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Carfilzomib-Dependent Selective Inhibition of the Chymotrypsin-like Activity of the Proteasome Leads to Antitumor Activity in Waldenstrom's Macroglobulinemia

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Abstract

Purpose: Primary Waldenstrom's Macroglobulinemia (WM) cells present with a significantly higher level of the immunoproteasome compared with the constitutive proteasome. It has been demonstrated that selective inhibition of the chymotrypsin-like (CT-L) activity of constitutive-(c20S) and immuno-(i20S) proteasome represents a valid strategy to induce antineoplastic effect in hematologic tumors. We therefore evaluated carfilzomib, a potent selective, irreversible inhibitor of the CT-L activity of the i20S and c20S in WM cells.

Experimental Design: We tested the effect of carfilzomib on survival and proliferation of primary WM cells, as well as of other IgM-secreting lymphoma cell lines. Carfilzomib-dependent mechanisms of induced apoptosis in WM cells, and its effect on WM cells in the context of bone marrow (BM) microenvironment have been also evaluated. Moreover, the combinatory effect of carfilzomib and bortezomib has been investigated. *In vivo* studies have been performed.

Results: We demonstrated that carfilzomib targeted the CT-L activity of both i20S and c20S, which led to the induction of toxicity in primary WM cells, as well as in other IgM-secreting lymphoma cells. Importantly, carfilzomib targeted WM cells even in the context of BM milieu. In addition, carfilzomib induced apoptosis through c-jun-N-terminal-kinase activation, caspase cleavage, and initiation of unfolded protein response. Importantly, the combination of carfilzomib and bortezomib synergistically inhibited CT-L activity, as well as caspase-, PARP-cleavage and GRP94 expression. Antitumor activity of carfilzomib has been validated *in vivo*.

Conclusions: These findings suggest that targeting i20S and c20S CT-L activity by carfilzomib represents a valid antitumor strategy in WM and other IgM-secreting lymphomas. *Clin Cancer Res*; 17(7); 1753–64. ©2011 AACR.

Introduction

Waldenstrom's Macroglobulinemia (WM) is characterized by the presence of lymphoplasmacytic cells in the bone marrow (BM) and the secretion of IgM monoclonal protein in the serum, indicating that WM cells present a

high protein turnover (1–3). Protein metabolism is a tightly regulated process, and inhibition of its turnover may lead to apoptosis in malignant cells, such as with proteasome inhibitors (4). The 26S proteasome represents a crucial key in the regulation of cellular homeostasis. It consists of 3 different catalytic activities known as chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L), which are encoded by the $\beta 5$, $\beta 2$, and $\beta 1$ subunits, respectively (4). In addition, cells of hematopoietic origin express the immunoproteasome, which retains the structural subunits of the constitutive proteasome but exerts its enzymatic activities through the catalytic subunits LMP7, MECL1, and LMP2 which form the i20S core (5–7).

Specific targets for proteasome degradation include several proteins involved in cell-cycle regulation, cell proliferation, programmed cell death, and stress response. These findings validate the importance of targeting the proteasome for cancer therapy. Indeed, a wide spectrum of compounds, both natural and synthetic,

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Translational Relevance

We have evaluated the antineoplastic activity of carfilzomib, a selective inhibitor of the chymotrypsin-like activity of both immunoproteasome and constitutive proteasome in Waldenstrom's Macroglobulinemia and found that carfilzomib is responsible for inducing an antitumor effect in WM cells, as shown both *in vitro* and *in vivo*. These findings provide the preclinical evidence for using carfilzomib in clinical trials in this disease.

has been identified as proteasome inhibitors, such as bortezomib (Millenium Inc.), NPI-0052 (Nereus Pharmaceuticals) and carfilzomib (Onyx Pharmaceuticals).

It has recently been demonstrated that B-cell malignancies are characterized by a preferential expression of the i20S. We have previously validated these findings in primary WM cells which are characterized by higher expression of the i20S immunoproteasome subunits as compared with c20S subunits and they contain a higher i20S content as compared with normal CD19+ B-cells. In addition, solid tumor cell lines (colon carcinoma, pancreatic carcinoma, and basal epithelial cell carcinoma) express low amounts of LMP7. These findings indicate that a selective inhibition of the CT-L activity of the proteasome, both at the constitutive and immunoproteasome level, represents a valid strategy to exert an antineoplastic effect in hematologic malignancies.(8) Indeed, the antitumor activity of carfilzomib has been also validated both *in vitro* and *in vivo* both in B-cell malignancies, and in solid tumors (9,10); but its potential antineoplastic effect in WM has not been evaluated yet.

We therefore evaluated the antitumor activity of the new irreversible, peptide epoxyketone, selective CT-L proteasome inhibitor carfilzomib in WM. Our findings demonstrate that carfilzomib inhibits the chymotrypsin-like activity of both the immunoproteasome (LMP7) and the constitutive proteasome (β 5) in WM cells, leading to reduced induction of cytotoxicity in primary WM cells, as well as programmed cell death in a caspase-dependent and caspase-independent manner, as shown by activation of c-jun-N-terminal kinase (JNK), and initiation of the unfolded protein response. Importantly, carfilzomib exerted cytotoxicity in WM cells, even in the context of BM milieu, where it overcame BM stromal cell-induced WM cell proliferation and inhibited the adhesion and migration of WM cells towards stromal cell-derived factor-1. Notably, carfilzomib exerted an additive effect in combination with bortezomib in inhibiting the β 5/CT-L activity of the proteasome, thus resulting in a synergistic induction of toxicity in WM cells. Importantly, carfilzomib-dependent antitumor activity has been confirmed *in vivo*. Taken together, these findings provide the preclinical rationale for using carfilzomib in WM.

Material and Methods

Cells

Primary WM cells were obtained from BM samples of previously treated WM patients using CD19⁺ microbead selection (Miltenyi Biotec) with over 90% purity, as confirmed by flow cytometric analysis with monoclonal antibody reactive to human CD20-PE (BD-Bioscience). The WM and the IgM secreting lymphoma cell lines (BCWM.1; BCWM.1-mCherry+; MEC.1; RL) were used in this study. (11) Peripheral blood mononuclear cells (PBMCs) were obtained from healthy subjects by Ficoll-Hipaque density sedimentation and CD19⁺ selection was performed as described above. All cells were cultured at 37°C in RPMI-1640 containing 10% FBS (Sigma Chemical), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (GIBCO). Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki protocol.

Reagents

Carfilzomib and proteasome active site binding protein (PABP) was provided by Onyx Pharmaceuticals, which was diluted in dimethyl sulfoxide (DMSO) and stored at 4°C until use, then diluted in culture medium immediately before use. The maximum final concentration of DMSO (<0.1%) did not affect cell proliferation and did not induce cytotoxicity on the cell lines and primary cells tested (data not shown). Bortezomib was obtained from Hospital Pharmacy. The c-Jun-NH2-kinase (JNK) inhibitor SP600215 was purchased from Calbiochem. Salubrinal was purchased from Axxora. The pan-caspase inhibitor Z-VAD-fmk was purchased from Promega. Recombinant IL6 and IGF1 were purchased from R&D.

Growth inhibition assay

The inhibitory effect of carfilzomib on the growth of WM cells, IgM secreting cell lines, and primary cells was assessed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Chemicon International) dye absorbance, as previously described (11).

DNA synthesis

DNA synthesis was measured by [³H]-thymidine ([³H]-TdR; Perkin Elmer) uptake, as previously described (11).

DNA fragmentation assay

Cell Death Detection ELISA (Roche Applied Science) was used to quantify DNA fragmentation, as per the manufacturer's instructions.

Immunoblotting

WM and IgM secreting cell lines were harvested and lysed using lysis buffer (Cell Signaling Technology) reconstituted with 5 mmol/L NaF, 2 mmol/L Na₃VO₄, 1 mmol/L PMSF (polymethylsulfonyl fluoride), 5 μ g/mL leupeptine, and

5 µg/mL aprotinin. Whole-cell lysates (50 µg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). The antibodies used for immunoblotting included: anti-caspase-3, -caspase-8, -caspase-9, -PARP, βcatenin, GRP94, phospho (p)-EIF2α, BIP, p-SAPK/JNK, -α-tubulin, and -β-actin antibodies (Santa Cruz Biotechnology).

Proteasome Constitutive Immuno Subunit ELISA assay (ProCISE)

The ProCISE assay was performed as previously described (8). In brief, human constitutive(c) proteasome 20S and immunoproteasome(i) 20S subunits (Boston Biochemical); monoclonal antibodies anti-β1, anti-β2, anti-LMP7, and anti-LMP2 (BioMol International); anti-MECL1 (Santa Cruz Biotechnology); anti-β5 (Covance Custom Product); and HRP-conjugated antibodies (Jackson ImmunoResearch and Zymed) were used. Baseline expression of each c20S and i20S subunits, and their modulation upon carfilzomib treatment, was tested on cell lysates prepared by incubating cell pellets in TE buffer (20 mmol/L TRIS pH 8.0, 5 mmol/L EDTA). Cell lysates were then incubated with PABP (5 µM) for 2 hours at 25°C. Samples were denatured with 8 M guanidine hydrochloride (Fisher Scientific) and subunits bound to PABP were captured with Streptavidin conjugated sepharose beads (GE Healthcare). Individual subunits were probed with antibodies specific to each subunit. Each subunit was measured as ng/mg total protein, according to the SuperSignal ELISA Pico Kit.

20S proteasome activity

The chymotrypsin-like activity of the 20S proteasome of primary WM tumor cells was determined by measurement of fluorescence generated from the cleavage of the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (suc-LLVY-AMC), as described (12).

Effect of Carfilzomib on paracrine WM cell growth in the BM

To evaluate growth stimulation and signaling in WM cells adherent to BM stromal cells (BMSCs), 3×10^4 BCWM.1 cells were cultured in BMSC-coated 96-well plates for 48 hours in the presence or absence of carfilzomib. DNA synthesis was measured as described (11).

Transwell migration assay

We performed transwell migration assay (Costar) using BCWM.1 cells in the presence or absence of 30 nM SDF-1, as described (12).

Adhesion assay

BCWM.1 cells were pretreated with carfilzomib for 4 hours, before an *in vitro* adhesion assay to fibronectin, following the manufacturer's recommendations (EMD Biosciences). Calcein AM was used to measure adherent cells, and the degree of fluorescence was measured using a

spectrophotometer (485–520). BSA-coated wells served as a negative control.

IgM detection

Serum IgM levels were quantified by ELISA (Human IgM ELISA kit, Zeptometrix), according to manufacturer's instructions.

In vivo studies

Severe combined immunodeficient mice (SCID) homozygous female mice (6 weeks old) were obtained from Charles River Laboratories. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Dana Farber Cancer Institute. BCWM.1-m Cherry positive cells were injected intravenously (i.v.) in SCID mice ($n = 5$ controls; $n = 5$ treated). Carfilzomib was formulated in an aqueous solution of 10% (w/v) sulfobutylether-β-cyclodextrin (Cydex) and 10 mmol/L sodium citrate (pH 3.5) for i.v. administration (5 mg/kg; twice/week, day 1–day 2; for 3 weeks), as previously reported (10). Vehicle consisted of cyclodextrin/citrate, as reported. 24 hours after day 2 of the third administration, BM were collected and analyzed by flow cytometry. Evaluation of tumor WM-mCherry⁺ cells and quantification of apoptotic cells in control vs carfilzomib-treated mice were performed by detecting the percentage of mCherry signal and by Annexin/DAPI staining and flow cytometry analysis, respectively.

Statistical analysis

Statistical significance of differences in drug-treated versus control cultures was determined using Student's *t*-test. The minimal level of significance was $P < 0.05$. Drug synergism was analyzed by isobologram analysis using the CalcuSyn software program (Biosoft), as described (11). Experiments have been repeated in triplicates. Error bars reported in the figures represent standard deviations.

Results

Carfilzomib selectively targets the CT-L activity of the proteasome in primary WM cells with a weak activity on other protease classes

We have previously demonstrated that primary tumor CD19⁺ BM-derived WM cells have a significantly higher level of the immunoproteasome compared with the constitutive proteasome, and that WM primary cells present with a significantly higher proteasome subunit expression compared with their normal cellular counterpart (13). Similar results were confirmed in BCWM.1 cells, as well as other lymphoma IgM secreting cells, such as MEC.1 and RL (13). We therefore investigated the activity of Carfilzomib, a novel irreversible, peptide epoxyketone, CT-L proteasome inhibitor, in targeting the CT-L activity in WM cells and other IgM secreting lymphoma cell lines. Cells were treated with increasing concentrations of Carfilzomib (2.5–10 nM) for 2 hours and exhibited significant inhibition of

the CT-L subunits of both constitutive proteasome ($\beta 5$) and immunoproteasome (LMP7; Fig. 1A; $P < 0.05$) in a dose-dependent manner, with minimal inhibition of the trypsin T-L and caspase activities (Fig. 1B and C), validating

the selectivity of Carfilzomib for the CT-L activity of the proteasome in WM. Importantly, Carfilzomib-induced inhibition of the CT-L proteasome activity was confirmed in primary CD19+ WM cells (Fig. 1D).

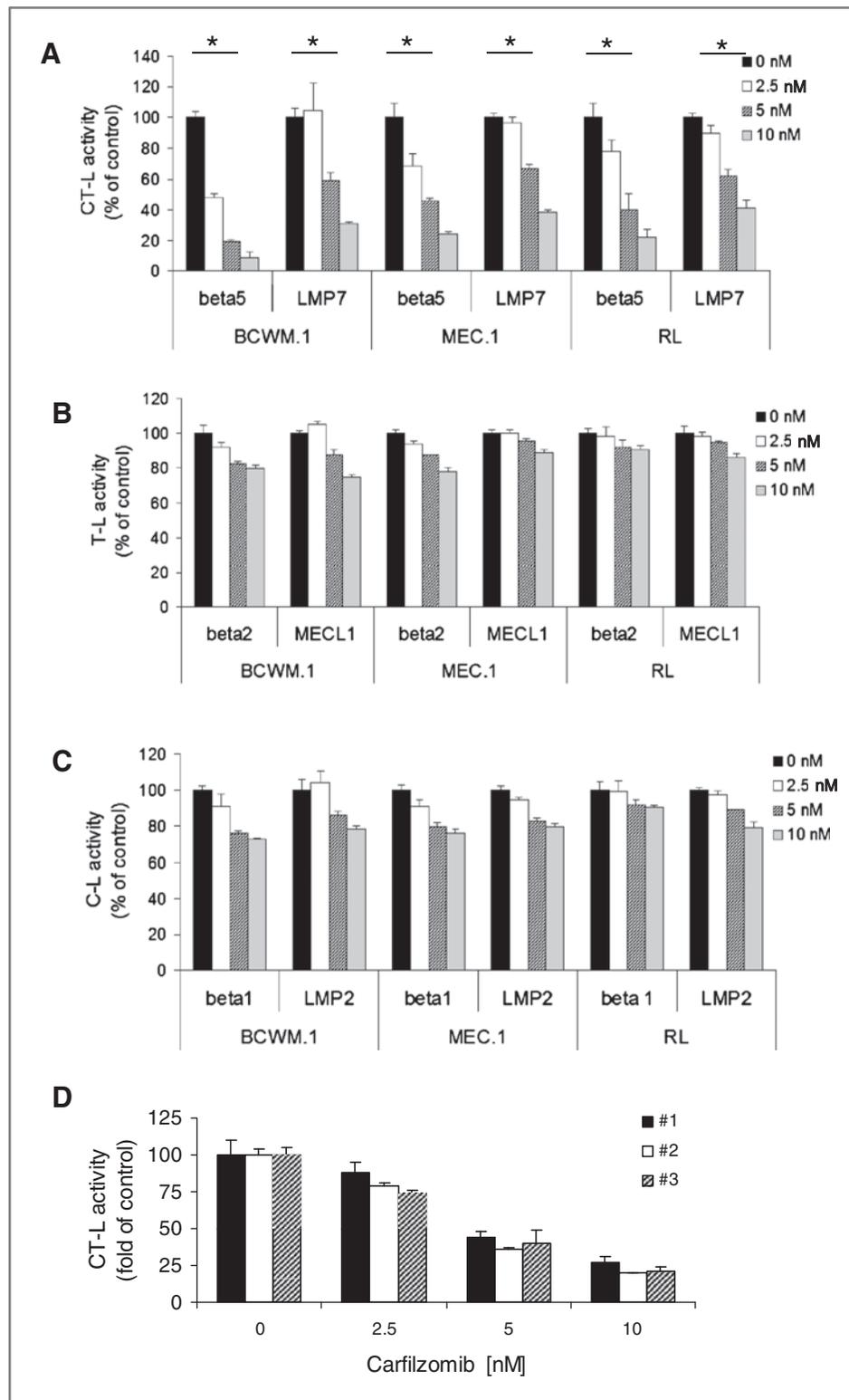


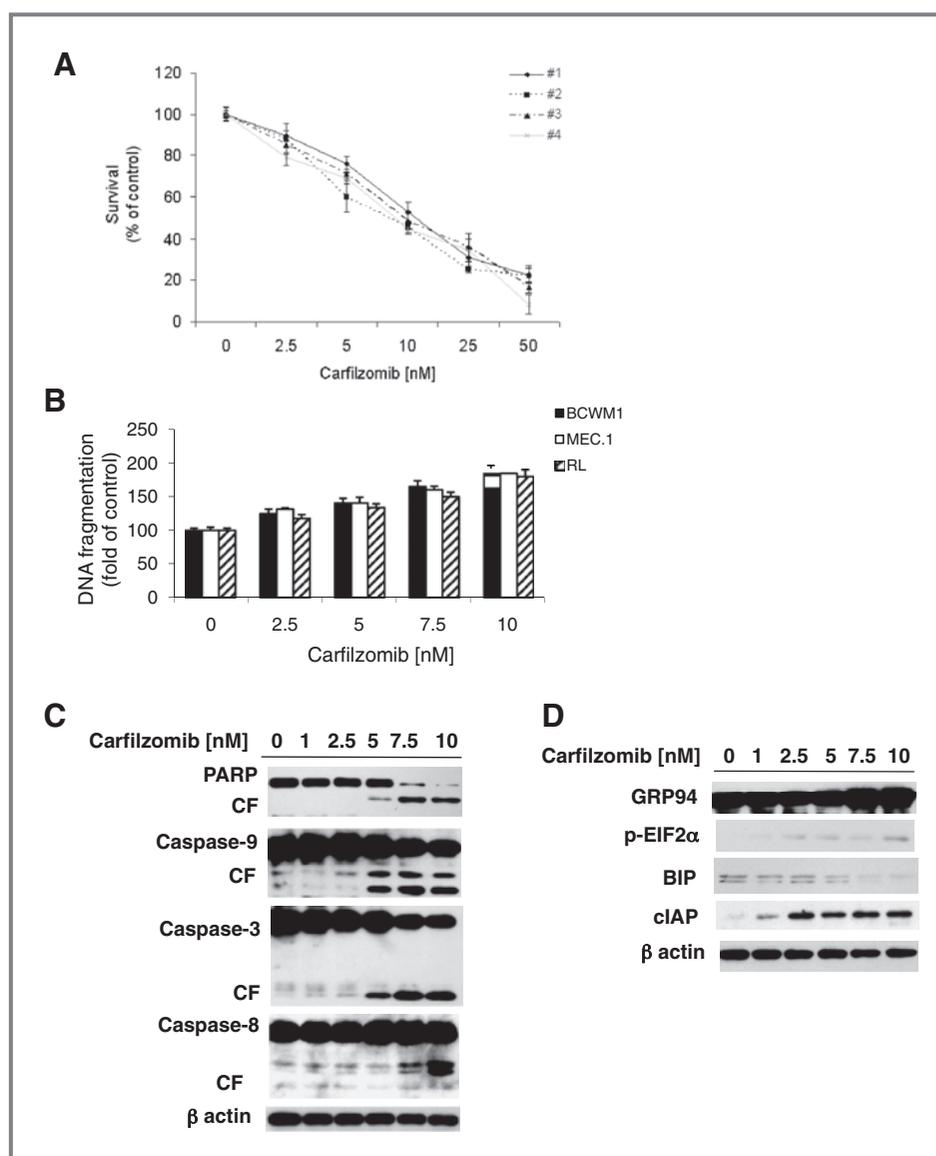
Figure 1. Carfilzomib selectively targets the chymotrypsin-like activity of the constitutive proteasome and immunoproteasome in WM cells and other IgM secreting lymphoma cell lines. A, B, and C, WM (BCWM.1) and IgM secreting lymphoma cell lines (RL; MEC.1) have been treated with carfilzomib (2.5–10 nM) for 2 hours and Carfilzomib effects on CT-L of constitutive proteasome ($\beta 1$, $\beta 2$, and $\beta 5$) and immunoproteasome (LMP2, MECL1, and LMP7) was assessed by ELISA protein lysates obtained from primary cells. Anti- $\beta 1$, $\beta 2$, $\beta 5$, -LMP2, -MECL1, -LMP7-primary, and HRP-conjugated secondary antibodies were used. Each subunits was measured as ng/ μ g total protein, according to the SuperSignal ELISA Pico Kit manufacturer's instructions. Experiments have been performed in triplicates. *, P values, all < 0.05 . D, Primary CD19+ tumor cells from 3 patients with WM were incubated for 2 hours in the presence of diluent or carfilzomib (2.5–10 nM). The chymotrypsin-like (CT-L) activity of the 20S proteasome of primary WM cells was determined by measurement of fluorescence generated from the cleavage of the fluorogenic substrate succ-LLVY-amc. Carfilzomib-induced modulation of CT-L activity has been expressed as fold of untreated samples. P values, all < 0.05 , but patient #1 0 nM vs. patient #1 2.5 nM (not significant). Bars represent standard deviation.

Carfilzomib exerts antitumor activity in WM cells and other IgM secreting lymphoma cells

The efficacy of Carfilzomib-dependent proteasome inhibition in targeting clonal IgM secreting cells was tested in primary WM CD19⁺ cells, and WM and IgM secreting lymphoma cell lines (BCWM1; RL; MEC.1), as well as in normal PBMC-derived CD19⁺ cells. We first evaluated the cytotoxic effect of Carfilzomib (2.5–50 nM) on primary WM bone-marrow derived CD19⁺ cells by MTT assay, and found that Carfilzomib induced cytotoxicity in a dose-dependent manner (IC₅₀: 10 nM; Fig. 2A). We subsequently validated Carfilzomib-induced toxicity in WM and IgM secreting lymphoma cell lines and found that Carfilzomib induced apoptosis in a dose-dependent manner, as assessed by DNA fragmentation (Fig. 2B). We next examined the molecular mechanisms whereby Car-

filzomib induces cytotoxicity in WM and demonstrated that Carfilzomib induced caspase-9, caspase-3, caspase-8, and PARP cleavage in a dose dependent manner (Fig. 2C). It is known that proteasome inhibition eradicates tumor cells, partly by initiating the unfolded protein response (UPR), a signaling cascade activated by the accumulation of misfolded proteins in the endoplasmic reticulum (ER; refs. 13,14). Previous reports indicate that induction of ER stress in WM cells may represent a valid therapeutic option in WM (15). We therefore sought to investigate the effect of Carfilzomib in modulating the expression of UPR components in WM cells as one of the mechanisms of cytotoxicity in WM cells. We found that Carfilzomib induced upregulation of UPR components, such as GRP94, together with increased phosphorylation of EIF-2 α (Fig. 2D). Similar results were confirmed at

Figure 2. Carfilzomib induces cytotoxicity in WM cells. **A**, Cytotoxicity was assessed by MTT assay in primary WM bone marrow-derived CD19⁺ cells (Carfilzomib 2.5–50 nM; 48 hours). Experiments have been performed in triplicates. **B**, BCWM.1, RL, and MEC.1 were cultured with carfilzomib (2.5–10 nM) for 48 hours and percentage of cells undergoing apoptosis was assessed by DNA fragmentation (*P* values: all <0.05, but BCWM1 and RL 0 nM vs. 2.5 nM (not significant)). **C** and **D**, BCWM.1 cells were cultured with carfilzomib (1–10 nM) for 12 hours. Whole cell lysates were subjected to Western blotting using anti-PARP, -caspase-9, -3, -8, - β -catenin, -GRP94, -phospho(p)-EIF2 α , -BiP, -cIAP, and - β actin antibodies.



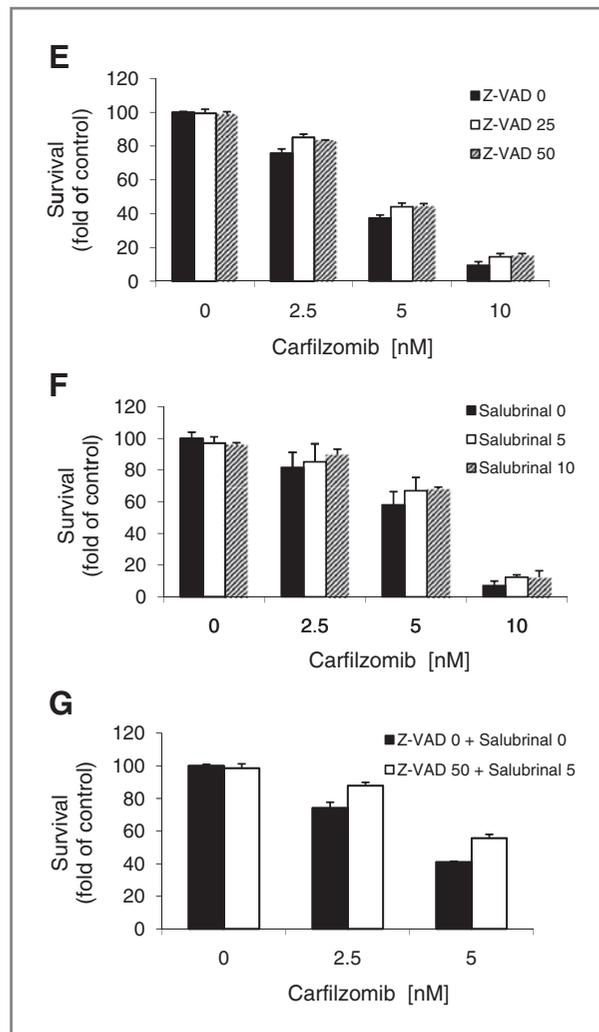


Figure 2. (Continued) E and F, BCWM.1 cells were treated with Carfilzomib (2.5–10 nM) for 48 hours, with or without Z-VAD-fmk (panel E; 25–50 μ M); or salubribral (panel F; 5–10 μ M). Cytotoxicity was determined by MTT assay. G, BCWM.1 cells were treated with carfilzomib (2.5–10 nM) for 48 hours, with or without the combination of Z-VAD-fmk (50 μ M) and salubribral (5 μ M). Cytotoxicity was determined by MTT assay. Bars represent standard deviation.

protein levels in other lymphoma IgM secreting cell lines (Supplemental Fig. 1).

Other mechanisms of carfilzomib-induced apoptosis in WM

Besides caspase activation, we found that carfilzomib induced apoptosis in WM cells in a caspase-independent manner, as demonstrated by down-modulation of BIP and upregulation of the antiapoptotic protein cIAP (Fig. 2D). We therefore explored other possible mechanisms of Carfilzomib-toxicity in WM cells. We first exposed WM cells to either the pan-caspase inhibitor Z-VAD-fmk (25–50 μ M) or to the ER stress-induced apoptosis protector salubribral (5–10 μ M); in the presence or absence of carfilzomib (2.5–5–10 nM). We observed that Z-VAD-fmk did not totally

overcome carfilzomib-induced cytotoxicity (Fig. 2E). Similar results were obtained in presence of salubribral (Fig. 2F) used either alone or in combination with Z-VAD-fmk (Fig. 2G), suggesting that other mechanisms may be responsible for Carfilzomib-dependent apoptosis in WM cells.

It has been reported that proteasome inhibition leads to induction of apoptosis through c-Jun N-terminal kinase (JNK) activation (16). We were able to show that carfilzomib triggered JNK activation in WM cells, as shown by upregulation of p-JNK1/2 (Fig. 3A). To better define the role of JNK activity in mediating carfilzomib-induced WM cytotoxicity, WM cells were treated with carfilzomib in either the presence or absence of the JNK inhibitor SP600125. Carfilzomib (5 nM, 7.5 nM)-induced cytotoxicity was inhibited upon SP600125 treatment. Similar results were validated on other lymphoma IgM secreting cell lines (RL; MEC1; Fig. 3B; $P < 0.05$).

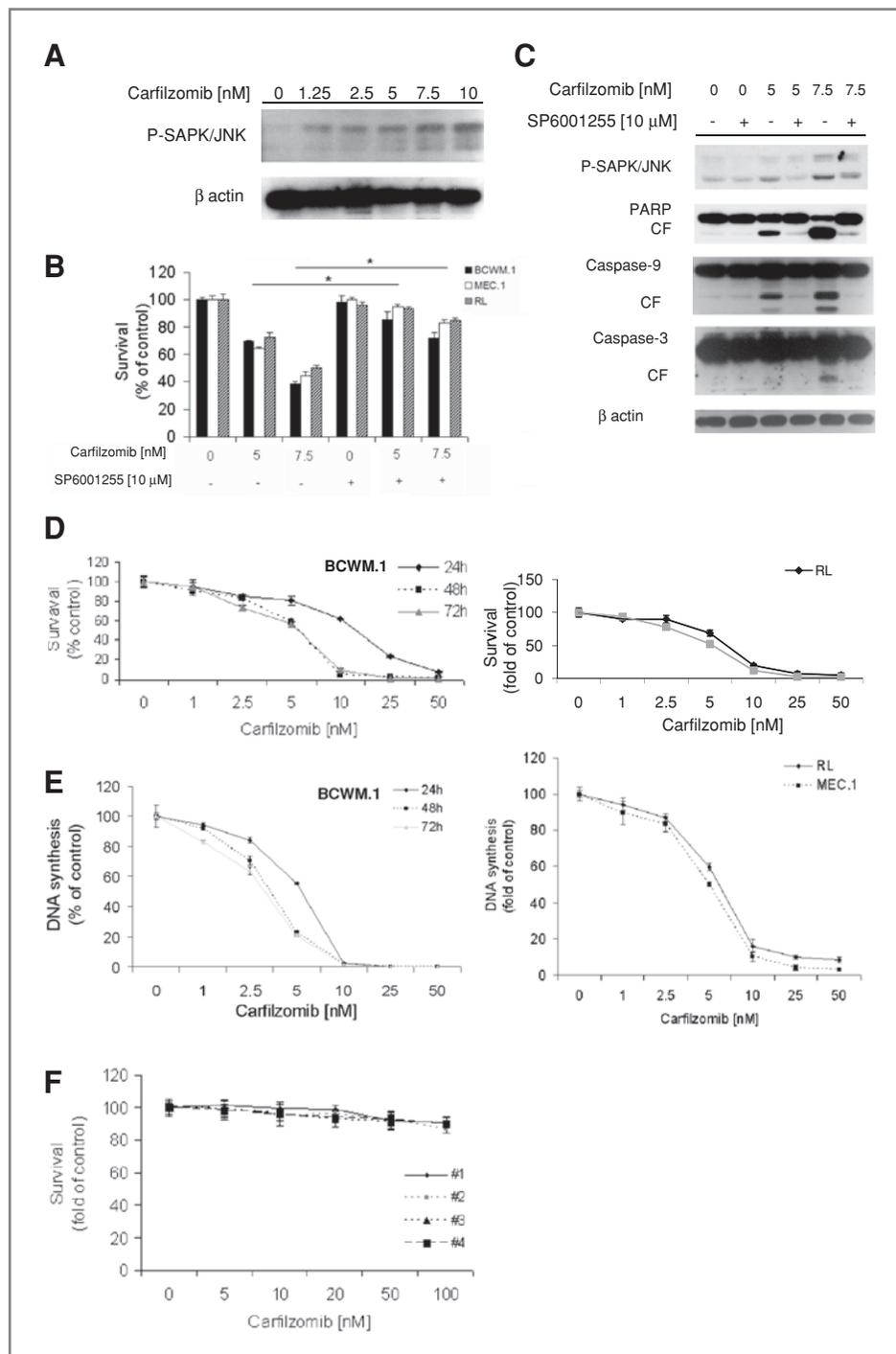
In addition, we demonstrated an inhibition of carfilzomib-dependent caspase-3, -9-, and PARP-cleavage (Fig. 3C), upon SP600125 treatment, confirming a role of JNK in regulating carfilzomib-dependent apoptosis in WM cells.

We further validated carfilzomib-dependent induction of antitumor activity in WM and IgM secreting lymphoma cell lines. Carfilzomib induced cytotoxicity in BCWM.1, RL, and MEC.1 in a dose-dependent manner (IC₅₀: 5–7.5 nM at 48 hours; Fig. 3D). Similarly, carfilzomib inhibited cell proliferation, as measured by [³H]-thymidine uptake assay, in a dose-dependent fashion (IC₅₀: 4–7.5 nM at 48 hours; Fig. 3E). In contrast, carfilzomib did not exert cytotoxicity on normal PBMC-derived CD19+ isolated from 4 healthy volunteers (Fig. 3F).

Carfilzomib targets WM cells in the context of bone marrow microenvironment

It has been clearly demonstrated that BM microenvironment confers growth and induces drug resistance in malignant cells (17). We therefore sought to determine the antitumor activity of carfilzomib in the presence of the BM milieu. We first investigated whether carfilzomib inhibits WM cell growth in the context of the BM milieu. BCWM.1 cells were cultured with carfilzomib (2.5–10 nM) in the presence or absence of BMSCs for 48 hours. The viability of BMSCs assessed by MTT was not affected by carfilzomib treatment (data not shown). Using [³H]-TdR uptake assay, adherence of BCWM.1 cells to BMSCs triggered a 35.9% increase in proliferation, which was inhibited by carfilzomib in a dose-dependent manner (Fig. 4A). We next evaluated the efficacy of carfilzomib in affecting migration and adhesion of WM cells. We first demonstrated that stromal derived factor-1 (SDF-1), one of the important regulators of migration in B-cells (18), induced migration in BCWM.1 cells at 30 nM SDF-1. To study the effect of carfilzomib on the migration of WM cells, BCWM.1 cells were incubated with carfilzomib (2.5–10 nM) for 4 hours. These doses and duration of incubation did not induce cytotoxicity in WM cells as confirmed by MTT (data not shown). Cells were then subjected to migration as

Figure 3. Carfilzomib-induced cytotoxicity is partially driven by activation of JNK and exerts antitumor activity in WM cells as well as in other lymphoma IgM secreting cells. A, BCWM.1 cells were cultured with carfilzomib (1.25–10 nM) for 12 hours. Whole cell lysates were subjected to Western blotting using anti-p-SAPK/JNK, and β -actin antibodies. B, BCWM.1 cells and IgM secreting lymphoma cells (RL; MEC1) were cultured with carfilzomib (5 nM, 7.5 nM) in presence or absence of the JNK inhibitor SP600125 (10 μ M) and cytotoxicity was assessed by MTT assay (*, P values: <0.05). C, BCWM.1 cells were cultured with carfilzomib (5 nM, 7.5 nM), in presence or absence of SP600125 (10 μ M) for 12 hours. Whole cell lysates were subjected to Western blotting using anti-p-SAPK/JNK, -PARP, -caspase-9, -3, and β -actin antibodies. D, Cytotoxicity was assessed by MTT assay in BCWM.1 cells and IgM secreting lymphoma cells RL, MEC.1, treated with carfilzomib 1–50 nM for 24–48–72 hours (BCWM.1) or 48 hours (RL, MEC1). E, DNA synthesis was measured by thymidine uptake assay in BCWM.1 cells and IgM secreting cell lines, RL, MEC.1 (48 hours), treated with carfilzomib (1–50 nM) for 24–48–72 hours (BCWM.1) or for 48 hours (RL, MEC1). F, Cytotoxicity was assessed by MTT in freshly isolated primary peripheral blood-derived CD19+ cells from 4 healthy donors, treated with carfilzomib 5–100 nM, for 48 hours. Bars represent standard deviation.



previously described.(11) Carfilzomib inhibited WM cell migration towards SDF-1, in a dose-dependent manner (Fig. 4B; $P < 0.05$). We also tested the effect of carfilzomib on the adhesion of WM cells and found that Carfilzomib exerted a dose-dependent inhibition of WM cell adhesion to fibronectin (Fig. 4C; $P < 0.05$). These results indicate the efficacy of carfilzomib in targeting WM cells, even in the context of BM milieu where it overcomes

BMSC-dependent proliferation, as well as adhesion and migration *in vitro*.

Combinatory effect of carfilzomib and bortezomib in targeting WM cells

Previous reports indicate that bortezomib mainly targets the CT-L activity and to a lesser degree the C-L activity (19); while carfilzomib acts as a selective inhibitor of the CT-L

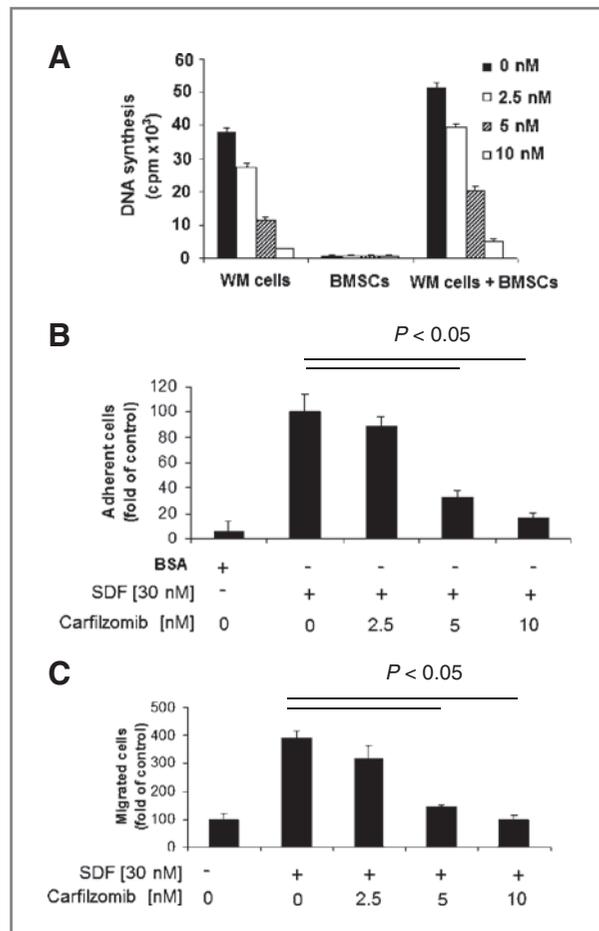


Figure 4. Carfilzomib targets WM cells even in the context of bone marrow milieu. **A**, BCWM.1 cells were cultured with control media and with carfilzomib (2.5–10 nM), for 48 hours, in the presence or absence of primary WM bone marrow stromal cells (BMSCs). Cell proliferation was assessed using [³H]-thymidine uptake assay. All data represent mean (\pm SD) of triplicate experiments. **B**, Adhesion assay with BCWM.1 in the presence or absence of carfilzomib (2.5–10 nM). BCWM.1 demonstrated increased adhesion in fibronectin-coated wells as compared with control. Carfilzomib inhibited adhesion in fibronectin-coated wells in a dose-dependent manner ($P < 0.05$). All data represent mean (\pm SD) of triplicate experiments. **C**, Transwell migration assay. SDF-1 (30 nM) was placed in the lower chambers and migration was determined after 3 hours. Transwell migration assay showing inhibition of migration of BCWM.1 in the presence of carfilzomib (2.5–10 nM). SDF-1 30 nM was placed in the lower chambers and induced migration as compared with control with no SDF-1. Carfilzomib inhibited SDF-1-induced migration in WM cells ($P < 0.05$). Bars represent standard deviation.

activity (17). Moreover, synergism between different classes of proteasome inhibitors in targeting WM cells has been demonstrated (12,20). We therefore sought to determine the effect of carfilzomib and bortezomib, either used as single agents or in combination, in targeting the CT-L activity of both constitutive proteasome and immunoproteasome. WM cells were treated with carfilzomib (2.5 and 5 nM), bortezomib (2.5–5 nM), alone or combination, for 2 hours. Carfilzomib showed a significant increase in inhibiting CT-L activity of the c20S (β 5; Fig. 5A) when combined

with bortezomib in WM cells. Specifically, carfilzomib (2.5 nM) induced inhibition of the β 5 activity in 53% of the treated cells, which was increased to 67% and 74% in the presence of bortezomib at 2.5 nM (combination index, C.I.: 0.97) and 5 nM (C.I.: 0.98), respectively, indicating an additive effect (Fig. 5B). Similar effects were also observed using carfilzomib at 5 nM. We therefore investigated whether the carfilzomib/bortezomib-dependent effect on targeting the CT-L activity could lead to either additive or synergistic induction of cytotoxicity on WM cells.

BCWM.1 cells were cultured with carfilzomib (5–7.5 nM) for 48 hours, in the presence or absence of bortezomib (5–10 nM). Carfilzomib showed significant cytotoxic effects when combined with bortezomib, as demonstrated using MTT assays at 48 hours (Fig. 5C). Carfilzomib (5 nM) induced cytotoxicity in 24.5% of BCWM.1 cells, which was increased to 67.3% and 86.2% in the presence of bortezomib at 5 nM (combination index, C.I.: 0.84) and 10 nM (C.I.: 0.59), respectively, indicating additive and synergistic activity, respectively. Similar results were obtained using bortezomib with carfilzomib 2.5 nM (Fig. 5C). Isobologram analysis, fractions affected, and the combination indexes for each of these combinations are indicated (Fig. 5D). Similar results were confirmed on other IgM secreting lymphoma cell lines (Supplemental Fig. 2).

To better define the mechanisms of combined carfilzomib plus bortezomib-induced WM cytotoxicity, we investigated the effect of carfilzomib (2.5–5 nM), either alone or in combination with bortezomib 10 nM, using immunoblotting after 12 hours treatment. Interestingly, we demonstrated that PARP-, caspase-9-, -3-cleavage and GRP94 upregulation were significantly higher using the combination compared with each agent alone (Fig. 5E). Importantly, carfilzomib/bortezomib-enhanced cytotoxicity was also confirmed on primary WM cells (Supplemental Fig. 3).

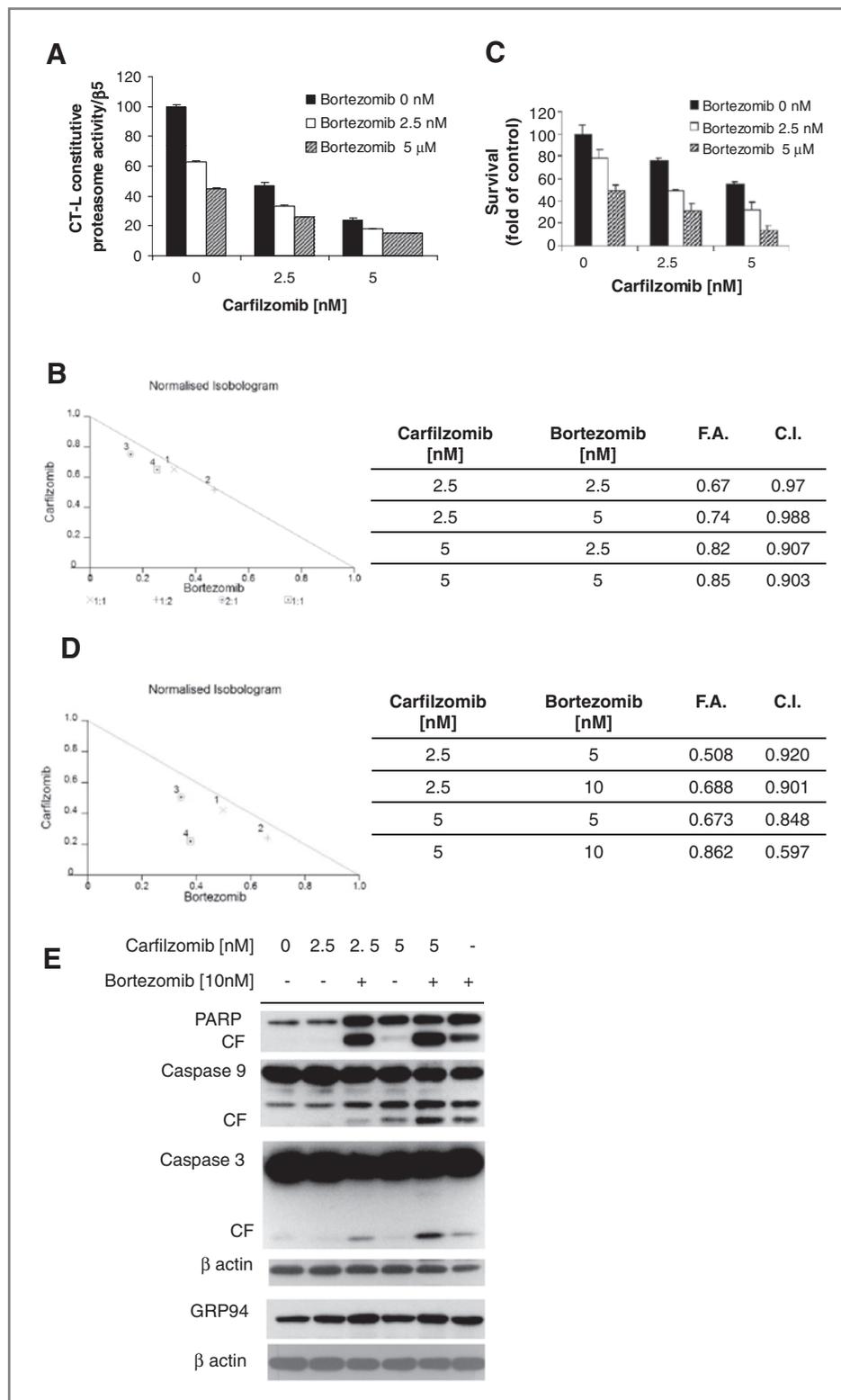
Carfilzomib targets WM cells *in vivo*

We next evaluated the antitumor activity of carfilzomib *in vivo*. SCID mice were treated with either vehicle ($n = 5$) or carfilzomib ($n = 5$) 5 mg/kg, day1–day2, for 3 weeks. BM cells and mononuclear cells were collected. Human WM cells (mCherry+) were detected by flow cytometry: we found that carfilzomib-treated mice presented with a significant lower number of tumor cells as compared with control mice (Fig. 6A; $P < 0.05$). In addition, WM-mCherry+ cells showed a significantly higher percentage of apoptosis in carfilzomib treated mice, as shown by annexin staining and flow cytometry analysis (Fig. 6B; $P < 0.05$). We next determined whether carfilzomib could affect IgM secretion from WM cells *in vivo*. Using a human IgM ELISA assay we showed that IgM secretion was significantly lower in the serum of mice treated with carfilzomib as compared with controls (Fig. 6C; $P < 0.05$).

Discussion

Bortezomib acts as a reversible inhibitor of the ubiquitin-26S proteasome pathway, and its antitumor activity has

Figure 5. Carfilzomib and bortezomib synergize in targeting WM cells. **A**, BCWM.1 cells were treated with carfilzomib (2.5 nM, 5 nM) in presence or absence of bortezomib (2.5 nM, 5 nM) for 2 hours; and effects on chymotrypsin-like activity (CT-L) of the constitutive proteasome ($\beta 5$) were evaluated by ELISA on protein lysates. Proteasome activity is expressed as fold of control (untreated cells). **B**, CalcuSyn software was used to determine presence or absence of synergism between carfilzomib and bortezomib in targeting the CT-L enzymatic activity. Isobologram, combination indices (C.I.) and fractions affected (FA) of the combination of carfilzomib and bortezomib are shown. All experiments were repeated in triplicate. **C**, BCWM.1 cells were cultured with carfilzomib (5 nM, 7.5 nM) for 48 hours, in the presence or absence of bortezomib (5 nM, 10 nM). Cytotoxicity was assessed by MTT assay. **D**, CalcuSyn software: isobologram, combination indices (C.I.) and fractions affected (FA) of the combination of carfilzomib and bortezomib are shown. All experiments were repeated in triplicate. **E**, BCWM.1 cells were cultured with carfilzomib (5 nM, 7.5 nM) in the presence or absence of bortezomib (10 nM) for 12 hours. Whole cell lysates were subjected to Western blotting using anti-PARP, -caspase-9, -3, -GRP94 and - β actin antibodies. Bars represent standard deviation.



been clearly demonstrated in a wide spectrum of hematologic malignancies, including WM, where bortezomib, either as single agent or in combination with rituximab, has shown a high response rate of about 80% in upfront or

relapsed and refractory WM (21,22). However, the rate of neuropathy induced by bortezomib in these patients was significantly higher than that observed in patients with Multiple Myeloma (MM) or other lymphomas due to

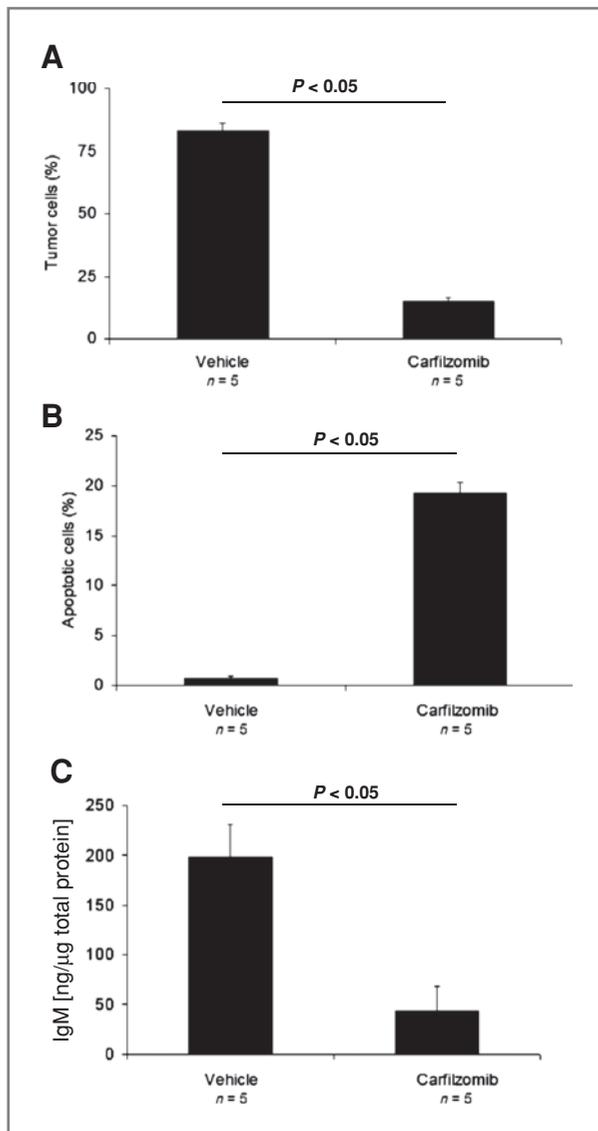


Figure 6. Carfilzomib targets WM cells *in vivo*. SCID mice have been injected intravenously (i.v.) with BCWM.1-mCherry⁺ cells, and treated with either vehicle ($n = 5$) or carfilzomib ($n = 5$; 5 mg/kg, day1–day2, for 3 weeks). Twenty-four hours after the last treatment, bone marrow cells and serum have been collected. **A**, Evaluation of tumor mCherry⁺ cells has been performed by detecting the percentage of mCherry signal (percentages represent average of $n = 5$ controls vs. average of $n = 5$ treated mice; $P < 0.05$). **B**, Quantification of apoptotic cells has been performed by Annexin/DAPI staining and flow cytometry analysis (percentages represent average of $n = 5$ controls vs. average of $n = 5$ treated mice; $P < 0.05$). **C**, Detection of human IgM levels has been performed by ELISA on serum collected from mice receiving either vehicle or carfilzomib (values represent average of $n = 5$ controls vs. average of $n = 5$ treated mice; $P < 0.05$). Bars represent standard deviation.

bortezomib-dependent inhibition of off-targets enzymes (21). Therefore, there is a need to develop proteasome inhibitors with a better neuro-toxicity profile.

Carfilzomib is a new irreversible, peptide epoxyketone proteasome inhibitor, that leads to a more sustained proteasome inhibition, with a minimal activity against off-

target enzymes, compared with the reversible inhibitor bortezomib (8). Carfilzomib has shown promising results in a Phase II clinical trial in patients with relapsed refractory MM, as well as in a recent phase I trial, demonstrating tolerability and clinical activity in multiple hematologic malignancies using consecutive-day dosing. Interestingly, it showed minimal neurotoxicity in the clinical trials performed to date (23, 24).

It has recently been demonstrated that carfilzomib-dependent selective inhibition of the CT-L activity of the i20S and c20S leads to significant antineoplastic effects, in a wide spectrum of hematologic tumors, such as Burkitt's lymphoma, T-cell leukemia and multiple myeloma, without inducing toxicity in nontransformed cells (8). Moreover, it has been shown that targeted inhibition of the immunoproteasome represents a valid antitumor strategy in plasma cell malignancies, such as in multiple myeloma, where it also overcomes resistance to conventional chemotherapeutics and nonspecific proteasome inhibitors that may lead to significant toxicity, due to their off-target activity (25).

In previous studies, we have characterized the distribution of the i20S and c20S subunits in WM primary cells and in their normal cellular counterpart, and described that primary WM cells express a higher amount of i20S subunits compared with c20S, and expression level of i20S components is higher than in normal cells.(13) We therefore sought to investigate the antitumor activity of carfilzomib in this disease. We demonstrated that clinically achievable concentrations of carfilzomib inhibited the CT-L activity of both i20S (LMP7) and c20S ($\beta 5$) in WM, resulting in increased toxicity in primary WM cells as well as in other IgM secreting lymphoma cell lines.

Specifically, carfilzomib-dependent inhibition of $\beta 5$ was reached at lower IC₅₀ doses as compared with the inhibition of LMP7: this could reflect the distribution of the CT-L subunits within WM tumor clone where LMP7 is higher as compared with $\beta 5$ (13).

Carfilzomib-induced toxicity in WM cells is supported by activation of caspase-dependent and caspase-independent mechanisms, as shown by the modulation of the UPR. Although under physiological conditions, UPR leads to accumulation and activation of misfolded proteins in the endoplasmic reticulum ER, thus resulting in cell survival (26) in tumor cells exposed to proteasome inhibitors the prolonged ER stress may induce cell apoptosis. Indeed, these studies demonstrated the carfilzomib-dependent initiation of the UPR in WM cells.

These findings indicate a possible different mechanism of action between CFZ and BTZ. Indeed, BTZ differs from tunicamycin and thapsigargin, known to be ER stress inducers, in that it is not associated with activation of UPR. For example, while it has been reported that BTZ inhibits tunicamycin-dependent upregulation of p-eIF2 α in pancreatic tumor cells, we found that CFZ was able to induce increase of p-eIF2 α , as well as increase of GRP94, another important member of the UPR machinery. Taken together these findings indicate that CFZ may be responsible for an

exacerbation of ER-stress induction, leading to WM cell death.

Other mechanisms of carfilzomib-induced caspase-independent apoptosis included the activation of JNK. Furthermore, by using the JNK inhibitor SP600215, we were able to show an inhibition of the carfilzomib-dependent induction of caspase-9, -3, and PARP cleavage.

Importantly, carfilzomib exerted antitumor activity in WM cells even in the context of BM milieu. This represents an important step in overcoming the BM-induced growth advantage in WM cells. This ability in targeting WM cell in the presence of primary WM stromal cells was also supported by the carfilzomib-induced inhibition of adhesion and migration, both essential for WM disease progression and dissemination.

We have previously shown the synergistic or additive effects between bortezomib and other proteasome inhibitors in WM, and similar results have been validated in MM (11,20). In these studies, we demonstrated that carfilzomib and bortezomib exerted an additive effect in targeting the CT-L activity of the proteasome. We further showed that the 2 agents synergistically induce cytotoxicity in WM cells. The mechanisms leading to synergistic effect by carfilzomib and bortezomib could be most likely multifactorial and not only due to their inhibitory effect on the CT-L activity of the proteasome. Indeed, we explored the mechanism of synergy for these 2 agents and demonstrated that they act synergistically by inducing a stronger cleavage of caspase-9, -3, and PARP, leading to synergistic toxicity in WM cells.

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These preclinical findings demonstrate for the first time that carfilzomib targets WM cells both *in vitro* and *in vivo*, due to its anti-CT-L activity of both immunoproteasome and constitutive proteasome, providing the framework for testing this novel irreversible CT-L inhibitor in this disease.

Disclosure of Potential Conflicts of Interest

IM Ghobrial, advisory board, Millenium/Takeda, Genzyme, Celgene, Onyx, and Novartis; AM Roccaro, advisory board, Roche.

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Author Contribution

A. Sacco, A. M. Roccaro, and I. M. Ghobrial designed the research and wrote the article; A. Sacco, B. Morgan, H. T. Ngo, A. K. Azab, Y. Liu, Y. Zhang, P. Quang, and P. Maiso performed research; A. Sacco, A. M. Roccaro, M. Aujay, G. C. Issa, and I. M. Ghobrial analyzed the data.

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