Trends in Parasitology

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Review

Pass the boron: benzoxaboroles as antiparasite drugs

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The development of new drug modalities has been facilitated recently by the introduction of boron as a component of organic compounds, and specifically within a benzoxaborale scaffold. This has enabled exploration of new chemical space and the development of effective compounds targeting a broad range of morbidities, including infections by protozoa, fungi, worms, and bacteria. Most notable is the recent demonstration of a single oral dose cure using acoziborole against African trypanosomiasis. Common and species-/structure-specific interactions between benzoxaboroles and parasite species have emerged and provide vital insights into the mechanisms of cidality, as well as potential challenges in terms of resistance and/or side effects. Here, we discuss the literature specific to benzoxaborole studies in parasitic protists and consider unanswered questions concerning this important new drug class.

Highlights

The benzoxaboroles are a new drug cohort.

Significant promise has been found for these compounds against many distinct pathologies.

Infectious diseases are a significant target of benzoxaboroles.

A small number of molecular targets appear to underpin mode of action.

CPSF3 and LeuRS are the major targets.

Benzoxaboroles – a new drug class to combat infectious disease

Campaigns against diseases caused by protozoan parasites have received significant good news in recent years. These advances include highly successful public health efforts to control, and nearly eliminate, human African trypanosomiasis, development of more effective vaccines against malaria[i], and several new drugs being licensed or entering late-phase clinical trials [1,2], albeit that cases of malaria have risen recently and case numbers of leishmaniasis remain stubbornly unchanged in parts of the world [3,4]. Amongst new drugs under consideration are the **benzoxaboroles** (see Glossary), a compound group that shows considerable promise as drugs against a range of parasites, including *Plasmodium*, *Toxoplasma*, and multiple trypanosomatids (Figure 1), which we discuss here.

Boron (atomic symbol B, atomic number 5, and atomic weight 10.81 $\pm$ 0.02), as an elemental crystal, is a metalloid and the first element of the boron group. Boron is comparatively rare as an element but more common as a mineral, for example, borax ($Na_2H_{20}B_4O_{17}$). Organoboron chemistry is complex, with boron incorporated into a wide variety of compounds. Bortezomib, a dipeptide boronic acid that inhibits the mammalian 26S proteasome [5], was approved by the Food and Drug Administration (FDA) in 2003 for treating multiple myeloma in the USA, and was the first boron-containing small molecule therapeutic to enter the clinic. Of specific relevance here are the benzoxaboroles, consisting of a five-membered oxaborole ring, a boronic acid heterocycle, fused to a benzene ring. The unique size and electron configuration of boron compared to carbon, nitrogen, and oxygen – the more common atomic constituents of such heterocyclic compounds – leads to distinct bond lengths and electron distribution within boron-heterocyclics, with unique potential structures for interactions with biological molecules and principally enzymes, and in particular both stability and activity as Lewis acids. Further, the trigonal planar geometry of the boron atom, common to the neutral form of boronic acids, induces a strain in the context of the oxaborole ring that is relieved upon water addition or formation of diol adducts, which explains the ability of benzoxaboroles to efficiently bind glycans at neutral pH (for further reading see [6]).

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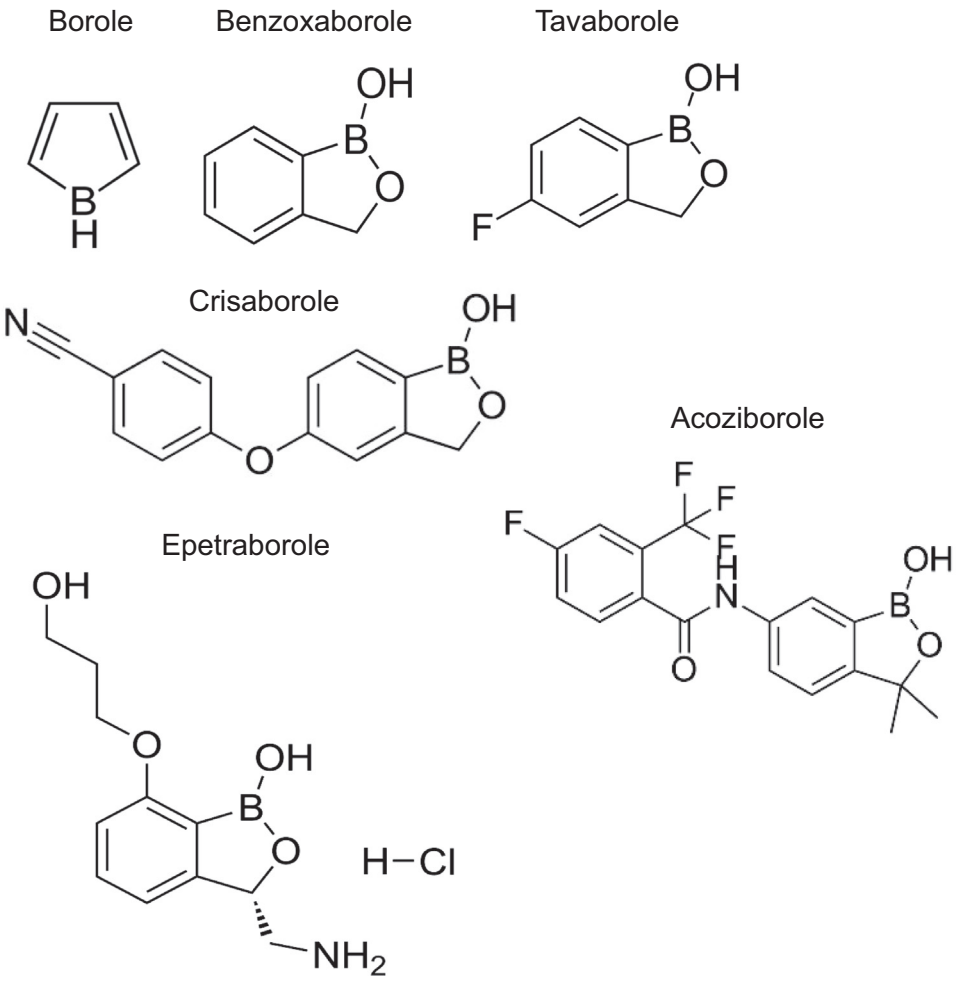

Figure 1. Examples of benzoxaborole and other organoboron compounds. Top left: Borole, the simplest organoboron compound. Top center: benzoxaborole, the scaffold behind the series of compounds discussed in this article. Top right: tavaborole, an effective antifungal. The fluorine adds polarity to improve solubility. Middle left: crisaborole, a PDE-4 inhibitor used in treatment of atopic dermatitis [26,27]. Middle right: acoziborole, a cleavage and polyadenylation specificity factor 3 (CPSF3) inhibitor and licensed for treatment of African trypanosomiasis [2]. Lower left: epetraborole, which is a leucyl-tRNA synthetase (LeuRS) inhibitor and effective against mycobacteria [55].

Benzoxaboroles were initially synthesized in 1957 [7], but only emerged as important medicinal chemistry agents about a half-century later, when Anacor Pharmaceuticals LLC, using an organo-boron based chemistry platform to create novel anti-infectives, discovered a new class of antifungal drug, 5-fluorobenzoxaborole, **Tavaborole** (trade name Kerydin, AN2690, B1(C2=C(CO1)C=C(C=C2)F)O) [8]. More recently, in 2016, the company was acquired by Pfizer driven by the invention of the non-steroidal anti-inflammatory drug crisaborole (trade name Eucrisa, AN2728), reviewed by the FDA at the time, for topical treatment for atopic dermatitis [9]. Anacor, facilitating wide access to boron-based chemistry and a diverse library of benzoxaboroles for use beyond market-oriented research [10,11], has left a legacy of a new drug class yielding several highly promising antibacterial and antiparasitic drugs and now exists as AN2, essentially a phoenix company that includes a number of former Anacor staff. Here, we summarize the

Glossary

Acoziborole: a member of the benzoxaboroles that has been developed for activity against African trypanosomes. The compound is capable of cure with a single oral dose, and of both stage 1 and stage 2 disease. This feature potentially ameliorates the requirement for lumbar puncture, as classically this invasive procedure is required for diagnosis of trypanosome invasion of the central nervous system.
Benzoxaborole: the eight-carbon bicyclic core structure that contains the boron atom. Otherwise, a collective name for the class of compounds that contain this core structure.
Boron: a metallic element with atomic number 5, a constituent of the benzoxaborole core. Differences between boron and carbon and the impact on structure and other properties are important for providing new modality.
Cleavage and polyadenylation specificity factor 3 (CPSF3): an endonuclease that is the catalytic component of the CPSF complex, which itself consists of eight or more subunits depending on species. CPSF is responsible for recognizing the polyadenylation signal and hence catalyzing mRNA maturation. CPSF3 is a common eukaryotic target of benzoxaboroles.
Drug target: the biomolecule, typically a protein, that is bound by, and inhibited by, the drug, leading to – in the case of infectives – suppression or elimination of the infectious agent. In many cases the drug target is unclear or is complex, but for benzoxaboroles a limited cohort of targets have been identified which suggest a level of specificity that is comparatively simple.
Leucyl-tRNA synthetase (LeuRS): a multi-domain enzyme responsible for charging the leucyl-tRNA with leucine.
Pre-prodrug: a form of the active compound that is pharmacologically inactive due to substituents that block its action. Conversion to the active form requires metabolism to convert the compound into a pharmacologically active drug. In the case of a pre-prodrug, two conversions are required.
Resistance: refers to the reduced effect of a drug, usually due to mutation of the drug's target, an enzyme that processes a prodrug, or other activities that otherwise increase sensitivity.

impressive development of benzoxaborole therapeutics and current understanding of their complex mechanisms of action with a focus on antiparasitic applications.

Tavaborole: marketed as Kerydin, it is an antifungal medication for the treatment of onychomycosis, fungal infections of the nail. The first benzoxaborole to be approved by the Food and Drug Administration (FDA) in the USA.

Antiparasitic benzoxaboroles

Drugs used to treat sleeping sickness have historically been limited in their efficacy due to challenges with administration, toxicity, **resistance**, or limited activity against the meningoencephalitic stage 2 disease due to insufficient central nervous system penetration [12]. There has been great progress in recent years, however, and several new anti-trypanosomal drugs have entered clinical trials [13,14]. In the case of sleeping sickness, both fexinidazole [1] and **acoziborole** (SCYX-7158/AN5568, CC2(C)OB(O)c3c2ccc(c3)NC(=O)c1ccc(F)cc1C(F)(F)F) [2,10,15] have successfully completed advanced Phase 2b/3 clinical trials. Acoziborole was progressed through preclinical and clinical development by Anacor, Scynexis, and the Drugs for Neglected Diseases initiative (DND*i*), based in the main on phenotypic screening strategies that considered a wide library of structurally diverse benzoxaborole derivatives. After an 18-month follow-up, acoziborole was determined to be safe and >95% effective in sleeping sickness patients when administered as a single oral dose (three 320 mg tablets). An extraordinary long half-life of around 40 days [2] helps to explain the single-dose efficacy of the new drug. Acoziborole now presents excellent new treatment options for Stage 1 and Stage 2 disease without the need for hospitalization, or painful and hazardous lumbar puncture for diagnosis. More recently, and as part of the ongoing ACOZI-KIDS trial, nine 11- to 14-year-olds have been effectively treated with 640 mg of acoziborole with no relapses observed, suggesting that acoziborole may soon be used in children as young as 1 year[ii].

Additional benzoxaborole compounds are now in trials against nagana in cattle [16], and are under development for the treatment of Chagas' disease [17], leishmaniasis [18,19], malaria and cryptosporidiosis [20–22]. Benzoxaboroles also display antiviral, antibacterial, and antifungal activity [23].

Molecular targets of benzoxaboroles

Many drugs, including anti-infectives, are used effectively without identification of their specific molecular **drug target(s)**. In such cases, however, challenges can arise for improving safety, efficacy and in monitoring and tackling resistance, if it arises, or in developing rational combination therapies, that is, targeting more than one cellular activity to reduce the risk of mutation leading to resistance. The most frequent benzoxaborole targets are **leucyl-tRNA synthetase** (**LeuRS**) and **cleavage and polyadenylation specificity factor 3** (**CPSF3**) (Figure 2), but selective activity was also identified amongst benzoxaboroles against additional targets (summarized in Figure 3): benzoxaborole and diazaborine derivatives inhibit the *Mycobacterium tuberculosis* enoyl-acyl carrier protein reductase InhA [24]. Bacterial targets are the *Pseudomonas aeruginosa* penicillin-binding protein [25], while bicyclic boronates inhibit bacterial β-lactamases [23]. Lastly, the 4-cyanophenyl benzoxaborole derivative crisaborole acts as a human phosphodiesterase 4 inhibitor [26,27].

The benzoxaborole, tavaborole, approved by the FDA following development for use against toenail fungal onychomycosis, inhibits fungal LeuRS by the formation of a covalent adduct with AMP [28], occupying the same site as post-transfer editing substrates and trapping the leucil-tRNA in the editing site (Figure 2). The same mechanism was exploited for the development of antibacterial benzoxaboroles [29,30] where this feature is particularly valuable as LeuRS constitutes a new target and hence avoids cross resistance with existing antibacterials, specifically common antibiotic resistance mechanisms associated with β-lactamases. Ganfeborole, which targets *M. tuberculosis* LeuRS, emerged as a candidate for the treatment of tuberculosis and, very recently, entered Phase 2a clinical trials [31,32].

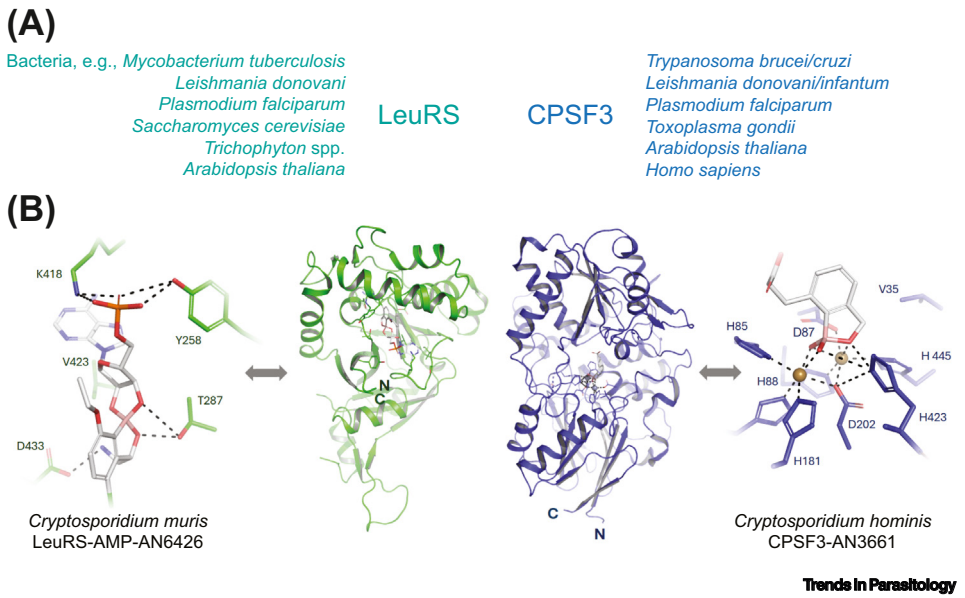

Figure 2. Major targets of benzoxaboroles. (A) Both leucyl-tRNA synthetase (LeuRS) and cleavage and polyadenylation specificity factor 3 (CPSF3) (teal and blue respectively) are highly conserved, albeit that CPSF3 is restricted to eukaryotes. Significantly, both targets have been identified in several organisms for distinct benzoxaboroles, suggesting that a small alteration in structure is sufficient to favor one target over the other. (B) The crystal structures of *Cryptosporidium hominis* CPSF3 (residues 1–482; pdb ID 6Q55) in complex with AN3661 [21] and the *Cryptosporidium muris* LeuRS editing domain with bound AN6426–AMP adduct (residues 254–541; pdb ID 5FOM) [33] are shown in blue and green cartoon representation, respectively. The respective ligand environments are in stick representation (boron is in pink) and the two zinc ions of the CPSF3 metallo-β-lactamase domain, participating in CPSF3–AN3661 binding, are indicated as spheres in sand, respectively. Hydrogen-bonds are drawn as dashed lines; only direct side-chain interactions with the respective inhibitor and via the zinc ions are depicted for clarity.

LeuRS was also identified as a target for benzoxaboroles that display activity against *Plasmodium falciparum* and efficacy against *Plasmodium* parasites in mouse models [20], as well as benzoxaboroles that display activity against *Toxoplasma* and *Cryptosporidium* [33]. Crystal structures of the LeuRS editing domains in complex with the respective benzoxaborole are available for various organisms [28–30,33,34], (Figure 2B). The mode of inhibition by the benzoxaborole adenosine adduct appears to be conserved between prokaryotic and eukaryotic LeuRS targets [35].

A wide range of other benzoxaboroles target CPSF3 in *Plasmodium* [22], *Toxoplasma* [36,37], *Trypanosoma brucei* [38–40], *Trypanosoma cruzi* [17,41], and *Leishmania infantum* and *L. donovani* [18,19]. CPSF3 is an RNA endonuclease and the key catalytic component of a eukaryotic-specific conserved large protein complex required for pre-mRNA processing and maturation. Thus, CPSF3 is indicated as an important, promising, and pharmacologically validated drug target in multiple parasites.

As acoziborole has advanced through clinical trials, and AN11736 was in veterinary trials against nagana, the mechanism of killing the parasites remained a mystery. Genome-scale overexpression screens in *T. brucei* were used to identify CPSF3 as a target of these drugs and three additional benzoxaboroles [38]. CPSF3 overexpression also rendered *T. brucei* less sensitive to other benzoxaboroles that rapidly inhibited mRNA *trans*-splicing [39,40]. Indeed, CPSF3 was subsequently shown to be a target of benzoxaboroles that are effective at killing the trypanosomatids that cause Chagas' disease [17], and leishmaniasis [18,19] – with efficacy demonstrated in a trial in naturally infected rhesus macaques for the former parasite. Significantly, in *T. brucei,* CPSF3 is rapidly degraded in benzoxaborole-treated cells, together with two additional

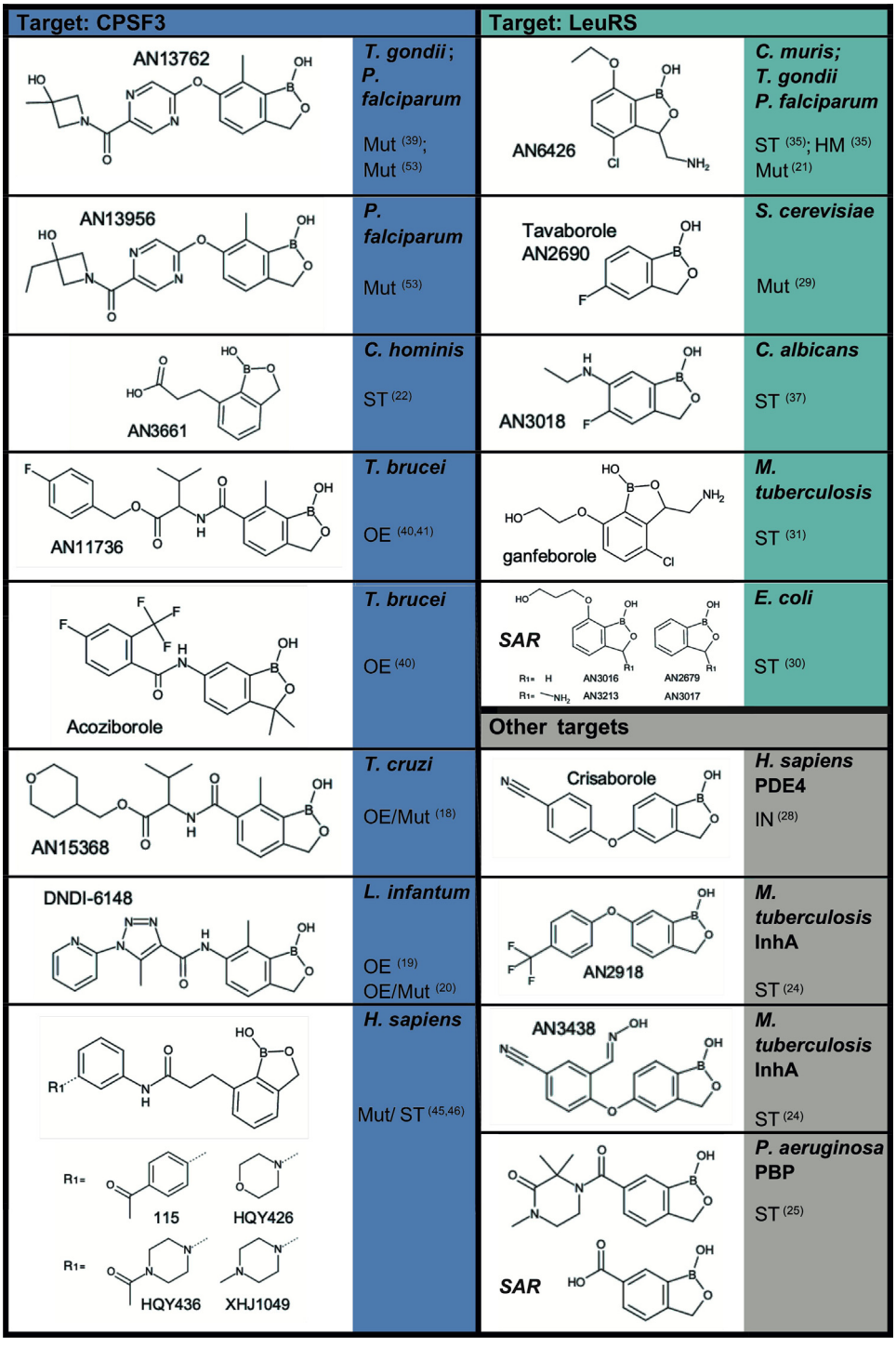

Figure 3. Cellular targets described for benzoxaborole derivatives. Summary of benzoxaboroles with experimental evidence (OE, target overexpression; Mut, target mutation, ST, experimental costructure; IN, *in vitro* inhibition; HM, homology model) for inhibition of CPSF3 (left), leucyl-tRNA synthetase (LeuRS) (top right) and other targets bottom right

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components of the mRNA maturation complex, CPSF100 and symplekin [42]. This augmented turnover is connected to SUMOylation, as RNAi-based knockdown screens link SUMO and components of the SUMOylation pathway to drug resistance, and for multiple distinct benzoxaborole structures, suggesting that SUMO-associated CPSF3 turnover may be central to benzoxaborole sensitivity in trypanosomes and possibly additional organisms [42]. Further, there is also a connection between differentiation/stress transcriptional changes and SUMOylation which may reflect the consequences of a blockade to mRNA processing [43].

CPSF3 is a conserved component of the eukaryotic mRNA processing machinery, and benzoxaboroles that target this critical component in the gene expression pathway are also under development for applications in oncology [44,45]. Here, inhibition of human CPSF3 endonuclease activity by benzoxaboroles, demonstrated to interfere with transcriptional termination, is exploited to selectively target tumors that are especially reliant on high transcriptional activity. Notably, increased transcriptional readthrough was also reported as a consequence of benzoxaborole AN3661 treatment in *Arabidopsis thaliana*, when used as a tool to elucidate CPSF3 functions in plants [46].

Co-crystal structures with benzoxaboroles have been obtained for *Cryptosporidium* [21] and human CPSF3 [45], revealing that the drug binds at the active site (Figure 2B), concordant with models for *T. brucei* CPSF3/benzoxaborole interactions [38]. Several benzoxaborole resistance-associated CPSF3 mutations have also been described (see later), providing further insight into this selective mechanism for inhibition of mRNA processing. Thus, benzoxaboroles targeting CPSF3 have emerged as potential therapies for the treatment of sleeping sickness, nagana, Chagas' disease, leishmaniasis, malaria, toxoplasmosis, cryptosporidiosis, and cancer.

Although CPSF3 is conserved across eukaryotes [47], divergence between CPSF3 orthologs clearly allows for selective targeting of pathogens in humans, primates, cattle, and mouse models. The interface between the metallo-β-lactamase and β-CASP domains of CPSF3 form the active site, where two critical Fe^{2+}, Zn^{2+}, or Mn^{2+} ions are coordinated by conserved His and Asp residues [48]. Gene editing revealed a CPSF3 $N^{232}H$ mutation linked to acoziborole resistance in *T. brucei* [38,49] and in *T. cruzi* [17]. Mutations at the equivalent $Y^{328}H/C$ position in *Toxoplasma* or $Y^{207}H$ in human cells [45] were also linked to benzoxaborole resistance, as were several further mutations in *Plasmodium* CPSF3 [22] and *Toxoplasma* CPSF3 [36,37]. These mutations, together with mutations in SUMO modification pathway proteins, present a theoretical risk of resistance in a therapeutic setting, but it also remains possible that any resistant cells will display fitness defects *in vivo* given the central and essential roles of both CPSF3 and SUMOylation. Notably, silencing SUMO leads to a greater drug resistance phenotype than either overexpression of CPSF3 or mutation of the enzyme to prevent acoziborole accessing the catalytic site, which suggests that inhibition of CPSF3 and its accelerated turnover both contribute towards acoziborole toxicity by the synergistic removal of mRNA processing capability [42]. It remains unknown if SUMOylation is a conserved component of benzoxaborole sensitivity in human cells.

Benzoxaboroles as (pre)prodrugs

Many drugs are delivered as prodrugs, frequently in a form where the active core is substituted with additional moieties that are enzymatically removed prior to acting at the target. Pathogen-

(PDE4, phosphodiesterase 4; enoyl-ACP reductase, InhA; PBP, penicillin binding protein). *SAR* denotes that further benzoxaborole derivatives for the respective target are described in the source publication. *C. albicans, Candida albicans*; *C. hominis, Cryptosporidium hominis*; *C. muris, Cryptosporidium muris*; *E. coli, Escherichia coli*; *H. sapiens, Homo sapiens*; *L. infantum, Leishmania infantum*; *M. tuberculosis, Mycobacterium tuberculosis*; *P. aeruginosa, Pseudomonas aeruginosa*; *P. falciparum, Plasmodium falciparum*; *S. cerevisiae, Saccharomyces cerevisiae*; *T. brucei, Trypanosoma brucei*; *T. cruzi, Trypanosoma cruzi*; *T. gondii, Toxoplasma gondii*. See also [17–21,24,25,27–30,33,34,37–39,44,45,52].

directed enzymes that metabolize prodrugs can substantially improve the therapeutic index by producing an active species within the pathogen cell and by concentrating that toxic species if it is inefficiently transported out of the pathogen.

Several distinct prodrug activation mechanisms have been described for benzoxaboroles (Figure 4). For example, a class of 4-aminomethylphenoxy-benzoxaboroles were identified as prodrugs that are activated in two steps, involving a host serum amine oxidase and then a *T. brucei* long-chain aldehyde dehydrogenase, which leads to trapping of the active adduct within the parasite [50]. Further, studies on a veterinary-active benzoxaborole revealed a distinct prodrug activation mechanism involving a serine carboxypeptidase in both *T. brucei* and in *T. congolense* [51]. A similar carboxypeptidase prodrug activation mechanism also operates in *T. cruzi* [17]. Unfortunately, both of the parasite enzymes involved in prodrug activation in these examples are dispensable for viability, at least in *in vitro* culture, raising the potential for the acquisition of resistance, but also providing genetic targets for monitoring resistance should the need arise. An esterase-based prodrug activation was observed for the antiplasmodial benzoxaborole AN13762 (Figure 4) by Prodrug Activation and Resistance Esterase (PARE) [52], that was previously found to facilitate pepstatin ester prodrug activation, and several AN13762 derivatives have been developed with improved *in vivo* properties [41]. Significantly, resistance genes were

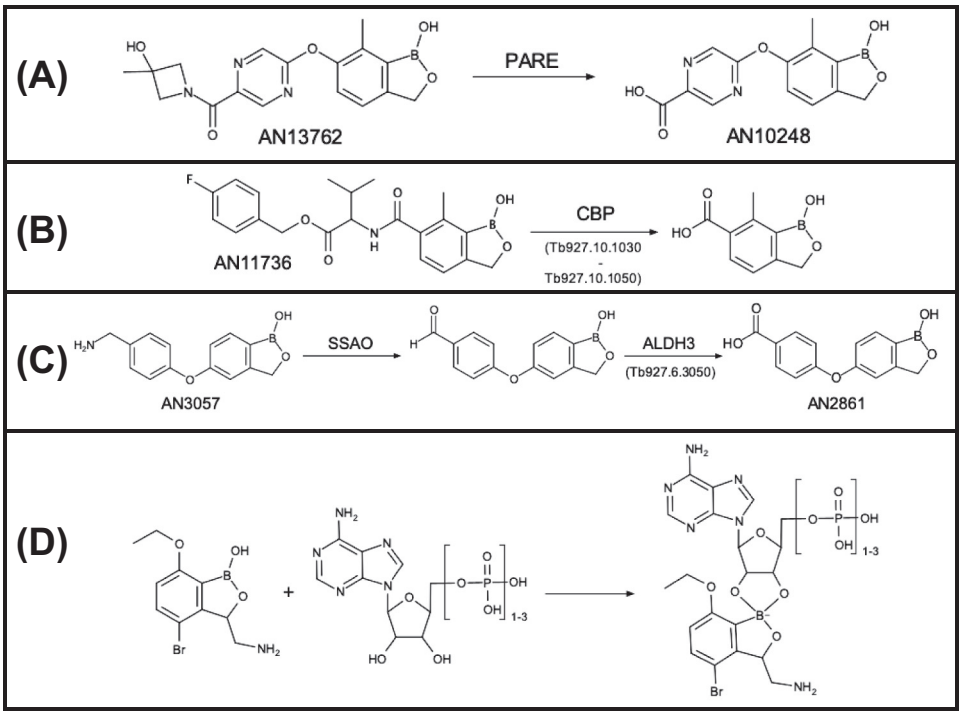

Figure 4. Prodrug activation pathways observed for benzoxaborole derivatives. (A) The Prodrug Activation and Resistance Esterase (PARE) processes the candidate antimalarial AN13762 into the active form AN10248 [52]. (B) *Trypanosoma brucei* encodes serine carboxypeptidases (CBPs) that cleave the ester bond in AN11736 and derivatives [51]. A similar prodrug activation pathway has been described in *Trypanosoma cruzi* for AN15368 [17], which carries the same ester linker (Figure 3). (C) Aminomethyl-benzoxaboroles were shown to be activated by a **pre-prodrug** activation cascade involving a semicarbazide-sensitive amine oxidase (SSAO) of the mammalian host and a *T. brucei* aldehyde dehydrogenase [50]. (D) Adduct formation with the adenosine ribose moiety, an established key mechanism of leucyl-tRNA synthetase (LeuRS) inhibition by tavaborole [28], was recently proposed as enzyme-free prodrug activation [35]: benzoxaboroles undergo a spontaneous cyclization reaction with ATP, AMP, or the terminal tRNA adenosine portion.

identified that are part of the SUMOylation pathway, suggesting that SUMOylation has a more general role in benzoxaborole sensitivity, and in common with trypanosomes [52]. LeuRS-inhibiting benzoxaboroles can also act as prodrugs as inhibition relies on adduct formation through cyclization with the adenosine ribose moiety of ATP, AMP, or the terminal tRNA adenosine portion [35].

Concluding remarks

Benzoxaboroles are having major positive impacts in clinical practice, and we now have a clearer view as to how these drugs work, why they are selective, and, in the context of infectious diseases, how resistance may emerge in the field. With two major common targets across eukaryotes, LeuRS and CPSF3, together with characterization of complex prodrug activation pathways, it may seem that we are close to a full understanding of benzoxaborole modes of action. However, this is likely premature (see Outstanding questions). The importance of prodrug pathways adds complexity to the manner in which benzoxaboroles can approach their target, and the breadth of cellular responses such as SUMOylation remain to be fully elucidated. As SUMO is associated with stress, understanding this in the host is potentially critical in fully assessing the toxicity or long-term impacts from benzoxaborole treatments.

The World Health Organization has proposed a target to interrupt disease transmission for African trypanosomiasis by 2030. An ambitious goal, but given the excellent new treatment options presented by fexinidazole and acoziborole, alongside affordable rapid diagnostic tests [53], this aim appears to be an increasingly achievable and sustainable goal; progress so far in reducing case numbers is well ahead of WHO targets. It will be important to ensure that these recently emerged diagnostics and drugs can be rapidly deployed when needed, as historical instances of sleeping sickness escalation are well documented and may be repeated [54]. The difference this time around is that excellent tools are in place that, if deployed effectively, should greatly improve prospects for management and control. The potential for these compounds to also furnish new therapeutics for additional kinetoplastid parasites as well as the Apicomplexan pathogens that present major public health challenges is considerable. Comparative low cost and stability of many benzoxaboroles offers significant advantages to deployment of this potent class of compounds.

Outstanding questions

Are apparent differences in responses to benzoxaboroles between animal and trypanosome cells due to distinct transcriptional mechanisms?

Is increased SUMOylation a consequence of CPSF3 inhibition?

Does benzoxaborole treatment in mammals evoke a SUMOylation response, and what are the consequences?

Validated targets for the benzoxaborole pharmacophore are surprisingly diverse; is there potential for polypharmacology?

Can pro-drug mechanisms be exploited to improve benzoxaborole efficacy, or will they prove problematic in terms of potential resistance?

Will benzoxaborole therapies emerge for other trypanosomal and apicomplexan parasites?

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Declaration of interests

The authors declare no competing interests.

Resources

[i]www.who.int/observatories/global-observatory-on-health-research-and-development/monitoring/who-review-of-malaria-vaccine-clinical-development

[ii]https://dndi.org/global-networks/acozi-kids/

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