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Auranofin Resistance in *Toxoplasma gondii* Decreases the Accumulation of Reactive Oxygen Species but Does Not Target Parasite Thioredoxin Reductase

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Auranofin, a repurposed FDA-approved drug originally designed to treat rheumatoid arthritis, has emerged as a promising anti-parasitic drug. It induces the accumulation of reactive oxygen species (ROS) in parasites, including *Toxoplasma gondii*. We generated auranofin resistant *T. gondii* lines through chemical mutagenesis to identify the molecular target of this drug. Resistant clones were confirmed with a competition assay using wild-type *T. gondii* expressing yellow fluorescence protein (YFP) as a reference strain. The predicted auranofin target, thioredoxin reductase, was not mutated in any of our resistant lines. Subsequent whole genomic sequencing analysis (WGS) did not reveal a consensus resistance locus, although many have point mutations in genes encoding redox-relevant proteins such as superoxide dismutase (*TgSOD2*) and ribonucleotide reductase. We investigated the *SOD2* L201P mutation and found that it was not sufficient to confer resistance when introduced into wild-type parasites. Resistant clones accumulated less ROS than their wild type counterparts. Our results demonstrate that resistance to auranofin in *T. gondii* enhances its ability to abate oxidative stress through diverse mechanisms. This evidence supports a hypothesized mechanism of auranofin anti-parasitic activity as disruption of redox homeostasis.

Keywords: gold, repurposing, anti-parasitic, resistance, redox, *Toxoplasma*, auranofin, superoxide

INTRODUCTION

For more than 50 years, the mainstay of treatment for acute toxoplasmosis has been a combination of a dihydrofolate reductase inhibitor and a sulfa antimicrobial. While this is currently a critical therapeutic strategy, currently approved drugs cannot eliminate latent parasites in cysts that are found in chronically infected individuals. Moreover, these drugs have significant bone marrow
toxicity, are suspected teratogens, and the emergence of resistance remains a potential threat to treatment. Repurposing FDA-approved drugs will accelerate drug discovery for neglected parasitic diseases. Most recently, aurano, a reprofilled drug that is FDA-approved for treatment of rheumatoid arthritis, has emerged as a promising anti-parasitic agent. It has antiproliferative activity against *Plasmodium falciparum* (Sannella et al., 2008), *Schistosoma mansoni* (Angelucci et al., 2009), *Leishmania infantum* (Ilari et al., 2012), and *Entamoeba histolytica* (Debnath et al., 2012), as well as other parasites with public health significance (Martinez-Gonzalez et al., 2010; Debnath et al., 2013; Tejman-Yarden et al., 2013; Bulman et al., 2015; da Silva et al., 2016; Hopper et al., 2016; Peroutka-Bigus and Bellaire, 2019). Despite this promising broad spectrum of anti-parasitic activity, the molecular target of aurano has only been indirectly implicated as thioredoxin reductase.

Thioredoxin reductase enzymes are found in archaea, bacteria and diverse eukaryotes and play a key role in reduction of disulfide bonds that is essential for cell replication and survival. In humans, inhibition of thioredoxin reductase 1 induces expression of heme oxygenase-1 to repress inflammation (Kobayashi et al., 2006).

We previously demonstrated that aurano reduces *T. gondii* infection of host cells *in vitro* and a single dose of aurano allows survival of chicken embryos infected with this parasite (Andrade et al., 2014). It is hypothesized that the anti-parasitic activity of aurano comes from inhibition of the thioredoxin reductase enzyme of these parasites, akin to its action on human cells (Kuntz et al., 2007; Debnath et al., 2012; Tejman-Yarden et al., 2013). This postulated mechanism of action explains why parasites treated with aurano accumulate ROS (Debnath et al., 2012). Intriguingly, aurano may improve infection outcome by decreasing pathogenic host inflammatory damage in addition to its direct inhibition of parasite replication.

In this paper we describe our work to investigate resistance to aurano in *T. gondii* parasites. This approach illuminates mechanisms of resistance that might arise during its use as an anti-parasitic. In some circumstances, identification of resistance mechanisms validates proposed drug targets [i.e., (McFadden et al., 2000)]. In this study, we found that resistance was not associated with changes to the *Toxoplasma* thioredoxin reductase gene and no other single locus emerged as a consistent site underlying resistance. However, we observed that resistant clones accumulated less ROS than their wild type counterparts, demonstrating that aurano resistance enhances oxidative stress responses.

**MATERIALS AND METHODS**

**Host Cell and Parasite Cultures**

Parasite clones and human foreskin fibroblasts (HFF) were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone), penicillin and streptomycin (50 μg/ml each) and 200 μM L-glutamine. We will refer to this complete medium as D10. *T. gondii* were maintained by serial passage in HFF monolayers at 37^°^C in a humid 5% CO₂ atmosphere, including RH parasites (National Institutes of Health AIDS Reference and reagent Repository, Bethesda, MD), RH tachyzoites expressing cytoplasmic yellow fluorescent protein (YFP) (kindly provided by M.J. Gubbels, Boston College, Boston, Massachusetts) (Gubbels et al., 2003) and Ku80 knock-out parasites (AKu80, kindly provided by V. Carruthers, University of Michigan Medical School Dexter, Michigan) (Huynh and Carruthers, 2009; Fox et al., 2009).

**Generation of *T. gondii* Clones Resistant to Aurano**

We mutagenized *T. gondii* with ENU as previously described (Pfefferkorn and Pfefferkorn, 1979; Nagamune et al., 2007). Briefly, approximately 1.5 × 10^7^ intracellular tachyzoites of wild-type RH strain *T. gondii* were mutagenized with ENU (N-Ethyl-N-nitrosourea, N3385-1G, Sigma Aldrich; 100–200 μg/ml) in DMEM without FBS for 2 h at 37^°^C. These parasites were washed and transferred to a new confluent HFF monolayer for selection with aurano (2.5 μM–3 μM) until normal pace of replication was observed (~2 weeks). Aurano resistant clones were single cell cloned by limiting dilution in 2 μM aurano. Isolated clones were propagated in D10 media with 1 μM aurano to maintain selection pressure without cytotoxic effects on host cells (data not shown).

We selected ten representative *T. gondii* clones from independent ENU-generated pools to investigate resistance. To assess aurano resistance, we modified a tachyzoite growth competition assay (Ma et al., 2008). Using wild-type YFP-expressing *T. gondii* parasites as a control, confluent monolayers of HFF cells were inoculated with approximately equal numbers (2 × 10^6^; 1:1 ratio) of ENU-mutagenized parasites and YFP-expressing wild-type RH parasites. Upon lysis, a new T25 flask with confluent HFF was inoculated with 0.25 ml of lysed parasites. Another 0.25 ml of lysed parasites was used for flow cytometry. The YFP fluorescence allowed us to differentiate wild-type and Aur^B^-parasites in the mixed populations to track their growth over serial passages. Host cell debris was removed by filtering of parasites with a 3 μm filter membrane. After parasites were collected by centrifugation (400g for 10 min), the pellet was resuspended in 1 ml PBS. The total number of parasites and the number of parasites expressing YFP were quantified by flow cytometry using a FACScalibur flow cytometer (Becton Dickinson) and analyzed with FlowJo software (Tree Star). The result of three independent experiments analyzing the replication fitness advantage between each aurano resistant clone and the wild-type parasites was analyzed with parametric paired t-tests. A two-tailed p-value <0.05 was considered statistically significant.

**Identification of Mutations in Auranofin-Resistant *T. gondii* Clones**

We extracted genomic DNA (gDNA) from aurano resistant parasites lines using the DNeasy Blood and Tissue kit (Qiagen). The thioredoxin reductase gene (TGGT1_309730, ToxoDB.org) was amplified from gDNA samples as two PCR fragments ~3.7 kb each. The 5’ half of the gene was amplified with primers GGACCTATGATTGCGCTTCCTGTG and GTCAATCCCATAATCGCGAGG, and the 3’ half with GTCTGCCTCTCCTGTTTTGCAGAAGC and CTCACCATCTCCAGAAGC. Sequencing
primers are 5TrxR 1R, 5TrxR 1F and 5TrxR 3F for the 5’ half, and TrxR F 8, 3TrxR 2F, 3TrxR 3F, and 3TrxR 4F for the 3’ half (Table S1).

To determine the presence of other SNVs in the coding region of our T. gondii auranoﬁn resistant clones, gDNA from 10 resistant clones and wild-type RH parasites was submitted for library construction and whole genome sequencing at the Genomic High Throughput Facility at University of California, Irvine. Briefly, genomic DNA (gDNA) was initially quantiﬁed with a Qubit DNA High Sensitivity kit. One hundred nanograms of gDNA was sheared to <300–500 bp in size using a Covaris S220 ultrasonicator (shearing conditions: duty Cycle 10%, Cycles/burst 200 and treatment time 55 sec) (Covaris, MA). The size and the quality of the resulting DNA fragments were analyzed with an Agilent DNA High Sensitivity chip. Ten nanograms of each sample’s fragmented DNA was end-repaired, poly adenylated and ligated to a 6bp DNA barcodes (Bioo Scientiﬁc NextFlex DNA barcodes) using a PrepX Complete ILMN DNA Library kit (Takara Bio, CA). The genomic library was built using the Apollo 324 system (Takara Bio, CA) to generate 520 bps inserts. To enrich the DNA libraries, 8 cycles of PCR ampliﬁcation using a Kapa library ampliﬁcation kit was used (cycling conditions: 98°C for 45 s, 8 cycles of 98°C for 15 sec, 60°C for 30 s 72° C for 30 s and then 72°C for 1 min) in an Apollo 324 system. The ultimate DNA concentration was calibrated using Qubit 2.0 dsDNA HS Assay kit and analyze with an Agilent DNA High Sensitivity chip. Each library was normalized to 2nM and then pooled equimolar amounts in the ﬁnal multiplex library that was sequenced on the Illumina HiSeq 4000.

The ten auranoﬁn resistant T. gondii clones and wild-type RH T. gondii were sequenced with HiSeq 4000 paired end 100 reads, quality analyzed, adapter and quality trimmed before alignment with the annotated reference genome of GT1 strain from ToxoDB.org (published 7/19/2013) (Gajria et al., 2008). After initial quality control with FASTQC, reads were trimmed using Illumina adapter sequences with the error probability of 0.05 and reads shorter than 20 bases were discarded. Single Nucleotide Variant (SNV) calling for the haploid genome had a coverage threshold of ≥5 fold. Each of the 10 mutant clones were compared to wild-type RH variant track and shared variants were ﬁltered out. Only SNVs in the coding regions of the 10 mutant clones are reported. Only genes containing SNVs with 100% frequency and ≥50% of read count and read coverage were considered in our ﬁnal analysis. Genomic DNA from each mutant clone was used to amplify the targeted SNV region (Table S1). Genomic sequences for all 10 mutant lines can be accessed NCBI, under the Bioproject PR1NA679812.

**Generation of Modiﬁed SOD2 Lines**

We generated a T. gondii clone with a 3’ knock-in YFP tag to TgSOD2 using a ligation independent cloning approach as previously described (Huynh and Carruthers, 2009). The mitochondrial distribution of our clone was conﬁrmed by ﬂuorescent microscopy using a Zeiss Axiostop with Axiovision camera and software. Co-localization of the TgSOD2-YFP with mitochondria was done by staining parasite mitochondria with an anti-F1B ATPase antibody (gift from Peter Bradley at UCLA).

We subsequently introduced the L201P mutation into the TgSOD2-YFP line using a modiﬁed CRISPR plasmid generated using plasmid pSAG1::CAS9-U6::sgUPRT as the scaffold (Shen et al., 2017; Addgene # 54467). Our plasmid had an additional DHFR-TM marker for pyrithiamine selection derived from pLoxP-DHFR-mCherry [Long et al., 2016], Addgene # 70147], and the two gRNA sequences were replaced with cggtataccgacagatccg and TAGTTTCCGTCAGTTCAAGG, selected by the Benchling software (Benchling.com).

The DNA sequence used for homology directed repair (HDR) was ampliﬁed with the primers GATAGTGTGTGA AGAGCACG and CTGCCGTACCAACTGFF from the LIC-YFP-SOD2 plasmid generated above. PCR amplicon contained the L201P mutation (CTA->CCA) generated with Q5 Site-Directed Mutagenesis kit (New England Biolab), as with the following modiﬁcations. To screen for positive CRISPR-Cas9 clones containing L201P, two silent mutations were created to generate new and unique restriction endonuclease sites close to Leucine 201: AflI on Leucine 180 (CTC->CTT, 62 bps upstream of L201P) and Xmnl on Serine 208 (TCA->TCT, 22 bps downstream of L201P). The PCR amplicon contained two additional deletions corresponding to the gRNA sites on endogenous SOD2 gene, in order to avoid SpCas9 protein cleaving the amplon for HDR. The sequence AGAGACC CGCGGG (including the PAM sequence cgg) was deleted on the HDR sequence corresponding to gRNA #1, and sequence CACTTACAGAGGTC (including the PAM sequence agg) was deleted on the HDR sequence corresponding to gRNA #2.

The L201P point mutation was introduced into the TgSOD2-YFP line through CRISPR-Cas9 (Figure S1). Transfection of the CRISPR-Cas9 plasmid and the HDR DNA was carried out as published previously, with minor modiﬁcations (Shen et al., 2017). Brieﬂy, 10⁷ of freshly lysed parasites with SOD2-YFP were ﬁltered and spun down at 400g and 18°C for 10 min. The pellet was resuspended with Cytosim buffer (10 mM KPO₄, 120 mM KCl, 5 mM MgCl₂, 25 mM HEPES, 2 mM EDTA, 2μM ATP and 5μM glutathione) up to 800μl, including 7.5μg of CRISPR plasmid and 1.5μg of HDR DNA. The mixture was transected using the ECM 630 Electron Cell Manipulator (BTX). Parasites were incubated into T25 ﬂasks after electroporation and switched to 2 μM pyrithiamine selection 24 h later. Transfected pools remained in pyrithiamine selection for 3 days, and then switched to D10 media without selection for one more day before single cell cloning. The gDNA for isolated clones were collected by the DNaseasy Blood and Tissue kit (Qiagen) and used to PCR amplify SOD2 with either primers SOD2 F/AmpR (for SOD2-YFP) or
primers SOD E2/SOD R (for SOD2 only, **Table S1**). PCR amplicons were digested with XcmI for screening and positive clones were sequence verified.

**Measurement of ROS Accumulation in *T. gondii***

To expose our parasites to oxidative stress, we harvested freshly lysed parasites and treated $5 \times 10^5$ parasites with 500 μM hydrogen peroxide ($H_2O_2$) for 30 min at 37°C, 5% CO₂, with or without 1 μM aurano. After incubation, ROS was detected using H₂DCFDA (Invitrogen). Each sample was incubated with 1 μM of H₂DCFDA and examined under a SpectraMax i5, (Molecular Devices California US); multimode microplate reader SoftMax Pro 7.0.2 Software) multimode microplate reader (excitation 494 nm; emission 522 nm) at 37°C for 15 min. For excitation, a single flash from a UV Xenon lamp was used for each well, and emission signals were recorded with a sensitivity setting of 100. The values are presented as relative fluorescence units. We compared ROS accumulation of each representative aurano resistant *T. gondii* clone to that of wild-type parasites by an unpaired t-test in three independent experiments. A two-tailed $p$-value $<0.05$ was considered statistically significant.

**Protein Alignment and Structural Models**

The predicted amino acid sequences for TgSOD2 (TGGT1_316330) and TgRNR (TGGT1_294640) were submitted to the SwissModel site to identify the optimal available protein structures for modeling (Gues et al., 2009; Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Waterhouse et al., 2018). The *Toxoplasma* sequences were threaded onto the structure of *Plasmodium knowlesi* SOD (PDB 2AWP) (Vedadi et al., 2007) and human RNR (PDB 3HND) (Fairman et al., 2011). Clustal Omega was used to align the amino acid sequences and EMBOSS was used to calculate percent identity and similarity. Information on MS peptide coverage was obtained from the ToxoDB database (Gajria et al., 2008).

**Expression Level of the Thioredoxin Reductase Gene in Mutants**

We used qPCR to quantify the expression level of thioredoxin reductase gene in both wild type and mutant parasites. RNA was extracted from parasite lines using the RNeasy Mini Kit (Qiagen), and then used for cDNA synthesis via iScript cDNA Synthesis Kit (Bio-Rad). The resulting cDNA samples were then subjected to qPCR with iTaq Universal SYBR Green Supermix (Bio-Rad) with the following gene targets: actin (TGGT1_209030, ToxoDB.org), GAPDH1 (TGGT1_289690, ToxoDB.org), thioredoxin reductase (TGGT1_309730, ToxoDB.org), and superoxide dismutase 2 (TGGT1_316330, ToxoDB.org).

The actin gene was amplified from cDNA samples as one PCR fragment ~60bp, with primers CACGAGAGGAGGATACG GCTTC and CGATGTCGCTAGAAGTCTCAG. The GAPDH1 gene was amplified from one PCR fragment ~120bp with primers CACCTTTGCAAGATGGTG and GACTGTC TTCAACGAGAAGGAG. The thioredoxin reductase gene was amplified a ~70bp PCR fragment using the primers CACAAAC ACACAACCCGGCG and GGTGACGGCTGAACAAAGTCG.

For the ~50bp fragment in superoxide dismutase 2 gene, primers GGCACACCGTGCTGATAAG and CGTGGTTCCATG CTTGTGCTG were used.

In order to quantify the amount of target transcript present, target CT values were normalized against actin CT values. The resulting ΔCT values were then compared through 2-way ANOVA between the different parasite strains surveyed.

**RESULTS**

**AurR *T. Gondii* Lines Have a Growth Advantage Over Wild Type Parasites**

To verify aurano resistance of the selected clones, we adapted a previously described competition assay (Ma et al., 2008). This was used in place of a normal dose-response assay because at aurano concentrations higher than 3 μM, the fibroblast monolayer begins to disrupt (data not shown), making the plaque assays not feasible. To confirm resistance to aurano in the AurR lines, we assessed their growth in competition with YFP-expressing RH strain parasites in control media and in media with 2.5–3.0 μM aurano. In competition assays, the inoculating parasite population is composed of 50% YFP-expressing wild type (sensitive) parasites and 50% of an AurR line verified by flow cytometry. The relative contribution of the individual populations was measured after each cycle of lytic growth in serial culture. If a parasite line increases to >50% of the population, it exhibits a growth advantage over the competing line. In the absence of aurano, AurR lines grew comparably or less well than the parental RH line that does not express YFP (Figure 1A). However, in the presence of 2.5 μM aurano, all AurR lines displayed significant growth advantage over YFP-expressing wild-type RH parasites (Figure 1B). The observations indicated that day 6 is the optimal day to capture the growth advantage of resistant lines under aurano selection because YFP-expressing wild-type parasites were eliminated from the culture. Given these results, we measured the ability of a larger set of AurR lines to compete with wild-type YFP-expressing RH strain parasites in the absence or presence of aurano by quantifying their contribution to the culture at 6 days (Figure 2). These results show that 9 of the 10 AurR lines outcompete the sensitive YFP-expressing RH line in the presence of 2.5 μM aurano. Line 6 has a distinct profile: it grows slowly in both control and aurano media, suggesting that it may evade aurano-mediated killing by protracted replication.

**AurR Lines Do Not Harbor Mutations in the Thioredoxin Reductase Gene**

Considering that thioredoxin reductase (Sannella et al., 2008; Angelucci et al., 2009; Debnath et al., 2012; Ilari et al., 2012) is the proposed target of aurano, we hypothesized that resistance in *T. gondii* would develop through mutations to the parasite thioredoxin reductase gene (TgTrxR, TGGT1_309730). Therefore, we amplified and sequenced the TgTrxR gene in 53 lines derived from parallel (independent) selections (not shown). We did not detect mutations (SNVs) in the thioredoxin reductase gene of any of the lines, indicating that aurano
resistance does not arise through modifications to TgTrxR that directly influence auranoﬁn binding or TgTrxR enzyme activity.

**AurR Lines Accumulate Less ROS**
The anti-parasitic activity of auranoﬁn relies on its ability to induce accumulation of ROS in parasites (Debnath et al., 2012). We exposed wild type RH strain parasites and four representative AurR lines to hydrogen peroxide (H₂O₂) for 30 min before measuring ROS accumulation with dichloro-ﬂuorescein (H₂DCDCF). All AurR lines displayed decreased accumulation of ROS relative to the parental RH line in both the absence and presence of auranoﬁn (Figure 3). For control, we also measured ROS accumulation in the absence of H₂O₂ and observed no ﬂuorescence signal (data not shown). This negative control is consistent with a prior study measuring ROS accumulation using the same dichloro-ﬂuorescein dye without the H₂O₂ (Lee et al., 2006).

**AurR Lines Harbor Diverse Mutations That Introduce Point Mutations**
Given that we did not observe mutations in *T. gondii* thioredoxin reductase, we performed whole genome sequencing (WGS) analysis of 10 independent *T. gondii* AurR lines. Our goal was to determine whether these *T. gondii* parasites harbor a common mutation(s) that underlies resistance to auranoﬁn. Analysis of coding sequences with 100% SNVs frequency and at least 50-fold coverage identiﬁed a variety of ~5 non-synonymous SNVs in the exons of each line. Selected SNVs observed in the WGS were validated by PCR ampliﬁcation and Sanger sequencing (primers in Table S1). We chose to speciﬁcally investigate the AurR line 8 to consider which SNV was most likely to underlie auranoﬁn resistance. Line 8 has 12 SNVs that introduce amino acid

![FIGURE 1](image1.png) | *Aur*R lines have a growth advantage under selective pressure with auranoﬁn. (A) In the absence of auranoﬁn, individual resistant lines grow as well or less well than the parental RH strain. (B) In the presence of 3 µM auranoﬁn, wild-type parasites are eliminated by day 6 (red box).

![FIGURE 2](image2.png) | Resistant *T. gondii* lines outcompete wild type parasites under auranoﬁn selection. AurR lines are represented as percentage of the total population of parasites after 6 days in competition with YFP-expressing wild type (sensitive) parasites, no drug media (white) and 2.5 µM auranoﬁn (black). Mean difference between parasite population in the absence of auranoﬁn and in the presence of auranoﬁn: 40.6; SD 14.6; two-tailed unpaired t-test *p* < 0.001.

![FIGURE 3](image3.png) | Auranoﬁn-resistant *T. gondii* parasites accumulate less ROS. Freshly lysed wild-type parasites were treated with 500 µM hydrogen peroxide (H₂O₂) for 30 min and accumulation of reactive oxygen species (ROS) was assayed with dichloro-ﬂuorescein H₂DCDCF. Auranoﬁn-resistant clones accumulated less ROS after H₂O₂ treatment compared to the wild-type RH parasites in the presence of H₂O₂ (white) or H₂O₂ + 1 µM auranoﬁn (black). (*) *p* < 0.05 when compared to RH or to RH+ Auranoﬁn (Au).
substitutions to diverse protein coding genes (Table 1). We used the community annotated ToxoDB database and BLAST analysis to investigate these loci. In particular, the availability of RNA-seq datasets permitted us to evaluate whether loci were expressed in the tachyzoite stage. In order to assess the significance of expressed loci, we used BLAST analysis to look for protein homologs and significant motifs and collected RNA-seq values and CRISPR screen scores for available loci. This information led us to focus on 2 of the loci (TGGT1_316330 and TGGT1_294640) as most likely resistance conferring. These loci encode a superoxide dismutase (SOD2) and the large subunit (R1) of ribonucleotide reductase (RNR). Both enzymes have strong fitness conferring effects in the deposited CRISPR screen data and well-conserved metabolic roles.

RNR removes the 2’-hydroxyl group of the ribose ring of nucleoside diphosphates to form deoxyribonucleotides from ribonucleotides which are used in DNA synthesis while SODs remove superoxide (O2•-) radicals by converting them into molecular oxygen and hydrogen peroxide. Since both TgRNR and TgSOD2 are conserved proteins, we threaded the amino acid sequence onto homologous crystal structures to locate the position of the amino acid substitutions. TgSOD2 is a Fe-type SOD and was previously demonstrated to localize to the tachyzoite mitochondrion (Brydges and Carruthers, 2003; Pino et al., 2007). SODs form dimers with shared coordination of two Fe(III) cofactors. The L201P mutation is located near to the key iron-coordinating residues: H111, E249 and H160 (Figure 4A) and is located in an α-helix. Substitution of a proline at this site likely causes the helix to bend, perhaps altering SOD enzymatic properties. TgRNR aligns well with human ribonucleotide reductase 1 (61.7% identity and 78.2% similarity). However, there is a 59 amino acid N-terminal extension to the Toxoplasma protein, with the conserved sequence beginning with M60 (Figure 4B). This leader does not represent mis-annotation of the N-terminus of the protein: there are MS peptides mapping to sequence, especially in samples that are from monomethylarginine and phosphopeptide-enriched datasets in ToxoDB. This is particularly interesting as arginine methylation is a modification associated with proteins that have roles in transcripational regulation, RNA metabolism and DNA repair. RNR use free-radical chemistry to convert ribonucleotides into deoxyribonucleotides. Significantly, ribose reduction requires generation of a free radical and RNR requires electrons donated from thioredoxin. Moreover, superoxide has been shown to inactivate RNR and yeast lacking either cytoplasmic or mitochondrial SODs have increased susceptibility to oxidative stress and RNR inactivation. The L166Q mutation is in a solvent exposed loop (Figure 4C), that is poorly conserved and not known to contribute to enzyme interactions.

**Resistance to Auranofin Is Not Conferred by an Isolated Mutation in TgSOD2**

The anti-parasitic effect of auranofin appears to stem from its molecule of gold, which is known to induce the accumulation of ROS in parasites (Debnath et al., 2012; Adeyemi et al., 2017). Since we hypothesized that a single mutation (SNV) suffices to generate resistance to auranofin, we analyzed the effect of the TgSOD2 L201P mutation on auranofin sensitivity. SOD2 is a *bona fide* enzymatic ROS scavenger and SOD2 is the sole mitochondrial superoxide dismutase in tachyzoites. To analyze the role of mutant SOD2 in auranofin resistance, we generated a *T. gondii* clone with a knock-in YFP tag for SOD2 that localizes to the tachyzoite mitochondrion as confirmed by co-localization with the mitochondrial marker F1B ATPase (Figure 5A). Subsequently, we generated two *T. gondii* SOD2.L201P lines: one lacking the YFP tag and one tagged with a C-terminal YFP fusion (Figure S1). Both lines were corroborated by Sanger sequencing. In the absence of Auranofin, both L201P lines grew less well than the TgSOD2-YFP wild-type line but were more fit than AurR line 8 (Figure 5B). Previous attempts to create SOD1 and SOD2 gene deletions failed (O’Dberg-Ferrat et al., 2000) and SOD2 is a fitness-conferring locus in a genomewide CRISPR/Cas screen (Sidik et al., 2016). It is noteworthy that the L201P mutation is associated with reduced fitness in the absence of superoxide stress (control conditions). In the presence of auranofin, only line 8 grows well, indicating that the single L201P mutation to TgSOD2 does not confer resistance. This may indicate that this substitution does not influence superoxide

**Table 1** | SNVs in AurR line 8.

| Rank | Mutation | Locus | Predicted protein | CRISPR | *RNA-seq | Motif | Index |
|------|----------|-------|------------------|--------|---------|-------|-------|
| 1    | L201P    | TGGT1_316330 | Superoxide dismutase SOD2 | -4.09 | 76.99 | SOD motif | 2.50 |
| 2    | L166Q    | TGGT1_294640 | Ribonucleoside-diphosphate reductase | -4.79 | 40.9 | R1 subunit, ribonucleotide reductase | 2.29 |
| 3    | Y95C     | TGGT1_233460 | SAC-related sequence SRS29B | -0.07 | 1809.46 | Major surface antigen p30 | 2.10 |
| 4    | Y637H    | TGGT1_288870 | Tetracopeptide repeat-containing | -1.66 | 36.52 | Predicted dehydrogenase | 1.81 |
| 5    | P1357-S1358A | TGGT1_310350 | PGAP1 family protein | -2.41 | 11.67 | Alpha/beta-hydrolase/PGAP1-like protein | 1.45 |
| 6    | D487G    | TGGT1_298820 | TBC domain-containing protein | -1.14 | 15.49 | Rab-GTPase-TBC domain | 1.25 |
| 7    | L606L    | TGGT1_271290 | Hypothetical protein | -2.02 | 8.1 | Alternative splicing regulator | 1.21 |
| 8    | F424L    | TGGT1_234250 | Hypothetical protein | -2.02 | 6.39 | SWAP/MAEBL | 1.11 |
| 9    | A574G    | TGGT1_290910 | Hypothetical protein | -1.39 | 7.63 | No putative conserved domain | 1.03 |
| 10   | D1983del | TGGT1_211310 | Hypothetical protein | -0.69 | 12.86 | RCC1 repeat | 0.95 |
| 11   | F563S    | TGGT1_260490 | Hypothetical protein | -1.62 | 5.34 | Herpes BLiF1 | 0.94 |
| 12   | P66P     | TGGT1_258340 | Hypothetical protein | 0.73 | 24.06 | No putative conserved domain | N/A |

*Transcriptome Gregory series at 2 h RH (log 2 FPKM+1). FPKM, fragments per kilobase of exon per million fragments mapped; N/A: not calculated due to dispensable (positive) CRISPR phenotype index.

Index Formula = Sum of Log10 of (−CRISPR phenotype index)+log10 (*RNAseq expression).
metabolism at all or that auranofin resistance also requires the mutant TgRNR enzyme. It is unlikely that these enzymes directly interact, as RNR enzymes are cytosolic while TgSOD2 is in the mitochondrial compartment. However, the SOD2.L201P T. gondii line accumulated ROS similarly to wild-type parasites, consistent with its auranofin-sensitive growth (Figure 5C).

DISCUSSION

Auranofin, is an FDA-approved drug for treatment of rheumatoid arthritis. More recently it has been proposed to be a promising anti-parasitic agent with activity against diverse parasites with public health significance. Despite this promising broad spectrum of anti-parasitic activity, the molecular target of auranofin has only been indirectly implicated as thioredoxin reductase. We previously demonstrated that auranofin decreases the parasite burden during a systemic infection with T. gondii using a chicken embryo model of acute toxoplasmosis (Andrade et al., 2014). Our preliminary observations suggest that auranofin exerts a dual effect: it modulates both parasite proliferation and host immunopathology. Herein, we report isolation and characterization of auranofin-resistant T. gondii lines. None of our resistant lines have mutations in the thioredoxin reductase gene. This may indicate that this enzyme is not the molecular target of auranofin in T. gondii; alternately, it may indicate that thioredoxin reductase cannot tolerate resistance-conferring mutations or is not the sole significant target in this organism.

During these investigations, we monitored the activity of auranofin on wild type parasites as a control condition relative to the resistant lines. Our observations suggest that auranofin enhances cumulative free radical damage, ultimately causing parasite death. Each of our auranofin resistant T. gondii lines carry several mutations (SNVs), suggesting a high threshold to develop auranofin resistance. This is a desirable characteristic for drug development. Importantly, resistant lines accumulate less reactive oxygen species (ROS) than wild-type parasites.

Chemical mutagenesis is a well-established and robust approach [i.e., (McFadden et al., 2000; Nagamune et al., 2007)] to identify the molecular targets of candidate anti-T. gondii drugs. This approach is also relevant to anticipating mechanisms of drug resistance. We isolated auranofin-resistant T. gondii lines derived from the RH strain. Resistant lines have increased replication relative to wild type RH parasites in competition assays carried out in the presence of auranofin. Lines with ≤5 accumulated SNVs (lines 1–4) appear to have a growth advantage over wild-type parasites in the absence auranofin. This probably reflects a fitness defect induced by expression of YFP in wild type parasites (Ma et al., 2008); these lines likely grow comparably to non-YFP expressing wild type parasites without auranofin present. In contrast, resistant lines that have >5 SNVs each compete poorly with wild type parasites in the absence of selection, suggesting that
accumulation of SNVs comes at a cost to parasite fitness. An alternative explanation to the isolation of multiple SNVs in each resistant clone is that aurano interferes with many processes and development of resistance requires alterations to more than one target. Another possibility to consider is that the expression level of thioredoxin reductase could change with aurano treatment. We measured the transcript levels of two redox genes, TrxR and SOD2, in both wild type and mutant line 8 (Figure S2). Aurano did not induce significant changes in the expression level of either TrxR or SOD2. While changes in TrxR transcription were not detected in the mutant line, it is possible that overexpression could lead to development of resistance. Future studies could investigate whether overexpression of thioredoxin reductase alone is sufficient to confer resistance to aurano.

Auranofin contains a molecule of gold in a 3,4,5-Triacetoxy-6-sulfanyl-oxan-2-yl methyl ethanoate scaffold and has been proposed to be a pro-drug (Chircorian and Barrios, 2004) that delivers gold to dithiol groups, like those found in proteins that bear thioredoxin-containing domains (Saccoccia et al., 2012). Although auranofin inhibits mammalian thioredoxin reductase (Gromer et al., 1998), the source of its anti-inflammatory activity remains ill-defined and may reflect inhibition of multiple targets. For example, macrophages decrease production of nitric oxide and pro-inflammatory cytokines in response to auranofin, which also inhibits cyclooxygenase-2-dependent prostaglandin E2 production (Yamashita et al., 2003). Previous studies using the parasite enzymes trypanothione reductase and glutathione-thioredoxin reductase incubated with auranofin demonstrated that co-crystals harbor gold bound to reactive cysteines in these proteins (Angelucci et al., 2009; Ilari et al., 2012; Parsonage et al., 2016). In addition, S. mansoni worms with decreased expression of thioredoxin glutathione reductase (SmTGR) exhibit diminished parasite viability upon exposure to auranofin. E. histolytica treated with auranofin upregulate transcripts of a gene encoding a protein resembling an arsenite-inducible RNA-associated protein (AIRAP) (Debnath et al., 2012). Interestingly, both arsenite and auranofin are inhibitors of thioredoxin reductase, implying that EhTrxR is a target of auranofin. Studies in both S. mansoni and E. histolytica corroborated the interaction of thioredoxin reductase with auranofin by mobility shift assays or other in vitro measures (Kuntz et al., 2007; Debnath et al., 2012). In these cases, the approach involved target validation guided by the chemical properties of gold rather than target identification through genetic resistance.
It was surprising to not identify changes to the thioredoxin reductase gene, given previous studies that implicate antioxidant enzymes containing thioredoxin or thioredoxin-like domains as a target for auranoﬁn in parasites. However, although S. mansoni and E. histolytica thioredoxin reductase antioxidant systems are essential for survival, a previous study indicates that T. gondii thioredoxin reductase is necessary for ROS metabolism (Xue et al., 2017). It should be noted that infection with 10^6 TgTrxR knockout parasites increases survival in mice by approximately 2 weeks relative to the parental RH line (Xue et al., 2017). In vitro, the null line exhibits reduced antioxidant capacity, invasion efﬁciency, and proliferation (Xue et al., 2017). These observations are in-line with the CRISPR ﬁtness score for thioredoxin reductase (-1.98) which places it as ﬁtness-conﬁrming but not essential.

Among the signiﬁcant SNVs found in our auranoﬁn resistant T. gondii clones, we directed our attention at a point mutation to the superoxide dismutase 2 (SOD2) gene. SOD2 is a bona ﬁde enzymatic antioxidant system that catalyzes the dismutation of superoxide anions into hydrogen peroxide and oxygen. The Toxoplasma genome contains three SOD genes. TgSOD1 is expressed in the cytoplasm of tachyzoites and TgSOD2 is located in the mitochondrion; TgSOD3 is speciﬁcally expressed in oocysts. TgSOD2 is a critical enzyme for tachyzoite survival; previous efforts to generate a TgSOD2 knockout line were not successful and its CRISPR ﬁtness score for (-4.09) indicates that it is strongly ﬁtness conferring and likely essential (Odberg-Ferragut et al., 2000). We hypothesized that the L201P mutation in TgSOD2 might increase its capacity to convert superoxide into hydrogen peroxide and oxygen to confer auranoﬁn resistance. To test this hypothesis, we generated several lines that harbor knock-in tag to wild type SOD2 and engineered both YFP-tagged and untagged lines that bear the L201P mutation. Consistent with previous characterization of TgSOD2 (Pino et al., 2007), both wild type and L201P TgSOD2-YFP lines exhibit a mitochondrial distribution. However, neither the tagged or untagged L201P TgSOD2 lines exhibit increased growth in 2.5 uM auranoﬁn, indicating that the single TgSOD2 mutation cannot confer resistance to this level of auranoﬁn or reduce accumulation of ROS in our assay.

In addition to the L201P TgSOD2 mutation, AurR line 8 harvests a mutation in the large subunit of TgRNR, an essential enzyme which generates deoxyribonucleotides that are required for genome duplication and parasite replication. TgRNR has a 59 amino acid N-terminal extension relative to other RNRs. Peptides from this extension are represented in phosphorylated and monomethylated MS datasets, suggesting that this domain may regulate RNR activity. RNRs perform free-radical chemistry, require electrons donated from thioredoxin and are susceptible to superoxide inactivation. Although the L166Q mutation is in an area that is not known to contribute to enzyme interactions, it may play a role diminishing TgRNR susceptibility to superoxide inactivation. While this mutation may indirectly contribute to resistance to superoxide, RNR enzymes do not directly contribute to redox homeostasis and the observed reduced accumulation of ROS in the AurR lines.

Our previous and current ﬁndings suggest that auranoﬁn decreases T. gondii viability through oxidative stress (Andrade et al., 2014). AurR lines exhibit an increased ability to scavenge ROS. We carried out preliminary studies to evaluate whether auranoﬁn resistant clones were resilient to the hydrogen peroxide damage. Since our observations were highly variable, we are currently generating parasites expressing redox sensors. The genetic studies presented here did not identify a consistent mechanism for acquisition of resistance by point mutations to a speciﬁc protein target. Our future studies will examine whether there are transcriptional, translational or post-translational changes that contribute to auranoﬁn resistance in these lines. For example, difference-gel electrophoresis and mass spectrometry have been used to identify differentially expressed proteins in sulfadiazine-resistant T. gondii isolates. While it was disappointing to not identify a single molecular target for auranoﬁn in T. gondii in our studies, this result likely indicates that an enhanced ability to scavenge ROS in the presence of auranoﬁn requires multiple changes to the parasite genome. Therefore, if auranoﬁn is repurposed as an anti-parasitic treatment, it would likely present an increased threshold to resistance in clinical contexts.

**DATA AVAILABILITY STATEMENT**

The sequencing data are publicly available at NCBI’s BioProject, with the accession # PRJNA679812.

**AUTHOR CONTRIBUTIONS**

CM and JT collected, contributed, and performed the analysis of the data and drafted the paper. SJ and SS collected and contributed the data. SR, YD, and JZ collected the data. NM contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.618994/full#supplementary-material

**Supplementary Figure 1** | CRISPR-Cas9 strategy for generating the T. gondii SOD2.L201P line. TgSOD2-YFP parasites incorporating the LIC plasmid (blue line) undergo CRISPR-Cas9 mediated homologous directed repair (HDR). The DNA amplicon used for HDR contains the L201P mutation (gold vertical line in exon 3) and two silent and unique restriction sites AflII and XcmI (black vertical lines in exon 3), which allow for screening of positive clones. Sequences corresponding to the two gRNA were deleted from the DNA for HDR (g1a and g2a, golden triangles). Each of the gRNA was present twice in TgSOD2-YFP parasites (g1a+b, g2a+b, red font), resulting in two T. gondii SOD2.L201P lines. For HDR after g1a/g2a cuts, a T. gondii SOD2.YFP.L201P line is generated (lower left). For HDR after g1a/g2b cuts, a T. gondii SOD2.L201P line without YFP or LIC plasmid (blue) is generated (lower right). Positive clones were screened with XcmI digest before sequence verification.

**Supplementary Figure 2** | Auranofin treatment does not change the expression of thioredoxin reductase nor superoxide dismutase 2 transcripts. RNA was harvested from freshly lysed parasites incubated in either control or 1μM auranofin containing media. Relative expression of SOD2 and TrxR genes normalized to actin. Parasites with or without exposure to auranofin, expressed similar transcripts amounts of TrxR and SOD2 with a range of -0.25 and Mann-Whitney U-Test. There were no statistical differences. This manuscript has been released as a pre-print at https://www.biorxiv.org/as (Ma et al., 2020).

**Supplementary Table 1** | Primers for SNV validation.

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