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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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Supplemental Information

Supplemental Figures

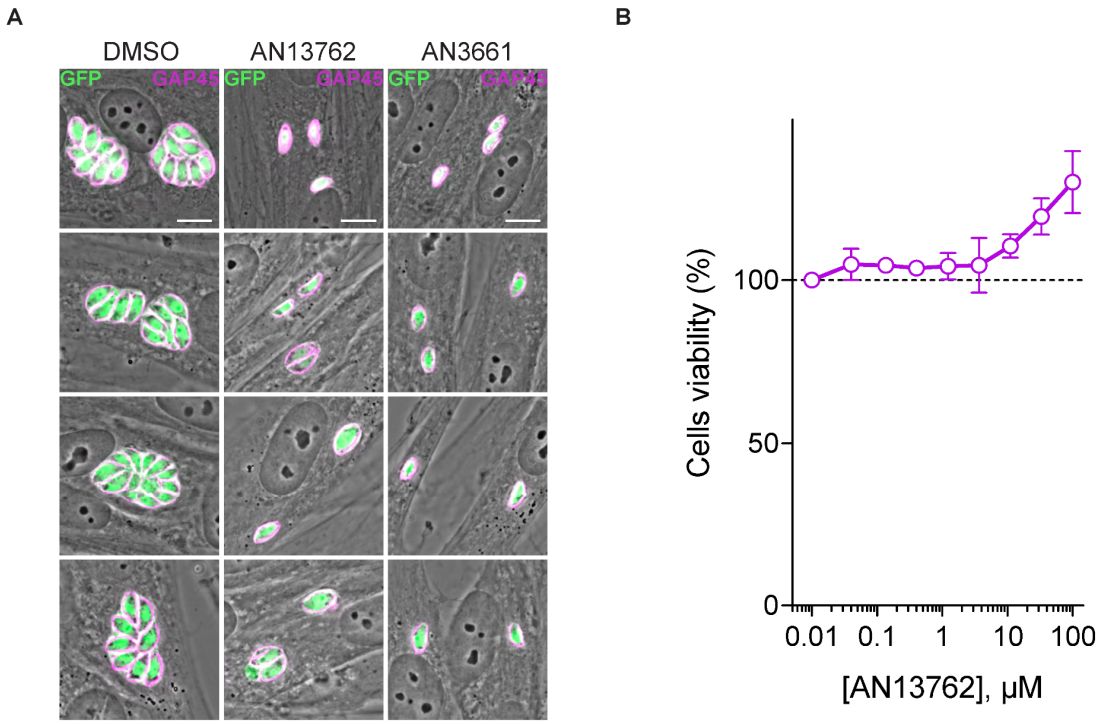

Figure S1. Activity of AN13762 against *Toxoplasma gondii,* Related to Figure 1. (A) Fluorescence microscopy showing intracellular growth of *T. gondii* parasites. HFF cells were infected with tachyzoites (RH Δ*ku80 UPRT::NLuc-P2A-EmGFP*) and incubated with 10 µM AN13762, 5 µM AN3661 or 0.1% DMSO as 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.

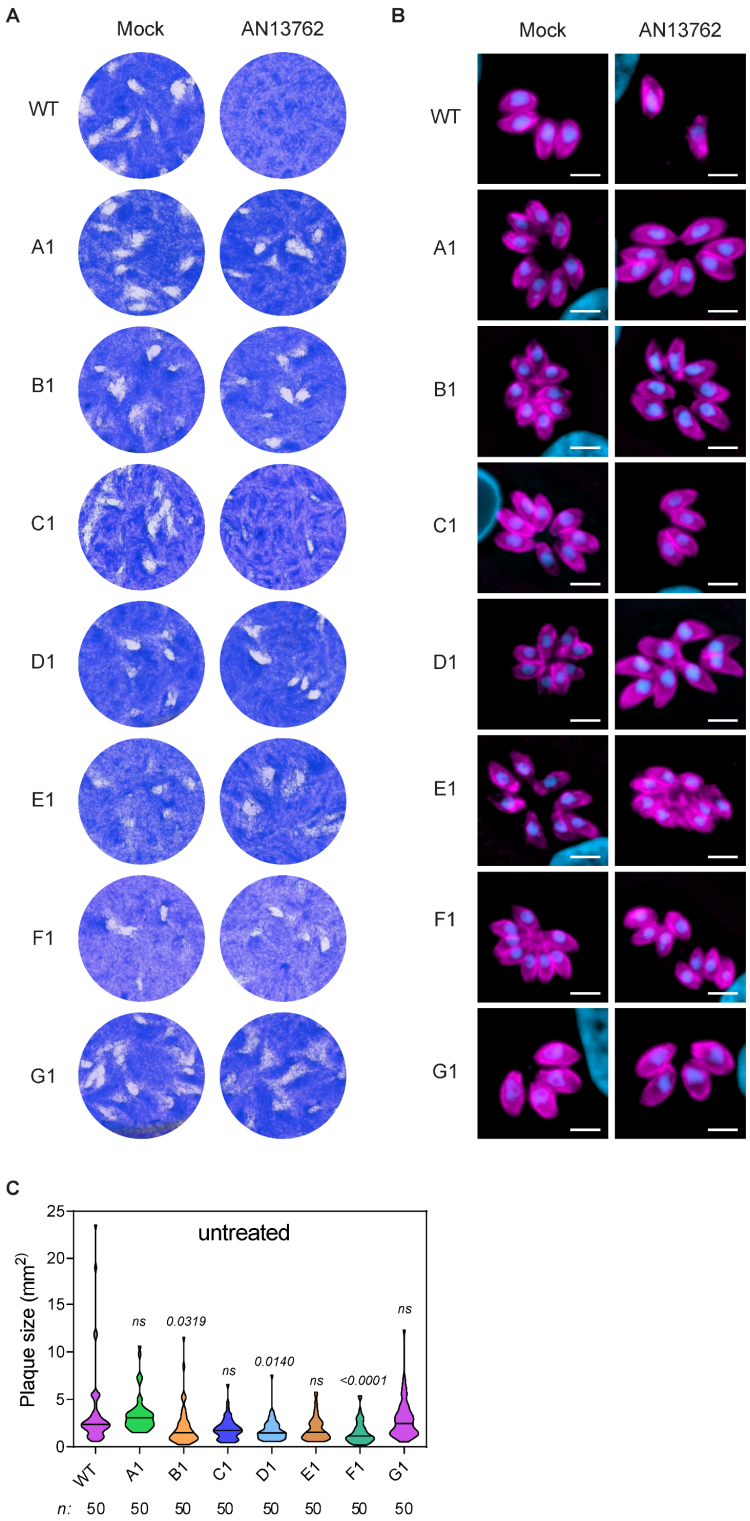

Figure S2. Activity of AN13762 against the EMS-induced drug-resistant lines, Related to Figure 2. (**A**) Plaque assay showing AN13762-resistant parasites forming plaques after 7 days of growth in the presence or absence of 10 µM AN13762. (**B**) Fluorescence microscopy showing intracellular growth of *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.

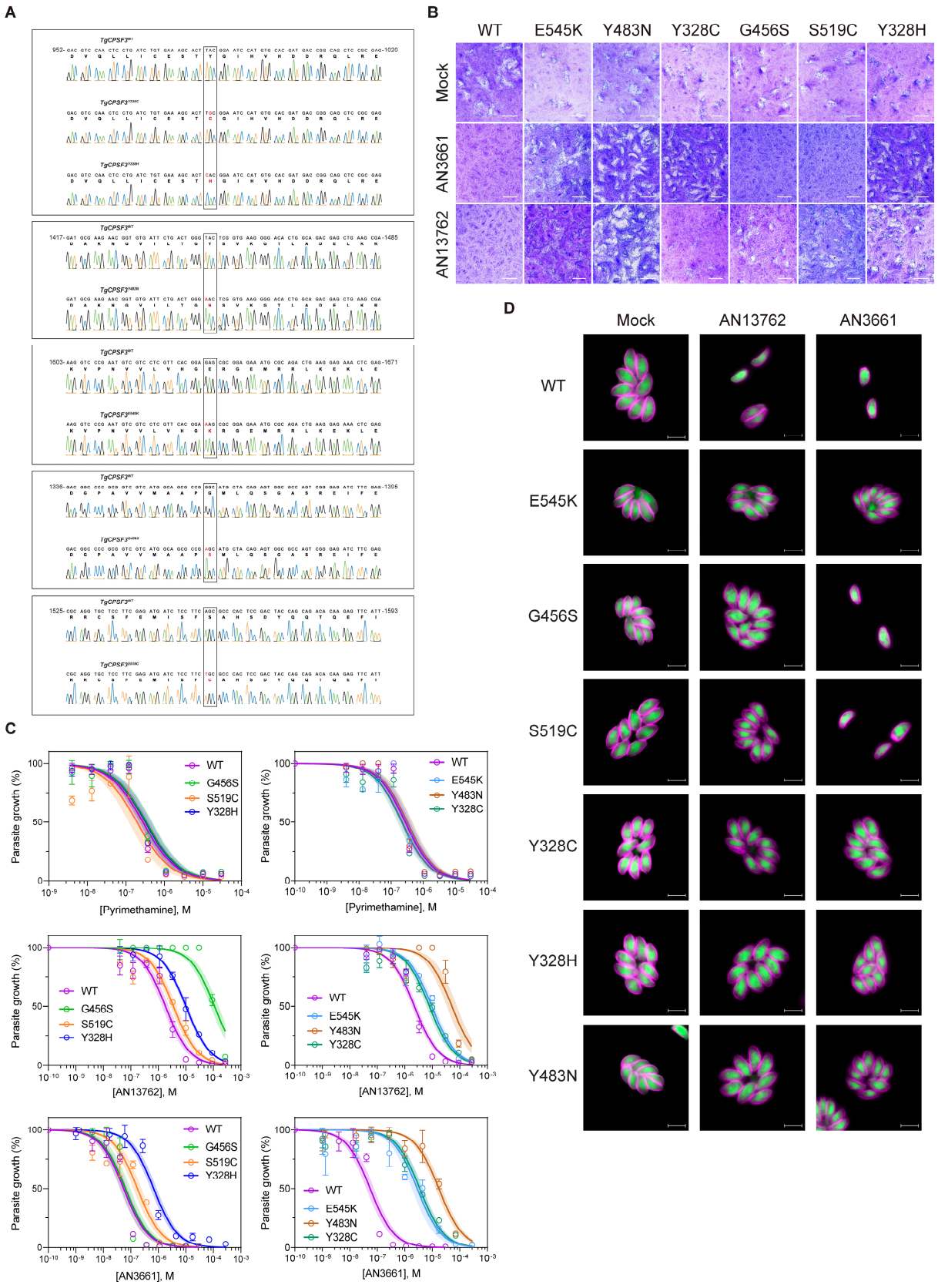

Figure S3. Activities of AN13762 and AN3661 against *CPSF3* edited parasites, Related to Figure 3. (**A**) Sanger chromatogram analysis showing *CPSF3* editing. Nucleotide positions relative to the ATG start codon on genomic DNA are indicated. (**B**) Effects of the compounds indicated on growth of the *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.

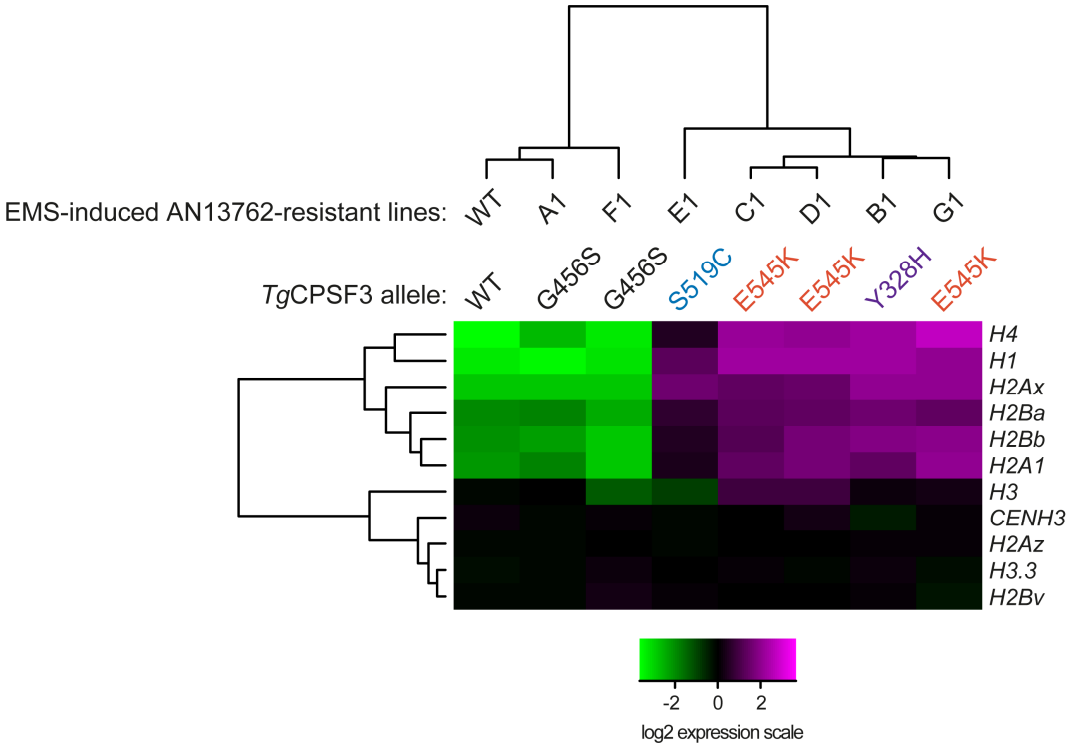

Figure S4. RNA-Seq analysis of genes encoding for histone subunits in WT and the EMS-induced AN13762-resistant strains (A1 to G1) of *T. gondii*, Related to Figure 2, Table S2, and Discussion. Heatmap of expression values obtained by RNA-Seq analysis of the indicated *T. gondii* genes. RPKM values were 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.

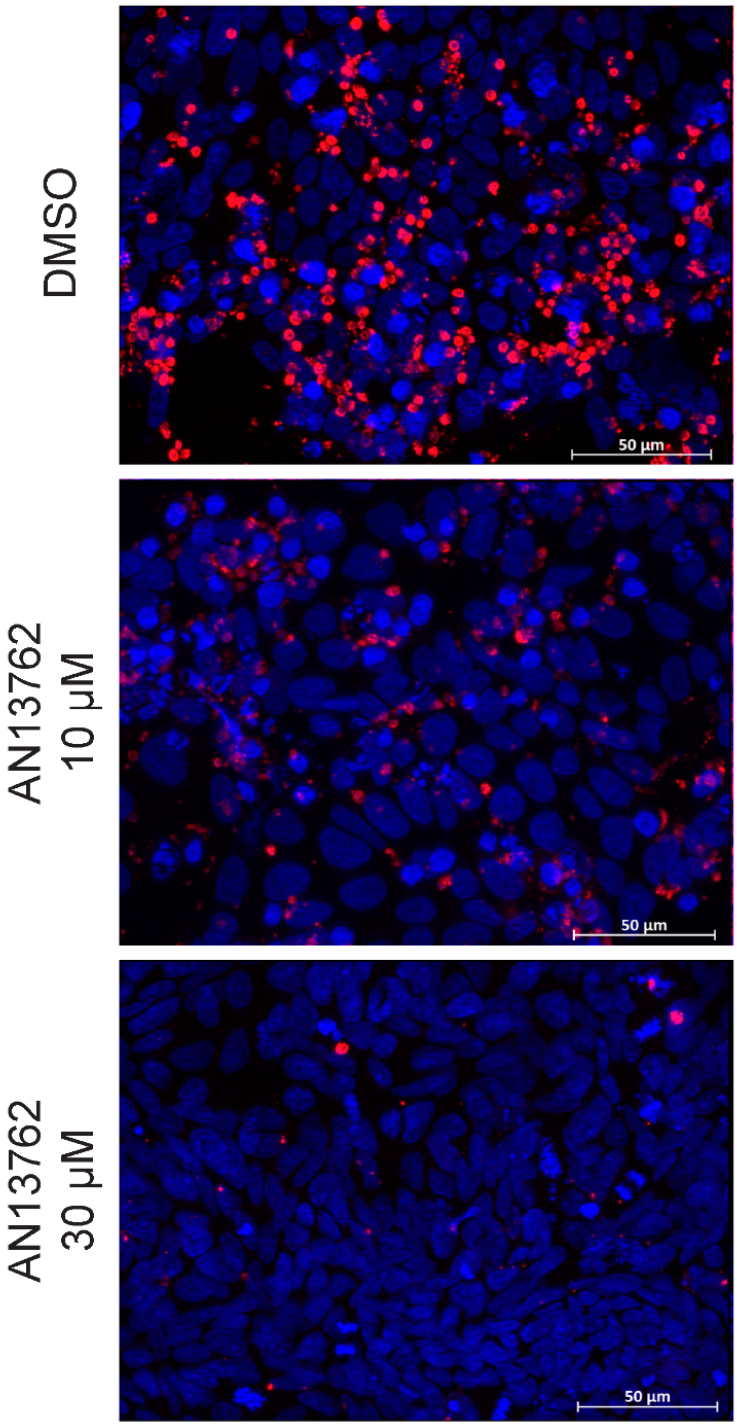

Figure S5. Activity of AN13762 against *Cryptosporidium parvum,* Related to Figure 5. Fluorescence microscopy showing intracellular growth of *C. parvum* parasites. Confluent HCT-8 cells were infected with freshly purified oocysts at a MOI of 1:1 of *C. parvum* INRAE Nluc strain in the presence of the 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).

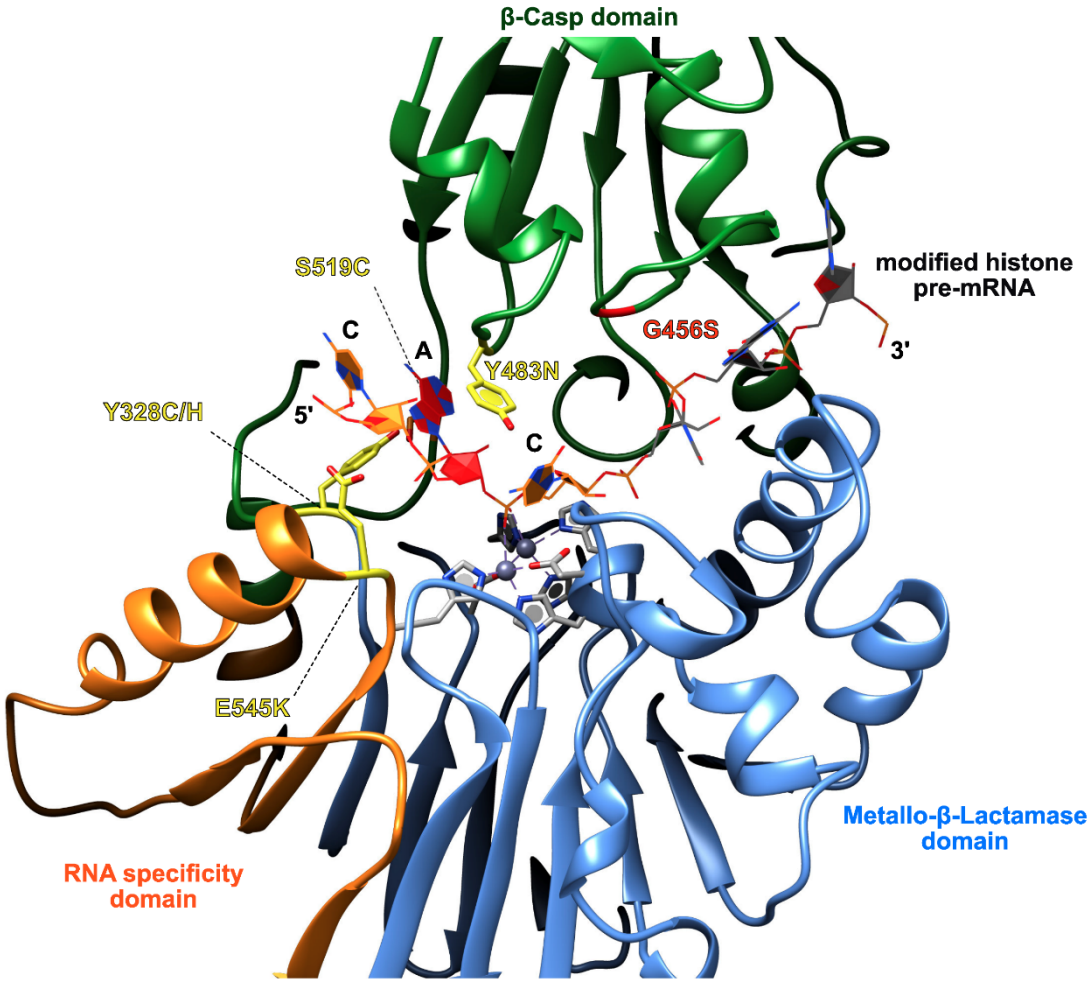

Figure S6. Oxaborole resistant mutations visualized within the human CPSF3 structure pre-catalytically bound to a modified histone H2A* pre-mRNA, Related to Figure 2 and Discussion. CPSF3 (extracted from the pdb-id: 6V4X) is displayed in cartoon fashion with the Metallo-β-Lactamase, β-Casp and RNA specificity 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, Invitrogen) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Invitrogen), 10 mM (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid) (HEPES) buffer pH 7.2, 2 mM L-glutamine, and 50 µg/mL of penicillin and streptomycin (Invitrogen). 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, ^{1}H-NMR).

Plasmids and primers

Oligonucleotides were ordered from Sigma-Aldrich. PCR amplifications were performed with KOD Xtreme™ 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 CPSF3^{G456S}-CRISPR-FWD and CPSF3^{G456S}-CRISPR-REV, CPSF3^{Y328H}-CRISPR-FWD and CPSF3^{Y328H}-CRISPR-REV, and CPSF3^{S519C}-CRISPR-FWD and CPSF3^{S519C}-CRISPR-REV (Table S1) were annealed and ligated into the pTOXO_Cas9CRISPR plasmid to create vectors used for construction of *T. gondii* recombinant for CPSF3^{G456S}, CPSF3^{Y328H} and CPSF3^{S519C}, 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 (P_{Tub8}) 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 EmGPF reporter proteins from the same mRNA transcript. The P_{Tub8}-Nluc-P2A-EmGFP-3'UTR SAG1 cassette was amplified by PCR using primers HR-UPRT-P_{Tub8}_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 EC_{50}**

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 $1:50^{th}$ 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. EC_{50} were determined using non-linear regression analysis of normalized data and assuming a sigmoidal dose response. EC_{50} 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*^{G456S}, *CPSF3*^{Y328H}, *CPSF3*^{S519C}, *CPSF3*^{Y328C}, *CPSF3*^{Y483N}, and *CPSF3*^{E545K} were generated by electroporation of the *T. gondii* RH NLuc strain with pTOXO_Cas9CRISPR vectors targeting the *CPSF3* coding sequence (sgCPSF3^{G456S}, sgCPSF3^{Y328H}, sgCPSF3^{S519C}, sgCPSF3^{Y328C}, sgCPSF3^{Y483N}, and sgCPSF3^{E545K}) and their respective donor single-stranded oligo DNA nucleotides (ssODNs) carrying respective nucleotide substitutions (CPSF3^{G456S}_donor, CPSF3^{Y328H}_donor, CPSF3^{S519C}_donor, CPSF3^{Y328C}_donor, CPSF3^{Y483N}_donor, and CPSF3^{E545K}_donor; Supplemental Table S1) for homology-directed repair. Recombinant parasites were selected with 10 µM AN13762 (*CPSF3*^{G456S}, *CPSF3*^{Y328H}, and *CPSF3*^{S519C}) or 5 µM AN3661 (*CPSF3*^{Y328C}, *CPSF3*^{Y483N}, and *CPSF3*^{E545K}, 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/JRj mice, Janvier, Le Genest-Dt-Isle, France; 7–9 weeks old). Mice were infected intraperitoneally with 10^3 tachyzoites of the virulent type I RH Nluc strain and the RH Nluc CPSF3^{E545K} or CPSF3^{G456S} 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 (10^3 tachyzoites per mouse).

Cryptosporidium EC_{50} 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 Nluc 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_{50} 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® 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 5×10^5 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 6.10^4 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, postfixed 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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