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TUTORIAL REVIEW

Boron-containing inhibitors of synthetases†

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The use of boron in small-molecule pharmaceuticals is increasing. Boron's ubiquitous occurrence in nature and the recent success of a boronic acid drug (Velcade®) in the clinic have alleviated many concerns over its use in pharmaceuticals. In addition, the unique physicochemical properties of boronic acids make them an attractive addition to the medicinal chemists toolbox. This *tutorial review* will discuss these properties and potential benefits for anyone interested in finding novel enzyme inhibitors. An exceptional class of boronic acids, the oxaboroles, will be highlighted and their properties and uses will be discussed in detail. Finally, the current paradigm for the reaction of boronic acids with enzyme nucleophiles will be summarized.

1. Introduction

Analysis of successful drugs and comparison to those that have failed in development have led to seminal papers, such as Lipinski *et al.*,[1] Gleeson,[2] and a recent review by Waring,[3] describing favorable physicochemical properties to guide medicinal chemists in their structural designs. These analyses essentially suggest that smaller, less lipophilic molecules will have a greater chance of being successful clinical candidates. As always, there are exceptions to these rules, but as demonstrated with the Lipinski rule of five, medicinal chemists are generally well served by taking note of these observed patterns. These patterns suggest that the volume of chemical space within which medicinal chemists can practice is limited. This constraint is typically in contrast with the goal of medicinal chemists to improve selectivity towards their therapeutic target and minimize off-target activity, which they achieve by adding substitutions to increase potency, usually resulting in larger, more lipophilic molecules. So how can a medicinal chemist be atom efficient yet increase molecular diversity? One solution is to introduce new atoms into drug molecules that provide a functional role not offered by other atoms. One such atom that can fulfill the roles of expanding molecular diversity and providing additional functionality is boron.

Boron contains an empty p-orbital which makes it a strong electrophile and a Lewis acid. It can readily form dative bonds with nucleophiles and in doing so transforms from an uncharged, trigonal-planar structure (Fig. 1) to an anionic, tetrahedral structure. This feature allows it to form dative

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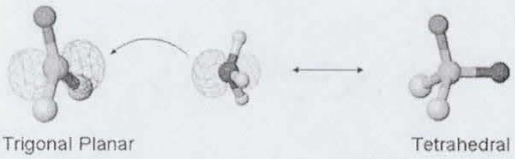


Fig. 1 Neutral, trigonal-planar boronic acid forms dative bonds with nucleophiles to generate an anionic, tetrahedral structure.

bonds with nucleophiles in enzyme active sites providing additional binding affinity. These dative bonds can provide greater stability than the non-covalent, hydrophobic interactions typical of most pharmaceuticals, yet they are also reversible, unlike the covalent bonds generated by suicide inhibitors. The attractive features offered by boron have not gone unnoticed by the pharmaceutical industry and the use of boron in pharmaceuticals has been expanding, with one boron-containing compound on the market, Velcade®, and several in clinical trials, which have been reviewed elsewhere.[4–6] Even so, because there are very few known natural products containing boron and very little experience in developing boron-containing therapeutics,[4] there are continuing concerns over its chemistry, pharmacology, and toxicology. Fortunately, these concerns are being dispelled as more boron-containing compounds make their way through clinical trials.

2. Boron in nature

Boron commonly exists in nature as boric acid and is an essential plant nutrient. It is found in food such as fruits, vegetables and nuts, and we consume 3–7 mg of boron daily in our diet. With a plentiful supply of boron available to cells, it's not surprising that natural products containing boron and functional roles for boron are currently being discovered. These discoveries are recent so it's likely that many more roles for boron remain to be uncovered. The first natural product found to contain boron was boromycin,[7] a polyether macrolide antibiotic active against Gram-positive bacteria (Fig. 2). The macrolide has a high affinity for boric acid in its hydrated form. Boromycin targets the cytoplasmic membrane of Gram-positive bacteria and causes a release of potassium ions.[8] Recently, boromycin was shown to have anti-HIV activity.[9]

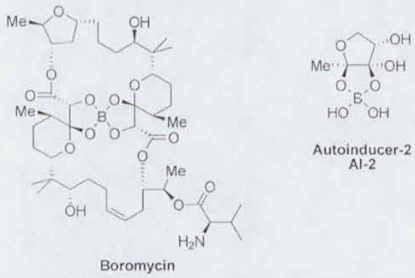


Fig. 2 Natural products containing boron. Boromycin, an antibiotic against Gram-positive bacteria, and AI-2, a bacterial autoinducer involved in quorum sensing.

Another boron-containing natural product was reported in 2002 when boron was found to be part of the natural product involved in quorum sensing.[10] Quorum sensing is a bacterial intercellular communication process that coordinates gene expression across cells to initiate events such as bioluminescence, biofilm formation, production of virulence factors, *etc*. This cross-talk occurs when bacteria release molecules, called autoinducers, into their environment. The boron containing autoinducer AI-2, a hydrated boric acid complex with a tetrahydroxy-dihydrofuran (Fig. 2), was identified as a ligand in an X-ray structure of the autosensor protein LuxP.[10] Regulation of quorum sensing as an approach to antimicrobial therapy was the subject of a recent review.[11]

Outside of natural products, the functional role of boron in plants has become more understood in recent years with research focused on observing phenotypic changes in response to boron deficiency. This has revealed that boron in the cell wall acts to bind polysaccharides. There is also evidence for boron in the maintenance of plasma membranes and metabolic pathways (reviewed in ref. 12). The mechanism of action of boron in these processes remains unknown; however, under the conditions of boron deprivation, it has been reported that reactive oxygen species accumulate, leading to oxidative damage.[13] The role of boron in animals and plants has been recently reviewed.[14]

3. Boron in therapeutics

Boric acid has a lethal dose (LD_{50}) similar to regular table salt, therefore boron is not an intrinsically toxic atom. Recognising boron's potential therapeutic index, the use of boron in therapeutics has increased in recent years. This expanded use of boron coincides with the expansion of organoboron chemistry that occurred over the last decade, for example, the Suzuki–Miyaura cross-coupling (ref. 15, reviewed in ref. 16 and 17).

Boronic acids have been used as electrophiles targeting active site residues of various enzymes including the proteasome (by Velcade®),[18] arginase,[19] serine proteases (including β-lactamase,[20] hepatitis C virus (HCV) protease,[21] and thrombin),[22] and others (Fig. 3).[23–26] Boron heterocycles have also been studied including diazaborines,[27] which inhibit the enzyme fatty acid biosynthesis I (FABI) through formation of an adduct with the cofactor nicotinamide adenine dinucleotide (NAD), although at the time of the original research, the target was unknown and structure–activity relationship studies were performed in the absence of this knowledge. More comprehensive reviews of boron-inhibitors have been published.[4–6,28,29]

4. Oxaboroles, chemistry and mechanism

The simplest oxaborole was first synthesized and characterized by Torssell[30] and was found to possess a high hydrolytic stability.[31] Historically, little attention has been paid to this remarkable class of boronic acids[32] as evidenced by the fact that almost half of the publications and patents mentioning oxaborole have been published since 2005. This new-found interest is likely the result of the exceptional sugar-binding properties of oxaborole under physiological conditions as

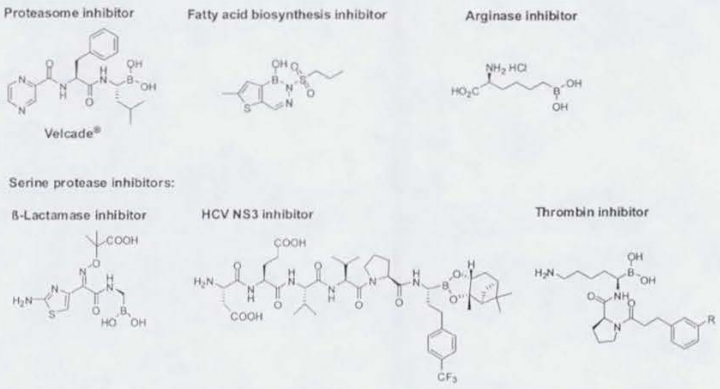


Fig. 3 Examples of boron-containing therapeutics, including Velcade®, the first FDA approved boron-containing therapeutic.

reported by Hall *et al.*[33,34] In addition, oxaboroles have recently been developed as clinical candidates, including AN2690 for the fungal infection onychomycosis[35] and AN2728 for the autoimmune disease psoriasis (Fig. 4).[36] AN2690 targets fungal leucyl tRNA synthetase (LeuRS)[37] and AN2728 targets the human phosphodiesterase-4 (PDE-4). This review will focus on AN2690 and the mechanism of action of the oxaborole against LeuRS.

The physical properties of oxaboroles differ from simple aryl-boronic acids mainly in their pK_a. Since boron has an empty p-orbital, electron withdrawing and inducing groups do not have such a great influence on the pK_a as they would on the electronics of a full p-orbital of nitrogen. However, cyclizing the boron into an oxaborole ring reduces the pK_a by 1–2 pK_a units (Fig. 5). This is believed to be due to the ring strain generated by a five-membered oxaborole ring when the boron atom is in the neutral, trigonal-planar form. The boron–oxygen bond is shorter than a carbon–carbon bond and this is the source of ring strain. This strain is relieved when the boron atom becomes hydroxylated to form the negatively

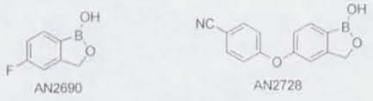


Fig. 4 Oxaborole compounds in clinical development.

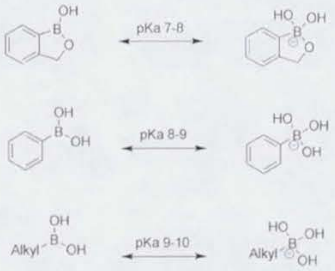


Fig. 5 pK_a of oxaborole compared to aromatic and aliphatic boronic acids.

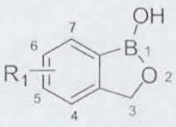
charged, tetrahedral species, thus reducing the bond angle from 120° to 109°. The overall effect is a lowering of the pK_a from ~9 for the corresponding phenyl boronic acid to approximately physiological pH. The boron in oxaboroles will therefore exist in equilibrium between its neutral acid (non-hydroxylated) and charged base (hydroxylated) forms with an approximately equal population of both at physiological pH. In contrast, the corresponding phenyl boronic acid will be mostly in its trigonal planar (non-hydroxylated) form with only ~1% in its tetrahedral form.

The study of aryl-boronic acids, such as phenyl boronic acid, reversibly binding diols has been studied for over 50 years with the first report by Lorand and Edwards.[38] Since then, they have been widely used as carbohydrate sensors.[29,39,40] Early physical studies of these reactions found that boronic ester formation was favored at high pH,[38,41–44] where the predominant (by a factor of at least 10^3–10^4) kinetically reactive species was the boronate anion.[45–47] Thus, it has been traditionally thought that adduct formation proceeded through the boronate anion almost exclusively.[44] However, a new consensus has emerged from more recent studies that demonstrate that the neutral, trigonal planar boronic acid species is the reactive species in solution.[48–51] Therefore, when the new paradigm is considered within the context of adduct formation at physiologically relevant pH, the implication is that oxaboroles can more readily form dative bonds than can the corresponding phenyl boronic acids.

AN2690 was the result of a medicinal chemistry program[52] with the objective to develop a small, water-soluble antifungal agent that could penetrate human finger- and toe-nail plates to treat onychomycosis both within the nail plate and in the underlying nail bed. Observed structure–activity relationships (SAR) were very restricted, allowing only small halogen substituents on the 5-position while all other substituents greatly reduced activity. The antifungal activity of AN2690 is broad, targeting dermatology pathogens *Trichophyton rubrum* and *Trichophyton mentagrophytes* as well as respiratory and invasive pathogens including *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* (Table 1).

The AN2690 program was conducted without knowledge of the target, LeuRS. The program used a purely phenotypic

Table 1 Minimum Inhibitory Concentration (MIC, $\mu g\ mL^{-1}$) of oxaboroles against common fungal pathogens. Structure–activity relationships shows the 5-fluorobenzoxaborole as the most active[52]

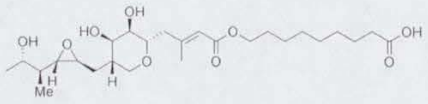


R1	*T. rubrum*	*T. mentagrophytes*	*C. albicans*	*C. neoformans*	*A. fumigatus*
4-F	16	16	64	32	32
5-F	1	1	0.5	0.25	0.25
6-F	16	32	16	32	8
7-F	16	16	32	32	4
5-Cl	1	2	1	2	1
5-Me	8	4	2	8	2
5-CF_3	8	8	16	16	8
5-CN	16	16	8	8	16
5-OMe	64	32	>64	>64	>64

approach, observing antifungal activity while screening for lack of cytotoxicity against mammalian cells. The mechanism of action was determined through reverse genetics and found to be a tRNA synthetase.[52] These enzymes are involved in protein biosynthesis and are responsible for attaching amino acids to the terminus of their corresponding tRNA molecules. The aminoacyl charged tRNA products are then used by the ribosome as substrates for protein synthesis. The ribosome matches the corresponding tRNA to the mRNA sequence.

There are 20 aminoacyl tRNA synthetases (RS's), one for each of the amino acids. It is imperative that the aminoacyl tRNA synthetase attaches the correct amino acid to the terminus of the correct tRNA since errors can lead to nonsense proteins. The ribosome does not proof-read the amino acid *versus* the anticodon of the tRNA and will indiscriminately insert the amino acid on the terminus of the tRNA onto the protein being synthesized. In order to maintain fidelity, 7 out of 20 aminoacyl tRNA synthetases have two domains, a synthetic domain and an editing domain, each with a unique active site. The synthetic active site charges the amino acid onto the tRNA and the editing active site hydrolyzes mis-charged tRNA's.

The tRNA synthetases belong to one of the two classes, Class I and Class II, depending on their structure. Class I RS's aminoacylate the 2′-hydroxyl group of the ribose ring of the terminal adenosine of tRNA whereas Class II RS's aminoacylate the 3′-hydroxyl group, with the exception of phenylalanine tRNA synthetase (PheRS).[53] Their central cellular function marks aminoacyl tRNA synthetases for therapeutic purposes and most activity in this area has revolved around inhibitors for anti-infective therapy, which has been reviewed.[54] To date, only one aminoacyl tRNA synthetase inhibitor, mupirocin, has been launched onto the market, although there are several promising candidates currently in clinical trials (Fig. 6). Mupirocin is a naturally occurring antibiotic, originally isolated from *Pseudomonas fluorescens*, and targets Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). Mupirocin works by inhibiting the synthetic active site of bacterial isoleucyl tRNA synthetase (IleRS), thus stopping the charging of Ile to $tRNA^{\bullet ILE}$ and shutting down protein synthesis. The success of



Mupirocin

Fig. 6 Mupirocin, the only marketed inhibitor of an aminoacyl tRNA synthetase (isoleucyl tRNA synthetase).

mupirocin has validated aminoacyl tRNA synthetases as an anti-infective target.

LeuRS is responsible for charging leucine onto $tRNA^{LEU}$ (Fig. 7). It is a member of the Class I tRNA synthetases and contains both synthesis and editing domains with active sites separated by approximately 30 Å. The synthetic active site of LeuRS displays high selectivity for leucine over 17 other amino acids. However, it is less able to select for leucine over valine or isoleucine and mis-incorporates these amino acids at a rate high enough such that nature has retained an editing domain to proof-read the amino acid charged onto $tRNA^{LEU}$. The editing domain will hydrolyze any aminoacyl-tRNA ester bond that fits into the active site pocket. This pocket has been designed to exclude leucine–tRNA, which cannot access the editing active site and is therefore released by the enzyme. By contrast, mischarged Val– and Ile–$tRNA^{LEU}$ fits into the editing active site pocket allowing for their hydrolysis. Thus the synthetic and editing active sites work together to select for leucine over the 19 other amino acids and maintain fidelity of protein synthesis.[55]

Microbiological and biochemical studies on the mechanism of AN2690 show that this compound specifically targets the editing domain of LeuRS.[37] It is non-competitive with leucine and ATP but does require tRNA for inhibition. It is a slow-tight binding inhibitor and, once the LeuRS–tRNA-inhibitor complex is formed, it is quite stable with a half-life of over 5 hours. Further structure–activity relationship studies confirmed that boron was essential for its activity as carbon analogs proved to be inactive. It was also demonstrated that the five-membered oxaborole ring was required for activity since neither the six-membered oxaborin nor corresponding acyclic boronic acids were active.

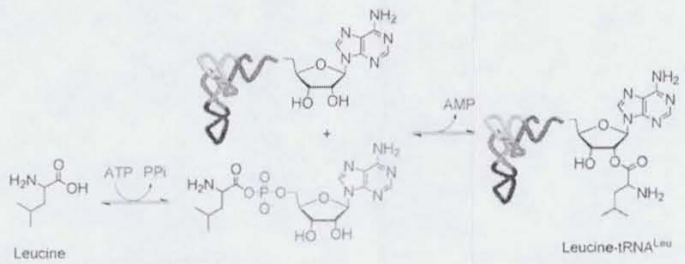


Fig. 7 Reaction catalyzed by leucyl tRNA synthetase is a two-step process. First, leucine reacts with ATP to form an activated leucyl-AMP ester with the loss of pyrophosphate, then a transesterification process relocates the leucine onto the terminal adenosine of $tRNA^{LEU}$ with the loss of AMP. The product, leucyl-$tRNA^{LEU}$, is then used by the ribosome for protein synthesis.

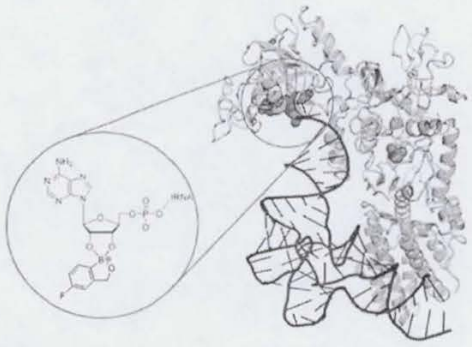


Fig. 8 X-Ray crystallography shows boron in a spiro complex with the *cis*-diols of the terminal adenosine of $tRNA^{LEU}$.

X-Ray crystallographic studies revealed that AN2690 forms a spiro adduct, tetrahedral about boron, with the terminal adenosine of the corresponding $tRNA^{LEU}$ in the editing active site[37] (Fig. 8). The boron has lost its original hydroxyl group and formed two new bonds with the 2′- and 3′-*cis* diols of the ribose. This complex locks the tRNA in the editing active site, preventing it from either leaving the enzyme or moving to the synthetic active site, where it would be aminoacylated. This adduct also blocks other tRNA's from binding to the enzyme thus rendering it inactive. This novel mode of inhibition has been termed the oxaborole tRNA trapping, or OBORT, mechanism

A striking feature of AN2690 is that it is a small molecule, having a molecular weight of just 152 a.m.u. This is typically considered too small to possess any significant binding affinity and indeed, many fragment-based libraries have members with molecular weights in excess of AN2690. Therefore, based on experience with fragment libraries, one would anticipate that AN2690 could sample many binding sites without expecting it to have any significant pharmacological potency. However, as can be seen with its mechanism of action, AN2690 is able to react with the terminus of $tRNA^{LEU}$ in the editing active site and in doing so increases its molecular weight approximately 170-fold (Fig. 8). This adduct occupies the entire tRNA binding site and the editing active site pocket, resulting in a very large inhibitor with a very high affinity for LeuRS.

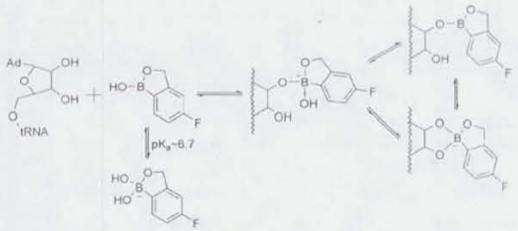


Fig. 9 Proposed reaction mechanism of AN2690 with $tRNA^{LEU}$ resulting in the spiro-product inhibitor of LeuRS.

The mechanism of action of AN2690, and its reaction with tRNA specifically, raises questions of indiscriminate reactivity and selectivity. Even though these complexes form spontaneously in neutral aqueous solution, they can exchange rapidly and have very weak association constants. In the simple case of oxaborole and D-ribose, the K_a is on the order of 4 M^{-1} at pH 6 (Tomsho and Benkovic, unpublished results). An important feature to note is that this AN2690–$tRNA^{LEU}$ complex was only observed in the editing active site and has not been observed in the synthetic active site. This fact leads us to speculate that the complex is only accumulated within the editing active site, where the small, hydrophobic pocket acts to stabilize and protect the complex from hydrolysis. These complexes hydrolyze rapidly in water and we believe that the environmental conditions of the LeuRS editing active site limit access of water to boron, thereby stabilizing the complex. The reason these complexes are not found elsewhere, whether in the synthetic active site of LeuRS or on some other tRNA synthetase, might be due to their accessibility to water and subsequent hydrolysis.

The current hypothesis for the chemical reaction mechanism follows (Fig. 9). In solution at physiological pH, AN2690 exists in an acid–base equilibrium, rapidly interconverting between its anionic, hydroxylated tetrahedral form and its neutral, trigonal-planar form. When the latter form is held in close enough proximity to the *cis*-diols of the tRNA, one of the ribose hydroxyl groups can add to the empty p-orbital to give the first intermediate borate ester. This step is likely to be rapidly reversible. The next step could go one of the two ways: elimination–addition or substitution. In the elimination–addition pathway, the boron hydroxyl group is lost as water

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