Virtual Screening and Biochemical Testing of Borocycles as Immunoproteasome Inhibitors

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Abstract

Inhibition of the immunoproteasome (iCP) offers new opportunities in the treatment of cancer, autoimmune disorders and neurodegenerative diseases. Inspired by the success of boronic acids as proteasome inhibitors we have complied a virtual library of commercially available 5- and 6-membered borocycles and performed a structure based virtual screening against the chymotrypsin-like (β5i) subunit of the iCP. The top scored docking poses were visually inspected to select compounds for experimental testing. Six compounds with 5-membered ring and another six compounds with 6-membered ring were subjected to biochemical tests. All compounds exhibited detectable inhibitory activity at 100 µM concentration and these are the first reported cyclic boronic acid inhibitors of the iCP. Structural variations including the ring size and the substitution of the borocycles and the substitution pattern of the attached aromatic ring resulted in no major variation of the inhibitory activity. We propose that the evaluation of larger cycling boronic acid libraries is needed to fully elucidate the potential of these structures.

Keywords

immunoproteasome, virtual screening, biochemical testing, borocycles

1 Introduction

The proteasome is a multiprotein complex which has multicatalytic properties. It assists in the degradation of damaged proteins into short peptide sequences and represents the crucial part of the ubiquitin-proteasome system [1–4]. It has proven to be a valuable pharmacological target for the treatment of various disorders such as cancer, inflammation and immune diseases [5, 6].

The proteasome can be divided into two parts: the 19S regulatory particle (19S RP) and the 20S catalytic core particle (20S CP). The latter consists of four heptameric rings, two internal β-rings and two external α-rings. Three β-rings are responsible for the catalytic activities, which are executed by the β1 (caspase-like), the β2 (trypsin-like), and the β5 (chymotrypsin-like) subunits [1–4].

The immunoproteasome (iCP) is an isoform of the 20S CP. It is expressed in response to a viral infection or after stimulation by pro-inflammatory cytokines, such as interferon γ [3]. In the immunoproteasome, β1i, β2i and β5i subunits are incorporated into internal β-ring instead of constitutive subunits. Selective inhibition of the iCP offers new opportunities in the treatment of inflammation and autoimmune diseases, hematologic malignancies and in several neurological diseases, where neuroinflammation is a hallmark of disease progression [3, 6]. Several inhibitors of the iCP were reported to date; however, of the majority have peptidic structures, which result in poor pharmacokinetic properties in most of the cases. Another notable issue is the limited selectivity against the CP [1, 6].

Despite that boronic acids are known in chemical literature since the 19th century, growing interest towards these structures can be witnessed in the last few decades [7]. Not only they act as important reagents in synthetic chemistry (Suzuki-coupling [8], Chan-Lam coupling [9], Petasis reaction [10], etc.), different kinds of boronic acids have been subject to intensive research in medicinal chemistry [11].

Boronic acid-based inhibitors have been reported for several enzymes. These are predominantly, but not exclusive, serine proteases, and the covalent binding to the catalytic serine residue has been proved or can be hypothesized [12]. Nowadays, significant efforts are devoted to the

design, synthesis and screening of boronic acid libraries. Dozens of hits against various targets were identified and a few drug discovery projects resulted in FDA-approved drugs (Table 1). For further insights into this topic, the reader is referred to excellent reviews focusing on boronic acids in medicinal chemistry [7, 11].

A large number of boronic acids have been investigated as CP and iCP inhibitors and bortezomib (Table 1) has been approved by FDA for the treatment of multiple myeloma. Inspired by the success of boronic acids as immunoproteasome inhibitors we have initiated a hit finding campaign with the objective of discovering cyclic boronic acids as inhibitors of the β5i subunit of iCP. This type of compounds is well known by medicinal chemists, and three cyclic boronic acid drugs have been approved by the FDA recently (Table 1). These include vaborbactam, a β-lactamase inhibitor, tavaborole, a leucyl-tRNA synthetase inhibitor, and crisaborole, a phosphodiesterase-4 inhibitor. Several other borocycles acting as β-lactamase inhibitors were identified [16–19], and Imperio et al. reported cyclic boronic acid sugar analogues which could function as potential agents in boron neutron capture therapy [20]. Nevertheless, to the best of our knowledge, no cyclic boronates were investigated as iCP inhibitors until the present work.
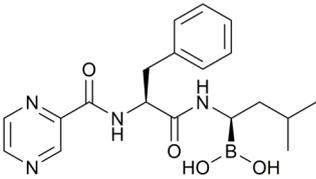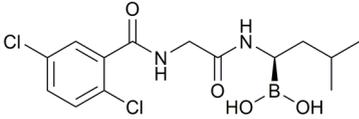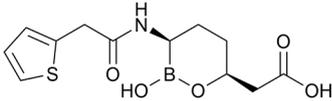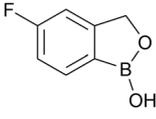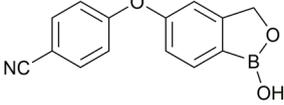
2 Methods
2.1 General chemistry methods
Compounds **1**, **2**, **3**, **4**, **5**, and **7** were purchased from Enamine Ltd, compounds **6**, **8**, **9**, **10**, **11**, and **12** were purchased from Chemspace and all were used for biochemical screening as supplied. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer at 295 K. The chemical shifts (δ) are reported in parts per million (ppm) and are referenced to the deuterated solvent used. The coupling constants (J) are given in Hz, and the splitting patterns are designated as follows:

- *s*: singlet;
- *d*: doublet;
- *dd*: double doublet;
- *t*: triplet;
- *td*: triplet of doublets;
- *m*: multiplet.

Analytical reversed-phase HPLC was performed on Thermo Scientific Dionex UltiMate 3000 UHPLC modular system, equipped with a photodiode array detector set to 254 nm. An Acquity UPLC® BEH Shield RP18 Column (2.1 × 100 mm; 1.7 μm) was used, thermostated at 40 °C, and flow rate was set to 0.3 mL/min. Compound solutions

Table 1 FDA-approved drugs containing boronic acid moiety

Structure	INN	Indication	Remark
	Bortezomib	multiple myeloma	proteasome inhibitor, approved in 2005 [13]
	Ixazomib	multiple myeloma	orally bioavailable proteasome inhibitor, approved in 2015 [14]
	Vaborbactam	bacterial infections	β-lactamase inhibitor, used in combination with meropenem, approved in 2017 [11]
	Tavaborole	onychomycosis	antifungal agent, approved in 2014 [15]
	Crisaborole	eczema	Phosphodiesterase-4 inhibitor, approved in 2016 [11]

were prepared as 0.5 mM in MeCN with 5 % DMSO, 1 μL of which was injected. For the determination of stability, 0.5 mM compound was incubated for 18 h in buffer (50 mM TRIS-Cl, 0.5 mM EDTA, pH 7.4, 2 % DMSO). After the incubation, 1 μL of this solution was injected. An eluent system of A (0.1 % TFA in H_2O) and B (MeCN) was used with gradient elution: 0–10 min, 5 % B → 80 % B; 10–12 min, 80 % B; 12–12.5 min, 80 % B → 5 % B.

2.2 ^{1}H NMR and HPLC analyses of compounds

N-((2,2-difluorocyclopentyl)methyl)-1-hydroxy-1,3-dihydrobenzo[*c*][1,2]oxaborole-6-carboxamide (**1**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.30 (s, 1H, BO<u>H</u>), 8.56 (t, *J* = 5.6 Hz, 1H, N<u>H</u>), 8.20 (d, *J* = 0.9 Hz, 1H, Ar-<u>H</u>), 7.91 (dd, *J* = 8.0, 1.7 Hz, 1H, Ar-H), 7.49 (d, *J* = 8.0 Hz, Ar-<u>H</u>), 5.04 (s, 2H, C<u>H</u>$_2$), 3.50 – 3.24 (m, 2H, C<u>H</u>$_2$, overlap with residual water peak), 2.23 – 1.43 (m, 7H, 3 × C<u>H</u>$_2$, C<u>H</u>); purity by HPLC: 91.0 %; stability in buffer: 99.2 % after 18 h.

2-Hydroxy-*N*-(1-hydroxy-3,4-dihydro-1*H*-benzo[*c*][1,2] oxaborinin-7-yl)-2-methyl-4-phenylbutanamide (**7**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.47 (s, 1H, BO<u>H</u>), 8.40 (s, 1H, Ar-<u>H</u>), 7.96 (d, *J* = 2.3 Hz, 1H, Ar-<u>H</u>), 7.67 (dd, *J* = 8.2, 2.3 Hz, 1H, Ar-<u>H</u>), 7.29 – 7.23 (m, 2H, 2 × Ar-<u>H</u>), 7.20 – 7.12 (m, *J* = 8.3 Hz, 4H, 3 × Ar-<u>H</u>), 5.72 (s, 1H), 4.05 (t, *J* = 5.9 Hz, 2H, CH$_2$C<u>H</u>$_2$OB), 2.82 (t, *J* = 5.8 Hz, 2H, C<u>H</u>$_2$CH$_2$OB), 2.79 – 2.70 (m, td, 1H, overlap), 2.48 – 2.37 (m, td, 1H, overlap with residual water peak), 2.04 (td, *J* = 13.0, 4.7 Hz, 1H), 1.81 (td, *J* = 13.0, 4.6 Hz, 1H), 1.39 (s, 3H, C<u>H</u>$_3$); purity by HPLC: 97.0 %; stability in buffer: 99.8 % after 18 h.

2.3 Residual activity determination

The biochemical screening of compounds was performed at 100 μM final concentrations in assay buffer (0.01 % SDS, 50 mM Tris-HCl, 0.5 mM EDTA, pH 7.4). To each compound, 0.2 nM human iCP (Boston Biochem, Inc., Cambridge, MA, USA) was added and incubated for 30 min at 37 °C. Afterwards, the reaction was initiated by the addition of Suc-LLVY-AMC (Bachem, Bubendorf, Switzerland) at 25 μM final concentration. This substrate was used to monitor the residual activity of the *β*5i subunit of the iCP. The reaction progress was recorded on the BioTek Synergy HT microplate reader by monitoring fluorescence at 460 nm (λ_{ex} = 360 nm) for 90 min at 37 °C. The initial linear ranges were used to calculate the velocity and to determine the residual activity.

2.4 Virtual screening

Commercially available borocycles were collected from eMolecules [21] building blocks and screening compounds. LigPrep by Schrödinger [22] was used to generate 3D conformations, tautomeric and ionization states from SMILES codes at pH 6–8, while retaining specified chiralities. The X-ray structure deposited as PDB entry 6E5B [23] was used for the virtual screening. The binding site is defined by the K and L chains, so all other chains were removed, as well as the covalently bound ligand. Protein Preparation Wizard [24, 25] was used to add hydrogen atoms, protonate residues at pH 7, refine the H-bond network and to perform a restrained minimization. The receptor's grid box required for docking calculations was centered on the corresponding co-crystallized ligand. Covalent docking was performed with CovDock program of the Schrödinger suite [22]. The pose prediction mode [26] with default setup was applied. The top scored docking poses were visually inspected to select compounds for experimental testing.

3 Results and discussion

The compounds selected for virtual screening were cyclic boronic acids with 5- and 6-membered rings as shown in Fig. 1.

304 and 100 compounds with 5- and 6-membered ring, respectively, were collected (Table S1 and Table S2 in the Supplement). Their covalent docking to the Thr1 residue of the *β*5i subunit of iCP resulted in various poses. Compounds that were oriented outward the binding site were not considered as potential hits. Several docked compounds adopted a binding mode resembling that of Ro19 (PDB: 5M2B), a non-peptidic *β*5i-selective inhibitor of human iCP [27]. These compounds oriented toward the Table S1 binding pocket whose different size in iCP and CP was proposed to be responsible for subtype selectivity [27]. Some other compounds adopted a position similar to that of peptid-like ligands that fit into the binding channel with main chain H-bonds and protrude into the subpockets by their sidechains. However, the docked cyclic boronic acids were unable to form the hydrogen bond pattern typical

Fig. 1 General formula of the borocycles with 5- and 6-membered rings

to peptide-like covalent ligands that are hydrogen bounded to Ser21 and Gly47 backbone amine and carbonyl, respectively. The binding modes proposed by covalent docking for compounds **1** and **7** are shown in Fig. 2.

Six compounds with 5-membered ring and another six compounds with 6-membered ring were selected for β5i inhibition assays. The selection was made by visual inspection taking into account docking scores and diversity of the selected compounds. The inhibitory activity was measured at 100 μM concentration and the residual activities are shown in Table 2.

All compounds exhibit detectable inhibitory activity at 100 μM concentration. The remaining enzyme activity was around 50 % suggesting IC_{50} values in the 100 μM range. The activities were fairly uniform for both the 5- and 6-membered borocycles with no apparent effect of the substituent of the aromatic ring. Out of the 12 compounds, nine contain amide functional group as this moiety was highly represented in the screened library owing to synthetic reasons. However, direct interaction between the amide group and the protein was observed only for some of the compounds in the docked binding mode.
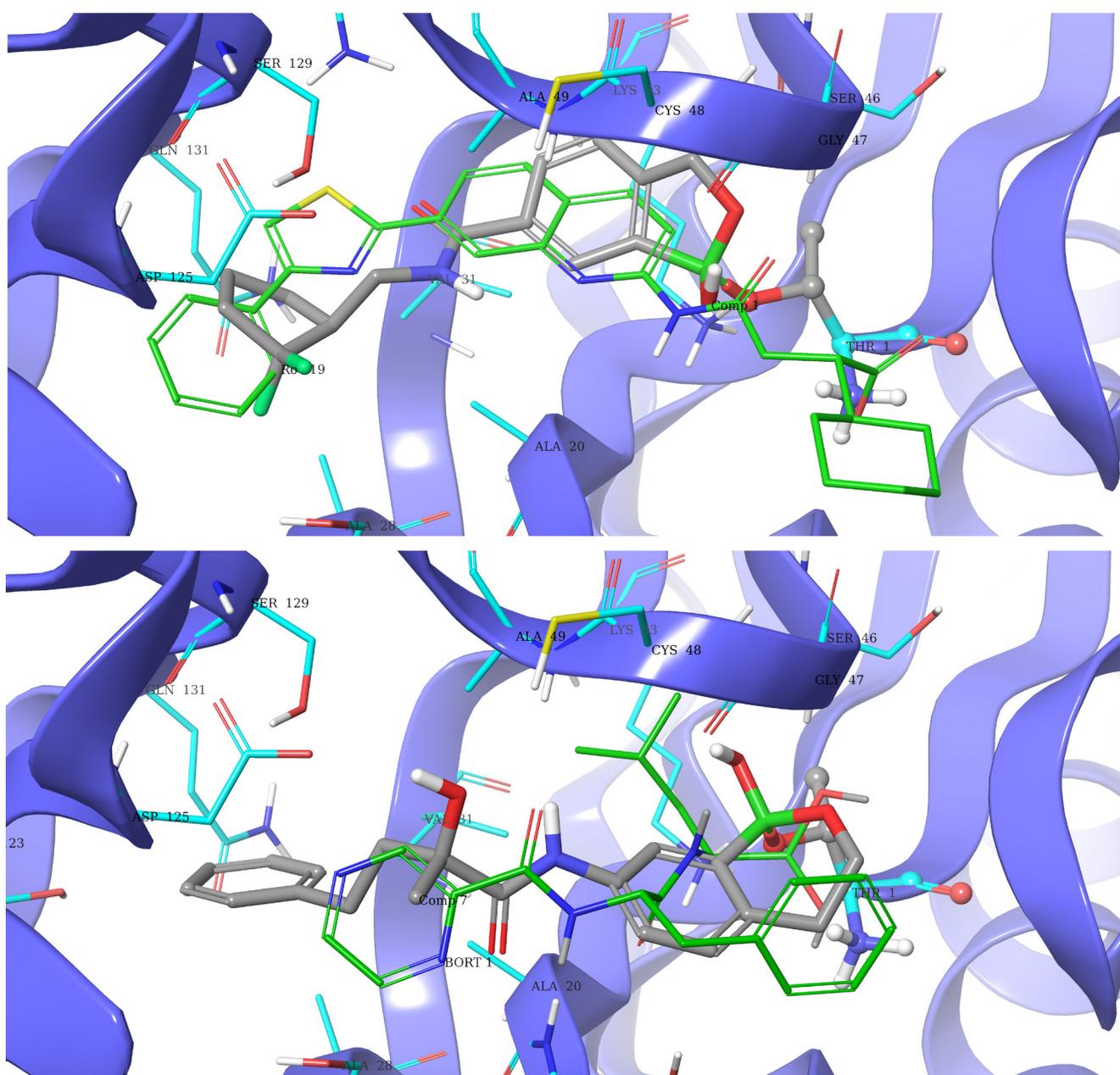

Fig. 2 Binding modes of compounds **1** (top) and **7** (bottom) as proposed by covalent docking. Cyclic boronic acid ligands are shown with grey carbon atoms in stick representation. The overlaid Ro19 (top) and bortezomib (bottom) ligands are shown with green carbon atoms in thin stick representation. Covalently labelled Thr1 is shown in ball-and-stick representation. See text for the discussion of the binding modes.

Table 2 Biochemical assay results for the virtual screening hits

Label	Structure	Residual activity (%) at 100 μM compound
1		39±12
2		44±16
3		49±14
4		50±9
5		71±7
6		55±20
7		39±1
8		77±9
9		53±17
10		59±20
11		55±13
12		53±1

The inhibitory activities of compounds with substituents on the borocycle (compounds **2**, **5**, **10**, and **11**) did not show a clear trend either.

Compounds **1** and **7**, which showed the strongest inhibition with residual activities values below 40 % (Table 2) were further analysed by ^{1}H NMR and HPLC to confirm their uniformity. In addition, buffer stability assay was performed for these two borocycles in order to evaluate the aqueous stability of the oxaborole (in compound **1**) and oxaborinin (in compound **7**) moieties. In both cases, the 18 h treatment of compounds in the assay buffer did not chemically alter the compounds.

Although covalent inhibitors most often exhibit higher than micromolar affinity toward their target, the observation that the fairly diverse set of compounds with similar cyclic boronic acid warheads has similar activity suggests that reactivity is the primary determinant for inhibitory activity. Moreover, neither the reactivity nor the compound recognition by the protein was significantly affected by the substitution pattern. Assuming covalent inhibition, the modest activity of these compounds suggests that either the electrophilicities of the cyclic boronic acid moieties are low, or that these warheads are not placed in an optimal position with respect to the OH group of the catalytic Thr1 in β5i active site.

4 Conclusions

We investigated a set of cyclic boronic acids as inhibitors of the β5i subunit of the immunoproteasome. The biochemical evaluation of twelve compounds selected by virtual screening showed activities in the micromolar range. We believe that further work with borocycles as proteasome inhibitors is warranted. Evaluations of larger libraries of compound are needed to fully elucidate the potential of these structures as inhibitors of either CP or iCP.

Acknowledgments

Abbreviations

FDA: Food and Drug Administration
iCP: immunoproteasome
INN: International Nonproprietary Name

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