in this study we have noted that the liver was relatively insensitive to proteasome inhibition compared to blood, lung, heart and adrenal gland [Demo et al., 2007]. However, radioactivity in the liver at 0.5 hours post-dose is quite high [Demo et al., 2007]. These disparate findings may be attributed to the competition between drug metabolizing enzymes and the binding to the proteasome within the liver. Indeed, the rate of carfilzomib disappearance in liver was more rapid relative to blood and other organs (lung, heart and adrenal gland) as demonstrated in the in vitro study (**Figure 5** and **supplemental data Figure 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 hours post-dose in rats. Carfilzomib is mainly eliminated in the form of metabolites, particularly peptidase cleavage products M14 and M15. In a previous report, high levels of radioactivity in urine and bile at 0.5 hours post-dose in the quantitative whole body autoradiography (QWBA) study were noted [Demo et al., 2007], which likely represents the rapid elimination of radiolabeled metabolites. The radioactivity, however, did not represent M14 and M15 as these two major metabolites do not contain the tritium labeled phenylalanine group. The total recovery of carfilzomib and monitored metabolites (M14, M15 and M16) in rat urine and bile was 26% and 31% of the dose, respectively, for a total of 57% within 24 hours post-dose in the present study using non-radiolabeled carfilzomib (**Table 4**). The incomplete recovery of the administrated 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 enucleated cells (erythrocytes and platelets), which cannot turnover their proteasomes, and the observed

dipeptides, 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 rapidly cleared from systemic circulation following i.v. administration to rats due to extrahepatic metabolism via peptidase 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 i.v. bolus and infusion administrations. The studies presented here help shed light on the disposition of this novel class of irreversible proteasome inhibitors.

Authorship Contribution

Participated in research design: J. Yang, Z. Wang, Y. Fang, J. Jiang, M. K. Bennett, C. J. Molineaux and C. J. Kirk

Conducted experiments: Y. Fang, Z. Wang, J. Jiang, F. Zhao, J. Yang and C. J. Kirk

Performed data analysis: J. Yang, Z. Wang, Y. Fang, J. Jiang, F. Zhao, H. Wong and C. J. Kirk

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

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Footnotes

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Legends for Figures

Fig. 1. Chemical structures of carfilzomib (PR-171), PR-054591 (IS) and ONX 0912.

Fig. 2. Major metabolic pathways of carfilzomib from metabolite profiling in rat plasma, urine and bile samples.

Fig. 3. Pharmacokinetics (PK) and pharmacodynamics (PD) of carfilzomib following single i.v. bolus or 30-minute i.v. infusion administraion to male Sprague Dawley rats. **A**. Plasma concentration over time profiles of carfilzomib following a single i.v. bolus administration at 2, 4 and 8 mg/kg. Plasma samples were collected prior to dose, immediately after dose (within 10 seconds) and at 1, 2, 5, 15, 30 and 60 minutes post-dose. **B**. Plasma concentration over time profile of carfilzomib following a 30-minute i.v. infusion at 8 mg/kg. Plasma samples were collected prior to dose, at 15 minutes post start of infusion (PSI), at the end of infusion and at 2, 5, 15, 30, 60 and 120 minutes post-dose. **C**. Proteasome inhibition and recovery following a single i.v. bolus or a 30-minute i.v. infusion of carfilzomib to Sprague Dawley rats. Rats received a single i.v. bolus (open column) or a 30-minute i.v. infusion (solid column) of carfilzomib at 8 mg/kg. Proteasome chymotrypsin-like (CT-L) activity was determined with Leu-Leu-Val-Tyr-AMC (LLVY-AMC) as substrate in lysates prepared from tissues or whole blood at 2 and 24 hours post-dose. For whole blood, CT-L activity was also measured at 15 minutes post start of infusion (PSI) (infusion only) and 15 minutes post-dose. Columns, mean activity relative to vehicle controls; bars, standard deviation (n = 3 per dose group).

Fig. 4. Plasma concentration over time profiles of carfilzomib and metabolites (M14, M15 and M16). Male Sprague Dawley rats received 2 mg/kg carfilzomib via a single i.v. bolus injection. Plasma samples were collected prior to dose, immediately after dose and at 1, 2, 5, 15, 30 and 60 minutes post-dose.

Fig. 5. In vitro stability of carfilzomib in rat blood and tissue homogenates (kidney, liver, heart and lung). Incubations were done at 37ºC with a carfilzomib concentration of 1 µg/mL. Aliquots of incubation mixture were taken at 2, 10, 20, 30, 60 and 90 minutes to determine the level of carfilzomib remaining.

Table 1. PK parameters of carfilzomib obtained from single i.v. bolus (2, 4 and 8 mg/kg) and 30-minute i.v. infusion (8 mg/kg) administrations to male Sprague Dawley rats.

Parameters:	i.v. Bolus Dose (mg/kg)			i.v. Infusion (mg/kg)
	2	4	8	8
First sampling time (min)	0.1	0.1	0.1	15
$t_{½}$ (min)	5 ± 0	17 ± 9	17 ± 3	10 ± 6
C_{max} or C_{ss} (µM)	16.4 ± 4.5	39.9 ± 3.2	42.9 ± 4.5	1.6 ± 0.4
AUC_{last} (min·µmol/L)	12.6 ± 2.9	28.5 ± 1.8	37.6 ± 2.8	35.8 ± 7.3
AUC_{inf} (min·µmol/L)	12.7 ± 2.9	28.6 ± 1.8	37.8 ± 2.8	35.9 ± 7.2
AUC_{inf}/D (min·kg·µmol/L/µmol)	4.6 ± 1.1	5.1 ± 0.3	3.4 ± 0.3	3.2 ± 0.7
CL (mL/min/kg)	229 ± 55	195 ± 12	296 ± 22	319 ± 66
V_{SS} (L/kg)	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	2.0 ± 0.5

Table 2. PK parameters of carfilzomib obtained from a single i.v. bolus dosing of carfilzomib (2 mg/kg) to Sprague Dawley rats pre-treated with ONX 0912 or formulation vehicle.

PK Parameters:	Pre-treatment with vehicle	Pre-treatment with ONX 0912
First sampling time (min)	0.1	0.1
$t_{½}$ (min)	4 ± 1	4 ± 3
C_{max} (µM)	20.8 ± 8.5	19.8 ± 3.2
AUC_{last} (min·µmol/L)	15.2 ± 5.6	14.7 ± 2.0
AUC_{inf} (min·µmol/L)	15.2 ± 5.6	14.8 ± 2.1
AUC_{inf}/D (min·kg·µmol/L/µmol)	5.5 ± 2.0	5.3 ± 0.7
CL (mL/min/kg)	199 ± 69	190 ± 27
V_{SS} (L/kg)	0.2 ± 0.1	0.2 ± 0.0

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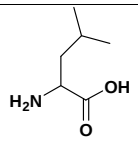
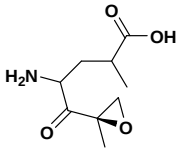
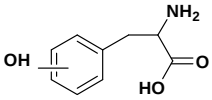
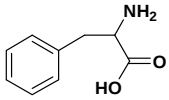
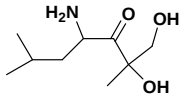
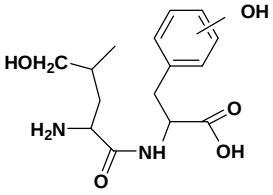
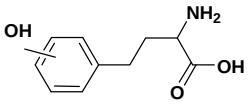
Table 3. Summary of carfilzomib metabolites identified in rat plasma, bile and urine samples.

Metabolite Code	Proposed Structure	MW (Da)	Rt (min)	Source[a]
M1[b]		131	10.4	RP, RU and RB
M2[b]		201	13.1	RP, RU and RB
M5[b]		181	13.1	RP, RU and RB
M7[b]		165	15.9	RP and RU
M8[b]		189	17.2	RP, RU and RB
M9[b]		310	16.7	RP and RB
M10		195	19.0	RU

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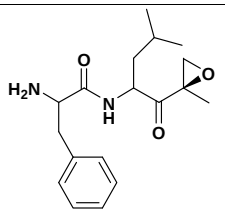
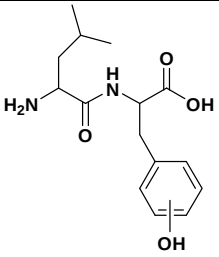
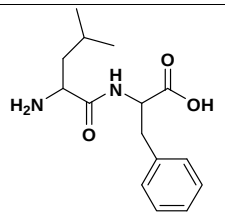
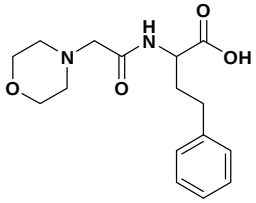
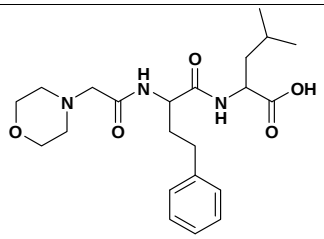
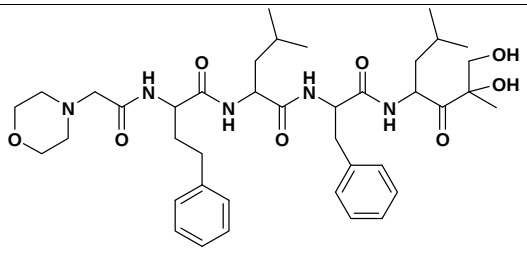
M11[b]		318	20.2	RP, RU and RB
M12[b]		294	21.2	RP, RU and RB
M13[b]		278	25.1	RB
0M14[b]		306	25.9	RP, RU and RB
M15[b]		419	33.0	RP, RU and RB
M16[b]		737	43.8	RP, RU and RB

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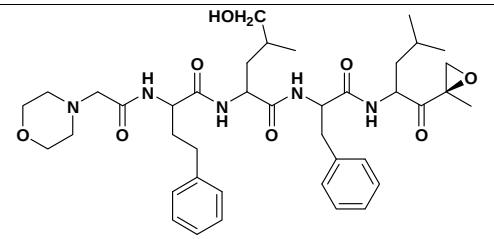
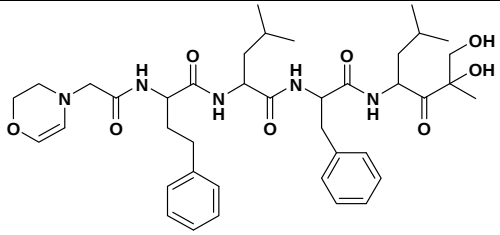
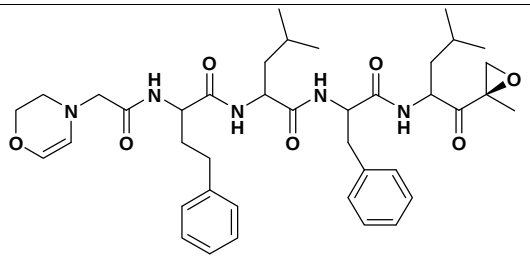
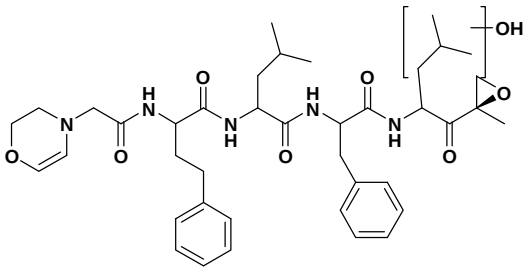
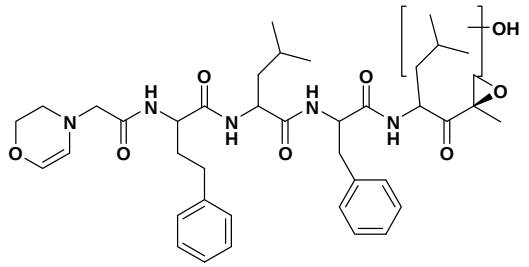
M17		735	51.1	RP and RU
M18		735	51.1	RU and RB
M19		717	59.3	RB
M20		733	46.2	RU and RB
M21		733	50.1	RU and RB

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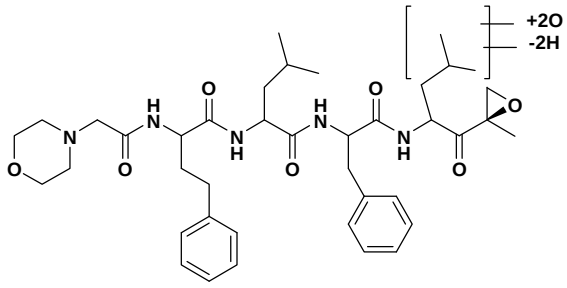
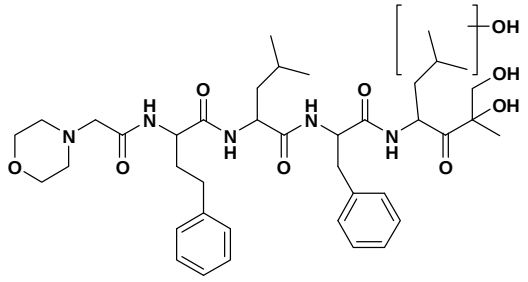
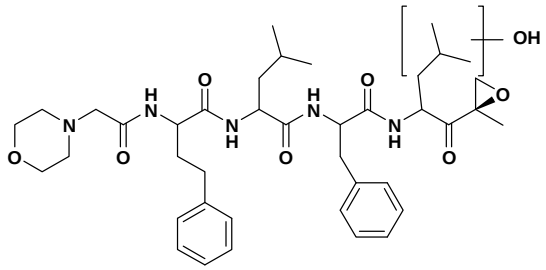
M22		749	42.5	RB
M23		753	39.1	RB
M24		735	44.4	RU

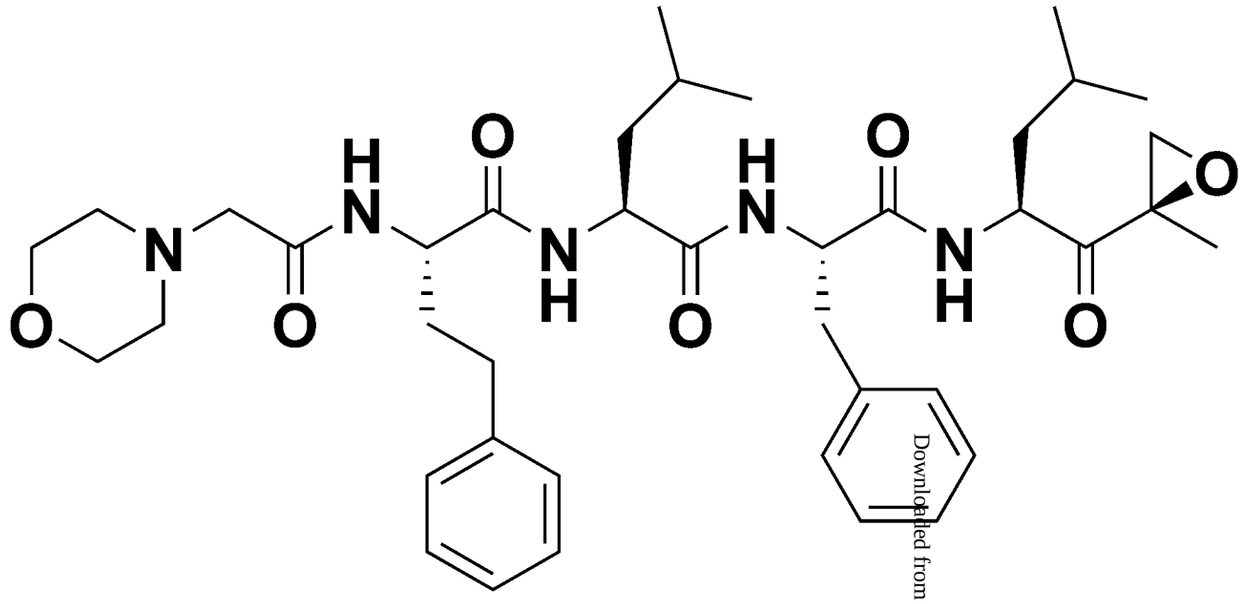
[a] RP = Rat plasma, RU = Rat urine, RB = Rat bile.
[b] These metabolites were also observed in monkey plasma samples obtained in a 9-month toxicity study.

Table 4. Percent (%) of carfilzomib dose detected in rat urine and bile following a single i.v. bolus administration of carfilzomib at 2 mg/kg.

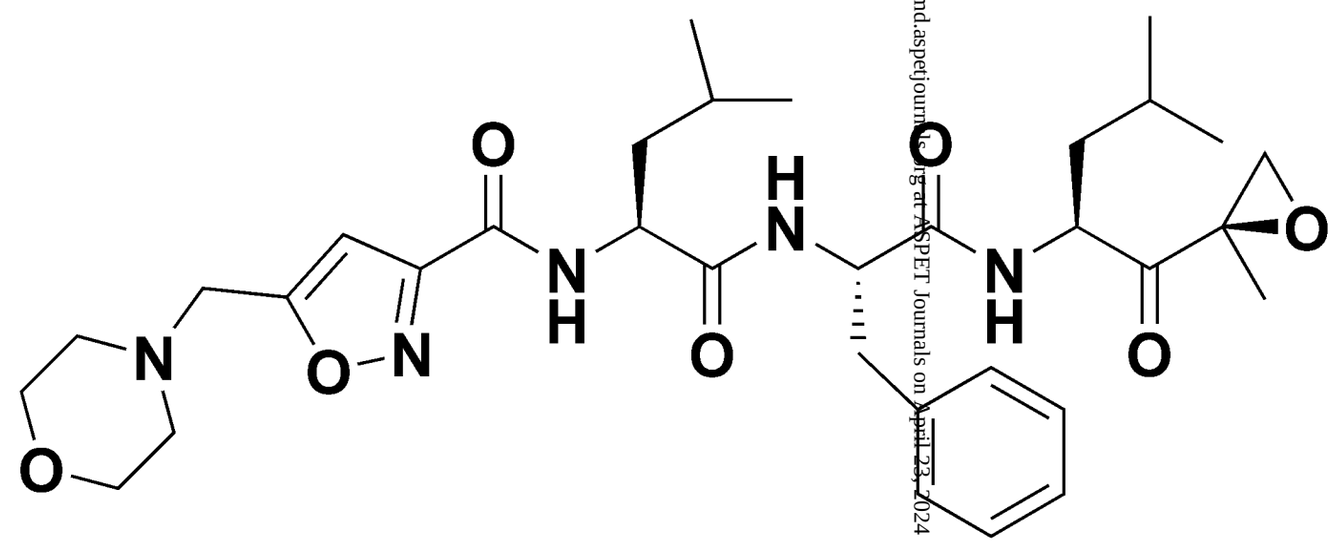
Analyte	Time (hr)	Percent (%) of dose (urine)	Percent (%) of dose (bile)
		Mean ± SD	
Carfilzomib	0–4	0.032 ± 0.021	0.017 ± 0.019
	4-8	0.005 ± 0.007	BQL
	8–24	0.003 ± 0.004	NA
M14	0–4	18.6 ± 10.6	12.9 ± 2.3
	4-8	3.86 ± 2.75	0.230 ± 0.108
	8–24	3.29 ± 1.29	NA
M15	0–4	0.164 ± 0.127	14.7 ± 2.1
	4-8	0.027 ± 0.034	0.076 ± 0.056
	8–24	0.045± 0.006	NA
M16	0–4	0.127 ± 0.100	2.64 ± 3.09
	4-8	0.027 ± 0.045	0.013 ± 0.010
	8–24	0.010 ± 0.006	NA
Total	0–24	26 ± 12	31 ± 5

NA: not applicable, bile samples were not collected from 8-24 hours.
BQL: below the quantitation limit.

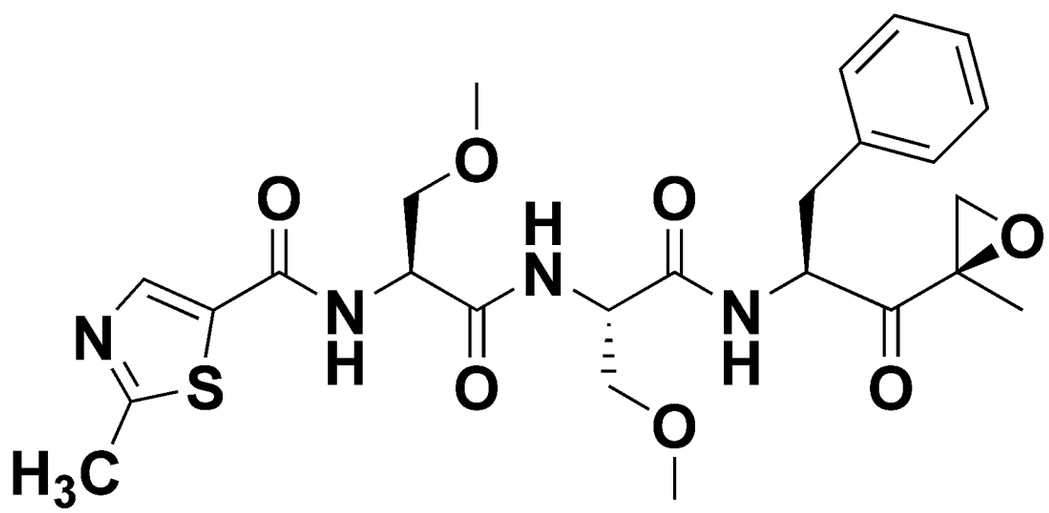
Figure 1



Carfilzomib



PR-054591 (IS)



ONX 0912

Figure 2

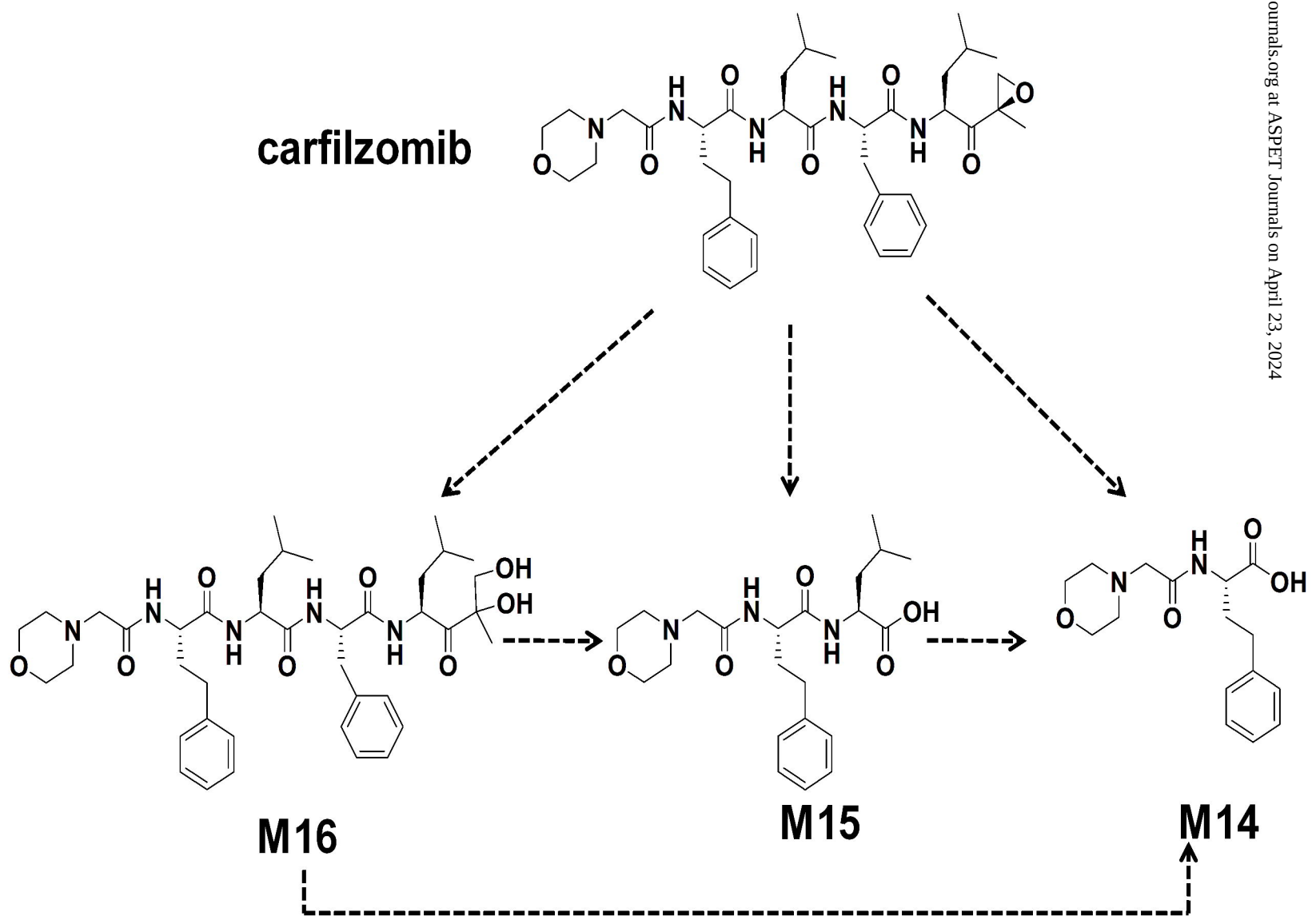


Figure 3

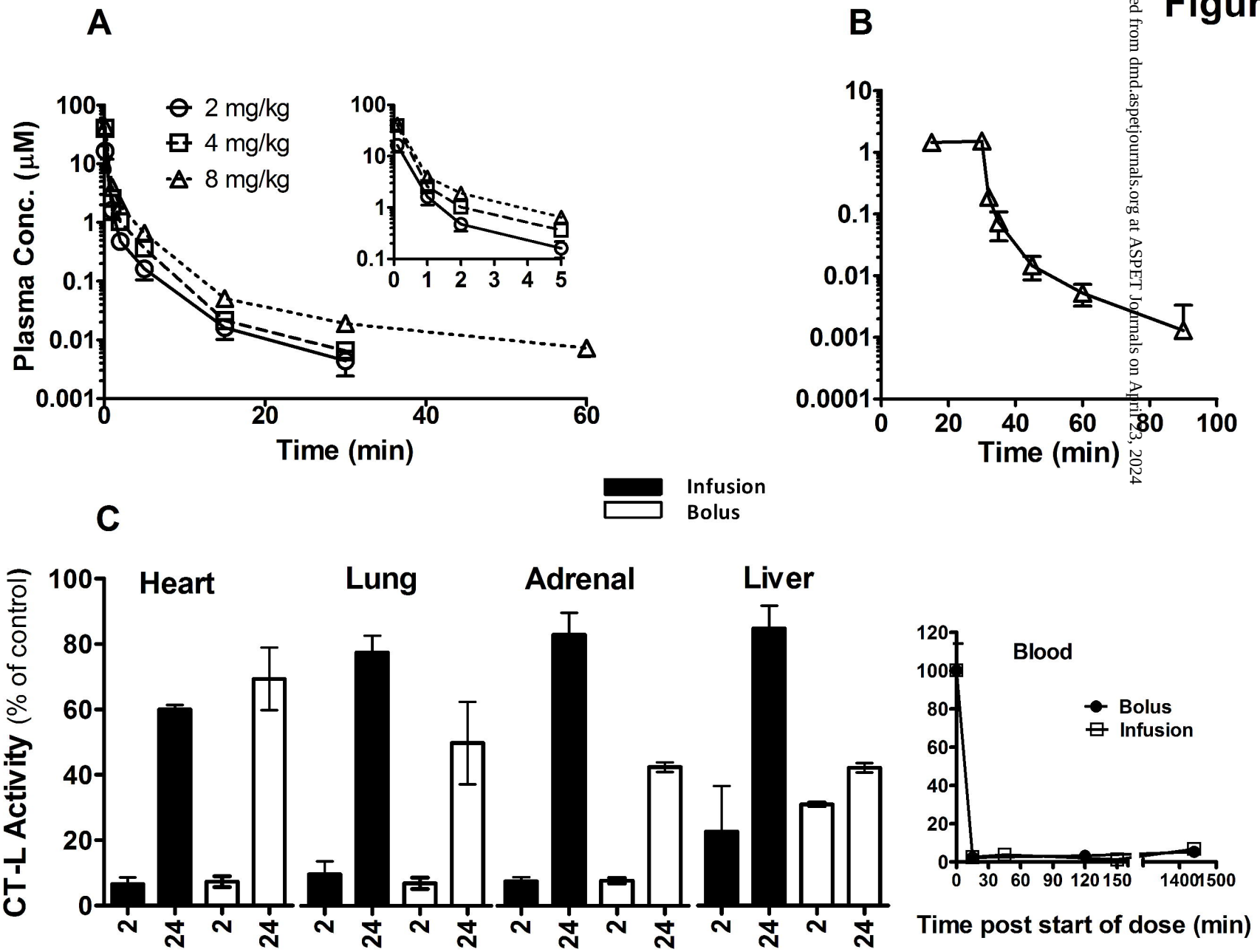


Figure 4

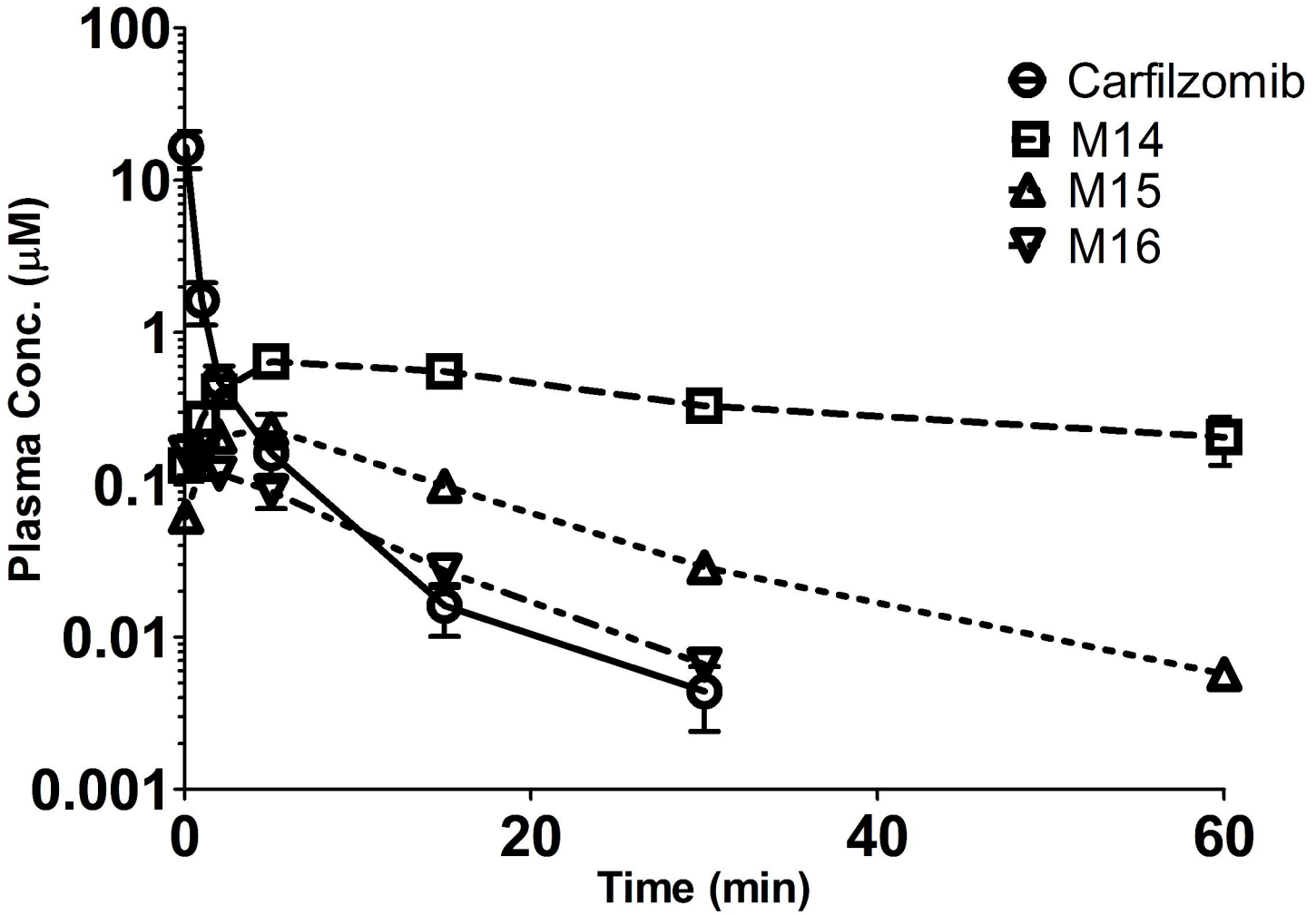
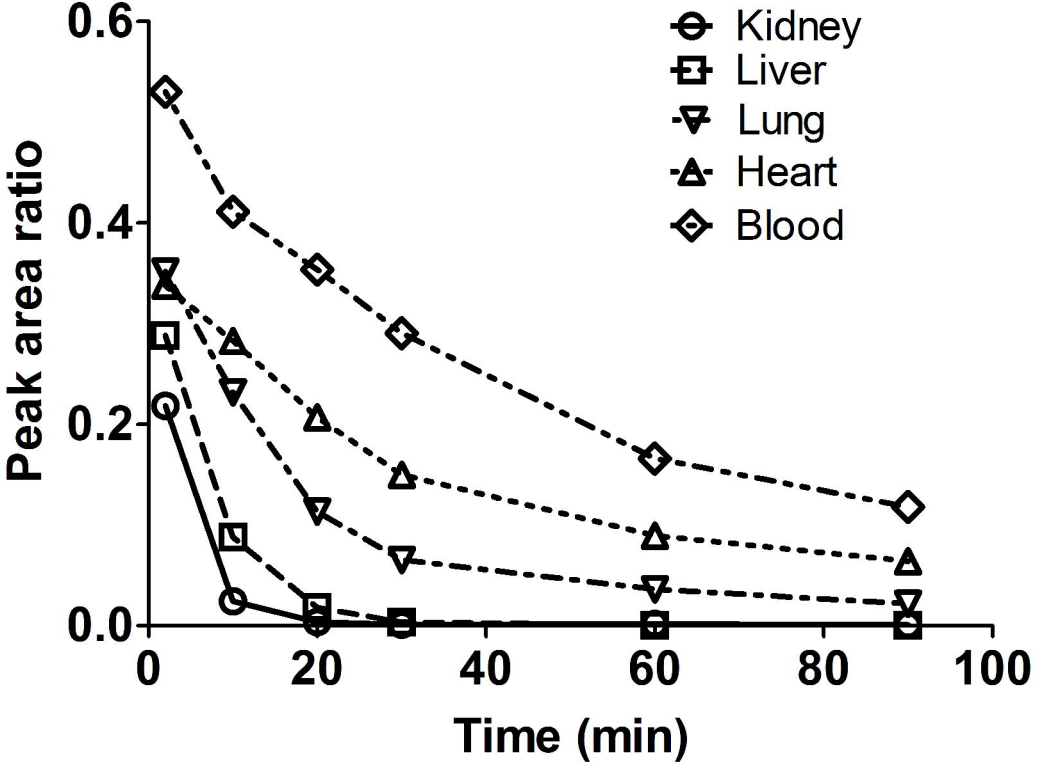

100
10
1
0.1
0.01
0.001
Plasma Conc. (μM)
0
20
40
60
Time (min)
Carfilzomib
M14
M15
M16

Figure 5


0.6
0.4
0.2
0.0
Peak area ratio
0
20
40
60
80
100
Time (min)
Kidney
Liver
Lung
Heart
Blood

Drug Metabolism and Disposition

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

Jinfu Yang, Zhengping Wang, Ying Fang, Jing Jiang, Frances Zhao, Hansen Wong, Mark K. Bennett, Christopher J. Molineaux and Christopher J. Kirk

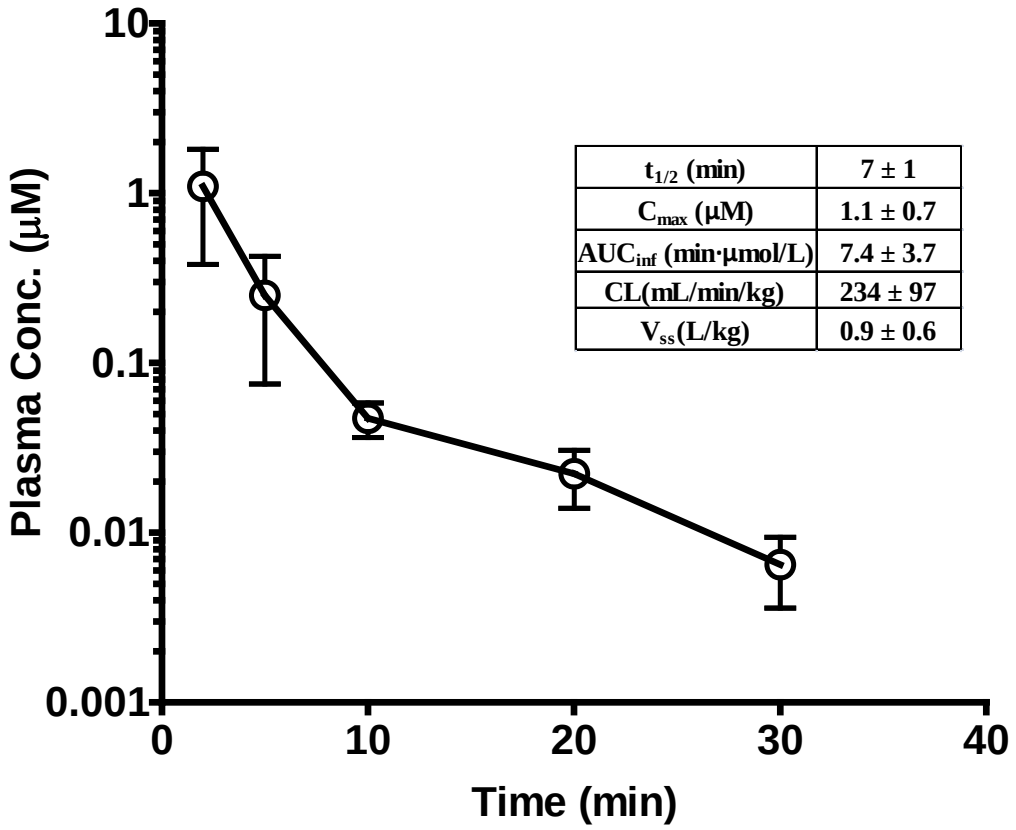


Figure S1. Pharmacokinetics (PK) of carfilzomib following single i.v. bolus administration to non-näive male cynomolgus monkeys at 1 mg/kg. Plasma samples were collected prior to dose, and at 2, 5, 10, 20, 30 and 60 minutes post-dose. Plasma samples immediately after dosing were not collected. Because of the sharp decline of carfilzomib concentration in plasma immediately after dosing, the clearance might have been overestimated and C_{max} underestimated with this blood sampling schedule.

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

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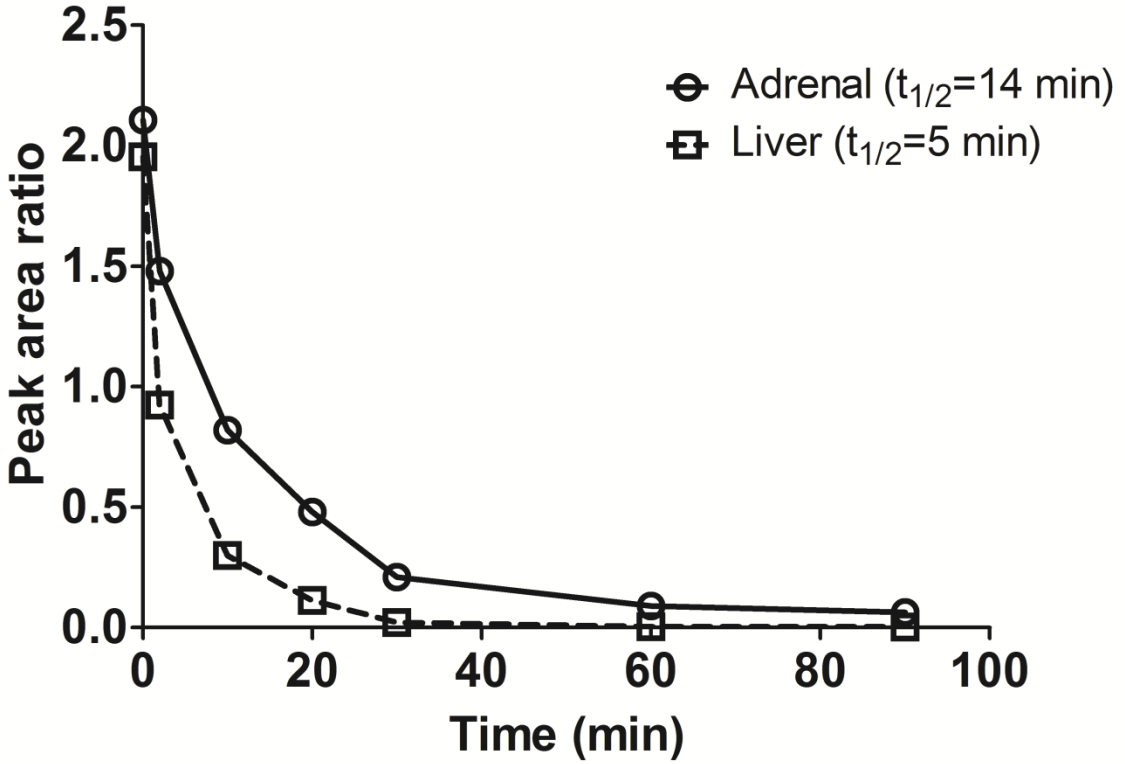

Figure S2. Stability of carfilzomib in rat liver and adrenal gland homogenates. Incubations were done at 37ºC with a carfilzomib concentration of 1000 ng/mL. Aliquots of incubation mixture were taken at 2, 10, 20, 30, 60 and 90 minutes to determine the level of carfilzomib remaining.

Drug Metabolism and Disposition

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

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PK parameters:	5 mg/kg i. v. bolus
$t_{½}$ (min)	18 ± 2
C_{max} (µM)	8.6 ± 3.3
AUC_{last} (min·µmol/L)	65.6 ± 20.1
AUC_{inf} (min·µmol/L)	65.6 ± 20.1
AUC_{inf}/D (min·kg·µmol/L/µmol)	9.7 ± 3.0
CL (mL/min/kg)	110 ± 31
V_{SS} (L/kg)	0.7 ± 0.3

Table S1. Pharmacokinetic parameters of PR-519 obtained from single i. v. bolus administration to male Sprague Dawley rats at 5 mg/kg