Target Identification of an Antimalarial Oxaborole Identifies AN13762 as an Alternative Chemotype for Targeting CPSF3 in Apicomplexan Parasites

HIGHLIGHTS

- AN13762 is active against T. gondii parasites
- Parasites resistant to AN13762 harbor mutations within TgCPSF3
- Mutations within TgCPSF3 confer resistance to AN13762
- AN13762 offers an alternative for targeting CPSF3 in Toxoplasma and Cryptosporidium
Target Identification of an Antimalarial Oxaborole Identifies AN13762 as an Alternative Chemotype for Targeting CPSF3 in Apicomplexan Parasites

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SUMMARY
Boron-containing compounds represent a promising class of molecules with proven efficacy against a wide range of pathogens, including apicomplexan parasites. Following lead optimization, the benzoxaborole AN13762 was identified as a preclinical candidate against the human malaria parasite, yet the molecular target remained uncertain. Here, we uncovered the parasiticidal mechanisms of AN13762, by combining forward genetics with transcriptome sequencing and computational mutation discovery and using Toxoplasma gondii as a relevant model for Apicomplexa. AN13762 was shown to target TgCPSF3, the catalytic subunit of the pre-mRNA cleavage and polyadenylation complex, as the antipan-apicomplexan benzoxaborole compound, AN3661. However, unique mutations within the TgCPSF3 catalytic site conferring resistance to AN13762 do not confer cross-protection against AN3661, suggesting a divergent resistance mechanism. Finally, in agreement with the high sequence conservation of CPSF3 between Toxoplasma and Cryptosporidium, AN13762 shows oral efficacy in cryptosporidiosis mouse model, a disease for which new drug development is of high priority.

INTRODUCTION
The Apicomplexa phylum contains intracellular single-celled parasites several of which are causative agents of animal and human diseases worldwide raising important public health problems (De Rycker et al., 2018). The group comprises important human pathogens such as Plasmodium, Toxoplasma, and Cryptosporidium responsible for malaria, toxoplasmosis, and cryptosporidiosis, respectively. For many of these diseases current treatments are suboptimal, and there are few or no alternatives available for some. Indeed, the current standard of treatment for Cryptosporidium infections, nitazoxanide, shows limited and immune-dependent effectiveness (Manjunatha et al., 2016). Although the current medication against Toxoplasma is quite effective, it has adverse side effects, particularly in immunocompromised patients, such as pyrimethamine-induced hematological toxicity and sulfonamide-induced skin rash, leukopenia, and thrombocytopenia (Dunay et al., 2018). In the case of malaria, emergence and spread of resistance to artemisinin-based combination therapy, the primary form of treatment, poses a constantly growing threat (De Rycker et al., 2018). Therefore, new classes of small-molecule drugs or drugs with novel modes of action are needed to overcome these limitations.

In an effort to optimize the efficacy of a novel class of boron-containing molecules against malarial parasites, the lead candidate AN13762 was identified in a phenotype-based screening (referred to as compound 46 in Zhang et al., 2017). Evaluation of pharmacokinetics showed that AN13762 has improved potency and metabolic stability, is orally bioavailable, and is equally potent across multidrug-resistant strains of Plasmodium falciparum, demonstrating no cross-resistance and a possible new mechanism of action. AN13762 has not exhibited either significant toxicology or cytotoxicity liabilities at any dose tested, and AN13762 was selected for preclinical development by Medicines for Malaria Venture in 2017 (Zhang et al., 2017). Although previous research on parental scaffold AN3661 identified Cleavage and Polyadenylation Specificity Factor 3 (CPSF3) as the direct target (Palencia et al., 2017; Sonoiki et al., 2017; Swale et al., 2019), a recent study investigating the resistance mechanisms of AN13762 in P. falciparum identified...
multiple components involved in prodrug activation or sumoylation and ubiquitination pathways along with PKCPSF3 suggesting that the latter was not the primary target (Sindhe et al., 2020).

The work described here was undertaken to shed light on the parasiticidal mechanisms of AN13762 using Toxoplasma gondii as a relevant representative of apicomplexan parasites. Here, we present evidence that AN13762 is effective against both T. gondii and Cryptosporidium parvum in vitro at low micromolar concentrations and in vivo in mouse models of toxoplasmosis and cryptosporidiosis, respectively. Using a forward genetic approach based on transcriptome sequencing, we identified its target as CPSF3, a common target of several benzoxaboroles such as AN3661, a compound active against apicomplexan parasites (Palencia et al., 2017; Sonoiki et al., 2017; Swale et al., 2019), or the trypanocidal compounds AN11736 and acoziborole (Wall et al., 2018). Importantly, several point mutations found in T. gondii CPSF3 conferring resistance against AN13762 were not effective against AN3661, suggesting a divergent mode of resistance mechanisms between CPSF3 and benzoxaboroles. Hence this work uncovers the molecular mechanism for the antiparasitic activity of a preclinical antimalarial candidate AN13762 and extends the clinical spectrum of activity of this chemotype to other life-threatening apicomplexan parasites.

RESULTS
AN13762 Is Active against T. gondii In Vitro and In Vivo

To assess the effectiveness of AN13762 against T. gondii parasites, growth of the type I reference RH strain was monitored within human foreskin fibroblasts (HFFs) treated with AN13762; its parental scaffold AN3661 as a positive control (Figure 1A); pyrimethamine, the standard of care for toxoplasmosis; or vehicle (DMSO). Efficient in vitro inhibition of T. gondii growth was repeatedly confirmed, with measured half maximum effective concentration (EC50) of 2.1 μM, which is almost 40 times higher than that of AN3661 (Figures 1B and 1C). Complete and sustained inhibition of growth was observed at 10 μM AN13762 without any adverse effects for the host cells (Figures 1Da and S1).

When AN13762 was administered orally for 7 days to T. gondii-infected mice, beginning on the first day following intraperitoneal injection of parasites, 100% of the animals survived the lethal infection by the highly virulent type I RH strain in contrast to untreated controls (Figures 1E and 1F). Second lethal challenges to the mice that survived the first infection confirmed that the initial 7-day treatment with AN13762 resulted in a protective immune response to subsequent T. gondii infection (Figures 1E and 1F), thus strengthening the biological and pharmacokinetic profile of AN13762 in animal efficacy studies. Altogether, these results indicate that AN13762 is effective against T. gondii both in vitro and in vivo allowing long-term cures in mouse model of acute toxoplasmosis with comparable efficacy to current treatment.

Selection of T. gondii Parasites Resistant to AN13762

In an attempt to shed light on the mechanism of action of AN13762, we performed a forward genetic screen combining chemical mutagenesis to isolate AN13762-resistant parasites and next-generation sequencing analysis to map mutations conferring drug resistance (Figure 2A). Central to our approach, we reasoned that the gene(s) that would be mutated in more than one independently mutagenized resistant clone might be relevant to the drug resistance mechanism and by this means alleviating the notoriously difficult molecular mapping of point mutations induced by mutagens. For this purpose, seven independent ethyl methanesulphonate (EMS) mutagenesis experiments were performed and the resulting mutagenized parasites were selected in the presence of 10 μM AN13762 (Figure 2B), which corresponds to approximately 5-fold the EC50 value. Resistant parasites were obtained from each of the seven mutagenesis experiments, whereas none of the non-mutagenized parasites survived the selection at 10 μM AN13762, attesting once more to the parasiticidal efficacy of this compound (Figure 2B). The resistant parasite lines were then cloned by limited dilution, and we selected a single clone from each mutagenesis experiment (named A1 to G1) for transcriptome sequencing by RNA sequencing (RNA-seq). All the resistant clones were able to grow and formed plaques when grown in the presence of 10 μM AN13762 (Figures 2C-2E and S2). In parallel, the parental strain was analyzed by RNA-seq and used as a reference to identify EMS-induced mutations. We use transcriptome sequencing as most drugs target expressed proteins, with levels of gene expression and mutations being part of the sequencing results.
Figure 1. Activity of AN13762 against Toxoplasma gondii

(A) Chemical structures of benzoxaborole leads AN13762 and AN3661.

(B) Dose-response curves for inhibition of T. gondii growth in vitro in response to increasing concentration of the indicated compounds. Confluent HFF monolayer was infected with tachyzoites of T. gondii RH strain expressing the NanoLuc luciferase (RH Δku80 UPRT::NLuc-P2A-EmGFP). The T. gondii strains used in this study are listed in Table S1. Data are presented as mean ± standard deviation (SD) of at least two independent biological assays, each with 3 technical replicates. Shaded error envelopes depict 95% confidence intervals.

(C) EC50 values of each biological replicate were determined by non-linear regression analysis. EC50 data are presented as mean ± SD from at least 2 independent biological replicates, each with 3 technical replicates.

(D) HFF cells were infected with tachyzoites (RH Δku80 UPRT::NLuc-P2A-EmGFP) and incubated with 10 μM AN13762, 5 μM AN3661, or 0.1% DMSO as control. Cells were fixed 24 h post-infection and then stained with antibodies against the T. gondii inner membrane complex protein GAP45 (magenta). The cytosolic GFP is shown in green. Scale bars, 10 μm. A complete dataset can be found in Figure S1.
Parasites Resistant to AN13762 Harbor Mutations within TgCPSF3

To map the EMS-induced mutations that confer drug resistance, the Illumina sequencing reads were aligned to the ~65-Mb T. gondii GT1 reference genome. The assembled sequences were analyzed to identify single nucleotide variations (SNVs), small insertions, or short deletions using the parental strain as a reference (see Transparent Methods). By focusing on mutations present in coding sequences, we identified a single gene, CPSF3 (Cleavage and Polyadenylation Specific Factor 3, TGGT1_285200), that harbored SNVs leading to amino acid substitutions in each of the seven drug-resistant lines that were not present in the parental strain (Figure 2F and Table 1). CPSF3 encodes a nuclear mRNA-processing endonuclease that functions in pre-mRNA maturation (Ryan, 2004), which has been previously identified as the target of several benzoxaborole compounds active against distantly related pathogens (Lunde et al., 2019; So-noiki et al., 2017; Swale et al., 2019; Wall et al., 2018), including T. gondii (Palencia et al., 2017). Importantly, four different mutations were identified (G456S, E545K, Y328H, and S519C; Figure 2G; Table 1), among which E545K conferred resistance against AN3661 in T. gondii (Palencia et al., 2017). Mutations span from the metallo-β-lactamase domain to the RNA specificity domain of CPSF3 (Figure 2G). Therefore, these data suggest that mutations in CPSF3 were responsible for resistance against AN13762.

Mutations within CPSF3 Confer Resistance to AN13762

To confirm that the CPSF3 mutations were sufficient to confer resistance to AN13762, we reconstructed each of the mutations identified in AN13762-resistant parasites into the sensitive parental wild-type strain using CRISPR/Cas9 system coupled to homology-directed repair for gene editing in T. gondii (Figure 3A) (Palencia et al., 2017). Thus, RHΔku80 parasites were co-transfected with a vector expressing the Cas9 endonuclease and synthetic guide RNA (sgRNA) and the corresponding homologous single-stranded donor oligonucleotides as repair template. After selection with AN13762, emerging resistant parasites were cloned, and DNA sequencing established that the mutations have been correctly inserted at CPSF3 locus (Figures 3B and S3A). Transfections with the Cas9 control vectors alone produced no surviving parasites. In the engineered parasites, we observed that the CPSF3 mutations E545K, G456S, S519C, and Y328H substantially decreased the sensitivity against AN13762 when compared with wild-type parasites (Figures 3C–3E and S3B–S3D). It is noteworthy that Y328H mutation had a significant effect on parasite growth in the absence of drug (Figure 3C, upper panel), suggesting that this mutation might affect basal activity of CPSF3 in tachyzoites, which is in line with CPSF3 being essential to parasite growth (Palencia et al., 2017; Sidik et al., 2016). In addition, the CPSF3-edited parasites harboring the mutations E545K or G456S were also resistant to AN13762 treatment in mice (Figure 1F). Altogether, these data confirm the primary role of CPSF3 mutations in conferring resistance to AN13762 and indicate that AN13762, in a similar fashion to AN3661, targets CPSF3 (Palencia et al., 2017; Swale et al., 2019).

AN13762-Resistant Mutations G456S and S519C Do Not Confer Cross-Resistance to AN3661

We had previously found that mutations in CPSF3 were conferring resistance to another oxaborole compound, AN3661 (Figure 2G, mutations Y328C, Y483N, and E545K, Palencia et al., 2017). To examine whether the AN13762-resistant mutations in CPSF3 confer cross-resistance to AN3661, we assayed AN3661 against reconstructed parasites harboring CPSF3 mutations E545K, G456S, S519C, and Y328H. As expected, the most prevalent mutation E545K that was identified in the mutagenesis experiments conducted against either AN13762 or AN3661 conferred resistance to both compounds (Figures 3C–3E and S3B–S3D). Note that the increase in resistance to AN3661 was more dramatic than for AN13762 (~100- and ~3-fold increase in EC50, respectively; Figure 3D). Very different results were obtained for G456S and S519C mutations, which did not allow parasite growth when exposed to 5 μM AN3661 (Figure 3C). The CPSF3ΔG456S mutation conferred the strongest resistance phenotype to AN13762 with a ~42-fold increase in AN13762 EC50 when compared with wild-type parasites, whereas sensitivity to AN3661 remained unaffected (Figures 3D and 3E). Of note, the latter mutations were not identified in the AN3661 screen, presumably reflecting their inability to protect against AN3661 at 5 μM. Conversely, the Y483N mutation identified in AN3661-resistant parasites conferred cross-resistance to
Similarly, mutations affecting the Y328 residue of CPSF3 decreased sensitivity to both compounds (Figures 3C and 3D). Altogether, these results further confirm the role of CPSF3 mutations in drug resistance and indicate a divergent mode of resistance between AN13762 and AN3661.

Molecular Docking Suggests a Divergent Resistance Mechanism between Oxaboroles

Multiple sequence alignments show a high overall sequence conservation within the metallo-β-lactamase (MBL), Beta-Casp, and RNA specificity domains of CPSF3 within apicomplexan parasites and humans (Figure 4A). One notable difference between the apicomplexan and human enzyme is the presence of an extended loop or “apicomplexan specific insert” whose length varies from 20 to 59 residues. However, conservation of the generated resistant SNVs to AN13762 within T. gondii CPSF3 coding sequence is absolute across species and appears close to the catalytic residues but is never directly involved in the coordination of the catalytic zinc atoms. Next, we visualized the resistance-conferring mutations within the recently obtained structure of Cryptosporidium CPSF3 (ChCPSF3) in co-crystal with AN3661 (pdb id 6Q55) (Swale et al., 2019). With the assumption that AN13762 interacts with a comparable geometry as the AN3661 benzoxaborole group, notably through the boron-driven octahedral coordination of the two catalytic zinc ions, we placed the AN13762 derivative in the same plane as AN3661 (Figure 4B–4D). Through this modeling, we did not generate any clashes with CPSF3, despite the much bigger size of AN13762 (13.4 Å in length against 7 Å for AN3661). When visualizing both AN3661 and AN13762 placement with regard to the resistance-conferring mutations, two important features can be noted. First, most of the mutations found (Y328C/H, E545K, S519C, and Y483N) that rescue parasites from both compounds are not directly observed in contact with the compound-binding site. Instead, the mutated residues are generally placed on loop regions lining the interfacial cavity between the RNA specificity domain and Beta-Casp domain. These resistance-conferring mutations probably act indirectly on the compound activity through either an allosteric mechanism preventing compound binding or by modifying RNA recognition by CPSF3 as these loop regions are believed to regulate RNA access and recognition (Sun et al., 2020). Second, the G456S mutation, which exclusively rescues T. gondii parasites from AN13762, is observed separated to the other resistance-conferring mutations. Because of its close proximity with the AN13762 pyrazinering and methylazetidine (2.2 Å distance), the G456S mutant probably introduces an important steric hindrance to AN13762 binding. AN3661, with a much shorter organic extension, does not come close enough for the mutation to have an effect on its binding and activity. As a result, the G456S mutant remains sensitive to AN3661 (Figure 3E).

AN13762 Is Active against Cryptosporidium In Vitro and In Vivo

The aforementioned data provide evidence that AN13762 targets CPSF3 enzyme. Given that it has been shown that CPSF3 is a bona fide target for inhibiting Cryptosporidium development (Swale et al., 2019),
EMS mutagenesis

Mutagenized parasites

Wild-type parasites

Drug-resistant parasites

Selection and cloning

GTTCACGGA

A

AG

TTCACGGA

A

AGC

TCACGGA

A

AGCG

CACGGA

A

AGCGC

ACGGA

A

AGCGCG

CGGA

A

AGCGCGG

GTTCACGGA

G

AG

TTCACGGA

G

AGC

TCACGGA

G

AGCG

CACGGA

G

AGCGC

ACGGA

G

AGCGCG

CGGA

G

AGCGCGG

RNA-Seq analysis

Recuring gene mutations

Transcriptome sequencing analysis by RNA-Seq

Independent mutagenesis

Cloning of resistant mutants

None

0 mM Mock

2.5 mM A

2.5 mM B

5 mM C

5 mM D

7 mM E

7 mM F

7 mM G

Selection with AN13762

AN13762

Mock

WT

A1

AN13762

Mock

WT

A1

Mock

AN13762

WT

A1

Phage size (mm2)

0

5

10

15

20

n.d.

n:

0

50

50

50

50

50

50

AN13762

Mock

WT

A1
we assessed the anticyryptosporidial activity of AN13762 in vitro and in vivo. The ability of AN13762 to inhibit C. parvum INRAE Nluc fast-growing strain in human ileocecal HCT-8 was assessed with its parental scaffold AN3661 as a positive control. Although less potent than AN3661, an efficient in vitro inhibition of C. parvum growth was repeatedly observed with AN13762 (EC50 13 \pm 9 \mu M) (Figures 5A, 5B and S5). AN13762 presented no detectable toxicity for the host cells, even at 100 \mu M (Figure 5C). AN13762 activity was therefore assessed in vivo in a neonatal mouse model. Seven-day-old neonates were orally treated with AN13762 mixed in carboxymethyl cellulose (CMC) 4 h after C. parvum infection and daily until 3 days post-infection (dpi). Parasite load was assessed in the intestine at 4 dpi by oocyst count and measuring Nluc activity representing transgenic expression by the INRAE Nluc strain. Both methods revealed an impressive and significant inhibition of parasite development as illustrated in Figure 5D and by scanning electron microscopy where only very scarce parasites can occasionally be found on the intestinal villi of treated mice. Remarkably, the enzymatic assay revealed a 4-log reduction in luminescence signals in treated mice, and oocysts were not detected by coproscopic intestinal material examination, which is much less sensitive than the former method. Altogether, these results indicate that AN13762 is effective against C. parvum both in vitro and in vivo and provide an additional drug presumably acting by a different mode of action than AN3661 to block CPSF3 activity.

**DISCUSSION**

Whole-cell phenotypic screening is an efficient approach in drug discovery that has led to the identification of numerous antimicrobial lead compounds, although the targets and mode of action remain unknown and challenging to determine. Although clinical development remains possible without this knowledge, lack of insight into the mechanism of action is one of the biggest obstacles for further medicinal chemistry optimization or to predict track drug resistance. Fortunately, a large variety of target deconvolution technologies are currently available. The approach developed here takes advantage of all the benefits of the EMS mutagenesis method, including its wide and mostly unbiased coverage of the genome with virtually all types of mutations (Farrell et al., 2014). In this work, by combining cost-effective RNA-seq based variant calling, computational mutation discovery and CRISPR/Cas9 genome editing, we identified CPSF3, the catalytic subunit of the pre-mRNA cleavage and polyadenylation complex, as the target of AN13762 in T. gondii parasites.

In eukaryotes, CPSF3 is key to the 3’ end processing of both polyadenylated and replication-dependent histone precursor mRNAs (Shi and Manley, 2015). These distinct 3’ ends are generated co-transcriptionally by specialized 3’ end processing machineries that recognize a conserved hexanucleotide AAUAAA and a downstream G/U-rich sequence on the 3’ end of nascent pre-mRNAs destined for polyadenylation or cleave histone mRNA precursors few nucleotides downstream of a highly conserved stem-loop structure (Marzluff et al., 2008). As a result, the majority of histone genes are expressed as nonpolyadenylated transcripts that...
mechanism of action of AN13762 in T. gondii where the mechanism of resistance is plural (Sindhe et al., 2020). In fact, while we were investigating the resistance against the anti-cancer agent JTE-607 (Ross et al., 2020), it is likely that the mechanism of resistance is shared. Possibly, the G330S and G456S mutations can only be effective for elongated molecules to come lethality but somehow favor histone pre-mRNA processing toward polyadenylation of transcripts that are otherwise barely detected using our poly(A)-selected transcript experiment settings. Interestingly, the mutations Y328H, E455K, and S519C are lining the channel accommodating the RNA substrate on CPSF3 (Figure S6), whereas the G456S mutation that is observed distant from the other mutations did not affect histone mRNA accumulation. It is noteworthy that the Y328 mutations significantly impacted the overall growth fitness (Figure 3C), suggesting a default in TgCPSF3Y328H/C activity. As the G456S mutation in T. gondii is equivalent to the G330S mutation found in the human CPSF3 counterpart conferring resistance against the anti-cancer agent JTE-607 (Ross et al., 2020), it is likely that the mechanism of resistance is shared. Possibly, the G330S and G456S mutations can only be effective for elongated molecules to clash with the compound thereby impeding binding without affecting recognition of the substrate. Yet further studies are required to determine whether the mutations in CPSF3 affect the access of the substrate to the catalytic site, complex assembly, or its conformational dynamics as shown recently by Sun et al. (2020). Altogether, these results underscore the advantage of using transcriptome sequencing to investigate mechanisms of drug action and to provide functional insight into the molecular biology of the target protein.

In mammalian cells, CPSF3 is embedded in a large multisubunit complex including CPSF1, CPSF2, CPSF4, CPSF7, cleavage stimulatory factor 1 (CSTF1), CSTF2, CSTF3, symplekin, and WDR33 (Dominski and Muzzaff, 2007; Ryan, 2004). A quite similar complex was purified in T. gondii (Table S2, Swale et al., manuscript in preparation), and the identified subunits were all predicted to be essential for tachyzoite growth in vitro (Sidik et al., 2016). No mutations with significant enrichment were found in the CPSF3 protein partners in the resistant strains, which is in agreement with our docking model based on Cryptosporidium hominis CPSF3 structural data where the oxaboroles are enfolded within the CPSF3 scaffold, presumably precluding any interaction with other components.

The mutations conferring resistance to AN13762 target TgCPSF3 catalytic site, a gold standard evidence for target confirmation of a bioactive small molecule. In the published structure of AN3661 bound to ChCPSF3, the oxaborole competes with the catalytic water molecules for zinc atoms, hence blocking the phosphate bond cleavage of the pre-mRNA substrate (Swale et al., 2019). Given the overall conservation of CPSF3 catalytic core in Apicomplexa and the high conservation of the residues involved in drug resistance, it is likely that AN13762 binds to this site and disrupts the pre-mRNA processing activity of TgCPSF3 that is essential for parasite growth.

Although it is clear that AN13762 targets CPSF3 in T. gondii, different results were observed in P. falciparum where the mechanism of resistance is plural (Sindhe et al., 2020). In fact, while we were investigating the mechanism of action of AN13762 in T. gondii, Sindhe and colleagues have shown that P. falciparum...
Figure 4. Docking Studies for Chemotypes AN13762 and AN3661

(A) Multiple sequence alignment of CPSF3 proteins from T. gondii (Tg), C. hominis (Ch), P. falciparum (Pf), and CPSF3 of H. sapiens (Hs). The domain architecture is indicated as follows: blue, metallo-β-lactamase; green, β-CASP; orange, RNA specificity domain; magenta, the insertion within the MBL (AN13762 AN3661). Y328C/H, G456S (T. gondii), E545K (C. hominis), Y483N (P. falciparum)*, and CPSF73 of H. sapiens (*). The domain insertion is indicated by asterisks. The highly conserved residues involved in the coordination of the zinc atoms Zn1 or Zn2 are indicated in gray. Mutations identified in parasites resistant to AN13762 or AN3661 are indicated in red and blue text, respectively.

Beta-Casp domain

RNA specificity domain

Apicomplexa specific insert

CPSF3

Zn1

Zn2

T. gondii

C. hominis

P. falciparum

H. sapiens

Zn1-2

G4565

Y328C,H

G4565

Y328C,H

Y432N

Y432N

E545K

E545K

Y483N

Y483N

Beta-Casp domain

RNA specificity domain

Apicomplexa specific insert

AN13762

AN3661

Figure 4. Docking Studies for Chemotypes AN13762 and AN3661

(A) Multiple sequence alignment of CPSF3 proteins from T. gondii (Tg), C. hominis (Ch), P. falciparum (Pf), and CPSF3 of H. sapiens (Hs). The domain architecture is indicated as follows: blue, metallo-β-lactamase; green, β-CASP; orange, RNA specificity domain; magenta, the insertion within the MBL (AN13762 AN3661). Y328C/H, G456S (T. gondii), E545K (C. hominis), Y483N (P. falciparum)*, and CPSF73 of H. sapiens (*). The domain insertion is indicated by asterisks. The highly conserved residues involved in the coordination of the zinc atoms Zn1 or Zn2 are indicated in gray. Mutations identified in parasites resistant to AN13762 or AN3661 are indicated in red and blue text, respectively.
resistance depends not only on the activity of Prodrug Activation and Resistance Esterase (PARE), an enzyme responsible for AN13762 processing, but also on enzymes involved in ubiquitination and SUMOylation pathways or PfCPSF3. The latter is responsible for the high level of resistance, thus suggesting that AN13762 or its refined derivative theoretically targets CPSF3 in malaria parasites as well. Whether AN13762 is processed in T. gondii is not known. However, as TgCPSF3G456S selectivity toward AN13762 is based on steric hindrance over the methylazetidine group, which is cleaved off upon processing by the esterase, it seems unlikely that such a modification occurs in T. gondii. Note that no mutations with significant enrichment were found in TGGT1_306330, the closest homolog to PARE in T. gondii (Table S2). Furthermore, as AN13762 processing is required for full antimalarial activity, it is tempting to speculate that the lack of intracellular activation explains the decreased sensitivity observed in T. gondii and Cryptosporidium (EC50 values are in the µM range, Figures 1C and 5B) relative to P. falciparum (EC50 values ranging from 18 to 118 nM, Sindhe et al., 2020).

Based on the catalytic core sequence homology between TgCPSF3 and CpCPSF3, both previously chemically validated targets for Toxoplasma and Cryptosporidium (Palencia et al., 2017; Swale et al., 2019), we successfully laid the groundwork for pathogen hopping. In this respect, AN13762 efficiently inhibits C. parvum, a species relevant to human health, in vitro and in vivo in mouse model of infection. These results appear to be even more important for the treatment of cryptosporidiosis, where druggable targets are scarce and there is a high demand for more efficient therapies. However, further work will be needed to demonstrate that AN13762 acts as a direct binder of the CpCPSF3 and inhibits its mRNA processing activity, thereby restricting the growth of parasites. The recent discovery of benzoxaborole-based chemistry has given rise to a series of compounds with great potential against various infectious agents, including trypanosomatids and apicomplexan parasites by targeting different molecular targets (De Rycker et al., 2018). Remarkably, multiple compounds with known or suspected anti-CPSF3 activity across different organisms share a similar benzoxaborole scaffold that could be a prerequisite to CPSF3 binding (Begolo et al., 2018; Lunde et al., 2019; Palencia et al., 2017; Wall et al., 2018). Interestingly, the oxaborole acoziborole can cross the blood-brain barrier (Nare et al., 2010), offering a therapeutic option to eradicate persistnt Toxoplasma cysts that are resistant to most, if not all, medications currently prescribed.

**Limitations of the Study**

Although our study is reasonably clear about AN13762 targeting CPSF3 in Toxoplasma and its activity against Cryptosporidium parasites, it remains possible that the mechanism of action in the latter is different and depends on prodrug-activating enzyme(s) such as PARE as described in Plasmodium species. Hopefully, recent advances in Cryptosporidium genetics will make it possible to carry out such investigations and genetically validate the CpCPSF3 molecular target in this organism (Vinayak et al., 2020).

**Resource Availability**

**Lead Contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alexandre Bougdour (alexandre.bougdour@inserm.fr).

**Materials Availability**

All unique materials generated in this study are available from the Lead Contact upon request.

**Data and Code Availability**

This study did not generate/analyze code.

The Illumina RNA-seq dataset generated during this study is available at NCBI GEO: GSE156685.
**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101871.
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AUTHOR CONTRIBUTIONS

F.L., M.-A.H., and A.B. conceptualized the research. A.B. supervised the research. V.B. designed and conducted the in vitro studies performed in T. gondii. C.S. performed structural modelings. M.-P.B.-P. and V.B. designed and conducted the in vivo experiments with T. gondii. A.B. computed and analyzed the RNA-seq data. F.L. supervised the work performed on Cryptosporidium. T.P. realized the in vitro and in vivo studies performed with Cryptosporidium. S.G. performed the electron microscopy study. V.B., C.S., and A.B. wrote the manuscript. Funding Acquisition, M.-A.H. and A.B. All the authors contributed to editing the final version of manuscript, discussed, and approved the results.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Target Identification of an Antimalarial Oxaborole

Identifies AN13762 as an Alternative Chemotype for

Targeting CPSF3 in Apicomplexan Parasites

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Figure S1. Activity of AN13762 against *Toxoplasma gondii*, Related to Figure 1. (A) Fluorescence microscopy showing intracellular growth of *T. gondii* parasites. HFF cells were infected with tachyzoites (RH Δku80 UPRT::NLuc-P2A-EmGFP) and incubated with 10 µM AN13762, 5 µM AN3661 or 0.1% DMSO as control. Cells were fixed 24 h post-infection and then stained with antibodies against the *T. gondii* inner membrane complex protein GAP45 (magenta). The cytosolic GFP is shown in green. Scale bars represent 10 µm. (B) Effect of AN13762 on host-cell viability. HFF cells were incubated for 72 h in the presence of increasing concentrations of AN13762. Percent viability compared to the untreated control is displayed as a function of compound concentration in micromolar concentrations. Data are presented as mean ± standard deviation (SD) of two independent biological assays, each with 3 technical replicates. Dotted line represents 100% viability.
Figure S2. Activity of AN13762 against the EMS-induced drug-resistant lines, Related to Figure 2. (A) Plaque assay showing AN13762-resistant parasites forming plaques after 7 days of growth in the presence or absence of 10 µM AN13762. (B) Fluorescence microscopy showing intracellular growth of T. gondii AN13762-resistant lines. HFF cells were infected by the indicated T. gondii strains in the presence or absence of 10 µM AN13762. At 24 h post-infection, cells were fixed and stained with antibodies against GAP45 (magenta) and Hoechst (blue) to detect IMC of parasites and nuclei, respectively. (C) Quantification of plaque sizes shown in (A) when cultured in the absence of AN13762. P-values corresponding to Kruskal–Wallis test with Dunn’s multiple comparisons with the wild-type (WT) strain are indicated. *ns*, not significative.
Figure S3. Activities of AN13762 and AN3661 against CPSF3 edited parasites, Related to Figure 3. (A) Sanger chromatogram analysis showing CPSF3 editing. Nucleotide positions relative to the ATG start codon on genomic DNA are indicated. (B) Effects of the compounds indicated on growth of the CPSF3 edited parasites as assessed by plaque assay. Plaque sizes were measured after 7 days of growth in the presence or absence of 10 µM AN13762 or 5 µM AN3661. (C) Dose–response curves for
inhibition of *T. gondii* growth *in vitro* in response to increasing concentration of the indicated compounds. Confluent HFF monolayer were infected with WT and the engineered CPSF3 mutant strains (G456S, S519C, Y328H, E545K, Y483N, Y328C) expressing the NanoLuc luciferase. Data are presented as mean ± standard deviation (SD) of n=3 technical replicates from a representative experiment out of at least two independent biological assays. Shaded error envelopes depict 95% confidence intervals. (D) Fluorescence microscopy showing intracellular growth of WT and the CPSF3 edited parasites (G456S, S519C, Y328H, E545K, Y483N, Y328C). HFF cells were infected with tachyzoites of the indicated *T. gondii* strains expressing the NLuc-P2A-EmGFP reporter gene and incubated with 10 µM AN13762, 5 µM AN3661 or 0.1% DMSO as control. Cells were fixed 24 h post-infection and then stained with antibodies against the *T. gondii* inner membrane complex protein GAP45 (magenta). The cytosolic GFP is shown in green. Scale bars represent 10 µm.
Figure S4. RNA-Seq analysis of genes encoding for histone subunits in WT and the EMS-induced AN13762-resistant strains (A1 to G1) of T. gondii. Related to Figure 2, Table S2, and Discussion. Heatmap of expression values obtained by RNA-Seq analysis of the indicated T. gondii genes. RPKM values were log2 transformed and mean centered using iDEP.90 (Ge et al., 2018). Hierarchical clustering of the samples and the selected genes are shown on top and on the left, respectively.
Figure S5. Activity of AN13762 against *Cryptosporidium parvum*, Related to Figure 5. Fluorescence microscopy showing intracellular growth of *C. parvum* parasites. Confluent HCT-8 cells were infected with freshly purified oocysts at a MOI of 1:1 of *C. parvum* INRAE NLuc strain in the presence of the indicated concentrations of AN13762 or 0.3% DMSO as control. Cells were fixed 48 h post-infection and then stained using rat antiserum generated against *C. parvum* (in red) and DAPI DNA-specific dye (in blue).
Figure S6. Oxaborole resistant mutations visualized within the human CPSF3 structure precatalytically bound to a modified histone H2A* pre-mRNA, Related to Figure 2 and Discussion. CPSF3 (extracted from the pdb-id: 6V4X) is displayed in cartoon fashion with the Metallo-β-Lactamase, β-Casp and RNA specificity domains colored respectively in blue, green and orange. Catalytic motif side chains are displayed in sticks and colored in grey. Mutated side chains conferring AN13762 and AN3661 resistance are shown as sticks and colored in yellow while the AN13762 exclusive resistant G456S is shown in red. Part of the modified H2A* histone pre-mRNA is displayed in a wire fashion with catalytic CAC cleavage motif bases highlighted in orange and red.
**Transparent Methods**

**Parasite strains and cell culture**

The *T. gondii* strains listed in the Table S1 were maintained by serial passage in HFF monolayers in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 10 mM (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (HEPES) buffer pH 7.2, 2 mM L-glutamine, and 50 μg/mL of penicillin and streptomycin. Cells were incubated at 37°C with 5% CO2 in humidified air. Human ileocecal adenocarcinoma cells (HCT-8) cultured in RPMI 1640 with glutamine supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, penicillin (50 U/mL), and streptomycin (50 μg/mL).

**Reagents**

The compounds 6-(2-((3-hydroxy-3-methylazetidin-1-yl)carbonyl)-pyrazinyl-5-oxy)-1,3-dihydro-1-hydroxy-7-methyl-2,1-benzoxaborole (AN13762) and 7-(2-carboxyethyl)-1,3-dihydro-1-hydroxy-2,1-benzoxaborole (AN3661) were purchased from ChemPartner (Purity ≥ 95% as determined by LC/MS, 1H-NMR).

**Plasmids and primers**

Oligonucleotides were ordered from Sigma-Aldrich. PCR amplifications were performed with KOD Xtremer™ Hot Start DNA Polymerase. Primers and plasmids used or generated in this study are listed in Table S1. The bicistronic vectors expressing the Cas9 genome editing enzyme and specific sgRNAs targeting the CPSF3 coding sequence were constructed as described previously (Curt-Varesano et al., 2016). Briefly, oligonucleotides CPSF3G456S-CRISPR-FWD and CPSF3G456S-CRISPR-REV, CPSF3Y328H-CRISPR-FWD and CPSF3Y328H-CRISPR-REV, and CPSF3S519C-CRISPR-FWD and CPSF3S519C-CRISPR-REV (Table S1) were annealed and ligated into the pTOXO _Cas9-CRISPR_ plasmid to create vectors used for construction of *T. gondii* recombinant for CPSF3G456S, CPSF3Y328H and CPSF3S519C, respectively.

**Generation of a *T. gondii* strain expressing the NanoLuc bioluminescent protein**

The construction carrying the Nluc-P2A-EmGFP coding sequences under the control of the Tub8 promoter sequence (Prub) was DNA-synthetized and cloned into pUC57-Simple vector by GenScript (DNA sequence provided in Table S1). Note that the P2A peptide sequence promotes a ribosomal skip, resulting in the stoichiometric expression of unfused Nluc and EmGFP reporter proteins from the same mRNA transcript. The Prub-Nluc-P2A-EmGFP-3’UTR SAG1 cassette was amplified by PCR using primers HR-UPRT-PPrub_F and UPRT-SAG1-RH_R and targeted to the UPRT locus as previously described (Shen et al., 2014). Briefly, the resulting amplicon was co-transfected with the plasmid pTOXO_Cas9-CRISPR::sgUPRT (Farhat et al., 2020) for homology directed repair at the UPRT locus. Recombinant parasites were selected with 5 µM of 5-fluoro-2-deoxyuridine (FUDR) and clones expressing both Nluc and EmGFP were isolated by limiting dilution.

**Immunofluorescence microscopy**

Cells grown on coverslips were fixed in 3% formaldehyde for 20 min at room temperature, permeabilized with 0.1% (v/v) Triton X-100 for 5 min and blocked in phosphate buffered saline (PBS) containing 3% (w/v) BSA. Samples were incubated for 1 h with primary antibodies (rabbit anti-GAP45 kindly provided by Pr. Dominique Soldati, University of Geneva) followed by the addition of secondary antibodies conjugated to Alexa Fluor 488 or 594 (Molecular Probes) to detect intracellular parasites. Nuclei were stained for 10 min at room temperature with Hoechst 33258. Coverslips were mounted on a glass slide with Mowiol mounting medium, and 0.25 µm Z-images stacks were acquired with an Axio Imager M2 fluorescence microscope (Carl Zeiss, Inc.). Images were processed with Icy 2.0 (icy.bioimageanalysis.org) using the EpiDEMIC plugin for blind deconvolution of each channel, separately. Maximum projection of deconvoluted stack images are shown.

HCT-8 cells grown on glass coverslips were infected with *C. parvum* INRAE strain at MOI=1. Three hours later monolayers were washed carefully and cells further incubated for 48 h in presence of AN13762 at 10 or 30 µM or equivalent concentration of DMSO (0.3%). After gentle washing the wells were immediately fixed for subsequent DAPI and α-*C. parvum* polyclonal rat antiserum (followed by anti-rat alexa fluor 568 conjugate) staining.
Plaque assays
Freshly egressed parasites were inoculated on a confluent monolayer of HFFs and grown for 7 days with or without the indicated compounds. Cells were fixed and stained with Coomassie blue staining solution (0.1% Coomassie R-250 in 40% ethanol and 10% acetic acid as previously described (Curt-Varesano et al., 2016).

Toxoplasma gondii in vitro measurement of EC50
The in vitro inhibitory activity of small compounds on T. gondii proliferation was determined as follows; 2,000 tachyzoites of T. gondii RH strain expressing the nanoluciferase (RH Nluc) were allowed to invade confluent HFF monolayer in a 96-well plate for 2h. Inhibitors (AN13762 and AN3661), along with pyrimethamine (minimum signal), were diluted in growth medium and added to the monolayers at various concentrations in triplicates (technical replicates) along with DMSO-treated controls (maximum signals). The assay was performed in a 100 µL final volume. After 48 h of growth at 37°C, the medium was removed and 50 µL PBS was added to each well. The NanoLuc assays were performed using the Nano-Glo® Luciferase Assay System according to manufacturer’s instructions (Promega). Lysis was performed in the wells by adding 50 µL Nano-Glo® Luciferase Assay Reagent containing 1:50th dilution of Nano-Glo® Luciferase Assay Substrate. After 3 minutes of incubation, luminescence was measured using the CLARIOstar® (BMG Labtech) plate reader. Bioluminescence values from the uninfected host cells was used to determine background signal. EC50 were determined using non-linear regression analysis of normalized data and assuming a sigmoidal dose response. EC50 values for each compound represent an average of at least two independent biological replicates. AN13762 cytotoxicity was assayed on HFF cells after 72 h of incubation using CellTiter-Blue Reagent® (Promega).

Toxoplasma gondii random mutagenesis
Parasites were chemically mutagenized as previously described (Palencia et al., 2017), with the following modifications. Briefly, ~10^7 tachyzoites (RH strain) growing intracellularly in HFF cells in a T25 flask were incubated at 37°C for 4 h in 0.1% FBS DMEM growth medium containing either ethyl methanesulphonate (EMS, ranging from 2.5 to 7 mM final concentration) or the appropriate vehicle controls (Figure 2B). After exposure to mutagen, parasites were washed three times with PBS, and the mutagenized population was allowed to recover in a fresh T25 flask containing an HFF monolayer in the absence of drug for 3–5 days. Released tachyzoites were then inoculated into fresh cell monolayers in medium containing 10 µM AN13762 and incubated until viable extracellular tachyzoites emerged 8–10 days later. Surviving parasites were passaged once more under continued AN13762 treatment and cloned by limiting dilution. Four cloned mutants were isolated each from 7 independent mutagenesis experiments. Thus, each flask contained unique SNV pools.

RNA-seq, sequence alignment, and variant calling
For each biological assay, a T175 flask containing a confluent monolayer of HFF was infected with RH wild-type or AN13762-resistant strains. Total RNAs were extracted and purified using TRizol (Invitrogen, Carlsbad, CA, USA) and RNeasy Plus Mini Kit (Qiagen). RNA quantity and quality were measured by NanoDrop 2000 (Thermo Scientific).

RNA-sequencing was performed as previously described (He et al., 2018), following standard Illumina protocols, by GENEWIZ (South Plainfield, NJ, USA). Briefly, the RNA quality was checked with TapeStation System (Agilent Technologies, Palo Alto, California, USA), and Illumina TruSEQ RNA library prep and sequencing reagents were used following the manufacturer’s recommendations (Illumina, San Diego, CA, USA). The samples were paired-end multiplex sequenced (2 x 125 bp) on the Illumina Hiseq 2500 platform and generated at least 40 million reads for each sample (Table S2).

The RNA-Seq reads (FASTQ) were processed and analyzed using the Lasergene Genomics Suite version 15 (DNASTAR, Madison, WI, USA) using default parameters. The paired-end reads were uploaded onto the SeqMan NGen (version 15, DNASTAR, Madison, WI, USA) platform for reference-based assembly and variant calling using the Toxoplasma Type I GT1 strain (ToxoDB-36, GT1 genome) as reference template. The ArrayStar module (version 15, DNASTAR, Madison, WI, USA) was used for normalization, variant detection and statistical analysis of uniquely mapped paired-end reads using the default parameters. The expression data quantification and normalization were calculated using the RPKM (Reads Per Kilobase of transcript per Million mapped reads) normalization method.

Variant calls were filtered to select variants present in coding regions with the following criteria: SNP% ≥ 90%, variant depth ≥ 30, and absent in the parental wild-type strain (Table S2). SNVs,
insertions and deletions present in regulatory or intergenic regions were filtered out as they are unlikely to contribute to drug resistance. Mutations were plotted on a Circos plot using Circa (OMGenomics.com).

**Toxoplasma gondii genome editing**

Targeted genome modifications were performed using the CRISPR/Cas9 system as described previously (Palencia et al., 2017). The recombinant parasites harboring allelic replacement for CPSF3<sup>G456S</sup>, CPSF3<sup>Y328H</sup>, CPSF3<sup>S519C</sup>, CPSF3<sup>Y328C</sup>, CPSF3<sup>Y483N</sup>, and CPSF3<sup>E545K</sup> were generated by electroporation of the *T. gondii* RH NLuc strain with pTOXO_Cas9CRISPR vectors targeting the CPSF3 coding sequence (sgCPSF3<sup>G456S</sup>, sgCPSF3<sup>Y328H</sup>, sgCPSF3<sup>S519C</sup>, sgCPSF3<sup>Y328C</sup>, sgCPSF3<sup>Y483N</sup>, and sgCPSF3<sup>E545K</sup>) and their respective donor single-stranded oligo DNA nucleotides (ssODNs) carrying respective nucleotide substitutions (CPSF3<sup>G456S_donor</sup>, CPSF3<sup>Y328H_donor</sup>, CPSF3<sup>S519C_donor</sup>, CPSF3<sup>Y328C_donor</sup>, CPSF3<sup>Y483N_donor</sup>, and CPSF3<sup>E545K_donor</sup>; Supplemental Table S1) for homology-directed repair. Recombinant parasites were selected with 10 µM AN13762 (CPSF3<sup>G456S</sup>, CPSF3<sup>Y328H</sup>, and CPSF3<sup>S519C</sup>) or 5 µM AN3661 (CPSF3<sup>Y328C</sup>, CPSF3<sup>Y483N</sup>, and CPSF3<sup>E545K</sup>, as described previously in (Palencia et al., 2017)) prior to subcloning by limited dilution, and allelic replacement was verified by sequencing of *T. gondii* CPSF3 genomic DNA.

**Toxoplasma gondii in vivo mouse therapeutic assays**

All animal procedures were conducted under pathogen-free conditions in compliance with established institutional guidance and approved protocols from the European Directive 2010/63/EU. We used randomization and blinding to treatment assignment to reduce bias in mice selection and outcome assessment. Two independent experiments were performed with three mice in each treatment group (female CBA/JR mice, Janvier, Le Genest- ... 7–9 weeks old). Mice were infected intraperitoneally with 10<sup>3</sup> tachyzoites of the virulent type I RH Nluc strain and the RH Nluc CPSF3<sup>E545K</sup> or CPSF3<sup>G456S</sup> mutant strains. These inocula routinely resulted in high mortality in control mice at 6–12 days post-infection. Treatments were initiated at day 1 post-infection and were continued for seven consecutive days. Treated mice were orally administered 40 mg/kg AN13762 or 200 mg/kg sulphadiazine (Sigma), as previously described (Palencia et al., 2017), both suspended in 1% (w/v) carboxymethylcellulose (CMC, Sigma) and 0.1% (v/v) Tween-80 (Sigma). In surviving mice, the protective immunity acquired against *Toxoplasma* conferred after the first challenge was confirmed by a lethal secondary challenge with the RH Nluc strain (10<sup>3</sup> tachyzoites per mouse).

**Cryptosporidium EC<sub>50</sub> determination and cell toxicity**

The *in vitro* inhibitory activity of small compounds on *Cryptosporidium* and cell toxicity were determined as described previously (Swale et al., 2019). Briefly, confluent HCT-8 cells were infected with freshly purified oocysts (multiplicity of infection (MOI) of 1:1) of *C. parvum* INRAE strain in the presence of different concentrations of AN3661 or AN13762. After 3 h, cell cultures were washed twice, and media were replaced with the same compound concentration and further incubated for 24 or 48 h. Culture supernatant was removed from the wells (six replicates for each concentration), and 200 µl of Nano-Glo lysis buffer containing 1:50 of Nano-Glow substrate (Promega) was added to the wells. After 3 min of incubation, luminescence was measured with GloMax-Multi+ (Promega) and analyzed with Instinct software. EC<sub>50</sub> was determined from dose response inhibition curve using with GraphPad Prism software. AN13762 cytotoxicity was assayed on HCT-8 cells after 24 h of incubation using an MTS assay (CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay, Promega).

**Cryptosporidium in vivo mouse therapeutic assays**

*In vivo* efficacy of AN13762 was assayed as described previously (Swale et al., 2019). Briefly, seven-day-old wild-type neonatal mice were infected by oral gavage with 5 × 10<sup>5</sup> oocysts of *C. parvum* INRAE strain and treated orally with 20 µL of treatment suspension [40mg/kg] in CMC or sham treated with 20 µL of vehicle solution (CMC). The degree of infection in individual neonatal mice was assessed by determining the number of oocysts by coproscopy (Thoma counting chamber; detection limit of 6.10<sup>4</sup> oocysts/small intestine) and Nluc activity in the intestinal contents.

**Scanning electron microscopy**

Ileal tissue samples were fixed by incubation for 24 h in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Samples were then washed in phosphate buffer, postfixied by incubation with 2% osmium tetroxide for 1 h, fully dehydrated in a graded series of ethanol solutions, and dried in hexamethyldisilane. Last, samples were coated with 40-Å platinum using a...
GATAN PECS 682 apparatus before observation under a Zeiss Ultra plus FEG-SEM scanning electron microscope.

**Homology modelling of *T. gondii* CPSF3 mutations and docking analysis of AN13762**

Homology modelling visualization of the *T. gondii* CPSF3 mutated residues was performed using the *Cryptosporidium hominis* CPSF3/AN3661 co-crystal structure (pdb id: 6Q55) as a structural model basis. *T. gondii* point mutations were depicted through direct sequence conservation while AN13762 docking was performed by manual placement of the oxaborole core onto the AN3661 backbone in Coot ([Emsley et al., 2010](#)) with no further energy minimization performed. Schematics were produced using Pymol (Schrödinger, LLC) and UCSF Chimera.

**Data and Code Availability**

The accession number for the RNA-Seq data reported in this paper is GEO: GSE156685.
Supplemental References

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