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New Peptidomimetic Boronates for Selective Inhibition of the Chymotrypsin-like Activity of the 26S Proteasome

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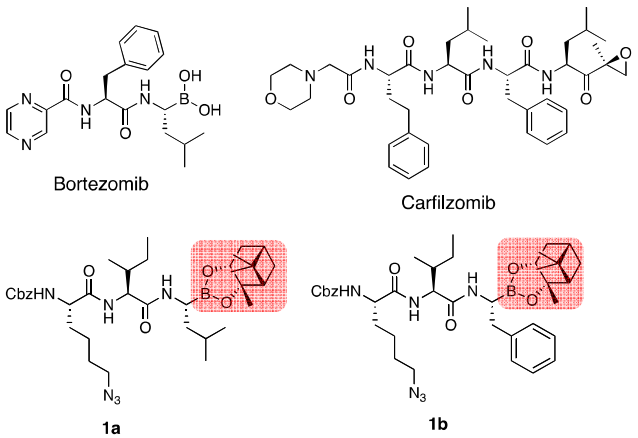
ABSTRACT: Proteasome is a large proteinase complex that degrades proteins via its three catalytic activities. Among these activities, the 'chymotrypsin-like' activity has emerged as the focus of drug discovery in cancer therapy. Here, we report new peptidomimetic boronates that are highly specific for the chymotrypsin-like catalytic activity of the proteasome. These new specific proteasome inhibitors demonstrated higher in vitro potency and selective cytotoxicity for cancer cells compared to benchmark proteasome inhibitors, bortezomib and carfilzomib. In breast cancer cell lines, treatment with **1a** or **2a** induced accumulation of the high molecular weight polyubiqutinated proteins at similar levels observed for bortezomib and carfilzomib, indicating that cancer cell death caused by **1a**/**2a** is chiefly due to proteasome inhibition.

Inhibition of the 26S proteasome is a recognised therapy for the treatment of certain haematological cancers, with bortezomib and carfilzomib (Figure 1) being FDA approved for the treatment of multiple myeloma. Several other proteasome inhibitors are currently in clinical trials.[1] These proteasome inhibitors share some common structural features, with a linear peptide backbone and a C-terminal electrophile that forms a covalent bond with N-terminal threonine of β1, β2, or β5 catalytic subunits of the 26S proteasome. Bortezomib is a dipeptide boronic acid that reversibly inhibits chymotrypsin-like (CT-L) activity of the 26S proteasome by preferentially binding to the active site of the β5 subunit.[2] However, at higher doses it also inhibits the caspase-like (C-L) and trypsin-like (T-L) activities associated with the β1 and β2 subunits respectively. Studies have shown that bortezomib has broad off-target inhibitory effect on other proteases, which are likely to contribute to its multiple clinical side effects.[3] In comparison, the C-terminal epoxyketone of carfilzomib is highly selective for β5 and β5i subunits with minimal cross reactivity to other proteases, allowing more sustained and specific inhibition of the 20S proteasome.[4] While carfilzomib has reduced side effects compared to bortezomib, its C-terminal epoxyketone is highly unstable *in vivo*, resulting in a short plasma half-life (5-20 min) and, therefore, low tissue distribution.[5] These shortcomings restrict the use of bortezomib and carfilzomib in treating multiple myeloma and mantle cell lymphoma. The FDA also approved a boronic citrate MLN9708 (ixazomib) in 2015 for treating multiple myeloma patients who have received at least one prior therapy.[6, 7] MLN9708 preferntially inhibits β5/β5i subunits and has significantly reduced cytotoxicities compared to another boronate (CEP-18770, delanzomib), which inhibits both β5 and β1 subunit.[8] Despite excellent *in vitro* efficacy in preclinical models, these inhibitors have so far failed to show a similar clinical benefit in patients with solid tumours.[9] This is likely associated with the low bio-stability and selectivity of carfilzomib and bortezomib.[10, 11] Thus, new proteasome inhibitors are required with improved overall anti-cancer efficacy, especially for the treatment of solid cancers.



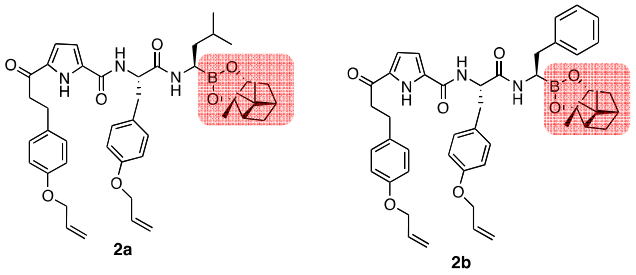


Figure 1. Structures of FDA-approved proteasome inhibitors bortezomib and carfilzomib and the target peptidomimetic boronate inhibitors **1a,b**, **2a,b**.

Here we report new peptidomimetic boronate-based 26S proteasome inhibitors (see **1a,b** and **2a,b** in Figure 1) that have high specificity for β5 catalytic subunit and low toxicity to non-malignant cells. Compounds **1a** and **2a** induced robust accumulation of high molecular weight proteins by inhibiting the 26S proteasome. Compared to bortezomib and carfilzomib, compound **1a** was significantly more toxic towards many cancer cell lines tested. Importantly, both compounds displayed less toxicity towards non-malignant cell lines. Previous reports[12, 13] have shown that the incorporation of a hydrophobic substituent, such as isoleucine, at P2 of peptidic aldehydes enhances selectivity for the CT-L activity over the T-L and C-L activities of the 20S proteasome. One such example (compound **3**, Figure 2) shows an excellent *in vitro* activity of 21 nM for CT-L. This peptidic aldehyde also bears a unique aliphatic azide at P3 to provide additional opportunities for hydrogen bonding interactions with the active site. It is worth noting that an azido group is known to be stable in biological environments[14] and it is found in FDA-approved drugs such as AZT.[15] Compound **4**, (Figure 2), with an O-allylated tyrosine at P2 and a pyrrole replacing the P3 residue and an associated peptide bond, also shows selectivity for the CT-L activity. The backbone pyrrole moiety reduces the peptide-like character of the inhibitor and defines the backbone into an extended conformation. However, C-terminal aldehyde-based peptidomimetics of type **3** and **4** are known to react with a variety of other proteases, e.g. chymotrypsin[16, 17], calpains[18, 19] and cathepsins.[20, 21] Here we replace the aldehyde with a boronic pinanediol ester (highlighted in pink, Figure 1), a group reported to provide similar activity toward CT-L activity of the 26S proteasome compared to the corresponding boronic acid,[22] while being easier to prepare and purify. The chiral ester also defines the absolute configuration of the P1 group introduced during synthesis and negates the need for a final and somewhat problematic deprotection to produce boronic acid. Target compounds **1a** and **2a** have a leucine at P1 as found in known proteasome inhibitors such as bortezomib and carfilzomib.[23-25] In comparison, compounds **1b** and **2b** have a phenylalanine at this position since the S_1 binding pocket of the CT-L activity of the immuno proteasome is known to favour the binding of a large hydrophobic group.[26] The boronates **1a,b** and **2a,b** were prepared as detailed in supporting information (see section f, supporting information).

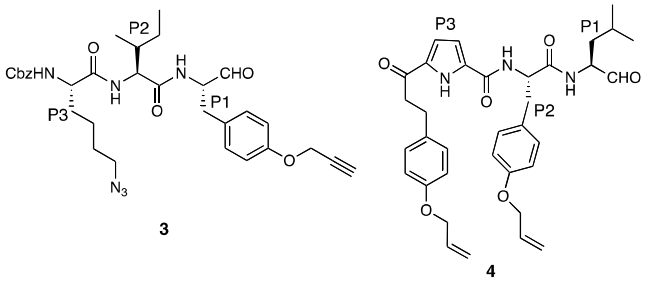


Figure 2. Structures of proteasome inhibitors reported by Abell et al. The amino acid residues of the inhibitors are defined according to nomenclature developed by Schechter and Berger.[27]

The 20S proteasome exhibits T-L, C-L and CT-L activities. Of the three activities, the CT-L activity carries out the bulk of the proteolytic breakdown, and is the most common target of pharmacologically designed proteasome inhibitors.[28] Therefore, we first evaluated whether compounds **1a,b** and **2a,b** were potent and selective for the CT-L activity, with the results shown in Table 1.

As expected, both bortezomib and carfilzomib were highly potent inhibitors of CT-L activity in this assay with IC_{50} values of 34.6 nM and 23.1 nM respectively. Bortezomib also significantly inhibited the C-L activity, which is consistent with a previous report.[29] The new peptidic boronates **1a,b** and **2a,b** were also highly active against the CT-L activity, with derivatives **1a,b** and **2a** proving to be more potent than bortezomib and carfilzomib. The most potent inhibitor in this series, **1a**, has an IC_{50} of 14.1 nM against the CT-L activity, which is more than 2-fold more potent than bortezomib. Unlike bortezomib, all of the compounds were at least 10-fold less active against the C-L activity compared to the CT-L activity. Compound **2a** was the most selective inhibitor of CT-L over C-L, with a 200-fold difference in activities. As per our design, compounds **1b** and **2b** with P1 Phe are more potent against the β5 subunit of the immunoproteasome in comparison to **1a** and **2a**, which have P1 Leu. Compound **1b**, with an IC_{50} of 13.8 nM, proved to be the most active inhibitor of β5i amongst all analogues and is slightly more potent compared to bortezomib. All compounds were also tested against chymotrypsin. The two most potent CT-L inhibitors, **1a** and **2a**, were inactive against α-chymotrypsin at the highest concentration tested (25000 nM). Compounds **1b** and **2b** showed some limited activity, presumably since they contain a Phe at P1. Somewhat surprisingly, bortezomib also showed limited activity.

Following administration of carfilzomib, patients display less of the "typical" toxicities associated with bortezomib, and this has been attributed to its higher selectivity for inhibiting CT-L activity over T-L, C-L as well as other

serine proteases' activities.[24] Therefore, the combination of excellent potency and high selectivity for the CT-L activity observed for the peptidic boronates **1a,b** and **2a,b** provides an opportunity to reduce side effects associated with the low subunit selectivity of bortezomib and limit drug resistance caused by the mutation in CT-L activity of the proteasome found in bortezomib-resistant cell lines.[30]

Table 1. Inhibition of rabbit 20S proteasome and bovine α-chymotrypsin by 1a,b, 2a,b, bortezomib and carfilzomib.

	IC_{50} (CT-L) (nM)[a]	IC_{50} (T-L) (nM)[a]	IC_{50} (C-L) (nM)[a]	IC_{50} (β5i) (nM)[a]	K_i (*b*Ct) (nM)[b]
1a	14.1 ± 4.2	>25000	1598.0 ± 98.3	117.8 ± 24.1	>25000
1b	21.0 ± 4.4	>25000	2448.3 ± 73.2	13.8 ± 1.9	7127.7
2a	20.9 ± 7.7	>25000	4179.0 ± 341.4	483.0 ± 112.4	>25000
2b	104.0 ± 15.9	>25000	3343.3 ± 416.1	113.3 ± 24.4	1309.8
borte zomib	34.6 ± 4.2	>25000	108.4 ± 34.0	16.8 ± 1.6	1824.2
carfil zomib	23.1 ± 4.4	>25000	>25000	ND[c]	>25000

[a] +/- Standard error of mean; n=3. [b]*b*Ct: bovine α-chymotrypsin, K_i values are the mean of three experiments. Variation between experiments is less than ± 10%. [c] ND = Not determined.

We next investigated whether the high potency and selectivity of **1a** and **2a** for CT-L activity translated into improved cytotoxic activity against cultured cancer cell lines. The cytotoxic LD_{50} across a panel of sarcoma, ovarian, breast and myeloma cell lines was determined using 7AAD assays following a 48 h exposure to titrations (0-5000 nM) of **1a**, **1b**, **2a**, bortezomib and carfilzomib. Preliminary data for compound **2b** against breast cancer cell lines showed limited cytotoxic activity and hence was excluded from further study (Figure S2, Supporting Information). Viability studies were also performed with a non-malignant breast epithelial cell line MCF-10A, a primary human lung fibroblasts cell line IMR-90, a primary human skin fibroblast line NDF and a normal human immortalised lymphoblastoid cell line LCL, thus allowing us to determine if the cytotoxicity of these compounds was cancer cell-specific.

Compounds **1a, 1b** and **2a** displayed potent *in vitro* cytotoxicity and dose-dependently decreased cell viability in all cell lines tested. Of the three inhibitors, in both solid and liquid cancer cell lines, compound **1a** consistently resulted in equal or greater cytotoxicity compared to bortezomib and carfilzomib. Myeloma cell lines are known to be highly sensitive to proteasome inhibitors. Consistent with this, the myeloma cell lines NCI-H929 and U266 showed the highest levels of sensitivity, with LD_{50} values of 0.0064 μM and 0.015 μM, respectively (see Table 2). Sensitivity to compound **1a** varied considerably between solid cancer cell lines, with LD_{50} values ranging from 0.035 μM in the RD-ES sarcoma cell line to 1.5 μM in the MCF-7 breast carcinoma cell line. In particular, compound **1a** induced cell death in the Ewing sarcoma cell line WE-68 (LD_{50} 0.035 μM) and ovarian cancer cell line SKOV-3 (LD_{50} 0.37 μM) at significantly lower doses compared to bortezomib (0.1 μM; 1.6 μM respectively, $p<0.01$; n=3). Recent evidence suggests that selective inhibition of the immunoproteasome-associated β5i and β1i activities is particularly effective against haematological cell lines as these predominantly express the immunoproteasome.[31] Despite compound **1b** being more active than **1a** against the β5i subunit, compound **1a** was more cytotoxic than **1b** against the myeloma cell line NCI-H929 and equally cytotoxic against the U266 cell line. (LD_{50} values of 0.0064 μM, 0.015 μM and 0.012 μM, 0.014 μM for compounds **1a** and **1b** respectively). This incongruity between subunit selectivity and cytotoxic LD_{50} is also observed for carfilzomib and may be a reflection of differential drug cell permeability. Importantly, the cytotoxicity of compounds **1a** and **2a** was more specific to cancer cells, compared to bortezomib and carfilzomib. Compound **1a** was approximately 3-fold, and compound **2a** 6-fold less toxic to non-malignant cells compared to bortezomib. The relative sensitivity of the cell lines to **1a** and **2a** was essentially identical, suggesting a common mechanism of cytotoxic action of each inhibitor in a particular cell line. Compound **1b**, in contrast, was much more cytotoxic against normal cell lines than **1a** and **2a**, deeming it unfit for therapeutic applications.

Previous studies report that inhibition of the proteasome causes stabilisation of the tumour suppressor p53, leading to activation of downstream pathways and as a consequence cancer cell cycle arrest or cell death.[32, 33] Therefore, to determine if the cell death observed upon treatment with **1a** and **2a** was influenced by p53 signalling, a pair of p53 wild-type and p53 mutant/null cell lines were used for each cancer type. There was no significant difference between the average LD_{50} values of the p53 wild-type and p53 mutant/null cell lines (Figure S2, supporting information), albeit there was considerable variation within each cancer type. In multiple myeloma and ovarian cancer, p53-proficient cell lines NCI-H929 and KGN were approximately 2-fold and 5-fold more sensitive than U266 and SKOV-3 cell lines in which p53 was mutated or null. However, in breast cancer cell lines the trend was reversed, with p53 mutated cell line MDAMB-468 being markedly more sensitive than MCF7 with wild-type p53. As the overall pattern of sensitivity of the cell lines was individually consistent across a small library of proteasome inhibitors (bortezomib, carfilzomib, **1a** and **2a**), it is possible that stabilization of p53 may mediate cytotoxic effects in a cancer or tissue dependent manner.

Table 2. Cytotoxicity of proteasome inhibitors against a panel of solid cancer cell lines or non-malignant cell lines.

Cell line	Histology/ origin	P53 status	LD_{50} (μM)				
			1a	**2a**	**1b**	Borte-zomib	Carfil-zomib
WE-68	Ewing sarcoma	wild-type	0.035* (±0.001)	0.065 (±0.007)	0.014 (±0.005)	0.1 (±0.01)	0.08 (±0.02)
RDES		mutant	0.035 (± 0.005)	0.065 (±0.01)	0.033 (±0.001)	0.04 (±0.002)	0.043 (±0.012)
KGN	Ovarian cancer	wild-type	0.065 (±0.02)	0.18 (±0.11)	0.048 (±0.023)	0.18 (±0.09)	0.45 (±0.15)
SKOV3		null	0.37* (±0.15)	1.5 (±0.3)	0.046 (±0.004)	1.6 (±0.4	0.32 (±0.11)
MCF7	Breast cancer	wild-type	1.5* (±0.5)	3* (±0.05)	0.079 (±0.047)	9.8 (±5.5)	4.5 (±3.5)
MDAMB468		mutant	0.03 (±0.004)	0.05 (±0.003)	0.024 (±0.004)	0.037 (±0.013)	0.33 (±0.11)
NCI-H929	Multiple myeloma	wild-type	0.0064 (±0.0002)	0.009 (±0.003)	0.012 (±0.001)	0.0066 (±0.0011)	ND
U266		mutant	0.015 (±0.001)	0.029 (±0.002)	0.014 (±0.001)	0.018 (±0.001)	0.06 (±0.01)
MCF10A	Immortalised non-malignant breast	wild-type	5 (±1.5)	9* (±3)	1.25 (±1.9)	1.5 (±0.6)	0.32 (±0.09)
IMR-90	Normal primary lung fibroblast	wild-type	0.2 (±0.1)	0.15 (±0.1)	0.008 (±0.05)	0.13 (±0.09)	0.13 (±0.02)
DSF	Normal skin fibroblast	wild-type	0.5 (±0.1)	1.08* (±0.12)	0.06 (±0.04)	0.48 (±0.2)	0.35 (±0.11)
LCL	B-cell lympho-blastoid	wild-type	0.03* (±0.001)	0.09* (±0.003)	ND	0.02 (±0.002)	0.03 (±0.006)

[a]Dose-response curves are provided in Supporting Information. *indicates statistical significance (<0.05) compared to bortezomib. ND-not determined.

Next, the accumulation of polyubiquitinated proteins in intact cells was analysed to verify that the observed cell death was a result of proteasome inhibition by compounds **1a**/**2a**. Cellular proteins destined for degradation are first "tagged" with multiple ubiquitin molecules to be recognised by the 26S proteasome. Therefore, inhibition of the proteasome results in rapid accumulation of high molecular weight polyubiquitin-conjugated proteins, which can be detected with an anti-ubiquitin antibody. Western blot analyses revealed that treatment with 35 nM of compounds **1a** and **2a** for 4 h substantially increased high molecular weight polyubiquitinated proteins in both MDA-MB-468 and MCF7 cell lines (Figure S3, supporting information). This observation excludes the possibility that reduced uptake of proteasome inhibitors by MCF7 is responsible for the cytotoxic insensitivity of this cell line. For both **1a** and **2a**, the extent of polyubiquitin accumulation was quantitatively similar to that observed using bortezomib. This is largely consistent with cytotoxic efficacies observed for these compounds. Defects or mutations in downstream signalling pathways that drive proteasome inhibitor mediated cell death are likely responsible for the variation in sensitivity seen across cell lines. However, the mechanism that drives cell death requires further assessment and falls outside the scope of this study.

In summary, we report examples of a new class of proteasome inhibitor **1a,b** and **2a,b** with improved *in vitro* activity against the purified enzyme and higher specificity for the CT-L activity compared to bortezomib. Inhibitor **1a** was shown to be significantly more cytotoxic against solid tumour cells compared to both bortezomib and carfilzomib, warranting further investigation *in vivo*. We also demonstrate that the observed cytotoxicity of compounds **1a** and **2a** was due to inhibition of the 26S as Western blot analysis of the cell lines treated with these compounds showed a significant accumulation of polyubiquitinated proteins as a result of decreased proteasome function. Thus compound **1a** is an attractive

drug candidate that offers potential benefits as it is predicted to possess reduced clinical side effects compared to the current chemotherapy agents bortezomib and carfilzomib.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

The preparation of boronates, experimental procedures, full characterization data copies of ^{1}H NMR and ^{13}C NMR spectra and HPLC traces for all new compounds, dose-response curve for the inhibition of purified 20S proteasome and cell cytotoxicity experiments. (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AMC, 7-amino-4-methylcoumarin; *b*Ct, bovine α-chymotrypsin; FDA, U.S. Food and Drug Administration; 7AAD, 7-aminoactinomycin D.

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