Proteasome Inhibitors with Pyrazole Scaffolds from Structure-Based Virtual Screening

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ABSTRACT: We performed a virtual screen of ~340,000 small molecules against the active site of proteasomes followed by in vitro assays and subsequent optimization, yielding a proteasome inhibitor with pyrazole scaffold. The pyrazole-scaffold compound displayed excellent metabolic stability and was highly effective in suppressing solid tumor growth in vivo. Furthermore, the effectiveness of this compound was not negatively impacted by resistance to bortezomib or carfilzomib.

INTRODUCTION

The proteasome is a key player in one of the most fundamental cellular processes in eukaryotes, the ubiquitin-dependent protein degradation pathway.1 The barrel-shaped 20S core of the proteasome consists of four stacked heptameric rings: two outer α-rings and two inner β-rings (Figure 1A). While the two outer α-rings serve mainly structural roles, the two inner β-rings have three catalytic subunits in each ring. In mammalian cells, there exist two main proteasome subtypes: the constitutive proteasome (CP) and the immunoproteasome (IP). These two proteasome subtypes differ by the incorporation of two distinct sets of catalytic subunits. The CP is expressed in all eukaryotic cells and plays key roles in many important intracellular processes, such as cell cycle progression, development, and inflammation. The CP contains the catalytic subunits β1, β2, and β5, which cleave peptide bonds after acidic (caspase-like, C-L), basic (trypsin-like, T-L), and hydrophobic (chymotrypsin-like, CT-L) residues, respectively.2

On the other hand, the IP is expressed in immune cells and can be induced in other cell types upon exposure to inflammatory cytokines, such as interferon-γ (INF-γ) and tumor necrosis factor α (TNF-α).3 The 20S IP core is structurally identical to the constitutive proteasome except for the incorporation of catalytic subunits β1i, β2i, and β5i instead of β1, β2, and β5, respectively (Figure 1B). The catalytic activities of the IP subunits β2i and β5i are relatively similar to those of their constitutive counterparts, possessing T-L and CT-L activity, respectively. In contrast, the replacement of β1 with β1i results in a change in the cleavage specificity from C-L to CT-L. Although the distinct catalytic subunits of the IP have been suggested to play roles in adding antigenic diversity to peptides generated from protein degradation,3–6 the catalytic subunits responsible for the CT-L activity (β5 and β5i) are thought to be most physiologically important and have been recognized as the key targets of bortezomib and carfilzomib.7,8

Figure 1. (A) The 20S proteasome is composed of four heptameric rings: two α-rings and two β-rings. (B) Schematic representation of subunit composition within a 20S proteasome core. Each β-ring contains three catalytic subunits. (C) The chemical structure of two FDA-approved proteasome inhibitors, bortezomib (Velcade) and carfilzomib (Kyprolis).

Given the crucial roles that proteasomes play in cell proliferation and survival, the strategy to exploit the proteasome as an anticancer target was initially met with skepticism. However,
the proteasome was finally validated as an anticancer target with the FDA approval of bortezomib (Velcade) in 2003, the first-in-class proteasome inhibitor for the treatment of relapsed multiple myeloma (MM).9 The FDA approval of a second-generation proteasome inhibitor carfilzomib (Kyprolis) in 2012 for the treatment of relapsed MM patients who have received at least two prior therapies including bortezomib firmly placed the proteasome as an exciting target in fighting cancer.10–12 The addition of these proteasome inhibitors to chemotherapeutic armaments has dramatically improved the therapeutic landscape for patients with MM.13 However, despite initial clinical successes of these drugs, intrinsic and acquired drug resistance poses major challenges. Additionally, these drugs have not been successful in treating patients with solid cancers despite numerous clinical trials and compelling evidence of anticancer activity in preclinical models of solid cancers including prostate cancer.14 This lack of efficacy in solid cancer patients has been generally attributed to poor in vivo stability, undesirable pharmacokinetic properties and unwanted toxicities, arising from the shared chemical features of these agents, namely, peptide backbones and electrophilic pharmacophores.13–22

Here, we report the development of novel proteasome inhibitors utilizing a non-peptide scaffold which was discovered via a virtual screening and medicinal chemistry approach. The identified lead compound, G4-1, which targets β5 and β5i, demonstrates excellent metabolic stability and is highly effective in suppressing tumor growth in a mouse xenograft model of prostate cancer. Furthermore, G4-1 is equally effective in killing parental cells and model cell lines with acquired resistance to bortezomib or carfilzomib.

## Experimental Section

### Virtual Screening.
Structure-based virtual screening was performed following a procedure reported previously by us.23 Briefly, the conformation of proteasomes for virtual screening was selected from the molecular dynamics trajectory of recently built homology model in complex with newly discovered highly potent peptide compounds.24 On the basis of favorable binding energy and optimal accommodation of the known inhibitors at the active site, the protein conformation at 341 ps was selected for the final screening. After docking 345 447 compounds included in the University of Cincinnati library, 288 compounds for the experimental validation were selected based on consensus scoring, force field based energy scoring functions (MM-PBSA and MM-GBSA and manual visualization of binding modes). Out of 288 compounds tested, 19 compounds were found to be active at 5 μM in a CT-L activity assay using the IP (for more detailed kinetics procedures, see Supporting Information). Among these compounds, G4 was found to potently inhibit the CT-L activity of the CP and IP. G4-1, an analogue of G-4, was prepared following a synthetic scheme described in Figure 2D and HPLC purification with purity greater than 98% (Supporting Information).

### Enzyme Kinetics Assays.
Initially, purified 20S human proteasomes (CP and IP, R&D Systems) were used to assess the in vitro inhibition of proteasome catalytic activities by G4 and its analogs. In our 96-well format assays involving a 100 μL total volume, 20S proteasomes (0.5 μg/mL) were incubated with G4, its analogs, or reference compounds (e.g., carfilzomib) in assay buffer (20 mM Tris-HCl, 0.5 mM EDTA, 0.035% SDS) for 30 min at room temperature. Reactions were initiated by the addition of individual subunit-selective fluorogenic substrates containing the AMC (7-amino-4-methylcoumarin) group. The following substrates were used: for β1 activity, Ac-nLPnLD-AMC (100 μM); for β2 and β2 activity, Boc-LRR-AMC (200 μM); for β5 activity, Ac-WLA-AMC (20 μM); for β5i activity, Ac-PAL-AMC (100 μM); for β5i activity, Ac-ANW-AMC (100 μM). The fluorescence of liberated AMC was measured over a period of 90 min at room temperature using excitation and emission wavelengths of 360 and 460 nm on a SpectraMax M5 fluorescence plate reader (Molecular Devices).

In separate experiments, cytotoxic extracts of RPMI-8226 or BxPC-3 cells were prepared according to the method of Kissel and Goldberg25 and used in place of purified proteasomes. The fluorescence of liberated AMC of the subunit-selective probe substrates was measured as described above.

### Jump Dilution Reversibility Assay.
In further investigation of the mode of proteasome inhibition by G4-1, a dilution assay was performed following a procedure previously reported.26 Briefly, RPMI 8226 cell lysates containing 30 μg of total protein were incubated with G4-1 (10 μM) in 20S proteasome assay buffer for 30 min at room temperature. Lysates treated with G4-1 were subsequently transferred to a semimicrocuvette, and the baseline proteasome activity was determined by measuring the hydrolysis rate of Suc-LLVY-AMC. After establishment of the baseline, lysates treated with G4-1 were rapidly mixed with the 20S proteasome assay buffer, yielding 25-fold dilution. Following this 25-fold dilution from 10 to 0.4 μM G4-1, the hydrolysis of Suc-LLVY-AMC was again monitored over approximately 30 min.

### Cell Culture.
Human cancer cell lines BxPC-3, H358, H-23, LNCaP, Panc-1, and RPMI 8226 were obtained from the ATCC (American Type Culture Collection) and maintained in the ATCC-recommended media, DMEM or RPMI 1640 supplemented with 10% fetal bovine serum (from Gibco, CellGro, and Atlanta Biologicals). BxPC-3 cells with acquired resistance to bortezomib or carfilzomib were established by growing them in the presence of stepwise increasing concentrations of the respective drug over a period of approximately 6 months. In order to determine the extent of drug resistance, cytotoxicity assays were performed using BxPC-3 sublines adapted to 60 nM bortezomib and 200 nM carfilzomib, respectively.

### Measurement of Cytotoxic Effects of G4 and Its Analogs.
The cytotoxic effects of G4 and its analogs were determined using CellTiter Glo assay or CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega). Adherent cells (Panc-1, LNCaP, BxPC-3, bortezomib- or carfilzomib-resistant BxPC-3 sublines) growing in log phase were plated at 7000–10000 cells per well. RPMI 8226 cells growing in suspension were collected by centrifugation and plated at 10 000 cells per well. Twenty-four hours after plating, media containing the test compounds were added to each well to deliver the intended final concentration. After 72 h, the cell viability was determined using the assay protocol recommended by the manufacturers. The resulting signals were quantified using a Veritas microplate luminometer or a SpectraMax M5 microplate spectrophotometer (Molecular Devices).

### Microsomal Stability Assay.
The metabolic stability profiles of G4-1 and reference compounds (carfilzomib and bortezomib) were assessed by monitoring the disappearance of the test compounds in
the presence of liver microsomes. A typical incubation mixture (100 μL total volume) for metabolic stability studies contained 1 μM test compounds, 0.5 mg/mL microsomal protein (pooled Balb/c mouse liver microsomes prepared in-house or BD UltraPool human liver microsomes), 100 mM Tris-HCl buffer (pH 7.4), and NADPH-generating system (5 mM isocitric acid, 0.2 unit/mL isocitric acid dehydrogenase, 5 mM magnesium chloride, 1 mM NADPH). After preincubation at 37 °C for 5 min, the reactions were started by addition of NADPH and further incubated for another 0, 5, 10, 20 min. For control experiments, NADPH and/or liver microsomes were omitted from these incubations. The reactions were terminated by adding 100 μL of ice-cold acetonitrile containing phenytoin (1 μM) as internal standard and keeping on ice for 30 min, followed by centrifugation at 16,100 g for 15 min to obtain the supernatant. Aliquots (5 μL) were then analyzed for substrate disappearance using liquid chromatography–tandem mass spectrometry (Agilent 1200 HPLC instrument interfaced with Applied Biosystems Qtrap 3200) equipped with an electrospray ion source.

In Vivo Efficacy Assay. Six-week-old male BALB/c athymic nude mice (purchased from Orient Bio Inc., SungNam, Republic of Korea) were maintained in accordance with the National Institute of Toxicological Research of the Korea Food and Drug Administration guidelines as well as the regulations for the care and use of laboratory animals of the animal ethics committee of the Konyang University. LNCaP cells (2 × 10⁶ cells/50 μL) were subcutaneously implanted into each animal. After the xenograft tumors had grown to a size of ~100 mm³, mice (n = 5/group) were dosed intraperitoneally twice a week for 4 weeks with G4-1 (5 mg/kg), carfilzomib (5 mg/kg), or vehicle only (8% DMSO in HP-Beta-CD and citrate). Tumor volumes (calculated using the following formula, (width)² × length/2) and body weights were measured every 4 days during the experimental period. At the end of the experimental period (at day 30), mice were euthanized by cervical dislocation and tumors were isolated and weighed.

## RESULTS AND DISCUSSION

Screening of a Small Molecule Library against the CT-L Activity of Proteasomes. The majority of currently available proteasome inhibitors including bortezomib and carfilzomib are small peptides with an electrophilic warhead. These electrophilic warheads such as boronates, epoxyketones, β-lactones, and vinyl sulfones directly target the hydroxyl nucleophile of the N-terminal threonine in the active site, blocking the proteolytic activity of proteasome via covalent modifications. Although the combination of a peptide backbone with an electrophilic pharmacophore can provide a relatively easy route to proteasomal inhibition, it may suffer from poor metabolic stability and side effects. To circumvent these concerns, increased efforts have been made recently to generate peptide activity targeting peptide or non-peptide, reversible proteasome inhibitors. However, no further development has been made to date, mainly because of low proteasome inhibitory potency or poor in vivo efficacy of these compounds.

In our current study, we employed a stepwise screening strategy to identify non-peptide proteasome inhibitors lacking reactive warheads with improved potency and efficacy against proteasomes (Figure 2A). Given that the CT-L activity–confering β5i and β5i subunits share a high degree of structural homology at the active sites, we performed a virtual screening of a library of 345,447 small molecules (provided by University of Cincinnati Drug Discovery Center) against the β5i subunit following a procedure described by us and selected 288 compounds based on their predicted fit into the active site of β5i.

These selected 288 compounds were then tested for their ability to inhibit the CT-L activity of proteasomes, and subsequently G4 was identified as a lead inhibitor (Figure 2A and Supporting Information S1). Since only a limited amount of G4 was available from the library, we synthesized G4 following the scheme described in Figure 2B. Using this synthetic G4 compound, we found that G4 preferentially inhibits the CT-L activity–confering β1i and β5i/β5i and C-L activity responsible β1 but not β2/β2i subunits which are responsible for T-L activity (Figure 2C). Consistent with its ability to inhibit the proteasome activity in vitro, G4 effectively induced cancer cell death in the low micromolar range (IC₅₀ ≈ 7 μM) (Figure 2D).

Investigation of the G4 Chemical Space. To probe structure–activity-relationship of G4 compound, we created a library of G4 analogs modified at rings A–D following synthetic procedures similar to that described in Figure 2B (Figure 3A,B; see Supporting Information for modified synthetic schemes) and measured their potencies against the CT-L activity of purified proteasomes. The enzyme kinetics studies demonstrated that modifications at rings A and B have significant impacts on inhibitory activity, G4-1 being the most potent inhibitor (Figure 3C and Supporting Information S2). In comparison, modifications at the ring D had little impact on the activity of G4. Interestingly, while most of these G4 derivatives inhibit the CT-L activity of proteasome, some such as G4-16 and G4-21 activated the CT-L activity of proteasomes, up to 200% at 10 μM (Figure 3C). The mechanism by which these compounds enhance the CT-L activity of proteasome is unclear at this time. While further target validation study is needed, our initial studies using these G4-1 derivatives showed an apparent correlation between the proteasome inhibitory activity and the anticancer effects of pyrazole-based G4 analogs, indicating that proteasome inhibition is likely to mediate the cytotoxic effects of G4 and G4-1 (Supporting Information S3).

We next further investigated the structure–activity relationship (SAR) by docking simulation of G4-1 to the β5 subunit of the proteasome. Our docking studies suggest that the improved
activity of G4-1 over G-4 may be attributed to hydrogen bonds created by the introduction of an amide linkage at the ring A (Figure 4A). Ring B is predicted to occupy the S3 subsite located at the interface between β5 and β6 subunits. Ring D of G4-1 is predicted to occupy the S1 subsite but not as deeply and fully as the Leu side chain of bortezomib (Figure 4B). Overall, G4-1 is predicted to occupy only two subsites, as compared to three for bortezomib (Figure 4B), and this predicted difference may be exploited to further improve inhibitory potency of G4-1.

**G4-1 as a Lead Proteasome Inhibitor.** Given its improved inhibitory potency against the CT-L activity of purified proteasomes and additional optimization potential (due to the amide linkage at the ring A), as compared to G4, we decided to further characterize G4-1 as a lead non-peptide proteasome inhibitor. It should be mentioned that although compounds G4-40, G4-41, and G4-42 showed the ability to potently inhibit in vitro CT-L activity (Figure 3C), we found that G-40, G4-41, and G4-42 were less potent or noncytotoxic in our initial cell viability assays, compared to our lead compound G4-1 (Supporting Information S2b), thus justifying the selection of G4-1 for further in vitro and in vivo studies. We first tested the ability of G4-1 to inhibit the activity of cellular proteasomes present in cell extracts, other than just purified proteasomes. As shown in Figure 5A, G4-1 was able to effectively inhibit the proteasomal CT-L and C-L activities of cell extracts. Similar to G4, G4-1 showed no activity against the T-L activity of cell extracts.

Given that the majority of currently available proteasome inhibitors, which contain peptide backbones, are prone to rapid in vivo inactivation, we next examined whether the pyrazole scaffold-based proteasome inhibitor G4-1 is metabolically more stable than those peptide-based drugs. Excitingly, G4-1 demonstrated excellent metabolic stability profiles in mouse and human liver microsomes, as compared to carfilzomib and bortezomib (Figure 5B). These results indicate that G4-1 is likely to have much improved in vivo stability compared to carfilzomib and bortezomib, which are known to undergo rapid metabolic inactivation.

Next, we investigated the mode of proteasome inhibition by G4-1 using a jump dilution reversibility assay previously reported. Preincubation of RPMI 8226 cell extracts with G4-1 for 30 min resulted in a nearly complete inhibition of proteasomal activity. However, after 25-fold dilution, the reaction rate increased exponentially with an inhibitor dissociation half-life of approximately 3.2 min (Figure 5C), indicating the reversibility of G4-1/proteasome interaction. As expected, G4-1 showed a good anticancer activity against a variety of cancer cell
lines including LNCaP prostate cancer cells (Figure 5D). Interestingly, the anticancer effect of G4-1 was not negatively impacted by acquired resistance to bortezomib or carfilzomib in cell line models (Figure 5E), which was generated by continuous drug exposure with stepwise increases in concentration (Supporting Information S4).

In Vivo Anticancer Activity of G4-1. Lastly, we investigated the in vivo anticancer efficacy of G4-1 using a xenograft mouse model. Given that one of important goals in the area of proteasome inhibitor therapy is to expand therapeutic benefits to patients with solid cancers, we used a solid cancer xenograft model of human prostate LNCaP cancer cell line following a procedure we previously reported. As shown in Figure 6A–C, G4-1 effectively suppressed tumor growth in vivo without apparent systemic toxicity in mice (for images of mice treated with G4-1, see Supporting Information S5). Unlike mice treated with carfilzomib which resulted in weight loss, mice treated with G4-1 maintained normal weight gain over the course of treatment (Figure 6D).

CONCLUSIONS

We have successfully developed the non-peptide, reversible proteasome inhibitor G4-1. This inhibitor utilizes a pyrazole scaffold and does not rely on an electrophilic warhead to mediate proteasome inhibition. In addition to its novel scaffold, G4-1 may represent an important advance due to its effectiveness in models of proteasome inhibitor resistance and metabolic stability. This is critical, as MM patients who are initially responsive to currently FDA-approved proteasome inhibitors almost inevitably develop resistance to those drugs. Therefore, G4-1 may open the door to an additional option for these refractory MM patients. Multiple clinical trials clearly demonstrated that the clinically approved proteasome inhibitors carfilzomib and bortezomib lack utility in the treatment of solid tumors due to their rapid metabolism, irreversible inhibition, sensitivity to resistance, and dose-limiting toxicities. Therefore, the metabolically stable pyrazole-based G4-1 provides an excellent lead that warrants further investigations of its activity against solid cancers. Additional studies regarding the pharmacokinetics and pharmacodynamics of G4-1 are ongoing.

ASSOCIATED CONTENT

Supporting Information

Synthesis procedures, NMR data, mass spectra analysis, biological experimental procedures, enzyme kinetics, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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