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New 26S Proteasome Inhibitors with High Selectivity for Chymotrypsin-Like Activity and p53-Dependent Cytotoxicity

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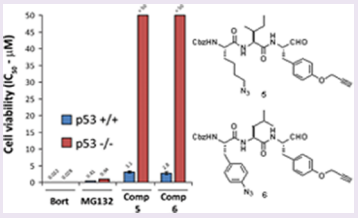
Supporting Information

ABSTRACT: The 26S proteasome has emerged over the past decade as an attractive therapeutic target in the treatment of cancers. Here, we report new tripeptide aldehydes that are highly specific for the chymotrypsin-like catalytic activity of the proteasome. These new specific proteasome inhibitors demonstrated high potency and specificity for sarcoma cells, with therapeutic windows superior to those observed for benchmark proteasome inhibitors, MG132 and Bortezomib. Constraining the peptide backbone into the β-strand geometry, known to favor binding to a protease, resulted in decreased activity *in vitro* and reduced anticancer activity. Using these new proteasome inhibitors, we show that the presence of an intact p53 pathway significantly enhances cytotoxic activity, thus suggesting that this tumor suppressor is a critical downstream mediator of cell death following proteasomal inhibition.



The 26S proteasome is a supramolecular protein assembly that plays a key role in the degradation of proteins that regulate the cell cycle, with its over activity being a key mechanism by which cells develop malignancy.[1] This protease consists of a 20S proteolytic component capped with a regulatory 19S complex at each end, the role of which is to unfold protein-substrates and stimulate proteolytic activity.[2,3] Proteolytic activity of the proteasome is mediated through three catalytic sites, including chymotrypsin-like (CT-L), trypsin-like (TL), and caspase-like (CP-L) subunits. The proteasome is responsible for providing the cell with a recycler function for damaged or misfolded proteins and thus has a critical role in the regulation of cell cycle and apoptotic pathways.[4]

The proteasome is typically hyperactive in malignancies,[4] with the dipeptidylboronic acid Bortezomib, (Figure 1)[5]

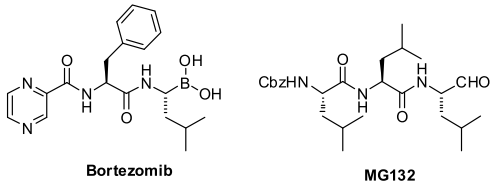


Figure 1. Structures of Bortezomib and MG132.

recently approved as a frontline treatment for multiple myeloma.[6] Despite its success, Bortezomib has some issues. Not all multiple myeloma patients respond to Bortezomib, and researchers are yet to identify biomarkers to predict its efficacy. Furthermore, those patients who do respond to Bortezomib almost uniformly develop resistance in the short term.[7] In addition, Bortezomib possesses undesirable side effects, is active against only a narrow range of blood malignancies, lacks specificity for cancer cells, and has a very narrow therapeutic window.[7]

Such clinical issues highlight an urgent need for more cancer-specific proteasome inhibitors and a better understanding of their detailed mechanisms of action and specificity profiles. One class of proteasome inhibitor that has unmet potential is the peptidic aldehydes, typified by MG132 (Figure 1).[8] While this inhibitor has potent cytotoxicity against cancer cell lines *in vitro*, it shares similar issues as Bortezomib; in particular, it lacks tumor cell specificity and hence is cytotoxic to normal tissues. Interestingly, both Bortezomib and MG132 are nonselective inhibitors, with activity against the CT-L, T-L, and CP-L subunits within the proteasome.

One of the key biological targets of the proteasome is the p53 tumor suppressor protein, the function of which is to restrict cell growth or induce cell death. It has been suggested that inhibition of the proteasome by Bortezomib, MG132, and other proteasome inhibitors results in enhanced p53 activity, which then drives tumor cell death. However, the exact role of p53 as a downstream mediator of Bortezomib-induced apoptosis remains unclear, with reports both supporting[9−11] and refuting[12−15] this notion. What we do know is that the p53 tumor suppressor is consistently maintained at low levels by the ubiquitin-proteasomal system. Pharmacological inhibition of the proteasome thus results in the rapid accumulation of p53 protein, subsequently inducing apoptosis in cancer cells.[16,17] The importance of p53 in the prevention of cancer development is highlighted by the fact that approximately 50% of all human cancers inactivate p53 through mutation of *TP53*, the gene encoding p53.[18,19] Although Bortezomib has been

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approved as a frontline treatment for multiple myeloma, it is unclear if myelomas harboring p53 mutations are resistant to this proteasome inhibitor. In general, p53 mutations in myelomas are associated with remarkably poor outcomes,[20] and as such it would seem likely that they are less sensitive to Bortezomib.

Previous reports suggest that potent inhibition of all three subunits of the proteasome resulted in profound non-specific cytotoxicity to non-transformed cells,[21] indicating that subunit specificity is an essential aspect of selective antineoplastic proteasome inhibitors. It was initially thought that the bulk of the proteolytic activity of the proteasome was mediated through the CT-L subunit[22,23] and that specific inhibition of CT-L catalytic activity was sufficient to drive cell death in hematological malignancies.[21] However, recent evidence suggests that the T-L and CP-L activities are not negligible for proteasome function. This was highlighted in a landmark study from the Kisselev laboratory demonstrating that co-inhibition of either T-L or CP-L activities with CT-L was essential to achieve maximal cytotoxicity of multiple myeloma cell lines.[24] Although these findings are critical to the development of more specific proteasome inhibitors, they are performed in neoplastic hematological cell lines that also express an immunoproteasome (an additional proteasome with similar subunits formed in many hematological cells). Therefore, it is currently unclear what specific proteasome subunit(s) should be considered as drug targets in solid tumors that lack an immunoproteasome.

Here, we report the synthesis and characterization of new tripeptide aldehydes based on MG132 that are designed for increased specificity for the CT-L activity of the proteasome. In contrast to the benchmarks, MG132 and Bortezomib, these analogues are highly specific inhibitors of the CT-L activity and show potent activity in sarcoma cell lines without non-specific cytotoxicity to normal non-malignant cells. Lastly, our findings suggest that the p53 pathway is a major effect in the ability of these novel proteasome inhibitors to induce cell death.

RESULTS AND DISCUSSION

Inhibitor Design. Although there are CT-L-specific inhibitors reported,[2,21,25−31] few of these have been extensively studied and tested as anticancer agents *in vivo*. Encouragingly, 2-aminobenzyl statine-based inhibitors, which display selectivity and reasonable potency for CT-L activity, show high antiproliferative activity in cell-based assays.[28] It has been reported that the introduction of a sterically bulky substituent at P2 of peptidic aldehydes enhances selectivity for the CT-L activity, where the corresponding S2 pocket is ill-defined and thought not critical for binding.[2,27] One such example, with a Asp(*t*-Bu) at P2, shows modest potency and selectivity for CT-L over T-L, CP-L, and also a cysteine protease calpain.[2] Further improvements are required if we are to better tailor the activity of peptidic aldehydes toward the CT-L subunit of the proteasome, while also reducing their activity against off-target proteases.

The inhibitors reported in this paper (compounds **1**−**6**, see Figure 2) are based on the structure of the benchmark peptidic aldehyde inhibitor MG132, which as discussed earlier shows high potency against all three proteasome activities (see Table 1). We chose to incorporate an acetylene-substituted aryl group at P1, as the corresponding S1 pocket of the CT-L subunit is known to bind hydrophobic groups.[2,32] By comparison, the importance of P3 to binding is less well studied, with the

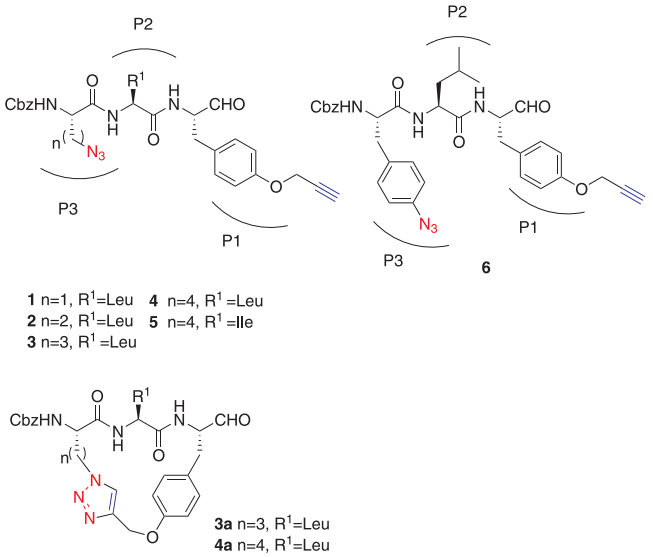

Figure 2. Tripeptide MG132 analogues.

makeup of the corresponding S3 binding pocket known to vary between the three different activities.[2] We saw this site as a relatively unexplored opportunity to introduce selectivity into the inhibitors.[26] In this paper we report the incorporation of an aliphatic azide (as in **1**−**5**) or an aromatic azide (as in **6**) at P3 as previously untested substituents at this position. These azides also allow cyclization to the acetylene of P1 *via* Huisgen cycloaddition,[33] in order to investigate the effect of constraining the backbone into a *β*-strand geometry (see compounds **3a** and **4a**). This geometry is known to favor ligand binding to the proteasome and indeed all other proteases.[34−36] While inhibitors of the proteasome reportedly adopt hydrogen bonds with the protease that are characteristic of binding in this geometry, unlike other proteases, the P2 group does not seem to form important contacts with the active site.[2] Presumably this accounts for the earlier discussed observation that the corresponding S2 pocket is not critical for binding to the CT-L subunit. In this paper, we chose to incorporate both Leu and a subtle variant (Ile) at P2 of our new inhibitors to allow direct comparison with MG132.

Synthesis of Tripeptide MG132 Analogues. The tripeptide aldehydes **1**−**6** were prepared by standard peptide coupling as depicted in Schemes 1 and 2.[33] Separate reactions of **7**−**10**[33] with Leu-O*t*Bu or IIe-O*t*Bu, in the presence of EDCI and HOBt, gave dipeptides **12**−**16**, the *tert*-butyl esters of which were hydrolyzed to give the carboxylic acids **17**−**21**. Coupling of each of these with the amino alcohol **11**,[33] in the presence of EDCI and HOBt, gave tripeptides **22**−**26** that were oxidized with Dess−Martin periodinane (DMP) to give the required acyclic aldehydes **1**−**5**, respectively. The tripeptides **24** and **25** were also cyclized on treatment with Cu(I)Br in CH_2Cl_2 to give **27** and **28**, which were oxidized with DMP to give **3a** and **4a**. An analogous reaction of the derivatives with a shorter tether (**22** and **23**) failed to give corresponding macrocycles, presumably because of steric strain associated with the corresponding smaller ring systems. The macrocycles of **3a** and **4a** had been shown in earlier work to constrain the geometry of the peptide backbone into the required *β*-strand geometry.[33]

Compound **6**, with an aryl group at both P1 and P3 was similarly prepared as shown in Scheme 2. Reaction of **29**[37] with

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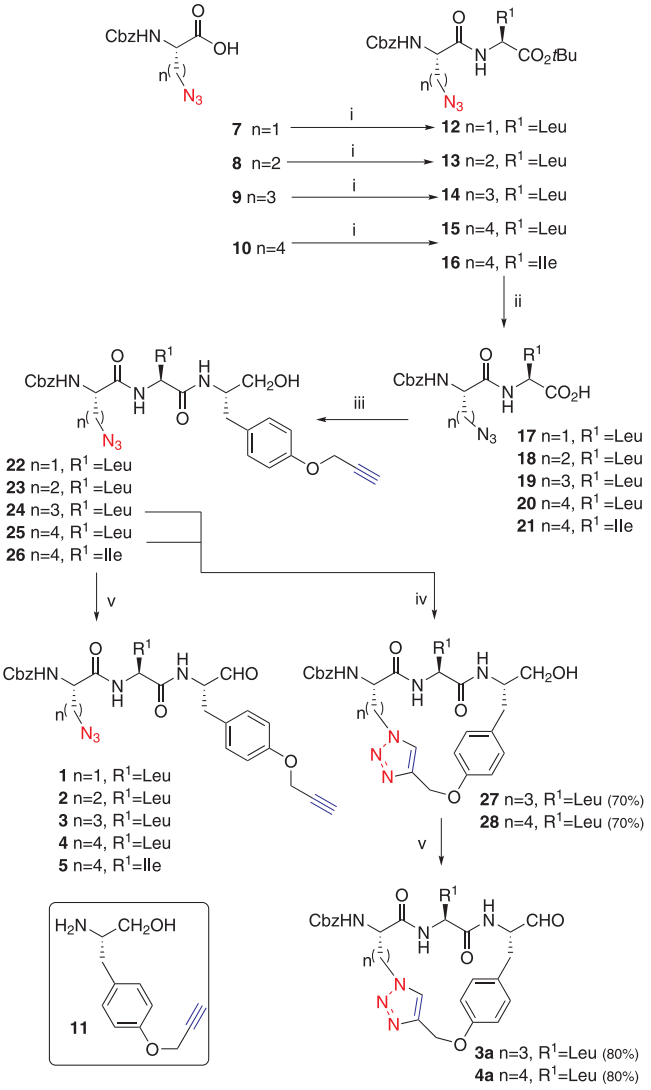
Table 1. Proteasome Inhibitory and Calpain II Activity (Standard Errors <5% of Mean)

				IC_{50} (nM)			
compound	P2	*n* (P3)	cyclic	CT-L	T-L	CP-L	Calpain II
1	Leu	1	N	34	>25,000	>25,000	107
2	Leu	2	N	355	>25,000	>25,000	324
3	Leu	3	N	54	>25,000	>25,000	780[e]
4	Leu	4	N	150	>25,000	>25,000	1,030[e]
5	Ile	4	N	21	>25,000	>25,000	389
6	Leu	*a*	N	23	>25,000	>25,000	nd[d]
3a	Leu	3	Y	917	>25,000	>25,000	137[e]
4a	Leu	4	Y	250	>25,000	>25,000	97[e]
MG132	Leu	*b*	N	1.2	1998	545	311
Bortezomib	Phe	*c*	N	35	>25,000[f]	438	>25,000

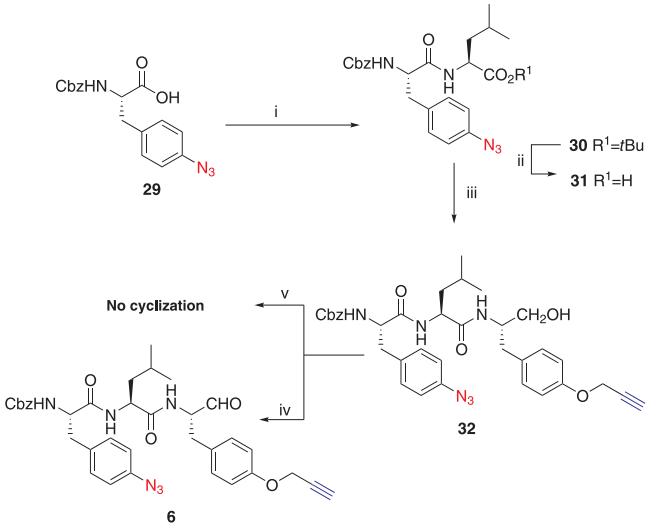
[a]Azido-Phe at P3. [b]Leu at P3. [c]No P3 amino acid. [d]nd = not determined. [e]Taken from ref 33. [f]Bortezomib activated T-L activity by 32% (Supplementary Figure S1).

Scheme 1[a]



[a]Reagents and conditions: (i) EDCI, HOBt, DIPEA, Leu-O*t*Bu or Ile-O*t*Bu, CH_2Cl_2, (80%, **12** and **13**; 78%, **16**); (ii) TFA, CH_2Cl_2, (85%, **17**,**18** and **21**); (iii) EDCI, HOBt, DIPEA, **11**, (75%, **22**; 74%, **23**; 75%, **26**); (iv) CuBr, DBU CH_2Cl_2, (70%, **27** and **28**); (v) DMP, CH_2Cl_2, (80%,**1** and **2**; 75%, **5**,80% **3a** and **4a**).

Scheme 2[a]



[a]Reagents and conditions: (i) EDCI, HOBt, DIPEA, Leu-O*t*Bu, CH_2Cl_2, 70%; (ii) TFA, CH_2Cl_2, 80%; (iii) EDCI, HOBt, DIPEA, **11**, 82%; (iv) DMP, CH_2Cl_2, 75%; (v) CuBr, DBU, CH_2Cl_2.

Leu-OtBu, in the presence of EDCI and HOBt, gave dipeptide **30** that was hydrolyzed to give **31**. Coupling of this carboxylic acid with the amino alcohol **11**, in the presence of EDCI and HOBt, gave tripeptide **32**, the aldehyde of which was oxidized with DMP to give the required aldehyde **6**. The attempted cyclization of **32**, on treatment with Cu(I)Br in CH_2Cl_2, failed to give the corresponding macrocycle, again presumably due to steric constraints of the associated ring.

Inhibition of the Proteasome. We initially determined the ability of the novel tripeptide MG132 analogues **1**−**6** and the macrocycles **3a** and **4a** to inhibit the three separate protease activities of the 20S proteasome (CT-L, T-L, and CP-L). The assays were performed *in vitro* using purified 20S proteasome/inhibitor mixtures, with the activity of each subunit of the proteasome assessed upon incubation with its respective target peptide. MG132 and Bortezomib were highly potent inhibitors of CT-L activity in this assay; see Table 1. However, MG132 also significantly inhibited the T-L and CP-L activities of the 20S proteasome, which is consistent with previous reports[25,27,38] The new peptidic aldehydes **1**−**6** were also highly potent against the CT-L activity, with derivatives **5** and **6** proving to be the most potent inhibitors (IC_{50} values of 21 and

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Table 2. Cytotoxicity of Proteasome Inhibitors against a Panel of Sarcoma Cell Lines or Normal Cell Lines (Standard Errors <5% of Mean)

	cancer cell lines IC_{50} (μM)[a]					normal cell lines IC_{50} (μM)[a]			
compound	WE-68	VH-64	STA-ET-1	TC-252	average	fibroblasts	osteoblasts	average	therapeutic window[b]
1	4.5	5.8	6.5	5.0	5.4	12.2	23.7	18.0	×3.3
2	4.7	4.9	5.4	2.3	4.3	29.4	13.6	21.5	×5.0
3	0.47	2.1	2.6	1.2	1.6	8.8	18.8	13.8	×8.7
4	10.8	1.9	2.6	1.6	4.2	39.5	10.4	25.0	×5.9
5	0.98	1.1	1.0	0.42	0.88	19.9	4.8	12.4	×14.1
6	1.1	1.9	2.4	0.64	1.5	45.4	11.6	28.5	×19.0
3a	30.5	12.8	6.2	5.4	13.7	>50	>50		
4a	18.0	>50	37.6	39.4	31.7	>50	>50		
MG132	0.68	0.68	0.59	0.49	0.61	2.8	2.7	2.8	×4.5
Bortezomib	0.02	0.04	<0.0008	0.01	0.02	0.02	0.28	0.15	×9.1

[a]Dose−response curves are provided in Supplementary Figure S2. [b]Therapeutic window represents the fold change in potency (IC_{50} value) of the proteasome inhibitor against the cancer cell line versus the normal cell line.

23 nM, respectively). However, unlike Bortezomib and MG132, all of the compounds (**1**−**6**) were inactive against both the T-L and CP-L activities of the proteasome up to the highest concentrations tested (25,000 nM). It is interesting to note that the introduction of Ile at P2 (see compound **5**) gave rise to a 7.5-fold increase in potency against the CT-L proteolytic activities relative to the direct MG132 analogue with Leu at P2 (see compound **4**). In addition, there is no apparent clear-cut preference for the length of tether (compare compound **1** to **4**), while the introduction of an aryl group at P3 (as in **6**) is well tolerated.

Somewhat surprisingly, the cyclized derivatives (**3a** and **4a**) were on average over 10-fold less potent against CT-L than their acyclic counterparts **3** and **4** (Table 1). Thus it appears that the proteasome prefers to bind a conformationally flexible acyclic ligand, rather than a structure that has its peptide backbone constrained into a β-strand conformation by cross-linking its P1 and P3 side chains. This contrasts other proteases such as calpains, where cyclization into a β-strand significantly increases potency (Table 1 and ref 33). Thus, cyclization of peptide aldehydes provides a novel avenue to dictate specificity between the proteasome and calpain proteases.

The combination of high potency and selectivity for the CT-L activity of the proteasome observed for the tripeptidic aldehyde compounds **1**, **3**, **5**, and **6** is rarely observed.[25] With the exception of a series of tripeptide-based vinyl sulfones[26] and some α-keto amides,[27] most inhibitors that show some selectivity for CT-L lack potency. Here, we show for the first time that the combination of high potency and selectivity for CT-L can be achieved with appropriate modification at P1 and P3 of MG132. Carfilzomib, a proteasome inhibitor in phase IIb trials, is the only proteasome in current clinical testing that has been experimentally shown to elicit specificity for the CT-L subunit.[21] However, such CT-L specificity was observed only at low doses of calfilzomib, as higher doses inhibited all three activities of the 20S proteasome. Interestingly this peptide-based inhibitor has a C-terminal epoxide, aryl groups at P2 and P4, and Leu at P1 and P3.

Tripeptide MG132 Analogues Specifically Kill Cancer Cells. We next investigated whether the combination of high potency and selectivity for CT-L, possessed by our inhibitors, translated into improved cytotoxic activity against cultured cancer cell lines. The viability of a panel of four sarcoma cell lines was determined following 48 h of exposure to a titration of concentrations of Bortezomib, MG132, acyclic compounds **1**−**6**, or cyclic compounds **3a** and **4a** (Table 2; Supplementary Figure 1). Parallel viability studies were performed using either normal primary human fibroblasts or primary osteoblasts, thus allowing us to determine if the cytotoxicity of these compounds was cancer cell-specific and hence determine an *in vitro* therapeutic window for each compound (Table 2).

Compounds **3**, **5**, and **6** were highly potent against cancer cells, with average IC_{50} values (1.6, 0.88, and 1.5 μM, respectively) comparable to that of MG132 (0.61 μM). In fact, these three compounds showed higher levels of cytotoxicity than their precursor MG132 in some cancer cell lines. Importantly and in contrast to MG132, the cytotoxicity of these compounds was more specific to cancer cells, with a significantly reduced toxicity to the normal cell lines. In particular, compound **6** showed 19-fold more potency against cancer cells over normal cells, effectively increasing the therapeutic window of MG132 by over 4-fold. In fact, both compounds **5** and **6** showed an increased specificity for cancer cell lines over normal cells compared to the two benchmark proteasome inhibitors, MG132 and Bortezomib. The macrocyclic compounds **3a** and **4a** were all significantly less potent against cancer cells compared to their acyclic counterparts. This is expected given their lower CT-L *in vitro* activity as discussed earlier.

The importance of specifically targeting the CT-L subunit as an anticancer therapy is still unresolved, with conflicting reports in the literature. For example, our finding of anti-neoplastic activity with CT-L specific inhibitors is consistent with a previous report from Parlati *et al.*[21] that demonstrated that specific inhibition of the CT-L subunit using carfilzomib drove selective cell death in multiple myeloma, non-Hodgkin's lymphoma, and leukemia cell lines. However, it is important to note that carfilzomib also inhibited LMP7, the similar subunit corresponding to the CT-L site in the immunoproteasome of these cells. In contrast, a comprehensive study from the Kisselev laboratory demonstrated that co-inhibition of the CT-L subunit with either the T-L or CP-L subunits was necessary to achieve maximal cytotoxic activity of multiple myeloma cell lines.[24] These observations are not consistent with our findings herein using sarcoma cell lines, thus potentially underscoring the fundamental differences in the proteasome function between solid tumors (sarcomas) and hematological malignancies (multiple myeloma). Furthermore, the mechanisms of action of proteasomal inhibitors may be disease-specific, in particular in relation to malignancies such as

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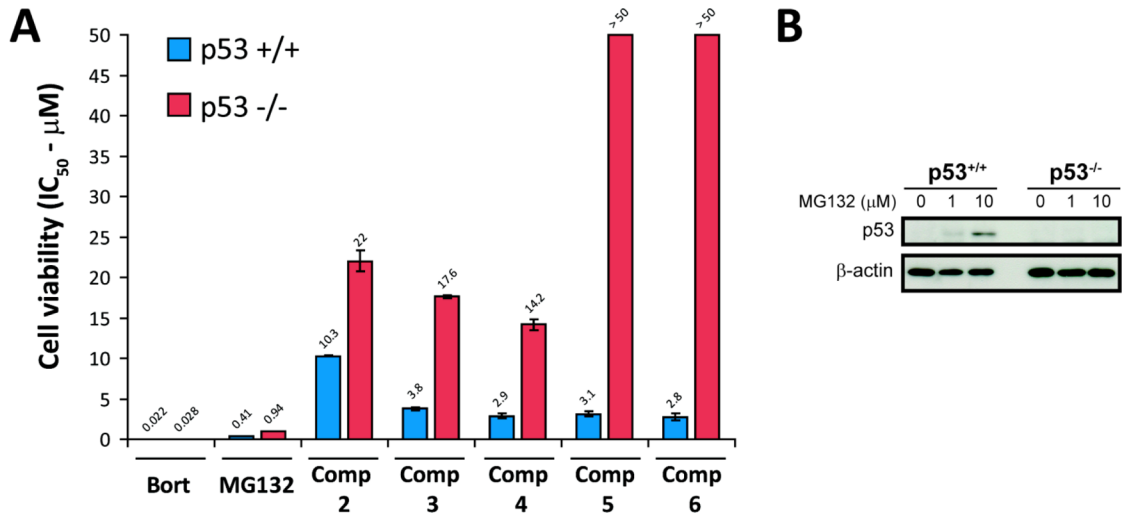

Figure 3. (A) Cell viability assays of MEFs $p53^{+/+}$ or $p53^{-/-}$ exposed to Bortezomib, MG132, or compounds **1**–**6**. Dose–response curves are provided in Supplementary Figure S3. Compound **1** was not assayed using this system. (B) Western blot analysis of p53 protein expression in MEF $p53^{+/+}$ or $p53^{-/-}$ following treatment with MG132 at indicated concentrations for 16 h. *β*-Actin is used as a loading control.

multiple myeloma that are associated with an excessive amount of mis-folded proteins and hence are more heavily reliant on the recycler function of the proteasome.

Thus the acyclic derivatives reported here have a superior therapeutic window compared to that of the benchmark inhibitors, MG132 or Bortezomib. Importantly, several of these new compounds combine a high potency for the CT-L activity of the proteasome with an improved ability to selectively kill cancer cells compared to MG132.

Tripeptide MG132 Analogues Mediate Cell Death in Part through the p53 Pathway. Although proteasome inhibitors have been translated from the bench to the clinic over the past decade, their specific mechanisms of action remain poorly understood. As discussed earlier, proteasomal overactivity in tumor cells has been attributed to aberrant degradation of numerous critical cancer-related proteins, including the pro-apoptotic tumor suppressor, p53. However, the role of p53 as a downstream mediator of cell death in response to proteasomal inhibition remains unclear. Therefore, we used our small library of CT-L-specific proteasome inhibitors (compounds **1**–**6**) to determine the role of p53 in their mechanism of cytotoxic action. For this purpose, we utilized mouse embryonic fibroblasts (MEFs) from transgenic $p53^{+/+}$ and $p53^{-/-}$ littermates. This isogenic pair of cell lines are either competent (MEF $p53^{+/+}$) or deficient (MEF $p53^{-/-}$) in p53 protein and are used here to assess a potential role for p53 as a downstream inducer of cell death in response to proteasomal inhibition.

The $p53^{+/+}$ MEFs were moderately sensitive to compounds **2**, **3**, **4**, **5**, and **6**, with IC_{50} values ranging from 2.7 to 10.3 μM (Figure 3A; Supplementary Figure S3). In contrast, MEFs lacking p53 ($p53^{-/-}$) were significantly less sensitive to the cytotoxic effects of these compounds, with deficiency of p53 rendering MEFs resistant to the cytotoxic activities of compounds **5** and **6**. A similar trend for p53-dependent cytotoxicity was observed upon treatment with MG132. MEF $p53^{+/+}$ cells were over 2-fold more sensitive to MG132 (IC_{50} value of 0.41 μM) as compared to the MEF $p53^{-/-}$ counterparts (IC_{50} value of 0.94 μM). Importantly, exposure of MEFs $p53^{+/+}$ to MG132 was also associated with increased p53 protein levels (Figure 3B), demonstrating that proteasomal inhibition results in accumulation of p53 protein in these cells. Collectively, the data demonstrate that inhibition of the proteasome using tripeptide aldehydes is associated with stabilization of biologically active p53, leading to cell death.

These studies on an isogenic pair of $p53^{+/+}$ or $p53^{-/-}$ cells demonstrate the contribution of p53 as a critical mediator of cell death following proteasomal inhibition. The role of p53 in this process was previously unclear, with reports suggesting that p53 is either essential[9−11] or dispensable[12−15] for the anticancer activity associated with proteasomal inhibition. Interestingly, the reports that refute a role for p53 as a cytotoxic mediator following proteasomal inhibition are restricted to hematological malignancies[12−15] and generally involve the use of panel cell lines with diverse genetic backgrounds that may compromise any p53-related response,[13−15] rather than an isogenic system as presented herein. Our conclusions of a p53-dependent mode of cytotoxic action is further strengthened by consistent results across a small library of proteasome inhibitors (Bortezomib, MG132, and compounds **2**–**6**) and are not limited to the use of a single proteasome inhibitor.[12−15]

In summary, we have shown that the incorporation of an azide group at P3 and a propargyloxyphenyl group at P1 in acyclic aldehydes results in compounds (see **1**–**6**) with a high degree of selectivity for CT-L over both T-L and CP-L. Further work is required to define the exact role of the azide group, but the effect is significantly more pronounced than that observed for the literature peptidic aldehyde MG132. Of general significance to future inhibitor design is the observation that the incorporation of Ile, in place of Leu, at P2 significantly increases potency against the CT-L proteolytic activity. Huisgen cycloaddition of the P3 azide with an aryl acetylene at P1 gave rise to macrocycles constrained into a *β*-strand geometry. Unlike reports for other proteases (particularly calpain II as discussed here), this gave compounds with reduced potency and also reduced anticancer efficacy. Thus, specific cyclization of peptide aldehydes provides a novel avenue to dictate specificity between the proteasome and other proteases.

The new acyclic CT-L specific inhibitors (compounds **1**–**6**) were significantly more specific for cancer cells compared to MG132. In particular, compound **6** showed a 4-fold improvement in its therapeutic window over MG132. Lastly, we have

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used this new panel of potent proteasome inhibitors to demonstrate a critical role of the p53 tumor suppressor protein as a mediator of the cytotoxic response associated with proteasomal inhibition.

METHODS

Chemicals. MG132 (Sigma-Aldrich, St Louis, MO, USA), Bortezomib (LKT Laboratories, St Paul, MN, USA), or MG132 derivatives were dissolved in 10 mM DMSO and stored at −20 °C.

***In Vitro* Proteasome Activity Assay.** Purified rabbit 20S proteasome and fluorogenic CT-L substrate (Suc-LLVY-AMC) were purchased from Boston Biochem (Cambridge, MA, USA). The T-L and CP-L fluorogenic substrates (Ac-RLR-AMC and Z-nLPnLD-AMC) were purchased from Enzo Life Sciences (Farmingdale, NY, USA). The 20S proteasome was diluted to 0.2 $\mu g/\mu L$ in 20S proteasome buffer (50 mM HEPES pH 7.6, 150 mM NaCl and 1 mM DDT) and stored at −80 °C. Purified 20S proteasome (8 ng) was preincubated with the indicated concentrations of inhibitors for 15 min and subsequently added to the AMC-labeled substrate peptide (50 μM) in assay buffer (25 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.05% NP-40, and 0.001% SDS (w/v)) at 37 °C for 2 h. Fluorescent substrate cleavage by the 20S proteasome was linear during this incubation time frame (Supplementary Figure S4). Hydrolyzed 7-amino-4-methylcoumarin (AMC) was subsequently detected with the FLUOstar OPTIMA microplatefluorometer at excitation/emission of 390/460 nm. The activity was estimated in relative fluorescence units and half of the maximal inhibitory activity of the proteasome is represented by IC_{50} values. A minimum of three biological replicates were performed for each data point.

Cell Viability Assays. Cell viability assays were performed as previously described.[39] Briefly, cells were seeded in 96-well microtiter plates at a density of 3×10^4 cells per well in the presence of the indicated chemical. Cells were harvested 48 h post-treatment, centrifuged at 1,300 × *g*, washed in phosphate-buffered saline (PBS), and stained with 7AAD solution (2 $\mu g/mL$) (7-amino-actinomycin-D, Invitrogen, Carlsbad, CA) for 10 min at RT. Viable cells were determined with the use of a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems) and analyzed with the use of FLOWJO (Tree Star, Inc.) and GraphPad Prism (GraphPad Software Inc.).

Cell Lines and Culture Conditions. WE-68 and VH-64 Ewing's sarcoma cells were kindly supplied by F. van Valen (Department of Orthopedic Surgery, Westfälische-Wilhelms-University, Germany). TC-252 and STA-ET-1 Ewing's sarcoma cells were kindly provided by G. Hamilton (Department of Surgery, University of Vienna, Austria) or P. Ambros (Children's Cancer Research Institute, St. Anna Children's Hospital, Vienna, Austria). Primary human embryonic fibroblasts or primary human osteoblasts were collected from the Women's and Children's Hospital (North Adelaide, South Australia, Australia) or Royal Adelaide Hospital with patient consent. Mouse Embryonic Fibroblasts (MEFs) and its p53-null derivative were kindly supplied by Guillermina Lozano (Department of Genetics, Anderson Cancer Centre, University of Texas, Houston, TX, USA).[40] WE-68 cell lines were grown in RPMI-1640 media, while human embryonic fibroblasts and MEFs were grown in Dulbecco's Modified Eagle's Medium (DMEM). Media was supplemented with 10% FCS, 1% PSG and 10 mM HEPES. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Western Blotting. Western blot analyses were performed as previously described. [41] Briefly, cells were harvested and lysed in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 50 mM NaF, 0.5% Triton-X-100, 0.5 mM Na_3VO_4 and 1x proteasome inhibitor (Roche, Indianapolis, IN, USA). Lysates were incubated on ice for 8 min and sonicated using a Vibra-Cell VCX130 (Sonics & Materials, Inc.) at 25% amplitude for 10 s. Protein concentrations were assayed with the bicinchoninic acid assay (Thermo Scientific, Massachusetts, USA) and subsequently resolved using a 10% SDS-PAGE gel. Proteins were transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences), blocked (10% milk/TBST; 30 min), hybridized with the appropriate primary or HRP-conjugated secondary antibody, and subsequently visualized using Enhanced Chemiluminescence (Amersham Biosciences).

Antibodies. Antibodies used included a mouse anti-β-actin (Sigma), anti-p53 (1C12, mouse specific) (Cell Signaling, Danvers, MA, USA), sheep anti-mouse IgG-HRP (Amersham Biosciences, Piscataway, NJ, USA), or donkey anti-rabbit IgG-HRP (Amersham Biosciences).

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and full characterization data for all new compounds, and copies of ^1H NMR and ^{13}C NMR spectra for all new compounds. 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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