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Development of peptide-based reversing agents for P-glycoprotein-mediated resistance to carfilzomib

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Abstract

Carfilzomib is a novel class of peptidyl epoxyketone proteasome inhibitor and has demonstrated promising activity in multiple clinical trials to treat patients with multiple myeloma and other types of cancers. Here, we investigated molecular mechanisms underlying acquired resistance to carfilzomib and a potential strategy to restore cellular sensitivity to carfilzomib. H23 and DLD-1 cells (human lung and colon adenocarcinomas cell lines) with acquired resistance to carfilzomib displayed marked cross-resistance to YU-101, a closely related proteasome inhibitor and paclitaxel, a known substrate of Pgp. However, carfilzomib-resistant cells remained sensitive to bortezomib, a clinically used dipeptide with boronic acid pharmacophore. In accordance with these observations, carfilzomib-resistant H23 and DLD-1 cells showed marked upregulation of P-glycoprotein (Pgp) compared to their parental controls and co-incubation with verapamil, a Pgp inhibitor, led to an almost complete restoration of cellular sensitivity to carfilzomib. These results indicate that Pgp upregulation plays a major role in the development of carfilzomib resistance in these cell lines. In developing a potential strategy to overcome carfilzomib resistance, we as a proof of concept prepared a small library of peptide analogs derived from the peptide backbone of carfilzomib and screened these molecules for their activity to restore carfilzomib sensitivity when co-treated with carfilzomib. We found that compounds as small as dipeptides are sufficient in restoring carfilzomib sensitivity. Taken together, we found that Pgp upregulation plays a major role in the development of resistance to carfilzomib in lung and colon adenocarcinomas cell lines and that small peptide analogs lacking the pharmacophore can be used as agents to reverse acquired carfilzomib resistance. Our findings may provide important information in developing a potential strategy to overcome drug resistance.

Keywords

acquired resistance; carfilzomib; P-glycoprotein; resistance reversal; small peptides

Introduction

The proteasome is a multiprotease complex found in all eukaryotic cells and plays a key role in regulating ubiquitin-dependent turnover of numerous proteins, including those involved in cell cycle progression, apoptosis, survival and stress response.^{1, 2} For this reason, many research efforts over the past decade have been dedicated to developing proteasome inhibitors as anticancer agents, resulting in the development of bortezomib (PS-341, Velcade®), a first-in-class proteasome inhibitor approved for the treatment of relapsed

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multiple myeloma and refractory mantle cell lymphoma. The successful development of bortezomib is followed by a number of next-generation proteasome inhibitors currently in preclinical and clinical development.^{3, 4} Among them, carfilzomib (PR-171), a tetrapeptide epoxyketone, is the furthest in clinical development.⁵ Compared to bortezomib, carfilzomib is shown to be highly specific for the proteasome and minimally inhibits other cellular proteases. This specificity of carfilzomib has been attributed to its improved toxicity profiles over bortezomib, a dipeptidyl boronate, which can inhibit non-proteasomal proteases, such as a serine protease HtrA2/Omi, and cause severe side effects such as peripheral neuropathy.^{5–7}

Further supporting promising potential of carfilzomib therapy, several investigations have now demonstrated that carfilzomib therapy (as a single agent or in combination with other chemotherapeutic agents) can be effective in treating hematopoietic malignancies and cancers of solid organs including non-small cell lung cancer and colon cancer.^{5, 8} However, it is also fully expected that resistance will emerge and cancer cells will not retain long-term efficacy with carfilzomib therapy. For epoxomicin (a prototypical peptidyl epoxyketone proteasome inhibitor isolated from an actinomycete strain), it has been shown that upregulation of P-glycoprotein (Pgp/MDR1) leads to cellular extrusion of epoxomicin and confers drug resistance.⁹ For carfilzomib, an early report described that human multiple myeloma cells resistant to doxorubicin are less sensitive to carfilzomib compared to their parental controls, suggesting the involvement of multidrug resistance (MDR)-related efflux pumps.¹⁰ Recently, a more detailed investigation was carried out using multiple cell lines stably expressing various MDR-related transporters and the results indicated that only Pgp, but none of the other MDR-related transporters, has the ability to extrude carfilzomib and to confer resistance.¹¹ However, it remains to be determined whether Pgp upregulation serves as a major mechanism for carfilzomib resistance in cancer cells exposed to prolonged carfilzomib therapy. In case of bortezomib, multiple resistance mechanisms have been reported; they include amplification/mutation of target proteasomal subunits,^{12–15} suppression of protein biosynthesis,¹⁶ alterations in ER stress responses¹⁷ or formation of stress granules.¹⁸ An early clinical trial with carfilzomib reported that patients who are refractory to bortezomib can be responsive to carfilzomib therapy, suggesting that resistance mechanisms for bortezomib may not overlap with those for carfilzomib.⁵ However, this has not been thoroughly examined.

In our current study, we investigated molecular factors involved in carfilzomib resistance by establishing carfilzomib-resistant lung and colon adenocarcinomas cell lines. Our results indicate that Pgp-mediated efflux plays a major role in acquired resistance of H23 and DLD-1 cancer cells to carfilzomib. As a proof of concept, we then set out to develop agents that can restore the sensitivity of cells to carfilzomib. We found that peptide analogs as small as dipeptides derived from the peptide backbone of carfilzomib can effectively restore carfilzomib sensitivity in our cell line models. These results indicate that small and minimally toxic peptide analogs may be used to overcome the resistance of cancer cells to carfilzomib or other drugs that develop Pgp-mediated drug resistance.

Experimental Section

Cell lines and reagents

Human cancer cell lines H23 (lung adenocarcinoma) and DLD-1 (colon adenocarcinoma) were obtained from American Type Culture Collection and maintained in the recommended culture media of RPMI-1640 supplemented with 10% fetal bovine serum (Clontech, Mountain View, CA) at 5% CO₂ and 37 °C. Carfilzomib and YU-101 were synthesized and purified as reported previously^{8, 19} and bortezomib was obtained from ChemieTek Inc. (Indianapolis, IN). Di-, tri- and tetra-peptide analogs of carfilzomib were prepared following

the standard peptide synthesis strategy.²⁰ Verapamil and paclitaxel were obtained from Sigma (St. Louis, MO). Pgp (F4) and BCRP antibodies were obtained from Sigma and GAPDH antibody was obtained from Cell Signaling (Danvers, MA). Vibrant® multidrug resistance assay kit containing calcein-AM was obtained from Invitrogen (Carlsbad, CA).

Establishment of carfilzomib-resistant cancer cell lines

H23 and DLD-1 cells were maintained with stepwise-increasing concentrations of carfilzomib over a period of 6 months. Initial concentrations of carfilzomib were 10 and 15 nM for H23 and DLD-1 cells and increased up to 500 and 1,000 nM over 6 months, respectively. The cells resistant to carfilzomib were termed H23/Carf and DLD-1/Carf.

Cell viability assay

H23/Carf, DLD-1/Carf and parental H23 and DLD-1 cells in logarithmic phase growth were seeded in 96-well plates at 5,000 – 20,000 cells/well in three or four replicates. After 24 hours, cells were treated with carfilzomib, bortezomib, YU-101 or paclitaxel at a series of concentrations for 72 h. Cell viability was measured using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). The IC₅₀ values were calculated by fitting the observed data to sigmoidal dose-response curves with variable slopes using GraphPad Prism 5.0 (La Jolla, CA).

Immunoblotting

Whole cell lysates were prepared in a lysis buffer (17 mM Tris, 50 mM NaCl, 0.3% Triton X-100, pH 8.0) containing protease inhibitors (Roche Applied Science, Indianapolis, IN). Cell lysates containing equivalent amounts of total protein were resolved by SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skim milk, membranes were probed with primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody. GAPDH was used as a gel loading control. Signals were visualized using enhanced chemiluminescence detection reagents.

Quantitative RT-PCR

Total RNAs (1 µg) from H23/Carf, DLD-1/Carf and parental H23 and DLD-1 cells were converted to single-stranded cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For quantitative RT-PCR analyses of MDR1 and BCRP transcripts, the following primer sequences were used; for MDR1, sense 5'-GTCCCAGGAGCCCATCCT-3' and antisense 5'-CCCGGCTGTTGTCTCCAT-3'; for BCRP, sense 5'-TGGCTGTCATGGCTTCAGTA-3' and antisense 5'-GCCACGTGATTCTTCACAA-3'; for β-actin, sense 5'-GCATCCTCACCCTGAAGTAC-3' and antisense 5'-GATAGCACAGCCTGGATAGC-3'. Quantitative RT-PCR was performed in triplicate using iCycler with the iQ SYBR-green Supermix (Bio-Rad). The conditions for quantitative RT-PCR were as follows: annealing at 65 °C with 40 cycles for MDR1 and β-actin; annealing at 55°C with 40 cycles for BCRP. The relative quantity of the transcripts were calculated by the formula $2^{-\Delta Ct}$, where ΔCt was determined by subtracting the average β-actin Ct value from the average target Ct value.

Synthesis of peptide analogs structurally related to carfilzomib

Tetra-peptides lacking an epoxyketone pharmacophore and its truncated peptides were synthesized by standard peptide coupling methods.²⁰ All intermediates and final products were validated by ¹H NMR and mass spectrometry.

Impact of peptide analogs on carfilzomib sensitivity in carfilzomib-resistant cells and their parental controls

In order to examine the resistance reversing effects of peptide analogs, H23/Carf or DLD-1/Carf cells were treated with peptide analogs (25 μ M) in the absence and presence of carfilzomib (500 nM for H23/Carf and 1000 nM for DLD-1/Carf). After 72 hours, cell viability was measured using the CellTiter-Glo luminescent cell viability assay and expressed as % viability relative to those treated with vehicle alone. With the selected peptide analogs (compounds 8 to 10), we examined whether these truncated peptides can restore carfilzomib sensitivity of H23/Carf or DLD-1/Carf cells in a concentration-dependent manner. Additional experiments were performed using compounds 8 to 10 to examine whether they can restore sensitivity to paclitaxel (2 μ M) in H23/Carf or DLD-1/Carf cells and whether they have any potentiating impact on cell killing by carfilzomib (15 nM) in the parental H23 and DLD-1 cells.

Comparison of peptide analogs for their inhibitory effects on the Pgp-dependent extrusion of calcein

The Pgp-inhibitory activity of peptide analogs was assessed using Vybrant® Multidrug Resistance Assay Kit (Invitrogen). Briefly, DLD-1/Carf and H23/Carf cells were plated onto 96-well plates (300,000 cells per well) in suspension. Cells were then pre-incubated with PBS, verapamil (25 μ M), or compounds 8 to 10 (25 μ M) for 15 min at 37 °C. Subsequently, calcein-AM was added to the cells at a final concentration of 0.25 μ M and the plates were incubated for 15 min at 37 °C. Cells were washed and cellular retention of calcein was assessed by measuring fluorescence (excitation 494 nm, emission 517 nm) using a fluorescence microplate reader (SpectraMax M5, Molecular Devices). Experiments were conducted in three replicates and the relative calcein retention was calculated by normalizing fluorescence signals from cells treated with compounds to those from cells treated with vehicle alone.

Statistical analyses

Results are expressed as means $\pm$ S.D. The statistical significance of the differences between groups was determined using either Student's t-test (with Bonferroni adjustment for multiple testing when appropriate) or one-way ANOVA (followed by the Newman-Keuls test or the Bonferroni test). All statistical analyses were carried out using GraphPad Prism (GraphPad Software).

Results

Development of H23 and DLD-1 cell lines with acquired resistance to carfilzomib

H23/Carf and DLD-1/Carf cells were established by maintaining H23 and DLD-1 cells with increasing concentrations of carfilzomib for approximately 6 months. H23/Carf and DLD-1/Carf cells were found to proliferate without any apparent cell death at carfilzomib concentrations of 500 and 1,000 nM, respectively. We determined the extent of carfilzomib resistance by measuring the IC₅₀ values of carfilzomib in inhibiting cell growth of these cell lines. As expected, IC₅₀ values for H23/Carf and DLD-1/Carf cells against carfilzomib were markedly increased; 1,300 vs 17.6 nM for H23/Carf and H23 cells (74-fold change) and 2,900 vs 32.9 nM for DLD-1/Carf and DLD-1 cells (88-fold change), respectively (Figure 1 and Table 1).

We have also determined whether H23/Carf and DLD-1/Carf cells are cross-resistant to YU-101, an epoxyketone-based proteasome inhibitor closely related to carfilzomib (of note, carfilzomib is derived from YU-101, by adding the more water-soluble morpholino group at the N-terminus).²¹ Not surprisingly, H23/Carf and DLD-1/Carf cells were found to be

highly cross-resistant to YU-101 (Figure 1 and Table 1). In contrast, H23/Carf and DLD-1/Carf cells remained quite sensitive to bortezomib, showing much less pronounced changes in their IC₅₀ values for bortezomib when compared to their parental controls. On the other hand, the carfilzomib-resistant cells displayed a high degree of cross-resistance to paclitaxel, a well-known Pgp substrate (>200-fold increase in the IC₅₀ values compared to parental controls).

Upregulation of P-glycoprotein as a major mechanism for acquired resistance to carfilzomib

In order to verify whether the decreased sensitivity of H23/Carf and DLD-1/Carf to carfilzomib and paclitaxel is indeed mediated by Pgp upregulation, we examined the cellular levels of Pgp and other MDR-related efflux pumps including BCRP (breast cancer resistance protein, *ABCG2*) and members of multidrug resistance proteins (MRP1, MRP2, MRP3). Our immunoblotting and RT-PCR analyses indicated that the protein and mRNA levels of Pgp are markedly upregulated in both H23/Carf and DLD-1/Carf cells, but the levels of BCRP remained unchanged (Figure 2). No detectable differences were found in the mRNA expression of MRP1, MRP2 or MRP3 (results not shown). We further examined whether Pgp upregulation is responsible for carfilzomib resistance using verapamil, a widely used Pgp transport inhibitor. Our results showed that verapamil can almost completely restore carfilzomib sensitivity in both H23/Carf and DLD-1/Carf cells (Figure 2C). Given the almost complete resistance reversal by verapamil, Pgp upregulation appears to be the major mechanism of carfilzomib resistance in these cell line models.

Small peptide analogs as resistance-reversing agents

Given that carfilzomib is a substrate of Pgp, we envisioned carfilzomib to be a good lead molecule in developing resistance-reversing agents. As a first step, we synthesized a small library of peptide analogs based on the peptide backbone structure of carfilzomib. All of the synthesized peptide analogs had benzyl ester groups at the C-terminus instead of the epoxyketone pharmacophore (Figure 3A). None of these peptide analogs, when treated alone, influenced cell viability of H23/Carf and DLD-1/Carf (open bars in Figure 3B). However when co-treated with carfilzomib (500 and 1,000 nM for H23/Carf and DLD-1/Carf, respectively, well below the IC₅₀ values in these carfilzomib-resistant cells), the peptide analogs (especially compounds 3 and 4) were able to partially restore carfilzomib sensitivity (Figure 3B). In order to improve their ability to reverse carfilzomib resistance, we replaced the N-terminus morpholino group of these carfilzomib fragments with a pyridine group (compounds 5 to 7, Figure 4A). The pyridine substitution was based on the recent report showing that peptidyl epoxyketones containing heterocyclic groups at the N-terminus have favorable interactions with Pgp.²² Indeed, we found that the compounds 5 to 7, which have the pyridine group at the N-terminus, are far more effective in restoring carfilzomib sensitivity than those with the morpholino group at the N-terminus (Figure 4B). The restored sensitivity is unlikely due to the toxicity of these compounds, in that when treated alone, these compounds had no effects on cell viability (open bars in Figure 4B). Next, we replaced the esterase-vulnerable benzyl ester with the esterase-proof Weinreb amide, yielding compounds 8 to 10 (Figure 5A). These substitutions further improved the ability of the peptide analogs to reverse carfilzomib resistance (Figure 5B). Again, the compounds 8 to 10, when treated alone, showed no major toxic effects in resistant cells (open bars, Figure 5B). In additional experiments, we confirmed that the resistance-reversing effects of compounds 8 to 10 are concentration-dependent in both DLD-1/Carf and H23/Carf cells (Figure 6A and 6B). Compounds 8 to 10 were also effective in reversing the cross-resistance of DLD-1/Carf and H23/Carf cells to paclitaxel (Figure 6C).

To further verify whether the resistance-reversing activity of these peptide analogs is related to their inhibitory effect on Pgp, we measured the impact of compounds 8 to 10 on the Pgp-mediated cellular extrusion of calcein. Consistent with the marked upregulation of Pgp in DLD-1/Carf and H23/Carf cells (Figure 2), the pre-incubation of the Pgp inhibitor verapamil led to approximately 610 – 750% increases in cellular retention of calcein in these carfilzomib resistant cell lines (Figure 6D). As a control, we have also examined the extent of changes in cellular retention of calcein in the parental DLD-1 and H23 cells. The results indicated that verapamil has little to no effect on cellular calcein retention in these parental cells (13 and 9% increases in the parental DLD-1 and H23 cells, respectively), suggesting that the Pgp activity is much lower in these parental cells. Preincubation of compounds 8 to 10 also led to substantial increases in cellular retention of calcein in DLD-1/Carf and H23/Carf cells (Figure 6D). All of compounds 8 to 10 showed p values <0.05 when compared to the vehicle control. However, when the Bonferroni-corrected p value threshold was used to account for multiple testing, only compounds 8 and 10 were found to be statistically significant in both DLD-1/Carf and H23/Carf cells.

Impact of small peptide analogs on the carfilzomib sensitivity of parental cell lines

In addition to reversing the acquired resistance to carfilzomib, the small peptide analogs of carfilzomib may potentially impact the sensitivity of parental cells to carfilzomib by influencing the basal expression/activity of Pgp. Indeed, our results indicate that the compounds 8 to 10 can potentiate the sensitivity of the parental DLD-1 and H23 cells to carfilzomib, although the extent of the potentiating effects was lesser than in carfilzomib-resistant cells (Figure 7A and 7B). These potentiating effects of compounds 8 to 10 were more pronounced in DLD-1 cells than in H23 cells, which may be related to cell line-dependent differences in the basal expression/activity of Pgp. The compound 8 was found to influence cell viability in H23 cells, but not in DLD-1 cells, when incubated alone (Figure 7B). Given that the compound 8 did not impact cell viability of H23/Carf and DLD-1/Carf cells (Figure 6A & 6B), the toxicity associated with compound 8 appears to be cell line-dependent.

Discussion

Carfilzomib is an epoxyketone-based proteasome inhibitor and has shown promising preclinical and clinical activity in multiple myeloma and other types of cancer.⁴ Compared to bortezomib, which also targets non-proteasomal proteases in cells, carfilzomib is highly specific towards the proteasome and shown to inhibit primarily the chymotrypsin-like activity of the proteasome via covalent modification. In early clinical trials, carfilzomib has shown improved toxicity profiles over bortezomib and anticancer activity even in patients who do not respond to bortezomib therapy.^{4, 5} These encouraging results with carfilzomib may lead to additional treatment options for blood cancers and cancers of other organs. However, as seen with many other chemotherapy agents, it is also expected that prolonged carfilzomib therapy is likely to result in the emergence of carfilzomib resistance. This led us to investigate molecular mechanisms for developing acquired resistance to carfilzomib and to explore a potential strategy to restore carfilzomib sensitivity.

In our present study, we report that Pgp plays a major role in acquired resistance to carfilzomib in lung and colon adenocarcinoma cell line models, extruding carfilzomib out of cells (Figure 2). In addition, we demonstrate that carfilzomib resistance can be reversed with co-treatment of small truncated peptides derived from the backbone of carfilzomib (Figures 3–6). While several types of peptides (especially with hydrophobic side chains such as bulky aromatic and alkyl groups) have been reported to interact with Pgp,^{23–26} they are typically of larger molecular size than peptide analogs developed in our current study or may encounter solubility problems due to bulky hydrophobic protecting groups at N- and C-

termini. Here, we report that molecules as small as dipeptide analogs can be used as resistance reversing agents. The favorable properties of these dipeptide analogs include having relatively good water solubility and no major toxicity. While the truncated peptide analogs have shown their resistance-reversing activity in our *in vitro* models, their efficacy *in vivo* may be affected by high *in vivo* clearance typically associated with peptide fragments. However, the recent study on the *in vivo* metabolism of carfilzomib supports that our peptide analogs may have adequate *in vivo* stability.²⁷ Following intravenous administration of carfilzomib to rats, Yang et al.,²⁷ detected the major carfilzomib metabolites, morpholino-homophenylalanine-leucine (M15) and morpholino-homophenylalanine (M14) as well as carfilzomib in plasma, urine, and bile. Both M14 and M15 were formed quickly as a result of peptide and epoxyketone hydrolysis and showed longer terminal half-lives in plasma than carfilzomib (the two metabolites made up 53% of total parental dose 24 hours post-administration). Given the close structural similarities between M14 and M15 with our peptide analogs, it is expected that the peptide analogs would have adequate *in vivo* stability in plasma.

Currently, further optimization of the peptide analogs is ongoing in order to improve the efficacy and potency in reversing carfilzomib resistance. During the past decade, substantial advances have been made in our understanding of the structure of Pgp and its binding modes with substrates of extremely diverse structures.^{30, 31} Along with that, there have been considerable efforts in predicting and designing Pgp substrates/inhibitors.^{32, 33} Using a recently reported prediction method (a support vector machine method available from <http://pgp.althotas.com>),³³ we tested whether our peptide analogs 1 to 10 are predicted to be Pgp substrates. Interestingly, the results predicted that all ten analogs are potential Pgp substrates. Although there is a possibility that all ten peptide analogs are indeed Pgp substrates at concentrations higher than we tested (i.e. > 25 μ M), our experimental results suggest that the chemical structures of these peptides at the N-terminus may be more important elements for Pgp interactions.

Our present study is mainly focused on cancer cell line models with acquired resistance to carfilzomib, but our findings can be applied to other types of cancer intrinsically resistant to carfilzomib via a Pgp-dependent mechanism. Interestingly, we also observed that the peptide analogs can, albeit to a much lesser extent, potentiate the effects of carfilzomib in the parental DLD-1 and H23 cells (Figure 7). These potentiating effects of the peptide analogs may depend on basal Pgp activity of the parental cell lines. Alternatively, these Pgp blockers may also be useful for improving oral bioavailability of proteasome inhibitors and other therapeutic agents that are Pgp substrates. In this regard, Zhou et al. recently reported the design of ONX0912, an orally active epoxyketone-based proteasome inhibitor through chemical modifications evading interactions with the intestinal Pgp.²² By evading the Pgp-dependent efflux in the intestinal epithelial barrier, ONX0912 was found to have the oral bioavailability of approximately 39% in rodents and dogs, much improved compared to carfilzomib.²² In addition to the approaches of modifying chemical structures to block Pgp interactions, co-administration of small peptide analogs may also be an attractive strategy to enhance oral bioavailability of these proteasome inhibitor drugs and other Pgp substrates.

In line with the early clinical evidence showing that carfilzomib can be effective even in patients refractory to bortezomib therapy,^{4, 5} our results support that cellular resistance to carfilzomib and bortezomib may occur via different mechanisms. We observed that DLD-1/Carf and H23/Carf show only a very modest level of cross-resistance to bortezomib (Figure 1). While we are not aware of any report directly examining bortezomib as a potential Pgp substrate, there are several reports indicating bortezomib as a poor substrate of Pgp even if it interacts with Pgp at all. For example, it was shown that Pgp-expressing leukemic cells (CEM/VLB) are markedly resistant to carfilzomib (114-fold increase in IC₅₀) compared to

the matching control, yet only a slight (4.5-fold) increase in IC₅₀ was observed for bortezomib.¹¹ Furthermore, in the same cell line models, the Pgp inhibitor P121 completely reversed carfilzomib resistance with little to no effect on bortezomib sensitivity.¹¹ Similar results were also reported by several other groups.^{15, 28, 29} Thus, it is unlikely that bortezomib is a good Pgp substrate and that Pgp upregulation contributes to bortezomib resistance.

In conclusion, we report that cancer cells can develop acquired resistance to carfilzomib by upregulating Pgp and that the acquired resistance may be overcome by peptide analogs structurally related to the peptide backbone of carfilzomib. These findings provide a potential strategy to overcome carfilzomib resistance or sensitize cancer cells to carfilzomib. Furthermore, the resistance reversing agents will be valuable as lead molecules in designing next generation of proteasome inhibitors and MDR reversing agents.

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Abbreviations

Pgp	P-glycoprotein
ABCB1	ATP Binding Cassette protein B1
BCRP	Breast Cancer Resistance Protein (ABCG2)
MDR	Multi-drug Resistance
MDR1	Multi-drug Resistance gene 1
Carf	carfilzomib, PR-171, (S)-4-Methyl-N-((S)-1-(((S)-4-methyl-1-((R)-2-methyloxiran-2-yl)-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-2-((S)-2-(2-morpholinoacetamido)-4-phenylbutanamido)pentanamide

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Fig 1.

Effects of carfilzomib, YU-101, bortezomib and paclitaxel on cell growth of carfilzomib-resistant cell lines, H23/Carf (A) and DLD-1/Carf (B) in comparison to their respective parental cell lines. The closed circles are for carfilzomib-resistant cells while the open circles are for their parental cell lines. Results are represented as means $\pm$ S.D.

Fig 2.

Upregulation of P-glycoprotein (Pgp) in carfilzomib-resistant cell lines is the major mechanism for carfilzomib resistance. **A:** immunoblotting analyses showing a marked increase in of Pgp expression in H23/Carf and DLD-1/Carf cells in comparison to their respective parental controls. **B:** RT-PCR analyses showing the upregulation of *MDR1* mRNA in H23/Carf and DLD-1/Carf cell lines in comparison to their respective parental controls. **C & D:** Inhibition of Pgp using verapamil (40 μ M) restores sensitivity to carfilzomib in H23/Carf and DLD-1/Carf cells. Results are represented as means $\pm$ S.D.

Fig 3.

Comparison of peptide analogs with differing lengths for their reversing effects on carfilzomib resistance **A:** Chemical structures of carfilzomib and structurally related peptide analogs, compounds 1 to 4. **B:** Co-incubation with peptide analogs leads to a partial reversal of carfilzomib resistance. H23/Carf and DLD-1/Carf cells were treated with 25 μ M of compounds 1 to 4 in the presence or absence of carfilzomib for 72 hours. Relative cell viability was measured using an ATP-based assay. Results are represented as means $\pm$ S.D. *, $p < 0.0001$, compared to the groups treated with vehicle alone, carfilzomib or peptide analogs alone, by the one-way ANOVA, followed by Bonferroni post testing.

Fig 4.

Comparison of peptide analogs with the pyridine substitution at the N-cap site for their reversing effects on carfilzomib resistance **A:** Chemical structures of di- and tri-peptide analogs, compounds 5 to 7. **B:** Co-incubation of di- or tri-peptide analogs with the pyridine group substitution (compounds 5 to 7) restores carfilzomib sensitivity to a greater extent than their counterparts with the morpholino group. H23/Carf and DLD-1/Carf cells were treated with 25 μM of compounds 5 to 7 in the presence or absence of carfilzomib for 72 hours. Relative cell viability was measured using an ATP-based assay. Results are represented as means ± S.D. *, $p < 0.0001$, compared to the groups treated with vehicle alone, carfilzomib or peptide analogs alone, by the one-way ANOVA, followed by Bonferroni post testing.

Fig 5.

Comparison of peptide analogs with the Weinreb amide substitution at the C-terminus for their reversing effects on carfilzomib resistance **A**: Chemical structures of di- and tri-peptide analogs, compounds 8 to 10. **B**: Co-incubation of di- or tri-peptide analogs with the Weinreb amide substitution (compounds 8 to 10) restores carfilzomib sensitivity to a greater extent than their counterparts with the benzyl ester group. H23/Carf and DLD-1/Carf cells were treated with 25 μM of compounds 8 to 10 in the presence or absence of carfilzomib for 72 hours. Relative cell viability was measured using an ATP-based assay. Results are represented as means ± S.D. *, $p < 0.0001$, compared to the groups treated with vehicle alone, carfilzomib or peptide analogs alone by the ANOVA, followed by Bonferroni post testing.

Fig 6.

Peptide analogs (compounds 8 to 10) show the ability to reverse acquired resistance to carfilzomib and paclitaxel and to inhibit Pgp-mediated extrusion of calcein. Results are represented as means $\pm$ S.D. **A & B:** Compounds 8 to 10 restore cellular sensitivity to carfilzomib in concentration-dependent manners in DLD-1/Carf (**A**) and H23/Carf cells (**B**). *, $p < 0.001$ compared to the groups treated with vehicle alone, carfilzomib alone or peptide analogs alone by the one-way ANOVA followed by Bonferroni post testing. **C:** Compounds 8 to 10 (12.5 μ M) can also reverse cross-resistance to paclitaxel (2 μ M) in DLD-1/Carf and H23/Carf cells. **, $p < 0.0001$, compared to groups treated with vehicle alone, drug alone, or peptide analogs only by the one-way ANOVA followed by Bonferroni post testing. **D:** Compounds 8 to 10 (25 μ M) lead to increased cellular retention of calcein, a P-glycoprotein substrate, in DLD-1/Carf and H23/Carf cells. *, $p < 0.05$ compared to the group treated with vehicle alone by Student's t-test; † $p < 0.0125$ (Bonferroni-corrected p value threshold).

Fig 7.

Peptide analogs (compounds 8 to 10) potentiate the sensitivity of the parental DLD-1 and H23 cells to carfilzomib. Results are represented as means $\pm$ S.D. **A & B:** Compounds 8 to 10 potentiate the effect of carfilzomib (15 nM) in the parental DLD-1 (**A**) and H23 (**B**) cells. *, $p < 0.001$ compared to the groups treated with vehicle alone, carfilzomib alone or peptide analogs alone by the one-way ANOVA followed by Bonferroni post testing.

Table 1

IC₅₀ values for carfilzomib, YU-101, bortezomib and paclitaxel in H23 and DLD-1 cells with acquired resistance to carfilzomib and their parental cell lines

Cell line	IC ₅₀ (nM)			
	Carfilzomib	YU-101	Bortezomib	Paclitaxel
H23	17.6	23.7	6.3	4.7
H23/Carf	1300	> 1000	57.1	> 1000
DLD1	32.9	37.7	19.8	5.5
DLD1/Carf	2900	> 1000	102	> 1000