In Vitro and In Vivo Therapeutic Efficacy of Carfilzomib in Mantle Cell Lymphoma: Targeting the Immunoproteasome

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Abstract

Mantle cell lymphoma (MCL) remains incurable due to its inevitable pattern of relapse after treatment with current existing therapies. However, the promise of a cure for MCL lies in the burgeoning area of novel agents. In this study, we elucidated the therapeutic effect and mechanism of carfilzomib, a novel long-acting second-generation proteasome inhibitor, in MCL cells. We found that carfilzomib induced growth inhibition and apoptosis in both established MCL cell lines and freshly isolated primary MCL cells in a dose-dependent manner. In contrast, carfilzomib was less toxic to normal peripheral blood mononuclear cells from healthy individuals. The carfilzomib-induced apoptosis of MCL cells was mediated by the activation of JNK, Bcl-2, and mitochondria-related pathways. In addition, carfilzomib inhibited the growth and survival signaling pathways NF-κB and STAT3. Interestingly, we discovered that expression of immunoproteasome (i-proteasome) subunits is required for the anti-MCL activity of carfilzomib in MCL cells. In MCL-bearing SCID mice/primary MCL-bearing SCID-hu mice, intravenous administration of 5 mg/kg carfilzomib on days 1 and 2 for 5 weeks slowed/abrogated tumor growth and significantly prolonged survival. Our preclinical data show that carfilzomib is a promising, potentially less toxic treatment for MCL. Furthermore, an intact i-proteasome, especially LMP2, appears to be necessary for its anti-MCL activity, suggesting that i-proteasome could serve as a biomarker for identifying patients who will benefit from carfilzomib. Mol Cancer Ther; 12(11); 2494–504. ©2013 AACR.

Introduction

Mantle cell lymphoma (MCL) is a morphologically distinct subtype of non–Hodgkin lymphoma characterized by the t(11; 14) (q13; q32) chromosomal translocation and cyclin D1 overexpression (1, 2). MCL responds poorly to standard chemotherapies; thus, novel therapeutic agents are urgently needed (3).

The ubiquitin/proteasome pathway, the principal intracellular protein degradation (4–6), is a novel chemotherapeutic target (7–9). This pathway plays an important role in cellular homeostasis, controlling essential functions such as cell-cycle progression, and the immune response (6, 10, 11). In particular, the proteasome pathway is important for activating NF-κB. NFκB activates numerous genes related to cell survival, apoptosis, and cell migration (12). Its persistent activity is associated with tumor formation, tumor growth, metastasis, and drug resistance in many cancer types, including B-cell non–Hodgkin lymphoma (NHL; refs. 13–15). Current therapeutic efforts to inhibit this central "switch" include using small molecules to block selected targets in the pathway (16). We previously showed that the first-in-class proteasome inhibitor bortezomib inhibits cell growth and induces apoptosis of MCL cells (17). In addition, bortezomib has been shown to have clinical efficacy in relapsed and refractory MCL (18, 19). However, its duration of response is limited, and peripheral neuropathy is a dose-limiting side effect (19, 20).

To develop more potent and less cytotoxic therapeutics, we investigated a longer acting proteasome inhibitor, carfilzomib (PR-171), as a novel compound for anti-MCL therapy. Carfilzomib is an epoxy ketone related to epoxomicin and is structurally and mechanistically distinct from bortezomib (21). Carfilzomib forms a covalent adduct with the N-terminal threonine of the proteasome’s β5 subunit (22, 23). Therefore, the recovery of proteasome function is limited and prolonged, limiting side effect (19, 20).
activity depends entirely on new proteasome synthesis, leading to specific and sustained proteasome inhibition. In a recent clinical trial in patients with hematologic malignancies, proteasome inhibition of greater than 80% was achieved at a tolerable dosage of carfilzomib (24).

Here, we investigated the efficacy of carfilzomib in MCL cell lines, patient primary cells, and xenograft mouse models. Our study showed that carfilzomib had a therapeutic effect on relapsed or refractory MCL and very low toxicity to normal lymphocytes. Our preclinical data show the feasibility of using carfilzomib in a clinical trial in patients with relapsed or refractory MCL.

Materials and Methods

Reagents
Carfilzomib was provided by Onyx Pharmaceuticals, Inc. and solubilized in dimethyl sulfoxide (DMSO) at 10 mmol/L for in vitro studies and in 10% Captisol (sulfobutylether-β-cyclohextrin) at 2 mg/mL for in vivo studies. Bortezomib (Millennium Pharmaceuticals, Inc.) was obtained from the pharmacy at the University of Texas MD Anderson Cancer Center and solubilized in 0.9% PBS as a stock solution. The pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD), the caspase-8 inhibitor Ile-Glu-Thr-Asp-CHO (IETD-CHO), the caspase-9 inhibitor Z-Leu-Glu(OMe)-His-Asp-(OMe) (Z-LEHD), and MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) were purchased from Calbiochem and dissolved in DMSO at a stock concentration. Fluorescein isothiocyanate (FITC)-conjugated Annexin V was purchased from Caltag Laboratories. FBS was purchased from Atlanta Biologicals. Phorbol-12-myristate-13-acetate (PMA) and ionomycin were purchased from Caltag Laboratories. Iodide was added. The tube was gently vortexed and incubated for 15 minutes at room temperature in the dark. After the incubation, 300 μL of binding buffer was added, and the samples were immediately analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson). The number of apoptotic cells was determined by counting Annexin V–positive cells.

Knockdown of LMP2 in Rec-1 MCL cells
Transient transfections of Rec-1 cells were conducted using the Neon transfection system (Invitrogen). Transfection experiments in Rec-1 cells were carried out in vitro using validated LMP2–specific siRNA and negative control siRNA (Ambion) and repeated at least twice to verify reproducible experimental results.

Western blot analysis
MCL cells were cultured with 5, 10, and 20 nmol/L of carfilzomib in the presence or absence of caspase inhibitors for 0, 6, 12, and/or 24 hours. The cells were harvested, washed twice with cold PBS, and lysed in a lysis buffer (Cell Signaling). The cell lysates were kept on ice for 30 minutes and centrifuged at 13,000 × g for 10 minutes at 4°C. To detect cytosolic cytochrome c, we used the Cytosol Fractionation Kit (BioVision) according to the manufacturer’s protocol to acquire cytosolic fractions. The protein concentration was determined by the Bradford assay (Bio-Rad). Fifty micrograms of sample proteins was mixed with the loading buffer and separated by 10% SDS-PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and incubated with primary antibodies. The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies and visualized with an enhanced chemiluminescence detection system.
Mino and Jeko-1 cells were incubated in media containing 0, 20, or 100 nmol/L of carfilzomib or 5 μg/mL of MG-132 for 6 hours. MG-132 is a reversible and cell-permeable proteasome inhibitor that was used as a positive control. Oct-1 is a ubiquitously expressed, octamer-binding transcription factor containing a homeobox subdomain (25). It was used as a loading control in EMSA. Nuclear extracts were prepared from these cells. Nuclear extract (5 μg) was incubated with a 32P-labeled NF-kB probe or Oct-1 probe (Promega) for 15 minutes at room temperature. The mixtures were then separated by electrophoresis on a 6% PAGE and visualized by autoradiography.

**20S proteasome assay**

20S proteasome activity was measured by monitoring the release of free AMC from the fluorogenic peptide Suc-Leu-Val-Tyr-AMC, using the 20S Proteasome Assay Kit (Boston Biochem). Briefly, total cell lysates (5 μg) were incubated with 1× reaction buffer (25 mmol/L HEPES, 0.5 mmol/L EDTA, 0.03% SDS, pH 7.6) and 10 μmol/L Suc-LVLY-AMC substrate. Changes in fluorescence intensity (excitation, 380 nm; emission, 460 nm) were monitored over time.

**MCL-bearing SCID/primary MCL-bearing SCID-hu mouse models**

Six- to 8-week-old male CB-17 SCID mice (Harlan) were housed and monitored in our animal research facility. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. For the MCL-bearing SCID mouse model, the SCID mice were subcutaneously inoculated on the right flank with 5 × 10⁶ Mino cells suspended in 50 μL PBS. After palpable tumors (≥3 mm in diameter) developed, the mice were treated with intravenous injections of vehicle control (10% captirol) or carfilzomib (5 mg/kg/d) twice a week for 5 weeks. Tumor burdens were evaluated weekly by measuring the tumor size. The primary MCL-bearing SCID-hu mouse model in which primary MCL cells are engrafted in subcutaneously implanted human fetal bone chips was generated as previously described (26). Briefly, approximately 4 to 6 weeks after human fetal bone chip implantation, 5 × 10⁸ purified primary MCL cells were injected directly into the subcutaneously implanted human fetal bone within the SCID-hu hosts, after the mice were anesthetized with ketamine (75 mg/kg) and xylazine (12.5 mg/kg; Lloyd). Mouse serum was collected weekly, and the level of circulating human β₂ microglobulin (β₂M) in mouse serum was used to monitor tumor burden using a human β₂M ELISA kit (Abcam). When human β₂M was detectable in mouse serum, carfilzomib was administered as described for the SCID mouse model. Mice were euthanized when they became moribund or when the subcutaneous tumors reached 15 mm in diameter of long dimension. Once the mice were sacrificed, subcutaneous tumor masses were taken out and subjected to immunohistochemical (IHC) staining.

**Statistical analysis**

All assays were conducted in triplicate and expressed as mean ± SE. Statistical significance of differences observed between experimental groups was determined by Student t test. For the animal experiments, overall survival was measured using the Kaplan–Meier method. P < 0.05 was considered significant.

**Results**

**Effects of carfilzomib on the growth and apoptosis in MCL cells and normal PBMCs in vitro**

The effect of carfilzomib on cell growth was initially established in a panel of 9 MCL cell lines (both classic and blastoid-variant) using 3H-thymidine incorporation assays. As shown in Supplementary Table S1, carfilzomib inhibited the growth of MCL cell lines at very low doses, with half maximal inhibitory concentration (IC50) values ranging from 1 to 25 nmol/L, with the exception of the Rec-1 cell line, which was refractory to carfilzomib, with an IC50 value of 280 nmol/L. For the carfilzomib-sensitive cells, carfilzomib inhibited the growth of MCL cell lines at a very low dose (P < 0.01; Fig. 1A). Importantly, carfilzomib inhibited the growth of primary MCL cells from patients (PT1, PT2, and PT3), with IC50 values ranging from 2 to 6 nmol/L (P < 0.01); however, one patient (PT4) was refractory to carfilzomib, even at a dose of 80 nmol/L (Fig. 1B). We also examined the effects of carfilzomib on normal PBMCs from 3 healthy blood donors. Carfilzomib did not affect the proliferation of resting PBMCs (Fig. 1C). However, carfilzomib significantly inhibited the proliferation of PBMCs activated by either ionomycin (1 μg/mL) plus PMA (300 ng/mL) or the anti-CD3 (5 μg/mL) plus anti-CD28 (1 μg/mL) monoclonal antibodies (P < 0.01; Fig. 1D). These data clearly show that carfilzomib inhibits the proliferation of most MCL cells and activated peripheral blood lymphocytes, without affecting the growth of normal resting PBMCs and carfilzomib-resistant MCL cells.

Next, we examined whether the effect of carfilzomib on cell growth inhibition was due to cell death. Representative carfilzomib-sensitive MCL cell lines were treated with increasing concentrations of carfilzomib for 48 hours and then analyzed for apoptosis using Annexin V–binding assays. The results showed that carfilzomib induced...
apoptosis in MCL cells in a dose-dependent manner (Fig. 1E). Carfilzomib also induced apoptosis in MCL cells in a time-dependent manner (Supplementary Fig. S1). We also treated MCL cells from patients with refractory or relapsed MCL with different concentrations of carfilzomib for 48 hours and assayed for apoptosis. Carfilzomib efficiently induced apoptosis in 3 representative primary MCL cells in a dose-dependent manner \((P < 0.01\), compared with vehicle controls; Fig. 1F). To examine whether carfilzomib is toxic to normal cells, normal PBMCs from 3 healthy volunteers were treated with different concentrations of carfilzomib for 48 hours. Increasing doses of carfilzomib did not significantly induce apoptosis of normal PBMCs \((P > 0.05\); Fig. 1F), indicating that carfilzomib selectively eliminates MCL cells but not normal blood cells.

**Carfilzomib induces apoptosis via a caspase-dependent signaling pathway**

To elucidate the signaling pathways contributing to carfilzomib-induced apoptosis in MCL cells, caspase
activation and PARP cleavage in cells treated with or without carfilzomib were assessed by Western blot analysis. Treatment of MCL cells with carfilzomib for 24 hours resulted in the dose-dependent activation of caspase-3 and cleavage of PARP (Fig. 2A). An optimal dose of 20 nmol/L carfilzomib was selected to treat MCL in a time-dependent manner. We found that carfilzomib induced activation and cleavage of caspase-3, caspase-8, caspase-9, and PARP after 12 hours of treatment (Fig. 2B). To further examine the carfilzomib-induced caspase-activated apoptosis in MCL, Mino and Jeko-1 cells were incubated with 20 nmol/L of carfilzomib for 0, 6, 12, and 24 hours. Whole-cell lysates were analyzed by Western blotting. C, percentages of apoptotic cells after treatment with the pan-caspase inhibitor Z-VAD, as measured by an Annexin V-binding assay. Average from 3 independent experiments is shown. D, representative histograms from an Annexin V-binding assay showing that the caspase-8 inhibitor IETD-CHO plus the caspase-9 inhibitor Z-LEHD, but neither alone, abrogated carfilzomib-induced apoptosis of MCL cells. In A and B, arrows indicate the cleaved forms of caspases (cCasp) and PARP.

**Figure 2.** Carfilzomib induces apoptosis in a caspase-dependent manner. A, carfilzomib induced activation and cleavage of caspase-3 and PARP in a dose-dependent manner. Mino and Jeko-1 cells were treated with 0, 5, 10, or 20 nmol/L carfilzomib for 24 hours. Whole-cell lysates were analyzed by Western blotting. B, time-dependent activation and cleavage of caspase-3, caspase-8, caspase-9, and PARP after treatment with carfilzomib. Mino and Jeko-1 cells were treated with 20 nmol/L of carfilzomib for 0, 6, 12, and 24 hours. Whole-cell lysates were analyzed by Western blotting. C, percentages of apoptotic cells after treatment with the pan-caspase inhibitor Z-VAD, as measured by an Annexin V-binding assay. Average from 3 independent experiments is shown. D, representative histograms from an Annexin V-binding assay showing that the caspase-8 inhibitor IETD-CHO plus the caspase-9 inhibitor Z-LEHD, but neither alone, abrogated carfilzomib-induced apoptosis of MCL cells. In A and B, arrows indicate the cleaved forms of caspases (cCasp) and PARP.

Carfilzomib activates JNK and mitochondria-associated signaling pathways

To examine whether carfilzomib-induced apoptosis in MCL involves mitochondria, we elucidated the involvement of Bcl-2, the best-characterized protein involved in the regulation of apoptotic cell death within mitochondria (27). As shown in Fig. 3A, treatment of MCL cells with carfilzomib activated the phosphorylation of Bcl-2. We next examined the signaling pathways upstream of Bcl-2. We found that JNK was activated after 6 hours of incubation with carfilzomib in Mino and Jeko-1 cells and remained highly activated at 12 to 24 hours (Fig. 3A). To
further elucidate the effects of carfilzomib-induced apoptosis after the phosphorylation of Bcl-2, we examined the release of the mitochondria-related apoptotic molecule cytochrome c (cyto c). Western blot analysis showed that carfilzomib enhanced the release of cyto c from mitochondria to cytosol after 12-hour incubation in both Mino and Jeko-1 cells (Fig. 3B). These results indicate that carfilzomib induces apoptosis of MCL cells via activation of the JNK and mitochondrial signaling pathways.

Carfilzomib inhibits the growth and survival signaling pathways in MCL cells

NF-κB is constitutively activated in MCL cells and plays an important role in cell survival and apoptosis (28). To determine the effects of carfilzomib on the NF-κB pathway, Mino and Jeko-1 cells were incubated with carfilzomib for different times up to 24 hours. Western blot analysis showed that carfilzomib blocked the phosphorylation of IkBα (pIkBα) and after 12 hours, pIkBα was significantly reduced (Fig. 3C). However, the total IkBα level was unchanged during 24-hour incubation with carfilzomib (Fig. 3C). Thus, carfilzomib likely inhibits NF-κB activity by blocking the phosphorylation of IkBα. An electrophoretic mobility shift assay showed that carfilzomib reduced the levels of nuclear NF-κB in MCL cells in a dose-dependent manner (Fig. 3D).

In addition, we found that carfilzomib also blocked other survival signaling pathways. We showed that carfilzomib rapidly blocked the phosphorylation of STAT3; after 12 hours of treatment, pSTAT3 was undetectable in both Mino and Jeko-1 cells (Fig. 3C). The results suggest that carfilzomib could block both NF-κB and STAT3 signaling pathways in MCL.

Carfilzomib-resistant MCL cells

As bortezomib was the first-generation proteasome inhibitor approved by the U.S. Food and Drug Administration for the treatment of relapsed or refractory MCL, we sought to compare the effect of carfilzomib with that of bortezomib in representative carfilzomib-sensitive/resistant MCL cells. We found that both carfilzomib and bortezomib inhibited the growth of the carfilzomib-sensitive MCL cell lines Mino, Jeko-1, and MAVER in a similar dose-dependent manner (P < 0.01; Fig. 4A–C). However, Rec-1, the carfilzomib-resistant MCL cell line, was highly sensitive to bortezomib (Fig. 4D). Similarly, the results from an Annexin V–binding assay showed that bortezomib, but not carfilzomib, induced apoptosis of Rec-1 cells in a dose-dependent manner (data not shown).

Expression of i-proteasome subunits is required for the anti-MCL activity of carfilzomib

To understand the mechanism(s) for the differential effects of carfilzomib and bortezomib in the Rec-1 cell line, we first evaluated the expression of the catalytic subunits of the 20S proteasome in MCL cell lines. Three representative MCL cell lines (2 sensitive to carfilzomib and 1 resistant to carfilzomib) were assessed for expression of the constitutive proteasome (c-proteasome) subunits β1, β2, and β5 and i-proteasome subunits LMP2, LMP7, and MECL-1 by Western blotting. In all 3 cell lines, the c-proteasome subunits as well as the 20S proteasome core were expressed (Fig. 5A). However, compared with the 2 carfilzomib-sensitive MCL cell lines Mino and Jeko-1, the carfilzomib-resistant MCL cell line Rec-1 lacked both the MECL-1 and LMP2 subunit expression and exhibited low LMP7 expression. The carfilzomib-sensitive...
Jeko-1 cells lack MECL-1 but expressed both the LMP2 and LMP7 subunits (Fig. 5A). Next, we tested whether the 20S proteasome is functional in the 3 MCL cell lines. We found that Mino and Jeko-1 cells exhibited 3- and 4-fold higher 20S proteasome activity than Rec-1 cells (Fig. 5B). These results suggest that the lack of i-proteasome subunits, particularly LMP2, in Rec-1 cells may lead to low 20S proteasome activity and may be responsible for the carfilzomib resistance in this cell line. To further confirm whether our data in MCL cell lines are consistent with the data from primary MCL cells, we analyzed the expression of LMP2 and β1 subunits in 11 primary MCL cases. We identified one primary MCL case, PT4, that did not significantly express LMP2 but expressed β1 (Fig. 5C), and PT4 MCL cells were found to be resistant to carfilzomib (Fig. 1B). Our findings suggest that i-proteasome subunit LMP2 could potentially be used as a marker to predict response to carfilzomib in MCL cells.

Next, we explore whether upregulation of i-proteasome expression could restore sensitivity in the carfilzomib-resistant Rec-1 cells. Stimulation of Rec-1 cells with IFNg for 48 hours increased protein level of i-proteasome subunit LMP2 but not the c-proteasome subunit β1 (Fig. 5D). Pretreatment of Rec-1 cells with IFNg sensitized Rec-1 cells to carfilzomib (Fig. 5E, P < 0.01). To provide direct evidence that upregulation of i-proteasome subunit LMP2 by IFNg was responsible for the observed sensitization, siRNA downregulation of LMP2 was applied before exposure to IFNg. After LMP2 knockdown (Fig. 5F), carfilzomib sensitization was attenuated (Fig. 5G, P < 0.01).

**Therapeutic effects of carfilzomib in the established MCL-bearing SCID/primary MCL-bearing SCID-hu mice**

To examine the anti-MCL effects of carfilzomib in vivo, an MCL-bearing SCID mouse model was used first. Mino cells (5 × 10⁶ cells per mouse) were subcutaneously inoculated into the right flank of SCID mice. When palpable tumors developed (≥3 mm in diameter), mice (6 per group) received either an intravenous injection of carfilzomib (5 mg/kg) or vehicle control twice a week for 5 weeks. Tumor growth was measured weekly until the tumor diameter reached 15 mm. Carfilzomib almost abrogated tumor growth (Fig. 6A, P < 0.01). Furthermore, carfilzomib significantly prolonged the survival time of the tumor-bearing mice (P < 0.01, Fig. 6B).

In the primary MCL-bearing SCID-hu mouse model, one injection of 5 × 10⁶ freshly isolated primary MCL cells was injected directly into human fetal bone implanted within the SCID-hu hosts. The level of circulating human β2M in mouse serum was used to monitor tumor burden in the SCID-hu mice. The intravenous administration of 5 mg/kg carfilzomib on days 1 and 2 per week, for 5 weeks inhibited tumor growth and prolonged survival in the primary MCL-bearing SCID-hu mice (P < 0.01, Fig. 6C and 6D).
Overall, these data clearly show that carfilzomib has antitumor effects in vivo. In addition, we observed that carfilzomib is well tolerated without severe side effects in the MCL-bearing SCID/SCID-hu mice. The IHC staining of tumor from the primary MCL-bearing SCID-hu mice showed that phosphorylated STAT3 was significantly detectable in vehicle control mice but not carfilzomib-treated mice (Fig. 6E).

Discussion

The current study showed that (i) the novel proteasome inhibitor carfilzomib is effective in killing MCL cells, both in vitro and in vivo; (ii) carfilzomib has low toxicity to normal PBMCs as well as in SCID/SCID-hu mice; (iii) carfilzomib is more selective in targeting i-proteasome than bortezomib in MCL cells; and (iv) carfilzomib can target multiple growth/survival pathways, leading to apoptosis in MCL cells.

The biologic significance of the ubiquitin/proteasome system in the control of cellular processes has been well-recognized; however, the pathophysiologic importance of i-proteasome, the inducible form of the proteasome, compared with the standard proteasome, has not been well-appreciated in cancer cells. i-proteasome was originally believed to function only in immune cells to improve the...
efficiency of MHC-I antigen presentation in adaptive immune responses after IFNγ stimulation (29, 30). More recently, it has become evident that i-proteasome is constitutively expressed and possesses broader biologic functions in various types of cancer including hematologic malignancies (31, 32). In this study, we found that the carfilzomib-sensitive MCL cell line Mino constitutively expressed i-proteasome subunits LMP2, LMP7, and MECL-1. We also tested the culture medium of Mino cells and showed that Mino cells did not secrete IFN-γ by autocrine signaling (data not shown). Therefore, the constitutive expression of i-proteasome subunits in carfilzomib-sensitive MCL cells is not IFN-γ dependent. Another carfilzomib-sensitive MCL cell line Jeko-1 expressed LMP2 and LMP7 but not MECL-1, indicating that MECL-1 is not related to carfilzomib resistance. By contrast, in the carfilzomib-resistant cell line Rec-1, i-proteasome subunits LMP2 and MECL-1 were not expressed and 20S proteasome activity was low, indicating that LMP2 is correlated with 20S proteasome activity and contributes to the sensitivity of MCL cells to carfilzomib. Our data of LMP2 expression and knockdown in carfilzomib-resistant Rec-1 cells suggest that MCL cells with LMP2 are more sensitive to carfilzomib than MCL cells that lack the subunit. Importantly, we also tested primary MCL cells from 11 patients and found that one patient (PT4) lacked LMP2 expression and was resistant to carfilzomib. We recently tested other LMP2-expressing MCL cells from these patients (PT7–PT11) and similar to samples PT1–PT3, we found all of them were sensitive to carfilzomib (data not shown). Therefore, our findings suggest that i-proteasome could potentially be used as prognostic biomarkers to identify patients who will benefit most from carfilzomib. In addition, the clinical studies show that carfilzomib is associated with lower rates of painful peripheral neuropathy than bortezomib (33, 34); however, i-proteasome may not link to this adverse event (35).

Our studies also showed that carfilzomib is very effective in MCL-bearing SCID/SCID-hu mouse models. Carfilzomib almost fully abrogated tumor growth and significantly prolonged the survival of tumor-bearing SCID mice. Furthermore, mice receiving intravenous injections of carfilzomib (5 mg/kg) had no significant side effects and survived more than 90 days without significantly increasing tumor burdens, indicating that carfilzomib is safe and effective in vivo. Of importance, our data showed that carfilzomib also inhibited tumor growth.
growth and prolonged survival in primary MCL-bearing SCID-hu mice, a model that more closely mimics the natural history of human MCL. Taken together, these results are in agreement with data from a phase I dose escalation study of carfilzomib in patients with hematologic malignancies. The phase I study showed that clinical responses were seen across a range of doses, indicating that carfilzomib has a wide therapeutic index. At the maximum tolerated dose, antitumor activity was observed at doses ≥11 mg/m², and responses occurred in patients who were refractory to bortezomib (24). In addition, there was an apparent reduction in the incidence of painful peripheral neuropathy, a dose-limiting side effect seen in patients treated with bortezomib (24). To our knowledge, our current study is the first to compare carfilzomib with bortezomib in terms of therapeutic effect and resistance. The striking difference we found is that carfilzomib but not bortezomib is dependent on i-proteasome for its anti-MCL activity. Because i-proteasomes are present mostly in immune or hematologic cells, carfilzomib is an ideal drug for treating hematologic malignancies such as MCL. Our finding that the carfilzomib-resistant MCL cells were highly sensitive to bortezomib suggests that i-proteasome is not the primary target of bortezomib, which likely has various targets as previously shown (36). This is likely why bortezomib is associated with severe adverse effects, including peripheral neuropathy, whereas carfilzomib is well-tolerated. In sum, our preclinical data further support the investigation of carfilzomib in clinical trials in patients with MCL.

Mechanistically, we showed that carfilzomib-induced apoptosis was associated with the phosphorylation of Bcl-2 and JNK and the release of cyto-c. Carfilzomib also blocked the phosphorylation of IκB and STAT3, revealing its broad effects on cell survival pathways. These results are consistent with the data obtained in myeloma cells (22). NF-κB, which is regulated by its interaction with inhibitory IκB proteins, is constitutively active in MCL cells (28, 37). In the cytosol, NF-κB is found in complex with IκB in an inactive state. Phosphorylation of IκB results in the release of NF-κB and its nuclear translocation (38). We found that carfilzomib inhibited constitutive activation of NF-κB by completely blocking the phosphorylation of IκB. This effect of carfilzomib on the NF-κB pathway likely contributes to its cell growth inhibition and apoptosis induction in MCL cells. Hideshima and colleagues reported that bortezomib downregulated IκB protein expression in multiple myeloma cell in a dose-dependent fashion (39). However, we did not detect a decrease in the IκB protein level within 24 hours of carfilzomib treatment. Rather, we observed phosphorylation of IκB within 6 to 12 hours of carfilzomib treatment. In addition, carfilzomib reduced the level of NF-κB in MCL cells in a dose-dependent manner. These results suggest that the carfilzomib-induced apoptosis in MCL cells is associated with its inhibition of NF-κB activity. We found that carfilzomib can also block other survival signaling pathways including the STAT3 pathway. STAT3, which possesses tumorigenic potential and anti-apoptotic activity, has been shown to be constitutively activated in MCL cells (40–43). However, how i-proteasome or its functions are related to these key signaling pathways is still unclear and requires further elucidation. Understanding the association between i-proteasome and these cell survival pathways is likely key for designing better therapeutic strategies for MCL, particularly refractory MCL.

The paradigm shift in cancer therapy has led to a focus on targeted therapy. However, it has become clear that multiple agents targeting multiple targets will be necessary to fully shut down the cell survival pathways. Discovering an agent that can target multiple growth/survival pathways while being highly selective for malignant cells, such as carfilzomib represents an ideal drug of choice. In conclusion, our results indicate that carfilzomib, the long-acting second-generation proteasome inhibitor, may be more selective, effective, and less toxic to patients with relapsed or refractory MCL.

Disclosure of Potential Conflicts of Interest
L. Zhang has commercial research grant from Onyx Research Funds. No potential conflicts of interest were disclosed by the other authors.

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