Molecular recognition with boronic acids—applications in chemical biology

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Abstract Small molecules have long been used for the selective recognition of a wide range of analytes. The ability of these chemical receptors to recognise and bind to specific targets mimics certain biological processes (such as protein–substrate interactions) and has therefore attracted recent interest. Due to the abundance of biological molecules possessing polyhydroxy motifs, boronic acids—which form five-membered boronate esters with diols—have become increasingly popular in the synthesis of small chemical receptors. Their targets include biological materials and natural products including phosphatidylinositol biphosphate, saccharides and polysaccharides, nucleic acids, metal ions and the neurotransmitter dopamine. This review will focus on the many ways in which small chemical receptors based on boronic acids have been used as biochemical tools for various purposes, including sensing and detection of analytes, interference in signalling pathways, enzyme inhibition and cell delivery systems. The most recent developments in each area will be highlighted.

Keywords Chemical receptors · Boronic acids · *cis*-Diols · Molecular recognition

Introduction

In a biological context, receptors are generally large protein structures which can bind specific ligands via specific molecular recognition processes. The binding sites of proteins—made up of amino acid residues and other species such as metal ions—have evolved for highly specific and often very strong interactions with their target molecule and are therefore useful biochemical tools [11, 21, 70]. Biological receptors

such as protein domains are often used as probes for the detection of their target molecule; one example is the use of antibodies to probe antigens in enzyme-linked immunosorbent assays (ELISA) [29]. These antibodies are conjugated to enzymes which induce a colourimetric [18] or luminescent [42] response, allowing the antigen to be quantified.

Synthetic receptors make use of a large range of possible chemical functional groups in order to bind to analytes with high affinity. Molecular scaffolds can be used to create three-dimensional binding pockets optimised to bind a given target. In artificial receptors, a wide range of functional groups can be incorporated to increase the host–guest complementarity which leads to strong and specific binding between the receptor and the analyte. Boronic acids are an example of such non-biological functional groups which can form reversible covalent bonds with 1,2- and 1,3-diols (as shown in Fig. 1). Boronic acids are known to bind to diol and polyol motifs which are present in saccharides and catechols; they can also form bonds with nucleophilic amino acid side chains such as that of serine.

Table 1 shows examples of several diol-containing biological molecules, their natural or biological receptor as well as artificial chemical receptors that have been reported to bind these specific molecules.

The use of chemical receptors versus their biological counterparts has many advantages. As well as often being more stable to changes in heat and pH, chemical receptors can be more readily modified to enhance activity and cell permeability. Reporter groups such as fluorophores can be incorporated, and this is often achieved more easily and with higher yield when using a chemical receptor rather than a protein or other biological receptor. The concentration of chemical receptors can be more easily controlled, and they act on native-state cells without the need to overexpress any proteins. Synthetic chemical receptors are therefore finding increasing applications in chemical biology and medicinal chemistry.

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Fig. 1 Boronic acids and their corresponding boronic esters can have a trigonal planar geometry (**1** and **3**, respectively) or upon reaction with a Lewis base, tetrahedral (**2** and **4**). They react with 1,2-diols by generating a stable cyclic ester (**3** and **4**). The pK_a of boronic acid affects the association constant of the cyclic ester. Ester formation will be favoured at $\text{pH} > pK_a$; at physiological pH, $K(\text{tet})$ will be higher than $K(\text{trig})$. The tetrahedral form (**2**) is therefore more useful in chemical receptors that are targeting biological species. Several methods are used to form a tetrahedral boron centre. Wulff-type boronic acids (such as **5**) make use of an intramolecular B–N dative bond, and boroxoles such as **6** are formed by an intramolecular five-membered ring, with a pK_a of around 7.2

The reversible, covalent bond between boronic acids and diols is affected by the substituents on both molecules [79].

The scaffold around the boronic acid profoundly changes its structure and reactivity; in general, the receptors which bind most strongly to diols contain Wulff-type boronic acids or boroxole motifs, and substitution of the aromatic ring may also increase the binding affinity towards diols [52]. In addition, 1,2-diols have a range of shapes and conformations: *cis* and *trans* diols have different reactivity with the same boronic acid [50], as do *cis*-1,2-diols on five- versus six-membered rings [28]. Aromaticity and ring strain are also contributing factors, with catechols reacting differently to cyclic aliphatic diols [71].

Boronic acids are Lewis acids and, as such, will accept lone pairs of electrons from Lewis bases. This is the basis of a number of receptors for fluoride, cyanide and phosphate anions. The reactivity of boronic acids and their use as anion sensors have been covered recently in reviews by Nishiyabu et al. [55], Wade et al. [75] and Guo et al. [26] (Fig. 2).

Recognition of mono-, oligo- and polysaccharides by boronic acids

The most common use of boronic acid motifs in molecular recognition is the sensing of mono- and polysaccharides. Saccharides are ubiquitous in biological systems and are a particularly challenging set of molecules to differentiate with chemical receptors due to their similarity in size and structure. Glucose, fructose, galactose and ribose (among others) all contain multiple 1,2- and 1,3-diols arranged around five and six-membered rings (see Fig. 3).

Monosaccharides are the building blocks of complex biomolecules such as cellulose and RNA. They can also be part of other large molecules such as steroids (to form ginsenosides) and peptides (forming peptidoglycan polymers). Long chains of monosaccharides can form poly-

Table 1 Summarising biological molecules, their natural recognition products and boronic acid-based chemical receptors

Molecule of interest	Biological receptor	Chemical receptor
Saccharides: monosaccharides	Lectins [19, 77]; antibodies [32]	Benzoboroxole [15]; tripodal boronic acid receptors [12, 84]
Saccharides: Thomsen–Friedenreich antigen	Lectins [19, 77]; antibodies [32]	Peptidyl boroxoles [58]
Saccharides: cell surface peptidoglycans	Lectins [19, 77]; antibodies [32]	Protein–boroxole adduct [17]; peptidyl boroxoles [10]; squarylium–boronic acid receptor [65]; optical sensors [36]
Phosphatidylinositol-(4,5)-bisphosphate	PLC δ 1-PH domain [43]; neomycin [62]	Pleckstrin homology domain mimetic [47]
Catecholamines	α - and β -adrenergic receptors	2-Anthrylboronic acid [81]; pyrene-based boronic acid receptor [34]
Catecholamines: dopamine	α - and β -adrenergic receptors	Boronic acid coumarin aldehyde [67]; carboxylic acid–boronic acid receptor (dopamine) [31]; functionalised nanoparticles [39]
Nucleotides	Kinase active sites, tRNA synthetase [8]	Tripodal boronic acid receptors [84]; phenylboronic acid (as sensing ensemble) [41]; proteasome inhibitor [3, 8]

Fig. 2 Reactivity of boronic acid towards various analytes

and oligosaccharides such as structural and storage polysaccharides and cyclodextrins.

Such complexity means that the differentiation of polysaccharides by small molecules remains a challenge.

Detection of Gram-positive bacteria

It has long been established that incorporating phenylboronic acids into a solid phase scaffold would aid the chromatographic separation of diol-containing molecules [22]. This was built upon in 1982 with Wulff's pioneering work on using methylaminoboronic acids and boroxoles for the separation of saccharides. Recently, this work has been put to use for the labelling and detection of Gram-positive bacteria, which possess a thick saccharide-rich peptidoglycan layer on the surface of the cell. For this purpose, Saito et al. [65] designed fluorescent receptor 8 which targets monosaccharides. The receptor has a reporter unit based on the squarylium fluorophore, and upon forming a boronate ester with the diol of a saccharide, a large enhancement in fluorescence is observed as aggregation of the receptor is disrupted. The addition of boric acid—which will compete with the receptor for binding sites—resulted in reduced fluorescence, showing that boronic acid is necessary for targeting the saccharide layer [65] (Fig. 4).

Receptor 8 was incorporated into a capillary electrophoresis–laser-induced fluorescence separation method and used to label and detect Gram-positive bacteria. The receptor targets the thick layer of saccharides by forming boronate esters,

labelling the cells and resulting in enhanced fluorescence. This sensitive technique had a low detection limit of only three cells [65].

Separation and sensing is not the only use of boronic acid-based motifs. The design of cell delivery agents is of increasing importance as many potential diagnostic or therapeutic tools are not taken up by the cell to a large degree, and this limits their usefulness.

A cell delivery system

A small number of studies have been carried out which use boronic acid motifs to increase the membrane permeability of small molecules. In the earliest of these, Gallop et al. [20] modified water-insoluble fluorophores with phenylboronic acid. The resulting compounds remained insoluble in water; however, in the presence of polyhydroxy-containing buffers, a boronate ester formed which passed through the cell membrane. Once inside the cell, the boronate ester hydrolysed and the fluorophores were deposited into hydrophobic pockets. In this case, only the boronate ester was soluble and the polyhydroxy component of the buffer was used to protect the boronic acid.

In a later study, the interaction between boronic acids and diols was again used to alter the electrostatic or lipophilic properties of drugs or fluorescent agents to enhance their permeation of model membranes [51]. By the addition of a boronic acid to the carbohydrate sialic acid, the lipophilicity

Fig. 3 Ring forms of saccharides glucose, fructose, galactose and ribose. All contain 1,2- and 1,3-diols

Fig. 4 Receptor **8** (SQ-BA), designed to detect monosaccharides

of the resulting boronate ester was increased and the uptake was enhanced [4].

Another recent use of boroxole–saccharide interactions for the delivery of small molecules and proteins into cells was the delivery of a small enzyme into cells [17, 33]. Often, large molecules are not taken up by the cell because they cannot get close enough due to the layer of charged saccharides surrounding it. The addition of saccharide-binding lectin proteins to a drug molecule or protein is a method that has been successfully used to bring drug molecules and pro-drugs, as well as whole proteins, into contact with the cell surface, where they were then transported into the cell [19]. Replacing lectins with a more specific boroxole–saccharide interaction has the advantage of increasing the strength and specificity of the drug–cell surface interaction, and the smaller size of the boroxole motif compared to the bulkier lectin means that the drug will experience less steric interference once it enters the cell. The boronation of the enzyme RNase enabled it to bind to glycans on the surface with high affinity, thus increasing the efficiency of its cellular delivery [17] (Fig. 5).

When the enzyme RNase A is present in the cytosol, it can degrade the RNA present there, causing cell death. To enhance the delivery of RNase A to the cytosol, the enzyme was boronated by attaching 5-amino-2-hydroxymethylphenylboronic acid at up to 11 sites on the protein via carbodiimide coupling. The resulting boronated enzyme was also labelled with a

Fig. 5 Boronated site on the RNase A enzyme can bind to the cell surface saccharides, enhancing the uptake of the enzyme into the cell [17]

fluorophore and retained partial activity (17 %). Measuring uptake by flow cytometry showed that the uptake of boronated enzyme was five times that of the non-boronated control. By the addition of large amounts of fructose (to which boroxole binds more strongly than the sialic acids present on the cell surface), this effect was reversed as the fructose outcompeted the sialic acid for boroxole binding sites. The presence of the enzyme in the cytosol was confirmed with a cytotoxicity assay. The increase in cell death in the presence of boronated RNase showed that the enzyme was active against cytosolic RNA. In contrast, cells proliferated normally in the presence of chemically inhibited the boronated enzyme, indicating that the source of toxicity was the enzymatic activity, not the boronation.

Boronation of a cytotoxic agent enhanced its uptake into the cell by binding to surface glycans. This process is similar to the use of lectin-based delivery systems, but boroxoles have higher affinity than lectins, and the possibility of using synthetic targeting motifs could lead to more specific drug targeting in the future, with increased specificity lowering the chances of off-target effects.

The interaction of peptidoglycans with lectins is known to mediate cell–cell adhesion. This process is often disrupted as one of the first steps in the metastasis of tumours as the cancer cell is released into the circulatory system before re-adhering to tissues at a secondary site [61]. Cell surface carbohydrates and their endogenous biological receptors are therefore of interest, and a number of tools have been developed to investigate this interaction. The progress of these has recently been reviewed by Walker et al. [76].

A specific receptor for a disaccharide biomarker

The Thomsen–Friedenreich (TF) disaccharide (Fig. 6) is a known tumour biomarker. Consisting of two linked galactose saccharides attached to a lipid or protein, this important antigen is scattered over the cell surface and is exposed and reactive in oncogenic cells [24]. As such, it is an important molecular recognition target for the diagnosis of cancer [30].

In order to synthesise a highly specific receptor for the TF antigen disaccharide, Pal et al. [58] created a small library of peptide receptors based on the binding site of saccharide-binding lectins (see Fig. 7). The ability of these peptides to bind to disaccharides was enhanced by the addition of two unnatural amino acid side chains comprising diol-binding boroxoles. Boroxoles are known to bind strongly to the 3,4-

Fig. 6 Structure of the TF antigenic disaccharide (R=protein or lipid)

Fig. 7 Generic structure of the solid phase library of peptidyl boroxoles. R_1 =1 of 20 amino acid spacers; R_2 =1 of 20 acyl capping groups

cis-diol of galactose [9], and the amino acids also contribute to binding via multiple hydrogen bond donor and acceptor interactions. By substituting the amino acid connecting the two boroxole units and the acyl capping group, a directed 400-member solid phase library was generated.

The amino acid spacer provided flexibility and pre-organisation of the two boroxole units, the relative position of which was optimised by the library. The boroxole functional groups bound strongly to the diols of the disaccharide, whilst the capping groups interact with the disaccharide via hydrogen bonding and CH- π interactions, where possible. Testing the binding affinity of the peptidyl boroxoles by competitive ELISA revealed 17 synthetic receptors that bind to the TF antigen, one of which was shown to be highly selective for Gal- β -1,3-GalNAc over other disaccharides (R_1 =4-methoxybenzene, R_2 =2-methyl-5-(*p*-tolyl)furan). This approach shows that incorporating a diol-binding boroxole motif into a library of disaccharide-binding peptides increased the binding affinity by approximately fivefold (boroxole-containing peptide: IC_{50} =20 μ M; control: IC_{50} =100 μ M) over the control, which lacked this strong, specific interaction.

Displacement assays as a measure of enzyme activity

Most chemical receptors lack a reporter group that can permit substrates to be directly detected. Therefore, the use of receptor–indicator pairs has emerged as a useful alternative. By monitoring the change in the UV–Vis spectrum of a dye as it is bound by a receptor and subsequently displaced by an analyte, indicator displacement assays enable binding constants between the chemical receptors and their analytes to be measured [53].

Several colourimetric dyes contain catechol moieties which enable boronic acid-functionalised molecules to bind to them, forming a five-membered boronate ester ring and changing the colorimetric properties of the indicator. The addition of a competitive analyte (e.g. one containing a diol) will displace the dye from the boronic acid binding site, returning it to its original colour and, hence, providing a parameter that can be measured spectroscopically (see Fig. 8).

Sensing ensembles such as these have been developed for a wide range of analytes including chiral diols [68], the anticoagulant oligosaccharide heparin [86], phosphosugars [12], nucleotides [84] and more [41, 54, 56, 83].

This method has been used as a measure of enzyme activity using diboronic acid receptor **9**, which forms a non-fluorescent complex when bound to a pyrene-based indicator (Fig. 9). The receptor releases the indicator as the enzyme produces the more strongly binding fructose product; the associated increase in fluorescence can be used to monitor the enzyme activity in real time [73].

Multicomponent sensing of saccharide-containing medications

The indicator displacement assays enable binding interactions to be quantified and are a valuable method of analysing the affinity and specificity of receptors. However, the synthesis and characterisation of chemical receptors that specifically bind to every monosaccharide and diol is a time-consuming and challenging process. Displacement assays have therefore been combined to form multicomponent sensing ensembles which provide much more information than single displacement assays. Multicomponent sensing ensembles make use of several combinations of receptors and dyes. Each analyte will have its own specific pattern of displacement, and in this way, complex mixtures can be deconvoluted. These sensing systems have been developed for many saccharide mixtures including monosaccharides and saccharide derivatives [16, 23], ginsenosides [85] and nucleotides [66].

In a new adaptation of the multicomponent sensing technique, Rout et al. [64] developed receptor **10** (Fig. 10), a probe containing multiple Wulff-type boronic acids appended to a proline amino acid with four different fluorophores attached. This was used to identify a diverse set of carbohydrate-based antibiotics often counterfeited. All of these molecules contain multiple 1,2- and 1,3-diols from saccharides and other polyol motifs. Upon the addition of this probe to a diol-containing target molecule, each boronic acid in the probe will bind to the target to a different degree. This results in a unique fluorescent profile as the photoelectron transfer, intramolecular charge transfer and Förster resonance energy transfer processes are altered in the presence of each analyte. Using principal component analysis, the authors were able to recognise the combinations of analytes with 93 % accuracy and identify unknown samples with 97 % accuracy.

The advantage of this approach is that only one molecule is necessary—the different reporter groups are all incorporated onto one scaffold, whereas with conventional multicomponent sensing ensembles, a number of receptors must be developed and used with several indicators. From the interaction of one molecule with an unknown mixture, a large amount of information can be obtained.

Fig. 8 Principles of a colourimetric indicator displacement assay with boronic acids

Molecular recognition of dopamine and catecholamines with boronic acids

Another 1,2-diol that has been shown to bind to boronic acids is the 1,2-benzendiols (catechol) moiety. In a biological context, this group is present in L-DOPA (the precursor to dopamine), dopamine itself and several metabolites thereof (Fig. 11). Dopamine is a neurotransmitter that is carefully regulated and is extremely important for many brain functions. Parkinson's disease is caused by a decrease in the amount of dopamine released into the synapse; decreased dopamine levels are also associated with a number of chronic neurological disorders including bipolar disorder and depression. Conversely, the buildup of dopamine levels can indicate that essential metabolites such as epinephrine and norepinephrine are not being formed. Research into the development of small chemical receptors to aid in the analysis of dopamine levels has been in progress for many years.

However, until recently, this work had been hindered by the lack of selectivity for dopamine over other similarly structured catecholamines.

Fluorescent receptors for catecholamines

There have been several attempts by researchers to develop chemical receptors that are capable of distinguishing between these molecules. In 1997, Yoon and Czarnik [81] developed a fluorescent turn-off sensor, receptor **11** (Fig. 12), which showed strong binding towards L-DOPA and dopamine and an impressive selectivity over glucose. However, a drawback of sensor **11** is that it did not show selectivity for L-DOPA over dopamine, unsurprising since the only recognition motif on the receptor was the boronic acid.

Secor and Glass [67] came closer to achieving selectivity in 2004 with the more promising receptor **12** (Fig. 13), which possessed both a methylaminoboronic acid group to bind to

Fig. 9 Principles of the use of Singaram et al. of fructose-binding receptor **9** and fluorescent dye to monitor the activity of the enzyme sucrose phosphorylase. As fructose is generated, the receptor binds to it

and the non-fluorescent (ground state) complex is disrupted. The resulting increase in fluorescence is used to monitor enzyme activity [73]

Fig. 10 Schematic representation of receptor **10**, a combinatorial probe created by Rout et al. which shows a unique fluorescence response in the presence of diol-containing medications. Principal component analysis is used to distinguish between analytes and identify combinations thereof

the 1,2-diol and a coumarin-appended aldehyde with the potential to form an imine with the free primary amine present in dopamine.

Upon the formation of an imine, coumarin experiences a bathochromic shift, and upon binding via boronic acid to the catechol fluorescence, quenching is observed; both of these techniques were used to establish the binding mechanisms. The receptor showed good selectivity for the primary catecholamines dopamine and norepinephrine over other substituents (epinephrine, a catechol-containing secondary amine, glucose, glucosamine and primary amine-containing amino acids). The success of this work indicated that by utilising two types of covalent interaction, a much more selective receptor could be designed.

Specific dopamine sensing

More recently, Tian and Shi used this method of two functional group interactions to develop a highly effective sensing system that is selective for dopamine over all other substituents tested (ascorbic acid, epinephrine, norepinephrine, glucose, lactose and DOPAC; L-DOPA was not mentioned) [39].

By using gold nanoparticles conjugated to both catechol-reactive boronic acid and primary amine-reactive succinimide, dopamine could be detected linearly down to 0.5-nM concentrations. As the nanoparticles bound to the catechol and primary amine groups, aggregation took place, causing visible change of the solution colour from red to purple.

Testing this system in a biological context, the authors were able to quantify the concentration of dopamine in a rat brain dialysate as 10 nM, a figure which agreed with previously reported values [35, 69]. Moreover, upon adding a dopamine reuptake inhibitor to the sample, dopamine was released from the transporting proteins. This effect was reflected in a further bathochromic shift of the UV–Vis spectrum of the nanoparticles. Conversely, the addition of a dopamine inhibitor reduces the detected concentration in the sample.

This work demonstrates selective detection of dopamine at physiological concentrations, with direct colourimetric read-out that is responsive to changes in dopamine levels as stimulated by various agents. Although no *in vivo* work has been currently reported, it is likely that such a promising tool will be extremely useful in probing brain function and neurological diseases related to excess or deficiency of dopamine.

Fig. 11 Structures of dopamine, precursor L-DOPA and metabolites DOPAC, norepinephrine and epinephrine

Fig. 12 Anthracene-based boronic acid receptor **11** binds to catechols

Molecular recognition of phosphatidylinositol bisphosphate with boronic acids

Recently, Vilar and Woscholski developed a novel small molecule that was capable of binding specifically to the lipid phosphatidylinositol-(4,5)-bisphosphate (PIP₂) [47]. Receptor **13** (the chemical pleckstrin homology domain mimetic; see Fig. 14) was shown to cross the cellular membrane and bind to its target with high affinity and specificity. The phospholipid PIP₂ possesses a six-membered carbon ring head group with adjacent hydroxyl groups which form a 1,2-diol as well as two phosphate groups and a long lipophilic tail which anchors it to the membrane of cells. In order to bind to this head group, receptor **13** was designed with boronic acid groups next to a methylamino motif to target the 1,2-diol. Urea groups were also incorporated into the chemical receptor and were proposed to act as hydrogen bond donors towards the phosphate groups (Fig. 15).

The position of the secondary amines created a Wulff-type boronic acid which formed a cyclic ester with the 1,2-diol at physiological pH. Receptor **13** bound to PIP₂ with low micromolar affinity, interfering in protein–lipid interactions by physically blocking access to the head group. This had the effect of inhibiting the activity of enzymes such as the phosphatase SopB. Phosphatase assays indicated that receptor **13** did not bind to other similar phosphatidylinositol phosphates, showing a high specificity for PIP₂, which makes it an extremely useful tool for probing the complex phosphatidylinositol phosphate network.

Although the chemical receptor lacks any fluorescent reporter groups, its effect in the cell could be tracked by the use of fluorescently labelled proteins. The pleckstrin homology (PH) domain which binds specifically to PIP₂ at the plasma

membrane was tagged with green fluorescent protein (GFP). In the absence of receptor **13**, a GFP-PLCδ1-PH domain was observed at the plasma membrane, where it bound to PIP₂. Adding receptor **13** displaced the GFP-PLCδ1-PH domain in a dose-dependent manner, and the fluorescent protein was observed in the cytosol instead of arranged at the membrane. Other PIP₂-dependent cellular processes were also disrupted by receptor **13**, including the uptake of transferrin by endocytosis, mitochondrial membrane integrity and the formation of actin stress fibres. All these data showed that the chemical receptor **13** was taken up by the cell, bound to PIP₂ and blocked protein–lipid interaction, disrupting PIP₂-dependent pathways in the cell.

Receptor **13** is a useful tool for exploring PIP₂-dependent cellular processes, many of which are linked to diseases including cancer [74], diabetes [14] and Lowe syndrome [45]. This work has identified PIP₂ as a potential drug target in the diagnosis and treatment of these disorders.

Enzyme inhibitors based on boronic acids

Boronic acids interact with amino acid residues that are able to donate a pair of electrons to the boron, including serine (side chain –CH₂OH), histidine (side chain methylimidazole) and lysine (side chain –(CH₂)₄NH₂). Due to the hydrophobic nature of many active sites, the boronic acid receptors can bind with high affinity to residues there, and inhibition is often irreversible. Figure 16 below indicates the amino acid side chains which are known to interact with boronic acids or boroxoles; however, it is possible that other amino acids such as threonine (side chain –CH(OH)CH₃) or cysteine (side chain –CH₂SH) could also interact in a similar way.

Fig. 13 Dopamine receptor **12** with dual recognition motifs; boronic acid forms an ester with catechol and aldehyde reacts with primary amine

Fig. 16 Side chains of histidine, serine and lysine are known to interact with boronic acids

regulatory proteins, transcription factors and tumour suppressors. In cancerous cells, this process is accelerated, and the subsequent lack of regulation allows the cell cycle to advance uncontrolled and results in tumour growth [2]. Inhibition of the 26S proteasome slows down this process and allows the cell cycle to progress at a more normal rate. Early proteasome inhibitors based on tripeptidyl aldehydes suffered from poor specificity as they inhibited also cysteine proteases such as cathepsin [1]. In an attempt to improve specificity and potency, Adams et al. changed the aldehyde

Fig. 18 Top Serine hydroxyl group attacks carbonyl of normal peptide, creating unstable transition state. Bottom Serine hydroxyl group attacks Lewis acidic boron on modified peptide, creating stable tetrahedral borate [38]

motif for other functional groups (chloromethyl ketone, trifluoromethyl ketone) and finally resulted upon the boronic acid tripeptides. Boronic acid forms a tetrahedral complex by accepting a lone pair of electrons from the threonine residue in the active site (Fig. 19), in a mechanism similar to that of serine protease inhibition.

Since boron interacts with sulphur only weakly, the peptidyl boronic acid inhibited cysteine protease in only a small amount, showing high specificity for the proteasome. The tripeptidyl boronic acid displayed strong binding towards the proteasome (low nanomolar K_i), and when the molecule was shortened to a dipeptidyl boronic acid, this affinity was maintained. Since serine proteases require longer peptide substrates, the dipeptidyl boronic acid had weaker inhibitory activity for this class of enzyme.

The peptidyl boronic acid PS-341 showed cytotoxic behaviour in vitro towards several cancer cell lines [3], causing

Fig. 17 Diagram developed from the PDB file of α -chymotrypsin co-crystallised with phenylethane boronic acid (PEBA) [72]. Inset, A boronate ester forms between PEBA and serine 195, part of the catalytic triad. Image generated using SwissPDB Viewer [25]

Table 2 Selected boronic acid-based enzyme inhibitors

Target	Inhibitor	Mechanism	Function	Notes
1. Serine proteases (General) (Philipp & Bender 1971)	Benzeneboronic acid:	Binds active site serine, histidine	Inhibits peptide hydrolysis by serine proteases (non-specific)	K_i (σ -chymotrypsin)= 196 μ M, K_i (Subtilisin)= 229 μ M
2. Chymotrypsin (serine protease) (Charles A Kettner & Shenvi 1984)	Ac-Boro-Phe-OH:	Substrate mimic (transition state analogue)	Inhibits cleavage of peptide substrates of chymotrypsin	K_i = 2.1 μ M
3. Thrombin (trypsin-like protease) (C. Kettner et al. 1990) (Lim et al. 1993)	Ac-Phe-Pro-BoroArg-OH:	Substrate mimic (transition state analogue)	Anticoagulant	K_i = 41 pM Selective for Thrombin over other trypsin-like serine proteases
4. Mn^{2+} 2-Arginase (Baggio et al. 1997)		Substrate mimic (transition state analogue)	Regulation of substrate availability for NO synthase and therefore smooth muscle activity	IC_{50} = 0.8 μ M
5. Proteasome 26S (Julian Adams et al. 1999)	(Velcade)	Binds active site serine	Antitumour activity by inhibiting hydrolysis of p21	K_i = 0.6 nM Approved by FDA
6. Aminoacyl-tRNA synthetase (LeuRS) (Rock et al. 2007) (Baker et al. 2011)	(AN2690)	Binds adenosine of substrate tRNA	Antifungal agent	IC_{50} = 2.1 μ M Currently in clinical trial
7. Serine- β -lactamase (Zervosen et al. 2012)		Binds active site serine, lysine	Inhibits antibiotic resistance	IC_{50} = 1.3 μ M. Monovalent (serine) & trivalent (2 Ser, 1 Lys) adducts

cell cycle arrest. In vivo studies showed that the compound reduced the volume of tumours, and so the compound was

enlisted in clinical trials. This dipeptidyl boronic acid has low molecular weight, high potency, and high efficacy in

Fig. 19 Bortezomib (Velcade) binding to the threonine residue in the active site of proteasome 26S

vivo and was therefore one of the first boronic acid-based molecules to be approved for use as an anticancer drug, now known as bortezomib and marketed as Velcade.

AN2690, an antifungal agent

The boroxole-containing compound AN2690 was discovered to be a potent antifungal agent by Baker et al. [6]. By phenotypic screening of a large library of modified boroxole compounds against fungal pathogens, AN2690 was highlighted as having broad-spectrum activity with low minimum inhibitory concentrations for all strains tested. The compound was then submitted for clinical trial as a potential treatment for fungal nail infections.

The mechanism of action of this compound was later elucidated by the same research group [63]. By engineering *Saccharomyces cerevisiae* resistant to AN2690, mutations were pinpointed to the *CDC60* gene which encodes the Leucyl-tRNA synthetase (LeuRS), an enzyme which is responsible for attaching the appropriate amino acid to tRNA (at the synthetic active site) and hydrolysing wrongly attached amino acids (at the editing active site). The compound was shown to inhibit the editing function in the presence of adenosine nucleotides. A series of crystal structures indicated that the inhibitor binds to the 3'-adenosine of the tRNA molecule via the boroxole, forming a boroxole-tRNA adduct that is “locked in” to the editing active site (Fig. 20).

This adduct is shielded from the aqueous environment and is therefore highly stable; whilst it is present in the editing

Fig. 20 Formation of the tRNA–boroxole complex which inhibits the tRNA synthetase LeuRS by blocking the active site of the enzyme [63]

active site, this function of the enzyme is inhibited. When boroxole binds the tRNA outside of the active site, the cyclic boronate ester is hydrolysed readily in the aqueous environment. This unusual inhibitor has a dual mode of action—it binds the substrate tRNA and also blocks the active site of the enzyme.

Toxicity

The boronic acid functional group in general has low toxicity [4, 13, 27]. However, when this group is accommodated into a larger molecule, the resulting compound may have effects specific to that structure which can cause cytotoxicity.

The main mechanism by which boronic acids are metabolised is deboronation, yielding boric acid. Although boric acid ($B(OH)_3$) is used in insecticides and herbicide, it has low toxicity in humans, with lethal doses comparable to that of sodium chloride, common table salt ($B(OH)_3$: $LD_{50}=2660$ mg/kg; NaCl: $LD_{50}=3,000$ mg/kg) [7, 78]. Deboronation can occur by a number of routes, the main ones being metal-catalysed hydrolytic cleavage [27, 48] and oxidative deboronation carried out by enzymes including cytochrome P450 [40, 44, 59]. Boric acid is then excreted by the kidneys without further metabolism [57].

Summary

The use of boronic acid, boronate ester and boroxole functional groups in chemical biology and medicinal chemistry has increased dramatically in recent years. These moieties have many advantages: boronic acids form strong, reversible covalent bonds to target diols—a process which has been extensively studied and characterised. The boron-containing groups themselves have low toxicity and therefore may be incorporated into molecules such as peptides without cytotoxic side effects. Their chemistry is extremely versatile, and as well as being a useful functional group for molecular recognition, boronic acids can fine-tune the reactivity of other nearby functional groups and interact with fluorophores to create reactive turn-off and turn-on sensors. The small size of the boronic acid group means that it can be incorporated into small molecules, inhibitors and chemical receptors; its specific activity permits selective and effective agents such as the proteasome inhibitor Velcade to be designed. The success of this molecule is likely to generate much more interest in the use of boronic acid-type functional groups in drug screening, detection and sensing systems, cell delivery, separation of biological analytes, and more.

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