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Title: Clinical Pharmacokinetics, Metabolism, and Drug-Drug Interaction of Carfilzomib

Authors: Jinfu Yang, Zhengping Wang, Christopher Kirk, Ying Fang, Melissa Alsina, Ashraf Badros, Kyriakos Papadopoulos, Alvin Wong, Tina Woo, Darrin Bomba, Jin Li, Jeffrey R. Infante

Affiliations: Onyx Pharmaceuticals, South San Francisco, California (J.Y., Z.W., C.K., Y.F., A.W., T.W., D.B., J.L.); H. Lee Moffitt Cancer and Research Center, Tampa, Florida (M.A.); Greenebaum Cancer Center, University of Maryland, Baltimore, Maryland (A.B.); The START (South Texas Accelerated Research Therapeutics) Center for Cancer Care, San Antonio, Texas (K.P.); Sarah Cannon Research Institute, Nashville, Tennessee (J.R.I.)

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Running Title: Clinical DMPK, and Drug-Drug Interaction of Carfilzomib

Correspondence: Jinfu Yang; Phone: (650) 870-1028; Email: jyang@calithera.com

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Discussion: 1,290 words

Abbreviations

ANOVA, analysis of variance; AUC_{0-12}, AUC from time zero to 12 h; AUC_{inf}, area under the plasma concentration-time curve extrapolated to infinity; AUC_{last}, area under the plasma concentration time curve from time zero to the time of last quantifiable concentration; C_{max}, maximum plasma concentration; CI, confidence interval; CL, clearance; CYP, cytochrome P450; DDI, drug-drug interaction; e^x, exponentiated; GLM, geometric linear model; HLM, human liver microsomes; IC_{50}, 50% inhibitory concentration; IV, intravenous; K_I, carfilzomib concentration that caused half the maximal rate of inactivation; k_{inact}, maximal rate of enzyme inactivation; LC MS/MS, liquid chromatography tandem mass spectrometry; LS, least-squares; M14, morpholino-hPhe; M15, morpholino-hPhe-Leu; M16, morpholino-hPhe-Leu-Phe-Leu-diol; MM, multiple

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myeloma; PK, pharmacokinetic; PPB, plasma protein binding; qPCR, quantitative polymerase chain reaction; RED, Rapid Equilibrium Dialysis; $t_{1/2}$, terminal half life; V_{ss}, volume of distribution at steady state.

Abstract

Carfilzomib, an irreversible proteasome inhibitor, has a favorable safety profile and significant anti-tumor activity in patients with relapsed and refractory multiple myeloma (MM). Here we summarize the clinical pharmacokinetics (PK), metabolism, and drug-drug interaction (DDI) profile of carfilzomib. The PK of carfilzomib, infused over 2–10 min, was evaluated in patients with solid tumors or MM. Metabolites of carfilzomib were characterized in patient plasma and urine samples. In vitro drug metabolism and DDI studies were conducted in human liver microsomes and hepatocytes. A clinical DDI study was conducted in patients with solid tumors to evaluate the effect of carfilzomib on cytochrome (CYP)3A activity. Plasma concentrations of carfilzomib declined rapidly and in a biphasic manner following intravenous administration. The systemic half life was short and the systemic clearance rate was higher than hepatic blood flow. Carfilzomib was cleared largely extrahepatically via peptidase cleavage and epoxide hydrolysis. CYP-mediated metabolism played a minor role, suggesting that co-administration of CYP inhibitors or inducers is unlikely to change its PK profile. Carfilzomib showed direct and time-dependent inhibition of CYP3A in human liver microsome preparations and exposure to carfilzomib resulted in reductions in CYP3A and 1A2 gene expression in cultured human hepatocytes. However, administration of carfilzomib did not affect the PK of midazolam in patients with solid tumors, and there were no safety signals indicative of potential drug interactions. We conclude that the rapid systemic clearance and short half life of carfilzomib limit clinically significant DDI.

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Introduction

The proteasome is a multicatalytic protease complex that plays a critical role in mediating the regulated degradation of intracellular proteins. The dipeptide boronate bortezomib (Velcade®, Millennium Pharmaceuticals, Inc., Cambridge, MA), is a reversible proteasome inhibitor first approved in 2003 for the treatment of multiple myeloma (MM) and mantle cell lymphoma (Bross et al., 2004) and has validated the proteasome as a therapeutic target in B-cell neoplasms. As a result, several next-generation agents have entered clinical trials (Bennett and Kirk, 2008; Dick and Fleming, 2010). The tetrapeptide epoxyketone carfilzomib is the first irreversible proteasome inhibitor to have been approved by Food and Drug Administration (FDA) for the treatment of relapsed and refractory multiple myeloma. It differs from bortezomib both in the duration of proteasome inhibition and in its selectivity for the unique class of 7 proteases that encompass the proteasome active sites (Demo et al., 2007; Arastu-Kapur et al., 2011). In several phase 2 studies, single-agent carfilzomib has demonstrated significant anti-tumor activity and a favorable safety profile (Martin et al., 2010; Siegel et al., 2012; Vij et al., 2012). Furthermore, preliminary data suggest that the combination of carfilzomib with lenalidomide and low-dose dexamethasone, with each drug administered at full doses and for an extended duration, was effective and well tolerated in patients with MM (Niesvizky et al., 2009). Preclinical studies in rats and monkeys have shown that carfilzomib is rapidly and extensively distributed and potently inhibits proteasome activity in a variety of tissues following intravenous (IV) administration (Yang et al., 2011). Carfilzomib has a systemic clearance (CL) greater than hepatic blood flow and a terminal half life ($t_{1/2}$) shorter than 30 min. The rapid elimination of carfilzomib is mediated primarily by metabolism via peptidase cleavage and epoxide hydrolysis (Yang et al., 2011), making carfilzomib a unique small molecule therapeutic agent.

Here we describe the pharmacokinetic (PK) and metabolic profiles of carfilzomib in patients with either solid tumors or MM. The major elimination pathways of carfilzomib were characterized in vitro using human hepatocytes and ex vivo using human plasma and urine samples. In vitro studies were performed to assess the effects of carfilzomib on the activity of cytochrome P450 (CYP) enzymes. A clinical drug-drug interaction (DDI) study is presented to evaluate the effect of carfilzomib on the PK of midazolam.

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Materials and Methods

All clinical trials were conducted according to Good Clinical Practice standards. The protocol, informed consent, and other relevant study documentation were approved by the appropriate Institutional Review Board at each participating site. All participants provided written informed consent in accordance with federal and institutional guidelines. Analyses of human plasma and urine samples, unless specified otherwise, were done using liquid chromatography tandem mass spectrometry (LC MS/MS) methods fully validated according to US Food and Drug Administration guidance in compliance with Good Laboratory Practice. Quality control (QC) samples covering concentrations across the calibration range were included in each analytical run to ensure accuracy, precision, and reproducibility. The percent deviation from nominal values (accuracy) for all QC samples were ≤ ±15% (≤ ±20% at lower limit of quantification) and the percent coefficient of variation (precision) were ≤ ±15% (≤ ±20% at lower limit of quantification). All samples were analyzed within the established stability period for sample collection and storage.

Pharmacokinetic studies

Plasma samples for PK analysis of carfilzomib were taken from patients participating in an open-label, phase 1b/2, multicenter study with relapsed solid tumors (PX-171-007; NCT00531284). Carfilzomib was administered to 3 patients intravenously over 2–10 min at a dose of 20 mg/m^2 on Days 1, 2, 8, 9, 15, and 16 of a 28-day cycle. Patients received 4 mg oral or IV dexamethasone before each carfilzomib dose for the first cycle. Plasma samples were collected on Days 1 and 16 of Cycle 1 prior to carfilzomib dosing, at the end of drug administration, and at 5, 15, and 30 min, and 1, 2, and 4 h after the end of administration. Samples were processed by solid phase extraction using Oasis® HLB 10 mg cartridges (Waters Corporation, Milford, MA) followed by LC MS/MS analysis to measure the plasma concentration of carfilzomib. A deuterated analogue (d_{10}-carfilzomib) was used as the internal standard for quantification with a calibration range of 0.100–200 ng/mL.

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PK parameter calculations, using the actual elapsed time relative to the start of infusion, including maximum plasma concentration (C_{max}), area under the plasma concentration time curve from time zero to the time of last quantifiable concentration (AUC_{last}), area under the plasma concentration-time curve extrapolated to infinity (AUC_{inf}), $t_{1/2}$, CL, and volume of distribution at steady state (V_{ss}), were carried out using noncompartmental methods (constant IV infusion model 202) in WinNonlin® Enterprise Version 5.2 (Pharsight, St Louis, MO), and statistical analyses were performed using SAS Version 9.2 (SAS Institute, Inc., Cary, NC).

Plasma protein binding

Plasma protein binding (PPB) of carfilzomib was determined using plasma samples collected in a phase 2, open-label, multicenter study in MM patients with varying degrees of renal dysfunction (PX-171-005; NCT00721734)(Niesvizky et al., 2011). In that study, patients received 15 mg/m^2 IV carfilzomib over 2–10 min on Days 1, 2, 8, 9, 15, and 16 of a 28-day cycle. If patients tolerated the first cycle of treatment, the dose was escalated to 20 mg/m^2 in Cycle 2. Plasma samples were collected at end of drug administration and 5 min after drug administration on Days 1 and 15 of Cycle 1 and Day 15 of Cycle 2. Plasma samples were dialyzed at 37°C against sodium phosphate buffer (pH 7.4) for 6 h using a Rapid Equilibrium Dialysis (RED) Device (Thermo Fisher Scientific, Inc., Rockford, IL). At the end of dialysis, aliquots of plasma samples were mixed with an equal volume of phosphate buffer, and aliquots of dialysates were mixed with an equal volume of blank plasma. Carfilzomib was then extracted by acetonitrile protein precipitation and analyzed using a non-validated LC-MS/MS method.

Metabolism

Plasma and urine samples collected in a separate phase 1 clinical trial (PX-171-002; NCT00150462)(Alsina et al., 2012) were used to characterize the metabolic profile of carfilzomib. In this trial, patients with relapsed and/or refractory hematologic malignancies received carfilzomib intravenously at 20 or 27 mg/m^2 following the dosing schedule described for PX-171-007. Plasma samples were collected pre-dose and at 5,

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15, and 30 min and 1, 2, and 4 h after administration, while urine samples were collected from 0 to 4 h post administration on Cycle 1 Day 1. Equal volumes of plasma or urine samples from 2–4 patients at each dose level and time point were pooled and analyzed by LC-MS/MS for metabolite profiling based on molecular mass and fragmentation patterns (XenoBiotic Laboratories; Plainsboro, NJ) as previously described (Yang et al., 2011). Structures of major metabolites, M14 (morpholino-hPhe), M15 (morpholino-hPhe-Leu), and M16 (morpholino-hPhe-Leu-Phe-Leu-diol), were further confirmed by authentic standards. The PK and excretion of M14, M15, and M16 were then determined in human plasma and urine samples collected in the PX-171-005 study. For PK, plasma samples were collected prior to dosing, at the end of the infusion, at 5, 15, and 30 min and 1, 1.5, 2, 4, 6, and 24 h post-dosing on Day 1 of Cycle 1. Samples were processed by protein precipitation and analyzed using a LC MS/MS method with a calibration range of 0.300□300 ng/mL for carfilzomib and 0.500□500 ng/mL for metabolites using deuterated analogues as the internal standards. For excretion, urine samples were collected from 0–5 h and 5–24 h post-injection on Day 1 of Cycle 1. Acetonitrile (25% of the sample volume) was added to the bulk urine samples to minimize potential binding of carfilzomib to the collection container. The treated samples were then aliquotted and analyzed over a concentration range of 4.00–2000 ng/mL for carfilzomib and M15, and 10.0–5000 ng/mL for M14.

Pooled human hepatocytes (XenoTech LLC, Lenexa, KS) suspended in Waymouth Medium (Sigma-Aldrich Co., LLC, St. Louis, MO) were incubated with 1 µM carfilzomib at 37°C in an atmosphere of 5% CO_2 at a final viable cell density of approximately 0.5 x 10^6 cells/mL in the presence and absence of known chemical inhibitors for each CYP isoform. Incubations with specific CYP substrates were also conducted as positive controls. At 0, 15, 30, 60, and 120 min, the reactions were quenched by adding 200 µL of acetonitrile containing appropriate internal standards. Concentrations of carfilzomib and major metabolites were determined using a non-validated LC-MS/MS method following protein precipitation.

In vitro CYP inhibition

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Human liver microsomes (HLM) (XenoTech LLC, Lenexa, KS) pooled from 16 donors of mixed genders were incubated at 37°C in duplicate with varying concentrations of carfilzomib (0.01–10 μM) to determine the inhibitory activity against 6 major human CYP isoforms (1A2, 2C8, 2C9, 2C19, 2D6, and 3A). The inhibitory potentials of M14, M15, and M16 on human CYP3A were assessed at concentrations of 0.04–30 μM. Substrates for the individual CYP isoforms were phenacetin (1A2; K_m =39 μM), amodiaquine (2C8; K_m =6.4 μM), diclofenac (2C9; K_m =5.9 μM), *S*-mephenytoin (2C19; K_m =36 μM), dextromethorphan (2D6; K_m =7.8 μM), testosterone (3A; K_m =100 μM), and midazolam (3A; K_m =3.8 μM). The microsomal protein concentration was 0.1 mg/mL, except for incubations with midazolam, where a protein concentration of 0.05 mg/mL was used. After 5 min, the reactions were quenched by protein precipitation with acetonitrile containing appropriate internal standards, and analyzed using validated LC-MS/MS methods (Rodrigues, 2008).

For IC_{50} measurements, determination of NADPH dependence and resistance to dilution, the concentrations of probe substrates were prepared to be approximately equal to their K_m values. For K_i measurements, the concentrations of midazolam were approximately equal to 0.3×K_m, K_m, 3×K_m, 6×K_m, and 10×K_m in the absence of carfilzomib or at carfilzomib concentrations ranging from 0.5–10 μM.

To determine the inactivation potency of carfilzomib on CYP3A, carfilzomib was preincubated in duplicate at 0, 0.5, 1, 3, 5, and 8 μM with pooled HLM (2.5 mg/mL for testosterone and 1.25 mg/mL for midazolam) and an NADPH-generating mixture (1 mM NADP, 5 mM glucose-6-phosphate and 1 unit/mL glucose-6-phosphate dehydrogenase) for 0, 5, 10, 20, and 30 min. After preincubation, 16 μL aliquots of the mixtures were diluted 25-fold with 50 mM potassium phosphate buffer containing 3 mM $MgCl_2$ and 1 mM EDTA (pH 7.4) and incubated with testosterone (200 μM) or midazolam (40 μM) and NADPH-generating mixture for 5 min to measure the residual enzymatic activity. K_I (a carfilzomib concentration that caused half the maximal rate of inactivation) and k_{inact} (maximal rate of enzyme inactivation) were determined.

In vitro CYP induction

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Human hepatocytes from 3 donors (BD Biosciences, Woburn, MA) were seeded at a density of ~2.0×10^5 viable cells per cm^2 in 24-well, collagen I-coated plates and maintained in Williams' Medium E (Sigma-Aldrich Co., LLC, St. Louis, MO) for 2 days prior to treatment with solvent control (0.08% DMSO), carfilzomib (0.1, 0.5, and 2.5 μM), rifampicin (20 μM, an inducer of CYP3A), or β-naphthoflavone (20 μM, an inducer of CYP1A2) for an additional 3 days with daily media and compound changes. After this, the medium was aspirated and replaced with fresh serum-free hepatocyte assay medium. Cells were incubated in triplicate with 200 μL of testosterone (200 μM) or phenacetin (100 μM), specific substrates for CYP3A and CYP1A2, respectively, for 30 and 60 min. The rates of 6β-hydroxytestosterone and acetaminophenol formation were measured by LC-MS/MS (Perloff et al., 2009). To test the potential inhibitory effects of carfilzomib on CYP catalytic activity, cells exposed to the positive control inducers were treated with fresh medium containing 2.5 μM carfilzomib for 30 min and washed once with drug-free medium prior to incubation with probe substrates for CYP1A2 and CYP3A activity measurement. Cellular toxicity assays were carried out using 3-[4, 5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT, Promega Corporation, Madison, WI), and expression of CYP3A and CYP1A2 mRNA was determined by quantitative PCR (qPCR).

Clinical drug interaction study

Patients (n=18) with solid tumors received a single 2 mg oral dose of midazolam on Day -7 followed by IV administration of carfilzomib at 27 mg/m^2 over 2–10 min on Days 1, 2, 8, 9, 15, and 16 of a single 28-day cycle. Patients also received a 2 mg oral dose of midazolam immediately following carfilzomib on Days 1 and 16. Plasma samples were collected pre-dose, at 5, 10, and 30 min, and 1, 2, 3, 4, 6, 8, 12, and 24 h post-midazolam dose on Days -7, 1, and 16. Midazolam concentrations in plasma were determined using automated liquid-liquid extraction with methyl *tert*-butyl ether followed by LC-MS/MS analysis across a calibration range of 0.100–100 ng/mL using d_4-midazolam as the internal standard. The PK profile of carfilzomib was determined as described above, using samples obtained on Day 1.

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PK analyses were performed via non-compartmental methods using WinNonlin® 5.2 (Pharsight Corporation, Mountain View, CA) to determine the midazolam pharmacokinetic parameters T_{max}, C_{max}, AUC from time zero to 12 h ($AUC_{0–12}$), AUC_{last}, AUC_{inf}, and $t_{1/2}$. $AUC_{0–12}$, instead of AUC_{last}, determined on Day 16 was used to compare with that on Day 1 because plasma samples were not collected at 24 h post dose on Day 16. Descriptive statistics for the plasma concentrations versus time as well as all PK parameters were calculated for each treatment [midazolam alone (Day -7) and co-treatment with carfilzomib (Days 1 and 16)]. Using the geometric linear model (GLM) procedure in SAS® (Version 9.2, SAS Institute, Inc., Cary, NC), analysis of variance (ANOVA) was performed on the *ln*-transformed AUC_{last} (for Day -7 and Day 1 only), AUC_{0-12} (For Day -7 and Day 16 only), AUC_{inf}, and C_{max} data at the alpha level of 0.05. Geometric mean ratios of midazolam with and without carfilzomib treatment (ie, Day 1 versus Day -7, and Day 16 versus Day -7) with 90% confidence intervals (CI) were calculated. Based on the analysis of *ln*-transformed data, the point estimates and 90% CI for the least-squares (LS) mean difference between treatments [(Day 1)–(Day -7)] were exponentiated (e^x) to obtain point estimates and 90% geometric CIs for the ratio [(Day 1)/(Day -7)] on the original scale.

As defined in the protocol, no clinically significant change in midazolam exposure was to be concluded if the 90% geometric CI of the ratio [(Day 1)/(Day -7) and (Day 16)/(Day -7)] of LS means from the ANOVA of the *ln*-transformed AUC_{last} [(Day 1)/(Day -7) only], AUC_{0-12} [(Day 16)/(Day -7) only], AUC_{inf}, and C_{max} were entirely within the standard equivalence range of 80–125%. Interactions were described as strong, moderate, or weak if AUC alterations were >5-fold, 2- to 5-fold, or 1.25- to 2-fold, respectively.

Results

Pharmacokinetics and metabolism of carfilzomib

Following IV administration of a dose of 20 mg/m^2 to patients with solid tumors, carfilzomib concentrations declined rapidly with time in a biphasic manner, and the majority of the drug was eliminated from the plasma compartment within 30 min (**Figure 1** and **Table 1**). Plasma CL was 146 ± 22 and 136 ± 53 L/hr on Days 1 and 16, respectively, exceeding hepatic blood flow (Kwon, 2001). Similar to results from an earlier

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in vitro rat blood partitioning experiment (Yang et al., 2011), preliminary data showed that carfilzomib had a low blood to plasma ratio in human blood (data not shown), indicating that the blood clearance is higher than the plasma clearance. These results suggest a significant contribution of extrahepatic mechanisms to carfilzomib elimination. No accumulation of carfilzomib was detected between doses, and exposure to carfilzomib was not changed upon repeat dosing. Carfilzomib was extensively protein bound in the plasma; PPB was 97.6–98.2%, independent of carfilzomib concentration across 21.6–7841 ng/mL and not affected by the status of renal function (data not shown).

Pooled plasma and urine samples derived from a phase 1 trial (Alsina et al., 2012) were used to determine the metabolic fate of carfilzomib following administration. A total of 15 metabolites were identified in human plasma and urine samples (**Supplemental Table 1**). The major metabolites (M14, M15 and M16) were derived from peptidase cleavage and epoxide hydrolysis of carfilzomib and their MS/MS spectra are shown in **Figure 2**. CYP-mediated metabolites detected only at very low levels. This correlated with the in vitro study in which the rate of carfilzomib metabolism in human hepatocytes was unaffected by the addition of inhibitors of the major CYP enzymes (**Supplemental Table 2**). Fourteen of the metabolites detected in human plasma and urine were also detected in animal studies, with only M6, a sulfated tyrosine detected at low levels in human urine samples, not observed in animal samples (Yang et al., 2011). M14, M15, and M16 were detectable in plasma immediately following carfilzomib administration (**Figure 3)**. In patients with normal renal function (creatinine clearance >80 mL/min), the median $t_{1/2}$ were 1.53, 1.47, and 0.64 h, and the relative exposures of the metabolites compared to the exposure of carfilzomib (mean AUC_{inf} ratio) were 1.20, 0.11, and 0.28 for M14, M15, and M16, respectively (**Supplemental Table 3**). Plasma levels of M16 were not affected by the degree of renal function; however, the relative exposures to M14 and M15 were about 7-fold and 1.8-fold higher, respectively, in patients receiving chronic dialysis relative to those with normal renal function (**Supplemental Table 3**). In urine samples collected from patients with normal renal function, M14 and M15 accounted for approximately 33% and 1.9% of the total carfilzomib dose (**Supplemental Table 4**), respectively, while carfilzomib accounted for <1% of the dose. M16 was determined to be a minor metabolite in urine and was not quantified in urine samples.

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In vitro inhibition and induction potential of carfilzomib on human CYPs

The epoxyketone pharmacophore of carfilzomib has the potential to covalently interact with and inhibit CYP enzymes. Carfilzomib's potential to alter the exposure of concomitantly administered medications was assessed in vitro. In the CYP inhibition study using HLM, the 50% inhibitory concentration (IC_{50}) values for CYP1A2, 2C8, 2C9, 2C19, and 2D6, were >10 µM (7194 ng/mL), the maximum concentration tested. This was greater than the mean C_{max} values from the clinical studies described above. In contrast, carfilzomib acted as a direct inhibitor of CYP3A (**Figure 4A**). The effects of carfilzomib were more pronounced when midazolam (IC_{50}: 1.7 µM) was used as the CYP3A substrate relative to testosterone (>10 µM) and the inhibition was competitive. The inhibitory effect of carfilzomib on human CYP3A was time-dependent. When carfilzomib was preincubated with HLM for 30 min, the IC_{50} value decreased from 1.7 to 0.49 µM when using midazolam as the substrate, and from >10 to 0.97 µM when testosterone was used as the substrate. This time-dependent inhibition required NADPH as a co-factor and was resistant to dilution. This suggests that carfilzomib is an irreversible or quasi-irreversible inhibitor of CYP3A **(Supplemental Figure 1)** and the time-dependent inhibition is not due to direct reaction of the epoxyketone with CYP3A. The K_I was determined to be 11 µM and the k_{inact} was 0.10 min^{-1} using both substrates.

Based on the in vitro inhibition results and the data on the exposure of carfilzomib in patients, we estimated the ratio of intrinsic clearance values (R values) of a CYP3A probe substrate in the absence and presence of carfilzomib using a basic model (US Food and Drug Administration, 2012). The R1 value for direct inhibition was approximately 4.5–5.7 using a total maximum plasma concentration of 5.9–8.0 µM (unpublished data and Supplemental Figure 2) with a carfilzomib dose of 27 mg/m^2. The calculated R2 value for time-dependent inhibition ranged from 84–540 using the maximum carfilzomib plasma concentration (above), the values of k_{inact} and K_I measured in this study, and the reported K_{deg} values for CYP3A4 (Yang et al., 2008).

Neither of the major circulating metabolites, M14 and M15, showed either direct or time-dependent inhibition of CYP3A at a concentrations up to 30 µM, whereas M16 showed time-dependent inhibition of

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CYP3A as the IC_{50} value decreased from >30 μM to 1.8 μM in the absence or presence of a 30-min preincubation (data not shown). The effect of carfilzomib on the activity and expression of CYP1A2 and 3A was evaluated by treating cultured primary human hepatocytes with varying concentrations of carfilzomib. CYP3A activity was decreased in a concentration-dependent manner following 3 days of treatment (**Figure 4B**). At a carfilzomib concentration of 2.5 μM, CYP3A4 activity decreased by 45–96%, and CYP1A2 activity dropped to below the limit of quantification in 2 of 3 hepatocyte cultures. Exposure to rifampicin (20 μM) or β-naphthoflavone (20 μM), known inducers of CYP3A and CY1A2, resulted in 14–50-fold or 9–47-fold induction of enzyme activity, respectively. In addition, cell viability was unaffected by the exposure to carfilzomib (data not shown), demonstrating the cell cultures were suitable for assessment of CYP induction. When rifampicin-treated hepatocyte cultures were incubated with carfilzomib at 2.5 μM for 30 min, only a 14–23% decrease in CYP3A activity was observed, suggesting that reduced enzymatic activity in human hepatocytes upon carfilzomib treatment for 3 days was unlikely to be due to enzyme inhibition.

Exposure to carfilzomib resulted in a concentration-dependent decrease in gene expression relative to solvent controls, with >95% decrease for CYP3A (3 donors) and ~40% decrease for CYP1A2 (1 donor) at 2.5 μM (**Figure 4C**). In contrast, exposure of cells to known CYP inducers resulted in increases in gene expression proportionate to the changes in enzymatic activity.

Because carfilzomib demonstrated an inhibitory effect on midazolam metabolism in HLM and reduced CYP3A activity and expression in human hepatocytes, a drug interaction study in patients with solid tumors was conducted to determine whether carfilzomib administration would alter the exposure of a CYP3A substrate in a physiological setting. Of 18 patients enrolled, 17 received at least 1 dose of carfilzomib, and 12 patients completed a full cycle of administration. **Figure 4D** depicts the mean plasma concentration versus time profiles for midazolam in samples taken prior to carfilzomib administration and on Days 1 and 16 of Cycle 1 of carfilzomib dosing. **Table 2** lists the PK parameters of midazolam. The 90% geometric CI of the ratios of midazolam exposure prior to carfilzomib dosing (Day -7) and after a single dose of carfilzomib (Day 1) fell within the equivalence range of 80–125% (**Supplemental Table 5**), indicating there was no clinically significant effect of carfilzomib on the PK of midazolam. Similarly, repeat dosing of carfilzomib

failed to demonstrate a major impact on midazolam exposure. Administration of carfilzomib to these patients resulted in systemic clearance similar to those described above (**Supplemental Figure 2**). In addition, no safety signals suggesting an over exposure to midazolam arose during the cycle of co-administration of the 2 compounds, providing further supporting evidence for a lack of a drug interaction (**Supplemental Table 6**).

Discussion

Carfilzomib is a potent, irreversible inhibitor of the chymotrypsin-like activity of the proteasome that displays rapid tissue distribution, high systemic clearance, and a short half life in animal models (Demo et al., 2007; Yang et al., 2011). In patients with MM, significant anti-tumor activity and a favorable safety profile have been reported (Martin et al., 2010; Siegel et al., 2012). However, the presence of a reactive pharmacophore makes an assessment of the PK, metabolism, and drug interactions of carfilzomib essential to understanding the practical clinical use of this promising new agent. The data presented here extend earlier preclinical findings and show that carfilzomib's characteristic PK profile results in a lack of drug interactions in patients.

In animals, following single doses of up to 48 mg/m^2, carfilzomib displayed rapid clearance from plasma and a nearly instantaneous formation of metabolites (Yang et al., 2011). Consistent with these observations, carfilzomib displays high systemic clearance and a short half life in patients with solid tumors. A similar PK profile was also observed in patients with hematological malignancies (O'Connor et al., 2009; Alsina et al., 2012) and MM having varying degrees of renal dysfunction (Niesvizky et al., 2011). The rapid clearance of carfilzomib is primarily mediated by metabolism instead of renal excretion. On the other hand, potent proteasome inhibition (≥80%) persists in blood after the drug is cleared systemically (O'Connor et al., 2009) due to the irreversible mechanism of target inhibition. Recovery of proteasome activity is independent of the duration of exposure to carfilzomib and is due to the rate of new proteasome synthesis (Meiners et al., 2003; Demo et al., 2007). These pharmacokinetic and pharmacodynamic properties are distinct from those of reversible inhibitors, where sustained drug exposure at or above the therapeutic concentration is necessary to achieve the desired duration of action (Singh et al., 2011). Therefore, the rapid elimination can minimize

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systemic exposure to carfilzomib and reduce potential off-target toxicities without affecting efficacy. This may partially account for the favorable safety profile of carfilzomib (Singhal et al., 2011).

The major metabolites (M14, M15 and M16) are inactive as proteasome inhibitors and are formed immediately following the administration of carfilzomib via peptidase cleavage and epoxide hydrolysis. The generation of these metabolites is likely to occur largely via extrahepatic mechanisms as supported by preclinical studies using tissue homogenates of lung, heart, and kidney (Yang et al., 2011). Additionally, there were very low levels of oxidative metabolites detected in plasma and urine samples, and the presence of CYP inhibitors did not affect the rate of carfilzomib metabolism in human hepatocytes, further suggesting that CYP enzymes play only a minor role in the clearance of carfilzomib. While a ~7-fold increase in the exposure of M14 in patients receiving chronic dialysis was noted relative to those patients with normal renal function, the lack of a reactive pharmacophore and the absence of an inhibitory effect on CYP enzymes suggest that this increase in exposure is not likely to be clinically relevant. The results from these metabolic studies suggest that carfilzomib can be co-administered with CYP inhibitors or inducers without altering its PK profile. In contrast, potent CYP3A inhibitors and inducers are known to have significant effects on the exposure of bortezomib in patients (Venkatakrishnan et al., 2009; Hellmann et al., 2011), and consequently, co-administration is not recommended.

Assessment of potential CYP inhibition is important in mitigating potential adverse drug effect to co-administered medications. This is particularly true for drugs such as carfilzomib with an electrophilic functional group. In HLM, carfilzomib induced direct and time-dependent inhibition of the metabolism of CYP3A substrates but had minimal effects on the other CYP isoforms. This inhibitory effect was minimal in cultured hepatocytes with elevated CYP3A activity when testosterone was used as the substrate. In a separate experiment, carfilzomib inhibited midazolam metabolism by 30–40% in hepatocytes, with no apparent trend toward time-dependent inhibition (data not shown). The apparent discrepancy in time-dependent inhibition observed in human liver microsomes and hepatocytes may be explained by the differences in the metabolism of carfilzomib in these two in vitro testing systems. The most abundant

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metabolite in human hepatocytes was the diol of carfilzomib (M16). On the other hand, CYP-mediated pathways, which are far less relevant in vivo, predominate in liver microsome incubations (data not shown). In cultured human hepatocytes, carfilzomib decreased the activities of CYP3A and 1A2 due to reductions in the expression of mRNA over a 3-day treatment. The ability of proteasome inhibitors to reduce CYP expression in vitro has been described previously (Zangar et al., 2003; Anwar-Mohamed et al., 2008; Zangar et al., 2008; Acharya et al., 2009), but the mechanism of this effect remains unclear.

Based on the in vitro inhibition results and the data on the exposure of carfilzomib in patients, we estimated the ratio of intrinsic clearance values (R values) of a CYP3A probe substrate in the absence and presence of carfilzomib using a basic model (US Food and Drug Administration, 2012). The results suggest potential drug-drug interaction in patients. In addition, carfilzomib also decreased CYP3A mRNA expression in cultured human hepatocytes, The clinical drug interaction study was therefore designed to assess both the effect of single- and repeat- dose administration of carfilzomib on CYP3A in solid tumor patients. The results of this study indicated that carfilzomib does not significantly alter the PK of midazolam following either single- or repeat-dose administration. Because midazolam is a highly sensitive CYP3A substrate (US Food and Drug Administration, 2012), it is reasonable to conclude that carfilzomib would not be expected to interact with other CYP3A substrates in vivo. Taken together, the results of the present study suggest that carfilzomib can be administered with other medications that are substrates of CYP enzymes without altering their exposure.

The lack of clinically significant drug interactions of carfilzomib with CYP3A may be attributed to the pharmacokinetic properties of carfilzomib. First, the drug is rapidly metabolized following IV administration with a short systemic half life. The mean plasma concentration at 5 min post - infusion was ~20% of the mean C_{max} and was further reduced to <1% by 30 min. Although the true intracellular hepatic concentration of carfilzomib is unknown, the exposure of CYP enzymes to intact carfilzomib is likely to be of a short duration. Furthermore, the major circulating metabolites, M14 and M15, are not inhibitors of CYP3A. Secondly, the NADPH-dependent oxidative metabolic pathway(s) responsible for time-dependent inhibition of CYP3A by carfilzomib and M16 in human liver microsomes were not significant in vivo. This is

supported by the lack of time-dependent inhibition in hepatocyte cultures. Finally, carfilzomib is highly bound to plasma proteins, further limiting the potential exposure of CYP enzymes to the free drug. Indeed, the level of proteasome inhibition in liver following IV administration of carfilzomib to rats was less than that seen in blood and other organs (Demo et al., 2007). Therefore, carfilzomib is unlikely to result in decreased mRNA expression of CYP isoforms in vivo as was seen in cultured hepatocytes.

In summary, carfilzomib displays high systemic clearance, a short half life, and rapid metabolism largely via extrahepatic peptidase cleavage and epoxide hydrolysis. CYP-mediated metabolism does not play an important role in the elimination of carfilzomib, therefore co-administration of carfilzomib with drugs that are potent CYP inhibitors or inducers is unlikely to alter its PK profile. Although exposure to carfilzomib resulted in modest inhibition of CYP3A activity in vitro in HLM and caused a decrease in CYP gene expression in human hepatocytes, clinically significant drug interaction was not noted in a study specifically designed to determine the effect of carfilzomib on CYP3A activity. Carfilzomib is a proteasome inhibitor with a distinct pharmacokinetic profile relative to bortezomib that may allow greater opportunity for general use in combination with other medications with less cause for concern regarding DDI.

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Authorship Contributions

Participated in research design: Yang, Wang, Kirk, Alsina, Badros, Papadopoulos, Wong, Infante

Administrative support for study: Woo, Bomba, Li

Conducted experiments: Yang, Wang, Alsina, Badros, Papadopoulos, Infante, Fang

Performed data analysis: Yang, Wang, Kirk, Fang, Wong, Infante, Fang, Wong

Wrote or contributed to the writing of the manuscript: Wang, Yang, Kirk, Alsina, Badros, Papadopoulos, Infante

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J.Y. and Z.W. contributed equally to this work.

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Legends for Figures

Figure 1. Pharmacokinetics of carfilzomib in patients with solid tumors.

Plasma concentration-time profiles of carfilzomib following dosing at 20 mg/m^2 on Days 1 and 16 (N=3) of Cycle 1. The concentrations are presented as mean concentrations ± SD.

Figure 2. MS/MS spectra of carfilzomib and its major metabolites (M14, M15 and M16)

A. Carfilzomib. B. M14. C. M15 and D. M16. All x axes are M/Z, all y axes are Relative Intensity

Figure 3. Rapid metabolism of carfilzomib in patients with multiple myeloma.

Plasma samples from patients receiving a single dose of 15 mg/m^2 of carfilzomib were analyzed for quantification of levels of 3 metabolites (M14, M15, and M16). Data are presented as mean concentrations ± SD. Inset shows metabolite concentration in the first 30 min following end of infusion.

Figure 4. In vitro and in vivo effects of carfilzomib on CYP enzyme activity.

A. Human liver microsomes were incubated in duplicate with varying concentrations of carfilzomib (0.01–10 μM) without pre-incubation (open circle) or with a 30-min pre-incubation (open triangle) prior to the addition of testosterone (left panel) or midazolam (right panel). Formation of the metabolites of the substrates was measured after 5 min, and data are presented as mean extent of substrate metabolism relative to 0.5% acetonitrile controls. B. Human hepatocytes from 3 donors were treated with 0.08% DMSO, 0.1, 1, and 2.5 μM carfilzomib, 20 μM rifampicin, or 20 μM β-naphthoflavone for 3 days followed by 30-min incubation with testosterone as a substrate for CYP3A, or by 60-min incubation with phenacetin as a substrate for CYP1A2. Data are presented as the mean activity (± SD) relative to DMSO treated samples from triplicate measurements for each donor. C. mRNA expression of CYP3A in the cells treated as in panel B was analyzed by qPCR. mRNA expression of CYP1A2 was tested using hepatocytes from an additional

donor treated as in B. Data are presented as the mean expression (± SD) relative to DMSO-treated samples from triplicate measurements for each donor. D: Midazolam plasma concentration-time profiles in patients with solid tumors were measured on Day -7 (prior to carfilzomib administration) and on Days 1 and 16 (following carfilzomib administration at 27 mg/m^2). Data are presented as mean concentrations ± SD.

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Tables

Table 1. Pharmacokinetic parameters (Mean ± SD, N=3) of carfilzomib following intravenous administration to patients with solid tumors at 20 mg/m^2

PK Parameters	**Cycle 1 Day 1,**	**Cycle 1 Day 16**
C_{max} (ng/mL)	2760 ± 520	3707 ± 1953
AUC_{last} (hr·ng/mL)	238 ± 36	301 ± 180
AUC_{inf} (hr·ng/mL)	238 ± 36	301 ± 180
$t_{½}$ (hr)	1.0 ± 0.6	1.1 ± 0.1
CL (L/hr)	146 ± 22	136 ± 53

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Table 2. Pharmacokinetic parameters of midazolam (Mean ± S. D.) in solid tumor patients alone (Day -7) or in co-administration (Day 1 and 16) with carfilzomib at 27 mg/m^2

PK Parameters	**Day -7 (N=17)**	**Day 1 (N=17)**	**Day 16 (N=12)**
C_{max} (ng/mL)	16.3 ± 7.6	15.7 ± 6.2	16.0 ± 7.7
AUC_{last} (hr·ng/mL)	49.8 ± 30.9	47.3 ± 26.4	48.4 ± 23.0
AUC_{0-12} (hr·ng/mL)	42.5 ± 23.2	40.3 ± 19.1	48.4 ± 23.0
AUC_{inf} (hr·ng/mL)[a]	54.3 ± 36.0	51.5 ± 30.3	59.9 ± 32.5
T_{max} (hr)	0.5 ± 0.2	0.7 ± 0.3	0.8 ± 0.7
$t_{½}$ (hr) [a]	6.6 ± 2.2	6.3 ± 1.9	5.5 ± 1.5

[a]N=11 on Day 16 for AUC_{inf} and $t_{1/2}$.

Figure 1

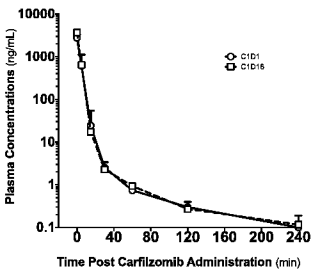
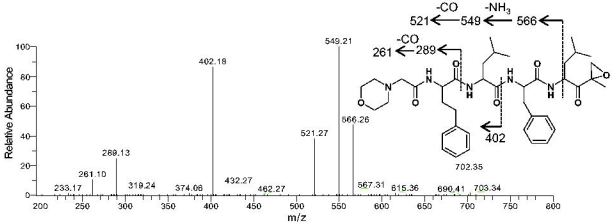
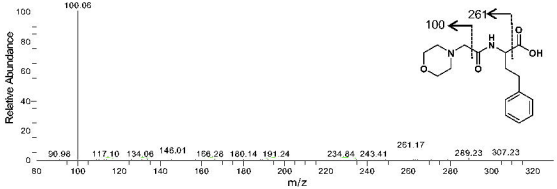

10000
1000
100
10
1
0.1
Plasma Concentrations (ng/mL)
C1D1
C1D16
0
40
80
120
160
200
240
Time Post Carfilzomib Administration (min)

Figure 2

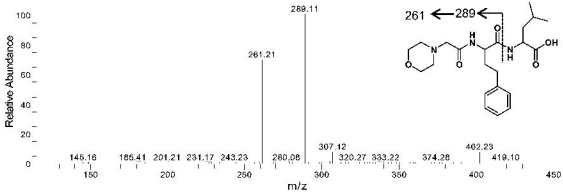
A



B



C



D

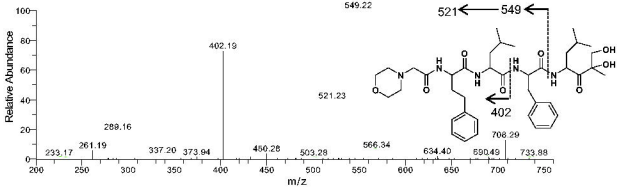


Figure 3

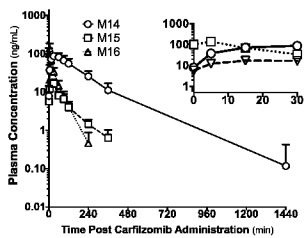
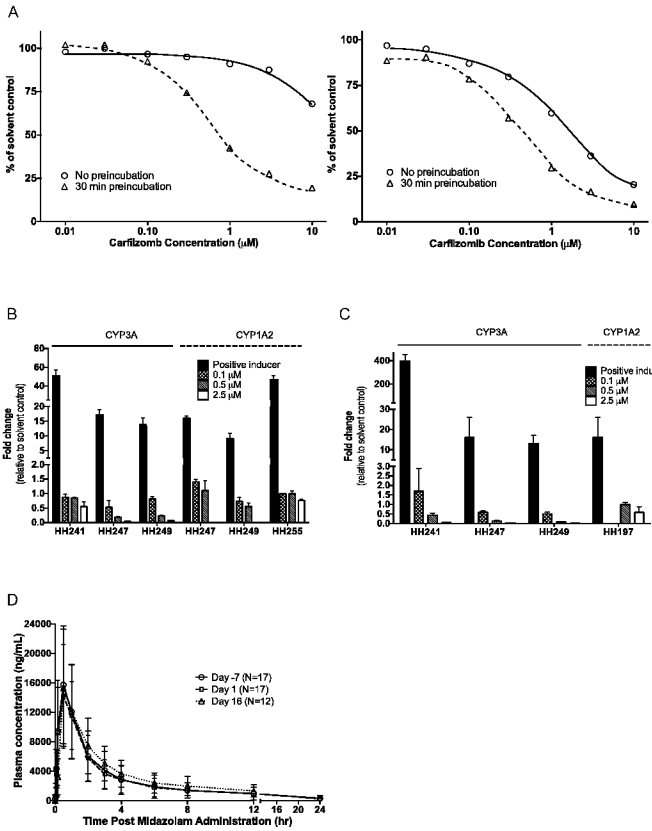

M14
M15
M16
Plasma Concentration (ng/mL)
1000
100
10
1
0.10
0.01
0
240
480
720
960
1200
1440
Time Post Carfilzomib Administration (min)
1000
100
10
1
0
10
20
30

Figure 4


A
% of solvent control
No preincubation
30 min preincubation
Carfilzomb Concentration (μM)
Carfilzomib Concentration (μM)
B
C
CYP3A
CYP1A2
Positive inducer
0.1 μM
0.5 μM
2.5 μM
Fold change (relative to solvent control)
HH241
HH247
HH249
HH247
HH249
HH255
HH197
D
Plasma concentration (ng/mL)
Day -7 (N=17)
Day 1 (N=17)
Day 16 (N=12)
Time Post Midazolam Administration (hr)