A potent antimalarial benzoxaborole targets a *Plasmodium falciparum* cleavage and polyadenylation specificity factor homologue

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Benzoxaboroles are effective against bacterial, fungal and protozoan pathogens. We report potent activity of the benzoxaborole AN3661 against *Plasmodium falciparum* laboratory-adapted strains (mean IC_{50} 32 nM), Ugandan field isolates (mean ex vivo IC_{50} 64 nM), and murine *P. berghei* and *P. falciparum* infections (day 4 ED_{90} 0.34 and 0.57 mg kg^{-1}, respectively). Multiple *P. falciparum* lines selected in vitro for resistance to AN3661 harboured point mutations in *pfcpsf3*, which encodes a homologue of mammalian cleavage and polyadenylation specificity factor subunit 3 (CPSF-73 or CPSF3). CRISPR-Cas9-mediated introduction of *pfcpsf3* mutations into parental lines recapitulated AN3661 resistance. PfCPSF3 homology models placed these mutations in the active site, where AN3661 is predicted to bind. Transcripts for three trophozoite-expressed genes were lost in AN3661-treated trophozoites, which was not observed in parasites selected or engineered for AN3661 resistance. Our results identify the pre-mRNA processing factor PfCPSF3 as a promising antimalarial drug target.

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Despite efforts to curb transmission, malaria remains an important global infectious disease, accounting for an estimated 212 million cases and 429,000 deaths worldwide in 2015 (ref. 1). Challenges to the control and elimination of malaria include widespread drug resistance in *Plasmodium falciparum*, the most virulent human malaria parasite. As older regimens are limited by resistance, artemisinin-based combination therapies have been adopted as the standard of care for the treatment of uncomplicated falciparum malaria. However, resistance to artemisinins has emerged in Southeast Asia2,3, and resistance has been seen to most artemisinin-based combination therapy partner drugs4. The development of new antimalarial agents with novel modes of action is urgently needed.

Benzoxaboroles are boron-containing compounds that have shown potent activity against a wide range of infectious pathogens, including bacteria5,6, fungi7 and protozoans8–12. The highly electrophilic nature of the boron component of these compounds leads to interaction with a variety of protein targets via reversible covalent bonds13,14, with identified targets including leucyl-tRNA synthetase (LeuRS)6,7, phosphodiesterase 4 (PDE4)15,16 and β-lactamase17. Tavaborole, the first benzoxaborole to receive FDA approval, is an inhibitor of fungal LeuRS that is used to treat onychomycosis18. Crisaborole, a PDE4 inhibitor, has completed phase 3 clinical trials for atopic dermatitis.

This manuscript reports the antimalarial profile and mechanism of action of another benzoxaborole, AN3661, which was identified from a screen of a benzoxaborole library against cultured *P. falciparum* asexual blood stage parasites19,20. AN3661 demonstrated potent antimalarial activity, and genetic and biochemical studies identified its target as a homologue of mammalian cleavage and polyadenylation specificity factor (CPSF) subunit 3.

Results

**In vitro characterization of AN3661 activity.** An *in vitro* screen of a benzoxaborole library19,20 against the multidrug-resistant *P. falciparum* W2 strain identified the 7-[(2'-carboxylic acid) ethyl] benzoxaborole AN3661 (Fig. 1a). AN3661 was active at nanomolar concentrations against *P. falciparum* laboratory strains known to be sensitive (3D7) or resistant (W2, Dd2, K1, HB3, FCR3 and TM90C2B) to standard antimalarial drugs, and it was similarly active in *ex vivo* studies of fresh Ugandan field isolates (Fig. 1b and Supplementary Table 1). There was no difference in AN3661 potency between drug-sensitive or -resistant parasite strains (Supplementary Table 2). AN3661 showed minimal cytotoxicity against mammalian cell lines, with the CC50 60.5 μM against Jurkat cells, and all other CC50 values greater than the highest concentrations tested (25 μM or above; Supplementary Table 3).

When cultured with the *P. falciparum* 3D7 strain, AN3661 reduced the number of viable parasites in a time- and dose-dependent manner (Supplementary Fig. 1a). Parasites exposed to 10 × IC50 concentrations of AN3661 reduced parasitemia with a rate of killing similar to that of pyrimethamine (Supplementary Fig. 1b). However, exposure to 30 × IC50 increased the rate of parasitemia.

Figure 1 | Activity of AN3661 against *P. falciparum* laboratory-adapted strains and field isolates. (a) Chemical structure of AN3661. (b) Susceptibility of laboratory strains and field isolates to AN3661. Bar graphs represent mean ± s.e.m. IC50 values. Each point in the dot plot represents one field isolate; the horizontal bar indicates the mean value.

Figure 2 | AN3661 efficacy in murine malaria models (a) AN3661 efficacy in *P. berghei*-infected mice. Mice (4–5 animals for each concentration tested) were treated orally with the indicated dosages of AN3661, chloroquine or vehicle daily for 4 days. Infections were monitored daily by Giemsa-stained blood smears, and mice were euthanized when parasitemias exceeded 50%. (b) AN3661 efficacy in *P. falciparum*-infected mice. Mice with ∼40% circulating human erythrocytes were intravenously infected with 2 × 107 *P. falciparum*-infected erythrocytes on day 0, and AN3661 was then administered by oral gavage at the indicated dosages for 4 consecutive days (arrows). Parasitemia was measured by flow cytometry daily until day 7. Mean parasitemias for three mice in each group are shown. Error bars represent s.e.m.
AN3661 activity in murine malaria models. When administered orally for 4 days to *P. berghei*-infected mice, beginning on the day of infection, AN3661 rapidly controlled parasitemias, with a 90% effective dose (ED90) 4 days after initiation of treatment of 0.34 mg kg\(^{-1}\). Daily dosages of 50 mg kg\(^{-1}\) and 100 mg kg\(^{-1}\) extended survival compared to untreated controls, and mice treated with 200 mg kg\(^{-1}\) per day demonstrated long-term cures (Fig. 2a). We also investigated compound efficacy against *P. falciparum* in a murine model using NODscidIL-2R\(^{null}\) mice engrafted with human erythrocytes and infected with *P. falciparum*\(^{21}\). When AN3661 was administered orally for 4 days, beginning on the third day of infection, the ED90 4 days after initiation of treatment was 0.57 mg kg\(^{-1}\) (Fig. 2b).

Stage specificity and morphology of treated parasites. Synchronized W2 strain parasites were exposed to AN3661 for 8-h intervals across the asexual blood stage cycle, and subsequent ring-stage parasitemias were compared to those of untreated controls. Inhibitory effects of AN3661 were greatest in early to middle trophozoite-stage parasites, as found with the reference drug chloroquine (Fig. 3a). Parasites treated with 370 nM AN3661, beginning at the early ring stage, appeared morphologically normal through late rings, but could not progress beyond the trophozoite stage, with the appearance of shrunken, pyknotic parasites (Fig. 3b).

**In vitro selection of parasites resistant to AN3661.** We quantified the ease of *in vitro* selection of resistance to AN3661 by subjecting \(10^8\)-\(10^9\) Dd2 strain parasites to 60 nM (2 × IC\(_{90}\)) AN3661. Regrowth was seen in two of three cultures with initial inocula of \(10^8\) parasites at days 45 and 56, one of three cultures with initial inocula of \(10^7\) parasites at day 23, and three of three cultures with initial inocula of \(10^8\) parasites, all on day 19 (Supplementary Table 4). These rates of resistance selection were similar to those observed for atovaquone.

To determine the target of AN3661, we employed two separate methods of generating resistant mutants. In the first, we pressured W2 and Dd2 strains of *P. falciparum* with increasing concentrations of AN3661 (37 nM–5 μM). In the second, a single concentration of AN3661 (170 nM) was applied to Dd2 parasites (Fig. 4a). Using the first method, for the W2 strain, 5 steps of continuous selection over 11 months led to a 200-fold decrease in sensitivity to AN3661 (Fig. 4b). After selection, W2 parasite lines with medium to high-level resistance (W2-R3, W2-R4 and W2-R5; with mean IC\(_{90}\) 0.7, 6.2 and 15.3 μM, respectively, compared to 31 nM for the W2 parental line) were cultured without drug pressure to test the stability of the resistance phenotype. In each case, revertant parasites were obtained that showed decreased resistance to AN3661 after culture without the compound. For W2-R3 parasites, the revertant (W2-R3\(_{rev}\)) had roughly the sensitivity of the parental W2 strain, but W2-R4 and W2-R5 revertants retained micromolar IC\(_{90}\) values indicating only partial loss of resistance. Results with Dd2 were similar to those with W2, with AN3661-resistant parasites obtained in 4 separate selections (mean IC\(_{90}\) 0.2–0.9 μM, compared to 22 nM for the Dd2 parental line, Fig. 4c). Selection for resistance to AN3661 was accompanied by only modest changes in sensitivity to the standard antimalarial chloroquine (Supplementary Table 5).

Whole-genome sequence analysis of AN3661-resistant parasites. Whole-genome sequence analysis and confirmatory dideoxy sequencing of W2 and Dd2 parental and AN3661-resistant clonal lines revealed both copy number variations (CNVs) and non-synonymous single-nucleotide polymorphisms (SNPs) in selected lines, compared to their parental lines (Supplementary Table 6). Importantly, every AN3661-resistant parasite line harboured mutations in *pfpsf3* (PF3D7_1438500). This gene encodes a
homologue of subunit 3 of the CPSF complex, which has been well characterized in various eukaryotes. In humans, this subunit has endonuclease activity and is referred to as CPSF-73, based on its molecular weight of 73 kDa. The predicted molecular weight of the \( P. falciparum \) homologue PfCPSF3 is 101 kDa. Dd2 parasites resistant to AN3661 had single PfCPSF3 mutations (T406I, Y408S, T409A or D470N; Fig. 4c). W2 parasites resistant to AN3661 demonstrated a more complex resistance trait: low-level resistant parasites had wild-type PfCPSF3, but high-level resistance was conferred by a single mutation, D470N, or a double mutation, H36Y/D470N.

We also applied whole-genome sequence analysis to revertant parasite lines that had been cultured without AN3661 after resistance selection. W2-R3 rev, which showed wild-type sensitivity to AN3661 (mean IC\(_{50}\) 23.4 nM), had lost the PfCPSF3 D470N mutation, but W2-R4 rev retained the double PfCPSF3 H36Y/D470N mutation. Low-level resistance to AN3661 observed in W2-R1 and W2-R2 was accompanied by amplification in the region of chromosome 5 (position 946,695–971,095) that encompasses \( pfmdr1 \) (PF3D7_0523000; Fig. 4b). This gene encodes an ABC transporter for which SNPs and CNV have been linked to altered sensitivity to a number of antimalarials.

\textit{pfcpfs3} gene editing recapitulated AN3661 resistance. To test the hypothesis that mutations in PfCPSF3 constitute the primary determinant of \( P. falciparum \) resistance to AN3661, we engineered Dd2 parasites to harbour either the T406I or Y408S mutations observed in our resistant lines. These experiments utilized a CRISPR-Cas9-based method of gene editing (Fig. 5a and Supplementary Table 7). Transfections yielded three parasite lines carrying the T406I mutation and two carrying the Y408S mutation. While most transfections included selection with 170 nM AN3661, importantly transfectant C4 (PfCPSF3 Y408S) did not, and rather was obtained using only WR99210 and blastidicin selection of the editing plasmids. All transfectants demonstrated 100% editing efficiency at the appropriate sites in
the genome, including silent mutations at the single-guide RNA (sgRNA)-directed Cas9 binding sites (Fig. 5b). In the engineered parasites, the PfCPSF3 T406I and Y408S mutations conferred a 12–50-fold decrease in AN3661 sensitivity, similar to changes in sensitivity in parasites that acquired these mutations after in vitro resistance selection (Fig. 5c). Selected and transfected parasites

**Figure 5 | CRISPR-Cas9-mediated pfcpss3 editing.** (a) Dd2 parental (WT) parasites were transfected with a dual-plasmid strategy in which one plasmid encoded the sgRNA, Cas9 nuclease and a dhfr selectable marker, and the other plasmid encoded a donor sequence containing either the T406I or Y408S PfCPSF3 mutations (in red), as well as synonymous mutations in the sgRNA-binding region. (b) Electropherograms showing unmodified Dd2 and genome-edited parasites. Grey boxes highlight nucleotides that differ from WT parasites. PfCPSF3 single-letter amino acid substitutions are indicated in red. (c) Susceptibility to AN3661 of the parental line (Dd2 WT), parasites selected in vitro (Dd2-Ra, Dd2-Rb and Dd2-Rc), and genetically modified lines (Dd2 transf. F1, F3, E1; Dd2 transf. C4, C4 cl.4, 7; Dd2 transf. D3; Dd2 transf. D3). Assay details are in Supplementary Table 1. Bar graphs represent mean ± s.e.m. IC50 values. The black bar denotes PICPSF3 WT, blue bars T406I, red bars Y408S and green bar T409A. Significance was determined using a two-tailed unpaired t-test, comparing transfected parasites with the parental Dd2 strain. ****P < 0.0001.
with the Y408S mutation were consistently less sensitive to AN3661 compared to parasites with the T406I mutation. These results confirm a primary role for pfcpsf3 mutations in conferring resistance to AN3661.

Resistance mutations cluster around the enzyme active site.

Amino acid sequence alignment showed that PfcPSF3 is a close homologue of human CPSF-73 (Fig. 6a), with 61% similarity and 39% identity (calculations did not include gaps in the alignment due to plasmodial insertions). A crystal structure of human CPSF-73 (PDB code: 2I7T) revealed that the enzyme belongs to the zinc-dependent metallo-β-lactamase (MBL) family, with MBL and β-CASP domains, and an active site at the interface of these domains containing two zinc ions24. Since this crystal structure appears to be in an inactive conformation, with the β-CASP domain occluding the active site, we generated a new model based on a crystal structure of the messenger RNA (mRNA) processing ribonuclease TTHA0252 from *Thermus thermophiles* HB8 (PDB code: 3IEM), an enzyme with 47% similarity and 27% identity with PfcPSF3. This structure was in an active conformation complexed with an RNA analogue in the active site. In the *T. thermophiles*-based PfcPSF3 model the β-CASP domain does not occlude the bi-metal active site, which is accessible to substrates and inhibitors (Fig. 6b). In both models, the identified PfcPSF3 resistance mutations T406I, Y408S, T409A and D470N clustered around the active site (Fig. 6b). This finding suggests functional relevance of the resistance mutations and supports the genetic evidence implicating PfcPSF3 as the antimalarial target of AN3661.

Molecular docking suggests oxaborole binding to PfcPSF3.

Considering the conformational complexity of the PfcPSF3 active site and limitations of forcefield parameters involving the boron atom, we docked AN3661 by matching important pharmacophore sites in the PfcPSF3 homology model based on *T. thermophiles*. The negatively charged tetrahedral oxaborole group from AN3661 was placed at the phosphate position at the cleavage site, interacting with the two catalytic zinc ions. Analogous interaction with metals has been seen in crystal structures of oxaboroles binding to PDE4 (ref. 16) and MBL NDM-1 (YR Freund, personal communication), with the oxaborole group acting as a transition state mimim and a phosphate mimic, respectively. The energy-minimized model of AN3661 at the active site of PfcPSF3 revealed the terminal carboxylate of AN3661 occupying an adjacent phosphate-binding site opposite R290 and Y252, forming a salt bridge and a hydrogen bond, respectively (Fig. 6b). Of the residues selected in AN3661-resistant parasites, Y408 and T409 were located at the entrance of the binding pocket in the β-CASP domain, in close proximity to the docked inhibitor, and D470 was positioned opposite Y408 in the RNA-metabolizing MBL domain (Fig. 6b). T406 was adjacent to R290, which directly interacts with the carboxylate from AN3661. It is interesting to note that in the *Toxoplasma gondii* CPSF-73 homologue, a Y328C (corresponding to Y252 in PfcPSF3) resistance mutation was selected under AN3661 pressure25, further supporting the hypothesis that this residue is important in AN3661 binding.

AN3661 inhibited the stability of *P. falciparum* transcripts.

The mammalian CPSF complex is required for the processing of newly synthesized transcripts (pre-mRNAs) to mature mRNA22,26,27. The cleavage of the 3′ end of pre-mRNA and subsequent addition of a poly(A) tail is necessary for mRNA stability and for export of mature mRNA from the nucleus to the cytosol, where it acts as a template for translation26. The observed similarity between PfcPSF3 and human CPSF-73 led us to predict that inhibition of PfcPSF3 would impact the stability of parasite mRNA transcripts. As AN3661-treated parasites failed to progress through the trophozoite stage (Fig. 3a), we suspected that a defect in mRNA stability would be most apparent in trophozoites. We thus examined transcripts for three trophozoite-expressed genes, falcipain-2 (FP2), 1-cys peroxiredoxin and purine nucleoside phosphorylase (PNP)28–30 in parasites cultured in the presence or absence of AN3661. Following a 4-h incubation with either AN3661 or the transcription initiation inhibitor actinomycin D, FP2, 1-cys peroxiredoxin and PNP transcripts were markedly reduced or undetectable in parental W2 and Dd2 parasites (Fig. 7a,e; Supplementary Figs 2 and 3). In contrast, parasites harbouring the PfcPSF3 mutations D470N, H367Y/D470N, T406I or Y408S selected with AN3661 or introduced with the CRISPR-Cas9 system demonstrated stable transcripts despite exposure to AN3661 (Fig. 7c,d,f–i). In all parasites examined, transcripts were unstable in the presence of actinomycin D, yet were unaffected by 4-h exposures to aminoglycosides or chloroquine antimalarials that inhibit unrelated modes of action (Fig. 7). Taken together, our data provide compelling evidence that AN3661 selectively inhibits PfcPSF3, leading to altered parasite mRNA processing and stability.

Discussion

Screening of a benzoxaborole library against cultured *P. falciparum* identified AN3661 as a potent antimalarial lead compound. Subsequent studies showed AN3661 to be active at low nanomolar concentrations against multiple *P. falciparum* strains that vary in their susceptibility to standard antimalarials and to be highly effective when administered orally to treat *P. berghei* and *P. falciparum* infections in mice. Multiple independently selected parasites with resistance to AN3661 contained mutations in the *P. falciparum* homologue of the endonuclease component of the CPSF complex that has been shown in other organisms to be responsible for pre-mRNA cleavage and polyadenylation24. Introduction of two of these PfcPSF3 mutations into wild-type parasites recapitulated resistance to AN3661, and modelling suggested that the mutations were in the PfcPSF3 active site at amino acids interacting with AN3661. Biochemical studies showed that treatment with AN3661 led to alterations in the stability of messages encoding three trophozoite proteins in wild-type, but not AN3661-resistant parasites. Collectively, these results support the conclusion that the primary target of AN3661 in *P. falciparum* is PfcPSF3.

CPSF is a multi-protein complex present in eukaryotic cells that is essential for processing of pre-mRNA to mRNA via cleavage and polyadenylation at the 3′-end of the pre-mRNA. In mammals, the coordinated activity of the CPSF complex and other proteins is initiated by interaction of CPSF-100 with an AAUAAA sequence located 10–30 bases upstream of the cleavage site22,24,26,31,32. Upon interaction with the AAUAAA sequence, the pre-mRNA undergoes site-specific endonucleolytic cleavage by CPSF-73 to generate the mRNA end, which then serves as a primer for synthesis of the poly(A) tail26. The endonuclease CPSF-73 is a member of the zinc-dependent MBL family, and contains 5 canonical MBL signature sequence motifs of histidine and aspartate residues, as well as a β-CASP domain24,26,33. The active site of CPSF-73 contains two zinc ions and is located at the interface of the MBL and β-CASP domains. PfcPSF3 is a homologue of mammalian CPSF-73 with conservation of the 5 MBL and β-CASP motifs.

Homology modelling of the structure of PfcPSF3 offered further support for its role as the target of AN3661. The
Figure 6 | AN3661 resistance mutations reside in the MBL and β-CASP domains of PICSPF3. (a) Conserved domains predicted by NCBI for PICPSF3 and its homologues in humans (Hs, gi:18044212), Toxoplasma gondii (Tg, KFG54681.1) and T. thermophilus (Tt, TTHA0252). R, RNA-metabolizing MBL domain. Amino acid numbers are shown for each protein. Asterisks denote amino acids predicted to bind the two zinc atoms in the MBL domain. Amino acids in red were mutated after drug selection. (b) Model of AN3661 (cyan) at the active site of PICPSF3 (MBL domain, mauve; β-CASP domain, green, RNA-metabolizing MBL domain, orange), built based on the crystal structure of T. thermophilus TTHA0252 (PDB code: 3IEM). Residues D470, Y408, T409 and T406, which were mutated after selection by AN3661, are shown as blue stick models. The negatively charged benzoxaborole group interacts extensively with the two catalytic zinc ions (grey), and the carboxylate side chain interacts with R290 and Y252, forming a salt bridge and hydrogen bond, respectively.
mutations seen in *P. falciparum* after selection for decreased sensitivity to AN3661 were in residues predicted to be in the enzyme’s active site and in proximity to AN3661. Remarkably, in parallel studies with *T. gondii*, a related apicomplexan parasite, selection of resistance to AN3661 was accompanied by mutations in the predicted active site of the *T. gondii* homologue of CPSF3 (ref. 25). Our docking model of AN3661 at the PfCPSF3 binding site is consistent with the structure-activity relationships established for this series of compounds as antimalarial agents. First, the oxaborole group is essential for activity, as replacement of a carbon atom for the boron resulted in an inactive compound20. Second, the carboxylic acid group is important for activity, although it can be replaced by other acidic groups such as a tetrazole19. The negatively charged tetrahedral oxaborole group serves as a unique ion chelator engaging the two zinc ions at the active site. The fact that AN3661 could be modelled into the bi-metal active site of PfCPSF3 is consistent with findings for other benzooxaboroles, which have been demonstrated to bind to bi-metal centres. In the active site of PDE4, which contains a zinc and a magnesium ion, the boron atom of AN2898 binds to the activated water molecule in the bi-metal centre and mimics the transition state of the substrate cAMP16. In the co-crystal structure of NDM-1 MBL and a benzoxaborole β-lactamase inhibitor, AN4483, the boron atom is coordinated to the bi-zinc centre, similar to the predicted binding conformation for AN3661 in PfCPSF3 (YR Freund, personal communication). These examples demonstrate the potential of the oxaborole group in benzoxaboroles such as AN3661 to act as a metal chelator capable of binding to a variety of enzymes containing bi-metal centres.

In our parasite drug pressuring studies, low-level resistance to AN3661 was associated with increased copy number for *pfmdr1*, which encodes a presumptive ABC transporter 23. Increased *pfmdr1* copy number has previously been associated with decreased sensitivity to the arylaminoalcohols mefloquine, chloroquine and artemisinin. Figure 7 | mRNA stability in the presence of AN3661. Northern blots shown are of parasite lines labelled as defined in Figs 3 and 4 (a-i) that were treated for 4 h with DMSO (untreated), actinomycin D, AN3661, artemisinin or chloroquine. RNA was then processed and the transcripts for purine nucleoside phosphorylase (PNP), 1-cys peroxiredoxin (1-CysPxn) and FP2 were detected and imaged as described in ‘Methods’ section. Levels of 28S rRNA were examined as a loading control. The figure shows results from a single experiment. Two additional experiments yielded the same results.
lumefantrine and halofantrine, and increased sensitivity to the 4-aminoquinolines chloroquine and amodiaquine. Alterations in pfmdr1 copy number may impact AN3661 accumulation at its intracellular site of action. However, high-level resistance to AN3661 was not associated with pfmdr1 copy number, suggesting that alterations in PICTFS3 are the dominant determinant for high-grade resistance.

Antimalarial drug discovery presents significant challenges. New antimalarials should meet multiple criteria, including excellent potency, ideally against multiple plasmodial species; oral bioavailability, ideally with cure after a single dose; safety in children and pregnant women; and low cost of production. New antimalarials must be highly selective.

Methods

Activity of compounds against cultured P. falciparum. P. falciparum strains (3D7, W2, Dd2, K1, HB3, FCR3 and TM90C2R; obtained from the Malaria Research and Reference Reagent Resource Center) were cultured by standard methods at 2–3% haematocrit in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen) supplemented with 0.5% Albumax II (GIBCO), 2 mM l-glutamine, 100 mM hypoxanthine, 5 μg ml⁻¹ gentamicin, 28 mM NaHCO₃, and 25 mM HEPES at 37 °C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂, except that for parasite reduction ratio studies (Supplementary Fig. 1) parasites were cultured at 2% haematocrit in RPMI-1640 medium supplemented with 0.5% Albumax II, 2% D-sucrose, 0.3% glutamine and 150 mM hypoxanthine.

In vitro dose-response assays were conducted in one of three ways. In the first, parasites were synchronized by treatment with 5% d-sorbitol and cultured in duplicate 96-well culture plates (200 µl per well) in the presence of serially diluted AN3661 or chloroquine (Sigma-Aldrich). Compound concentrations ranged from 0.056 to 1,000 nM. Control wells contained ≤ 0.2% dimethylsulphoxide (DMSO).

For the second method, cultures were exposed to a range of drug concentrations, with final DMSO concentrations ≤ 0.2%, for 72 h. Live parasites were stained with SYBR Green I (Invitrogen) and 100 mM MitoTracker Deep Red (Invitrogen), quantified by flow cytometry analysis on a BD Accuri C6 and analysed using FlowJo software.

In the third method, parasites were cultured at 2% haematocrit and 0.5% parasitemia were exposed to a range of drugs for 48 h, and parasitemia was then quantified by [³H]-hypoxanthine incorporation. IC₅₀ values were calculated by nonlinear regression using GraphPad Prism 6.0 software.

Ex vivo dose response against P. falciparum field isolates. The activity of AN3661 was tested against fresh P. falciparum isolates using an enzyme-linked immunosorbent assay directed against the histidine-rich protein-2, as previously described. These isolates were collected in 2012 from children with malaria living in Tororo, Uganda, before receipt of drug treatment.

Cellular cytotoxicity assay. Studied mammalian cells (all received from authenticated by, and noted to be Mycoplasma-free by the American Type Culture Collection, see Supplementary Table 3) were seeded into 96-well plates at 2 × 10⁴ cells per well in 100 µl growth medium (RPMI-1640 medium with 10% foetal bovine serum and 2 mM l-glutamine) with 10-fold serial dilutions (0.1 nM–100 µM) of AN3661; control wells had the same concentration of DMSO (0.25%). Plates were incubated at 37 °C in 5% CO₂ for 72 h. MTS (20 µl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) was then added for 4 h, and absorbance was determined at 490 and 690 nm. Inhibition of cell viability was calculated based on the formula:

\[\% \text{ inhibition of cell viability} = 100 - \left(\frac{\text{OD}_{\text{treated}} - \text{OD}_{\text{untreated}}}{\text{OD}_{\text{treated}}}\right) \times 100\]

In vitro parasite reduction ratio. This assay used limiting dilution to quantify the number of parasites that remained viable after various durations of treatment. P. falciparum 3D7 parasites (at 2% haematocrit and 0.5% parasitemia) were incubated with AN3661 for 24, 48, 72, 96 or 120 h. After appropriate time points, the compound was removed by washing, and parasites were serially diluted in 96-well plates and allowed to continue growing after fixing with 2% formaldehyde. The number of viable parasites after various incubation times with compounds was determined after 21 and 28 days by measuring [³H]-hypoxanthine incorporation in threefold serial dilutions of cultures, as previously reported. Human biological samples were sourced ethically, and their research use was in accord with the terms of informed consents.

Activity of AN3661 in murine models of malaria. Protocols for studies of murine P. berghei infection were approved by the University of California, San Francisco Institutional Animal Care and Use Committee. Female Swiss Webster mice (6–8 weeks of age; 18–20 g) were infected intraperitoneally with 6 × 10⁸ P. berghei-infected erythrocytes (passaged from a donor mouse) and then treated, beginning 1 h after inoculation, with AN3661 (in 55% polyethylene glycol 300, 25% propylene glycol, 20% water) or chloroquine (in water) by oral gavage once daily for 4 days. Treatment group assignments were allocated randomly. Investigators were not blinded to treatment groups. Negative controls were treated with vehicle only. Infections were monitored by daily microscopic evaluation of Giemsa-stained blood smears. ED₅₀ values, based on cure comparisons of treated and control animals on the fourth day after the initiation of treatment, were calculated using GraphPad Prism 6.0 software. Mice were euthanized when parasitemias exceeded 50%.

Studies of murine P. falciparum infection were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals. In vivo efficacy against P. falciparum was conducted as previously described. Age-matched female immunodeficient NOD.Cg-Prkd⁻/⁻Il2rg⁻/⁻Il19⁻/⁻Sz/Ta mice (8–10 weeks of age; 22–24 g) supplied by Charles River, L’Arbresle, France, under license of The Jackson Laboratory, Bar Harbor, ME, USA were engrafted with human erythrocytes (Roswell Park Memorial Institute – Carriage Cell Division, Blood Bank in Madrid, Spain) by daily intraperitoneal injection with 1 ml of a 50% haematocrit erythrocyte suspension (RPMI-1640 (Invitrogen), 25 mM HEPES (Sigma), 25% decomplemented AB⁺ human serum (Sigma) and 3.1 mM hypoxanthine (Sigma)). The sample size per experimental group was 3, calculated as the minimum required to detect 50% reduction in parasitemia compared to a vehicle-treated control group, assuming a value for α (confidence level) of 0.05 and a value for β (power) of 0.9. Mice with > 40% circulating human erythrocytes were intravenously infected with 2 × 10⁹ P. falciparum PDS0/300751-infected erythrocytes (day 0). AN3661 was then administered by oral gavage for 4 consecutive days, beginning on day 3 after infection. AN3661 was administered in 1% carboxymethylcellulose 0.1% Tween 80 in water before administration. Treatment group assignments were allocated randomly. Investigators were not blinded to treatment groups. Parasitemia was measured by flow cytometry in samples of peripheral blood stained with the fluorescent nucleic acid dye SYTO-16 (Molecular Probes; S-7578, 5 μM) and anti-murine erythrocyte TER119 monoclonal antibody (Becton Dickinson 553673; 10 μg ml⁻¹) in serial 2 μl blood samples taken every 24 h until assay completion. The ED₅₀ was estimated by fitting a four parameter logistic equation using GraphPad Prism 6.0.

Stage specificity assay. The stage-specific activity of AN3661 and chloroquine was analysed as previously described. Highly synchronous W2 strain P. falciparum (synchronized by treatment with 5% D-sorbitol) were cultured in triplicate wells in 96-well culture plates with 370 nM AN3661 or 1.3 μM chloroquine for 8 h intervals, beginning at the ring stage. Control cultures contained equivalent concentrations of DMSO (<0.2%). At the end of each interval, the cultures were washed three times and resuspended in culture media without drug. After 48 h, when control parasites were at the ring stage, the cultures were treated with AN3661 or vehicle, and uninfected and infected erythrocytes were replaced once a week. For all selections, parasitemia was monitored by microscopy, and recrudescence parasites were cloned by limiting dilution.

Selection of parasites with decreased sensitivity to AN3661. For step-wise selection, triplicate 10 ml cultures containing 6 × 10⁸ asynchronous W2 strain or a clonal population of the Dd2 strain were incubated with step-wise increasing concentrations of AN3661. W2 parasites were subjected to 37 nM AN3661 for 48 h, 75 nM for 43 days, 300 nM for 90 days, 1 μM for 73 days and finally 5 μM for 73 days. Dd2 parasites were subjected to 37 nM AN3661 for 31 days, then 200 nM for 73 days. For single-step resistance selection with Dd2, 2 × 10⁴ parasites in duplicate or 2 × 10⁵ parasites were incubated with 170 nM AN3661. For the first six days, parasitemia was monitored by microscopy and media containing drug was changed daily. Then, media was changed every 2 days, and fresh erythrocytes were replaced once a week. For all selections, parasitemia was monitored by microscopy, and recrudescence parasites were cloned by limiting dilution.
Whole-genome sequencing. To prepare genomic DNA, synchronized *P. falciparum*-infected erythrocytes (100 mL 2% hematocrit and 10% parasitemia) were treated with 0.2% saponin for 5 min on ice to lyse erythrocytes, followed by 3 washes in PBS. Parasite pellets were lysed in 150 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.1% sarkosyl (Sigma-Aldrich) and 200 mg ml⁻¹ proteinase K (QiaGen) overnight at 37°C. The samples were then subjected to extraction with phenol/chloroform/isomyl alcohol (25:24:1) at pH 7.9 (Ambion), treatment with 200 mg ml⁻¹ RNAse A (1 h at 37°C), two additional phenol/chloroform extractions, one chloroform extraction and then ethanol precipitation. All phenol/chloroform extractions were done using light phase lock tubes (5 Prime). Genomic DNA libraries were prepared from 100 ng DNA using the Nextera DNA Sample Prep Kit (Illumina) according to the manufacturer’s instructions, with the exception that the number of cycles was 6 and the bridge amplification step was at 65°C for 6 min. Each library was barcoded with unique sets of two indices from the Nextera Index Kit (Illumina) to allow multiple samples to be run on one flow cell. Next, fragments of 360–560 bp were extracted and collected using Lab Chip XT (Caliper Life Sciences) according to the manufacturer’s instructions. The fragments were amplified by limited-cycle PCR using Kapa HiFi DNA polymerase (Kapa Biosystems) with dNTPs with an 80% AT coding bias (6 cycles of 95°C for 10 s, 58°C for 30 s, 65°C for 6 min). Primers used for both PCR steps are detailed in Supplementary Table 8. Library concentrations were determined with a DNA Bioanalyzer (Agilent), and libraries were pooled at concentrations of 2 nM per well. Library preparations were then completed and sequenced at the UCSC Center for Advanced Technology, following by sequencing on a HiSeq 2000 system (Illumina). Sequence data for each library were aligned to the 3D7 reference genome using Bowtie40, discarding reads with (Illumina). Sequence data for each library were aligned to the 3D7 reference genome using Bowtie40, discarding reads with

Homology modelling of PCPF3 and docking analysis of AN3661. Sequences were from NCBI for human CPSF-73 (gi:18044212), PlasmoDB for PCPF3 (PF3D71438400) and PDB for T. thermophiles TTHA0252 HB8 (PDB code: 3JEM). Sequences were aligned using the NCBI BLAST Clustal Omega method and Maestro Prime (Schrodinger, LLC). Similarity and identify scores were calculated using Maestro, not including alignment gaps due to PCPF3 insertions. Crystal structures of human CPSF-73 (PDB code: 2I7T) and *T. thermophiles* HB8 (PDB code: 3JEM) served as templates to build homology models of PCPF3 using Maestro. The tetrahedral form of AN3661 was generated using the Optimization Program for Liquid Simulations 2005 forcefield and manually docked into the active site of the TTHA0252-derived homology model of PCPF3, mimicking the binding mode of benzoxaborole analogues observed from the crystal structures of *Mbl* NDM-1 (YR Freund, personal communication). The final docking model was obtained by minimizing the complex with a water solvation model and maintaining a fixed protein backbone.

RNA stability assay. Ring stage *P. falciparum* W2 (10–12 h post-invasion) or Dd2 (16–18 h post-invasion) parasites were cultured at 10–15% parasitemia and 2% hematocrit in a volume of 20 mL. Cultures were exposed to 100 nM AN3661, 600 nM chloroquine, 70 nM artemisinin or actinomycin D at either 80 μg ml⁻¹ for W2 or 20 μg ml⁻¹ for Dd2. Control cultures were exposed to DMSO at <0.01% final concentration. After 4 h of drug treatment, parasites were frozen in liquid nitrogen and stored at −80°C. Total RNA was subsequently extracted using the Trizol Plus RNA purification kit (Purelink RNA mini kit, Ambion) according to the manufacturer’s instructions. Briefly, the frozen parasitized RBC pellets were dissolved in pre-warmed Trizol reagent, followed by chloroform extraction. The aqueous phase was mixed with ethanol to obtain a final ethanol concentration of 35% and total RNA was washed, and eluted from the provided spin columns according to the manufacturer’s instructions. For Northern blots, 20 μg of total RNA was separated on 1.3% formaldehyde agarose gel and transferred to a Hybond N+ membrane (Amersham) and baked in a vacuum through capillary action. For hybridization, RNA was detected using DNA probes labelled with biotin-16-DUTP (Roche Diagnostics) (Supplementary Table 8). Northern blot signal was detected using Typhoon Trio imager (Amersham biosciences) and quantified using ImageQuant software.

Data availability. All relevant data are available from the corresponding author upon request.

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