Drug Resistance in *Toxoplasma gondii*

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**INTRODUCTION**

*Toxoplasma gondii* (*T. gondii*) is a remarkably successful protozoan parasite that belongs to the phylum Apicomplexa (*Tenter et al., 2000*). It is estimated that up to one-third of the world's human population is infected with *T. gondii* (*Montoya and Liesenfeld, 2004*). In addition, toxoplasmosis is considered as the third most common food-borne parasitic infection requiring hospitalization (*Vaillant et al., 2005*).
Toxoplasma infection in humans mainly occurs through two ways: (1) ingestion of tissue cysts with raw or undercooked meat. (2) Consumption of oocysts with contaminated food, water, vegetables, fruits, etc. Congenital transmission from mother to fetus is also possible when a woman gets an infection during pregnancy (Moncada and Montoya, 2012; Sepúlveda-Arias et al., 2014).

In most immunocompetent people, infection with T. gondii is usually asymptomatic. But in immunocompromised or congenitally infected patients without proper treatment, severe diseases may occur (Moncada and Montoya, 2012; Wang et al., 2017). In a recent global meta-analysis study, high odds ratios (ORs) was reported for Toxoplasma infection in HIV/AIDS patients especially in Asia and Africa and in cancer patients in Asia (Wang et al., 2017). However, in South America, severe ocular toxoplasmosis is higher than in many other parts of the world (Glasner et al., 1992).

The population structure of T. gondii consists of three main clonal lineages; Type I (including a highly virulent RH strain), Type II (including ME49 and PRU, avirulent strains), and Type III (including avirulent strains like NED) (Howe and Sibley, 1995).

Type II is the predominant type of clonal lineage that infects humans and animals in Europe and in North America. However, more recent studies in South America have documented the discovery of genetically atypical (non-clonal) strains of T. gondii isolated from human patients, which caused much more dramatic clinical symptoms compared with their European counterparts. Thus, the number of strains, or isolates, not to be categorized as type I, II, and III clonal lineages has increased strongly, and has meanwhile outnumbered those who are conventionally categorized (Shwab et al., 2014). These atypical strains also influenced immunocompetent individuals, and there are reports of abortions in Toxoplasma-positive pregnant women due to atypical T. gondii strains. Phenotypically, atypical T. gondii field strains are completely different from their European counterparts and from laboratory-adapter strains used as models for studies on T. gondii biology and the efficacy of novel compounds in drug development programs (Shwab et al., 2014).

Recommended drugs for treatment or prophylaxis of toxoplasmosis are limited to combinations of pyrimethamine (PYR) and sulfadiazine (SDZ). Unfortunately, these drugs have severe side effects such as neutropenia, leucopenia, severe platelet count decrease, thrombocytopenia, and hypersensitivity reactions (Porter and Sande, 1992; Rajapakse et al., 2013; Montazeri et al., 2015). Additionally, these drugs are related to some uncommon reactions as well, including agranulocytosis, Stevens–Johnson syndrome, toxic epidermal necrolysis, and hepatic necrosis, which may be fatal in patients with toxoplasmosis (McLeod et al., 2006).

In a retrospective review, 62% of patients treated with PYR, SDZ, and leucovorin showed a high rate of toxicity and a number of side effects which required a change in the therapeutic regimen in 44% of patients (Porter and Sande, 1992).

Drugs, such as azithromycin, clarithromycin, spiramycin, atovaquone, dapsone, and cotrimoxazole (trimethoprim-sulfamethoxazole), have also been used to treat clinical toxoplasmosis; however, they are poorly tolerated and have no effect on the bradyzoite form of the parasite (Montazeri et al., 2017b, 2018).

Also, there have been several reports on failures of the long-term treatment of toxoplasmic encephalitis, chorioretinitis, and congenital toxoplasmosis with antifolate, particularly among AIDS patients (Jacobson et al., 1996; Bossi et al., 1998; Villena et al., 1998). Hence, there is controversy whether these failures are related to pharmacological parameters (drug intolerance, poor compliance, and malabsorption) and/or to the development of drug-resistant parasites or a lower susceptibility of the T. gondii strain (Menecur et al., 2008). T. gondii parasite has an exceptional adaptive potential which renders it “resistant,” but the mechanism of resistance, or adaption, has not been completely elucidated (Kropf et al., 2012).

In a previous study by Ouellette, the basic mechanisms of parasite drug resistance in malaria, leishmaniasis, sleeping sickness, and common helminthiases were evaluated (Ouellette, 2001). In another study by McFadden et al. resistance was investigated as a tool to investigate old and new drug action sites in Toxoplasma parasite (McFadden et al., 2001). However, previous studies have demonstrated that drug resistance in T. gondii is not yet a major problem in human population (McFadden et al., 2001; Sims, 2009); recently, studies have focused on finding safe drugs with novel mechanisms of action for toxoplasmosis that are both efficacious and nontoxic for patients (Aladay and Doggett, 2017; Montazeri et al., 2017a; Daryani et al., 2018).

It should be noted that various studies have been published reporting drug resistance in T. gondii (Table 1). The fact that drug resistant forms of T. gondii strains can contribute to human disease could raise a concern for treatment failure in the future (Silva et al., 2017). This review is focused on the available knowledge, encompassing information on anti-Toxoplasma drug resistance including mechanisms of resistance and drug target in parasite.

**PYRIMETHAMINE RESISTANCE**

Clinically, acute toxoplasmosis is usually treated with a combination of PYR and SDZ. These drugs inhibit important enzymes for pyrimidine biosynthesis in the parasite [dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS)] and have a remarkable synergistic activity against parasite survival and replication. DHFR is also present in humans so that the treatment with DHFR inhibitors may induce a folate deficiency state, which is probably responsible for hematological side effects and embryopathies (Rajapakse et al., 2013). Therefore, in order to limit adverse hematological events, these treatments are administered with folic acid (Aladay and Doggett, 2017).

However, these pathways are not essential for T. gondii viability, as studies showed that in resistant strains of T. gondii to 5-fluorodeoxyuridine, adenosine arabinoside, and 6-thioguanine, the parasite has pyrimidine analogs, isolated in vitro were viable despite defects in the crucial nucleotide synthesis.
# Table 1: List of the studies that evaluated drug resistance in T. gondii.

| No | Drug Description | Treatment (dose/route and time) | In vitro/in vivo | Acute/Chronic | Host/Cell line | Strain | Resistant strain | Focus of the study | Method | Main results | Resistance mechanism | References |
|----|------------------|--------------------------------|-----------------|---------------|----------------|--------|-----------------|-------------------|---------|--------------|---------------------|------------|
| 1  | Anticoccidial drugs | 0.001–100 µg/ml | In vitro | Acute | HFF | RH | – | Resistance development in vitro | Incorporation of [3H]uracil and plaque assays | The resistance in T. gondii by attempting to select mutants in vitro from parasites mutagenized with ethylnitrosourea was explored | – | Ricketts and Pfefferkorn, 1993 |
| 2  | Aprinocid and arprinocid-N-oxide | 360 µg/orally | In vitro/in vivo | Acute | Swiss mice/HFF | RH | R-And<sup>R</sup>-1 | A mutant resistant to arprinocid-N-oxide | Incorporation of [3H]uracil and plaque assays/survival rates | A parasite mutant, R-And<sup>R</sup>-1 was isolated that was 16–20-fold more resistant to arprinocid-N-oxide than was the wild type RH T. gondii. This mutant was not resistant to arprinocid in vitro | – | Pfefferkorn et al., 1988 |
| 3  | Artemisinin | 35 µg/mL | In vitro | Acute | HFF | RH | – | The mechanism of action | Incorporation of [3H]uracil | Mutants resistant were selected to better understand its inhibitory effects on T. gondii | – | Berens et al., 1998 |
| 4  | Artemisinin | 2, 4, 12, or 300 µg/ml | In vitro | Acute | HFF | RH, clone 2F | KN200-1, KN200-6, and STL500-10A | The molecular mode of action | Microneme secretion assays, calcium monitoring, sequencing, and qRT-PCR | Calcium homeostasis is the mechanism of action of artemisinins against apicomplexan parasites | Altering in calcium homeostasis | Nagamune et al., 2007 |
| 5  | Atovaquone | 1 µM | In vitro | Acute | HFF | ME49 | R4, R5, R7, and R32 | The mechanisms of resistance | Incorporation of [3H]uracil, RT-PCR<sup>3</sup> and northern blot analysis | Atovaquone interfered with electron transport at the cytochrome bc<sub>1</sub> complex in T. gondii | Q<sub>0</sub> domain of cytochrome bc<sub>1</sub> complex | McFadden et al., 2000 |
| 6  | Atovaquone | 25nM | In vitro | Acute | Non-fermentable medium | RH | M129L and I254L | Molecular basis of resistance | Measurement of oxygen consumption, ubiquinol-cytochrome c reductase activity and molecular modeling | With the two mutations from T. gondii, M129L and I254L, we have a database of 13 point mutations surrounding the atovaquone binding site | A hydrophobic region of the binding pocket of the cytochrome bc<sub>1</sub> complex | Kessl et al., 2006 |

(Continued)
| No | Drug | Treatment (dose/route/and time) | In vitro/ in vivo | Acute/ Chronic | Host/Cell line | Strain | Resistant strain | Focus of the study | Method | Main results | Resistance mechanism | References |
|----|------|--------------------------------|-------------------|--------------|---------------|--------|-----------------|-------------------|--------|--------------|---------------------|------------|
| 7  | Atovaquone (566C80) or decoquinate | 0.1 and 0.01 µM | In vitro | Acute | HFF | RH | Deg^{R}1 and Atc^{R}1 | The mechanisms of resistance | Incorporation of ^{3}H|uracil, plaque assays, and oxygen uptake | De novo pyrimidine synthesis was not the major biochemical target of atovaquone and decoquinate | Pfefferkorn et al., 1993 |
| 8  | Atovaquone and SDZ | 6.25, 12.5, 25, 50/40, 80, 160, and 320 mg/kg/day/10 days/gavage | In vivo | Acute/ Chronic | Swiss Webster mice | RH, SAF, EGS, D4, D7, CH1, and CH3 | – | The efficacy of drugs for the treatment of mice infected with six strains isolated in Minas Gerais, Brazil | PCR-RFLP, survival rates, the presence of brain cysts, ELISA, and bioassay | Type I strains was more resistant to atovaquone | Alves and Vitor, 2005 |
| 9  | Azithromycin, spiramycin, or clindamycin | 100 ng/ml | In vitro | Acute | HFF | RH | Cln^{R}2, Azi^{R}1, or Spr^{R}1 | The mechanism of action | Incorporation of ^{3}H|uracil, plaque assay, and mitochondrial function measured by oxygen uptake | Mitochondrial protein synthesis was not the target of clindamycin or azithromycin | rRNA genes of the 35-kb genome | Pfefferkorn and Borotz, 1994a |
| 10 | Clindamycin | Up to 100 µg/ml | In vitro | Acute | HFF | RH | Cln^{R}2 | The mechanism of action | Incorporation of ^{3}H|uracil and plaque assays | A difference between the wild type and Cln^{R}2 in a mitochondrial ribosomal protein or in methylilation of mitochondrial rRNA was seen | A mitochondrial ribosomal protein or mitochondrial rRNA genes | Pfefferkorn et al., 1992b |
| 11 | Diclazuril | 0.0025, 0.005, 0.01, 0.1, and 1.0 µg/ml | In vitro/ in vivo | Acute | HFF/Mice | RH, 2 tissue cyst formers, GT-1, and WTD-3 | Dic^{R}1 | A resistant mutant | Transmission electron microscopy assays/survival rates and cysts count in mice brains | Dic^{R}1 mutant of the RH strain, resistant to 1.0 µg/ml diclazuril | – | Lindsay et al., 1995 |
| No | Drug                          | Treatment (dose/route/and time) | In vitro/in vivo | Acute/Chronic | Host/Cell line | Strain            | Resistant strain | Focus of the study | Method                         | Main results                                                                 | Resistance mechanism                          | References              |
|----|-------------------------------|---------------------------------|------------------|---------------|----------------|----------------|------------------|------------------|-------------------|--------------------------------|-------------------------------------------|--------------------------|
| 12 | Fosmidomycin                  | 100 mg/kg/10 days                | In vitro/in vivo | Acute         | HFF/ Webstermice | RH             | –                | The mechanisms of resistance | PCR, sequencing, immunofluorescence, and western blotting assays | Toxoplasma DOXPD pathway is essential in parasites that are highly fosmidomycin resistant | Target DOXP reductoisomerase           | Nair et al., 2011          |
| 13 | FUDR                         | 20 µg/ml                        | In vitro         | Acute         | HFF            | RH             | FUDR^2,1        | The mechanisms of resistance | Plaque assays, autoradiography, and a modified Schmidt-Thannhauser fractionation | The FUDR-resistant was resistant to wildtype T. gondii, fluorouracil, and fluorouridine | Pyrimidine salvage pathways       | Pfefferkorn and Pfefferkorn, 1977 |
| 14 | 5-FUDR and araA^1            | 20 µg/ml                        | In vitro/in vivo | Acute/Chronic | Mice/HFF       | C strain       | Genetic recombination with T. gondii | Immunofluorescence, plaque, enzyme, isotopic, and spectrophotometrically protein assays/cysts count in mice brains | Genetic recombination can readily be demonstrated with suitable mutants of T. gondii | –                           | Pfefferkorn and Pfefferkorn, 1980 |
| 15 | FUDR, HU, araA, and SF^h      | FUDR, 10^{-5}; HU, 2.4 x 10^{-4}, araA, 3 x 10^{-4}, SF, 2.7 x 10^{-7} M | In vitro/in vivo | Acute/Chronic | Mice/HFF       | C strain       | Genetic recombination between two different drug-resistant mutants of T. gondii | Plaque assays/cysts count in mice brains | The gene for FUDR resistance phenotypically suppressed the gene for HU resistance | –                           | Pfefferkorn and Kasper, 1983 |
| 16 | 1-Hydroxyquinolones          | 10–100 nM                       | In vitro         | Acute         | HFF            | RH             | N302S            | T. gondii TgDHODH^2 | PCR, sequencing, plasmid cloning, cDNA synthesis, replication assay, and Enzyme kinetics | The mode of action of HDQ on the T. gondii physiology appears to be a combination of the inhibition of energy metabolism and an inhibition of de novo pyrimidine synthesis | Restoration of de novo pyrimidine biosynthesis | Hegewald et al., 2013 |
| No | Drug | Treatment (dose/route/ and time) | In vitro/ in vivo | Acute/ Chronic | Host/Cell line | Strain | Resistant strain | Focus of the study | Method | Main results | Resistance mechanism | References |
|----|------|-------------------------------|-----------------|----------------|----------------|--------|-----------------|------------------|--------|--------------|----------------------|------------|
| 17 | Monensin | 2 ng/ml/24 h | In vitro | Acute | HFF | RH strain lacking a functional hpt gene | – | Isolation of a *T. gondii* mutant resistant to monensin and the drug-resistant phenotype | Plaque assays, PCR, cloning of TgMSH-1, Southern blot, and immunofluorescence assays | Disruption of TgMSH-1, an MSH in *T. gondii*, confers drug resistance | Disruption of mitochondrion TgMSH-1 | Garrison and Arrizabalaga, 2009 |
| 18 | 1NM-PP1 | 250 or 1,000 nM/3 weeks | In vitro | Acute | Vero cells | PLK/DUAL and PLK/hgprt | PLK/DUAL res.1 and PLK/DUAL res.2 | The mechanism of resistance to 1NM-PP1 | PCR, sequencing, invasion, cell division, calcium-induced egress, and plaque assays | TgMAPK1 as a novel target for 1NM-PP1 activity | The mutation in TgMAPK1 | Sugi et al., 2013 |
| 19 | Oryzalin | 0.5 or 2.5 μM | In vitro | Acute | HFF | RH | 49 independent resistant *T. gondii* lines | The mechanisms of resistance | PCR and sequencing | Toxoplasma resistance to oryzalin is associated with point mutations to α-1-tubulin | α-1-tubulin | Morrisette et al., 2004 |
| 20 | Oryzalin | 0.5 or 2.5 μM | In vitro | Acute | HFF | RH | – | Identification of resistance mutations confer resistance in Toxoplasma | PCR, sequencing immunofluorescence staining and flow cytometry | Mutations to α-1-tubulin confer dinitroaniline resistance at a cost to microtubule function and Toxoplasma fitness | α-1-tubulin | Ma et al., 2007 |
| 21 | Oryzalin | 0.5 μM | In vitro | Acute | HFF | RH | 46 resistant *T. gondii* lines | The development of new anti-parasitic therapies | PCR, sequencing immunofluorescence staining and flow cytometry | 46 *T. gondii* lines were isolated that have suppressed microtubule defects associated with the G142S or the F52Y mutations by acquiring secondary mutations | α-1-tubulin | Ma et al., 2008 |
| 22 | PYR<sup>k</sup> | 1 μM | In vitro | Acute | HFF | RH | M2, M3, M4, M2M3, M2M4, and M3M4 | The mechanisms of resistance | Incorporation of [3H]uracil, plaque assays, PCR, and sequencing | Analogous amino acid substitutions have been identified in the Toxoplasma enzyme that confer drug resistance to transfected parasites | Analogous amino acid substitutions in amino acid | Donald and Roos, 1993 |

(Continued)
| No | Drug | Treatment (dose/route/and time) | In vitro/ in vivo | Acute/ Chronic | Host/Cell line | Strain | Resistant strain | Focus of the study | Method | Main results | Resistance mechanism | References |
|----|------|--------------------------------|-----------------|---------------|----------------|--------|-----------------|-------------------|--------|-------------|----------------------|------------|
| 23 | PYR  | 0, 5, 10, 15, or 20 µM         | In vitro        | Acute         | HFF            | RH, P(LK), and Veg | W25R, L98S, and L134H | The potential role of dhfr polymorphisms | Incorporation of [3H]uracil, POR, and sequencing | PYR is a potent inhibitor of DHFR and three resistance mutations were identified, at amino acid residues | Analogous amino acid substitutions in amino acid residues | Reynolds et al., 2001 |
| 24 | PYR, atovaquone, and SDZ | 0.002–1/0.001–0.5/0.0005–100 mg/kg/gavage | In vitro, in vivo | Chronic      | MRC-5, THP-1, MRC-5, THP-1, and 10 strains from patients with congenital toxoplasmosis | B1, RMS-1995-ABE, and RMS-2001-MAU | The susceptibilities of T. gondii strains belonging to various genotypes to drugs | Specific enzyme-linked immunosorbent assay, qRT-PCR, POR, and direct sequencing | A higher variability was found for SDZ, with a possible resistance of three strains | – | – | Meneceur et al., 2008 |
| 25 | PYR, 5-fluorouracil, and 5-fluorocytosine | 1 µM | In vitro | Acute | HFF | RH | – | The development of improved model genetic systems | DNA extraction, [3H]Xanthine incorporation, southern blot, and western blot analysis | Exogenously supplied cytosine or uracil rescued the growth of CD transgenic T. gondii that were cultured in the presence of cytotoxic concentrations of pyrimidine compounds | DHFR-TS* gene | Fox et al., 1999 |
| 26 | SDZ  | 0–10 mM                        | In vitro        | Acute         | Mice/ Tissue culture | RH | R-SuR-5 and Swa-20 | Identification of SDZ-resistant strains of T. gondii in likely sources of human infection | PCR and sequencing, expression, and purification of protein | The human-derived allelic form encoding the SDZ-resistant enzyme was found in T. gondii associated with a fatal infection | Amino acid residues corresponding to DHPS-407 | Aspinall et al., 2002 |
| 27 | SDZ  | 0, 75, and 1,000 µg/mL/72 h    | In vitro        | Acute         | Vero cells     | RH and ME49 | RH, R-SDZ, ME-49, R-SDZ, TgA 103001, TgH 32006, and TgH 32045 | Identification of genotypic and/or phenotypic markers of SDZ resistance | PCR, qRT-PCR, and nucleotide sequence | T. gondii SDZ resistance is not related to three ABC genes, TgABC B1, TgABC B2, and TgABC C1 | – | Dolwa et al., 2013a |

(Continued)
| No | Drug | Treatment (dose/route and time) | In vitro/ in vivo | Acute/ Chronic | Host/Cell line | Strain | Resistant strain | Focus of the study | Method | Main results | Resistance mechanism | References  |
|----|------|-------------------------------|-----------------|----------------|----------------|--------|------------------|------------------|--------|--------------|-----------------------|------------|
| 28 | SDZ  | 0, 75, and 1,000 µg/mL/72h     | In vitro        | Acute          | Vero cells     | RH and ME49 | RH-R, SDZ, ME-49-R, SDZ, TgA103001, and TgH32006 | The development of two SDZ-resistant strains | ELISA and enzyme immunoassay | IC₅₀-values of SDZ were higher than 1,000 µg/mL for the two natural resistant strains (RH-RSDZ and ME-49-RSDZ) | – | Doliwa et al., 2013b |
| 29 | SDZ  | 0, 75, and 1,000 µg/mL/72h     | In vitro        | Acute          | Vero cells     | RH and ME49 | TgA103001, TgH32006, and TgH32045 | The mechanisms of resistance | Q-RT-PCR, western blot, Real-time qRT-PCR, DIGE, sypro ruby staining, and mass spectrometry analyses | SDZ resistance in T. gondii resistant strains was isolated from clinical cases | Differentially expressed proteins | Doliwa et al., 2013c |
| 30 | SDZ  | 0.2–2 µM/pip | In vitro/in vivo | Acute          | Swiss white mice/HFF | RH | R-SuF3-5 | SDZ resistance in R-SuF3-5 mutant of T. gondii | Incorporation of [³H]thymidine and plaque assays' survival rates | R-SuF3-5 was resistant to SDZ in vitro and in vivo | Inhibit the synthesis of dihydropteroic acid and the synthesis of dihydrofolic acid | Pfefferkorn et al., 1992a |
| 31 | SDZ  | 500 mg/L/ orally/10 days, 100, 200, or 300 mg/kg/ip/6 days | In vivo | Acute/ Chronic | Swiss mice | RH and ME49 | TgCxBr/RN3 (Cx3) and TgPgBr/RN1 (Pg1) | Identification of the pathogenicity and phenotypic SDZ resistance | Parasite isolation/survival rates, ELISA, PCR-RFLP, PCR, and sequencing | The Cx3 and Pg1 isolates showed SDZ resistance | – | Oliveira et al., 2016 |
| 32 | SDZ  | 80, 160, or 320 mg/Kg/day/gavage/10 days | In vivo | Acute          | Swiss mice | RH, GTI, ME49, VEG, TgCTBr03, 07, 08, 11, and 16 | TgCTBr11 | Identification of polymorphisms and profile of resistance to SDZ | PCR-RFLP, survival rates, cyst count, and ELISA assay | TgCTBr11 isolate presented a profile of resistance to SDZ | – | Silva et al., 2017 |
| No | Drug                          | Treatment (dose/route and time) | In vitro/in vivo | Acute/Chronic | Host/Cell line | Strain | Resistant strain | Focus of the study | Method | Main results | Resistance mechanism | References |
|----|-------------------------------|---------------------------------|------------------|---------------|---------------|--------|------------------|-------------------|--------|--------------|----------------------|------------|
| 33 | SDZ, atovaquone, diamidine,   | 0.01, 0.1, and 0.5 mM/1.5, 50,  | In vitro         | Acute         | HFF or Vero cells | PLK    | –                | Incorporation of $[^{[3]}$H]uracil, SDS-polyacrylamide gel electrophoresis, and western blots | The drugs targeted to mitochondria will cause wild type parasites to differentiate from tachyzoites to bradyzoites | –           | Tomavo and Boothroyd, 1995 |
|    | rotenone, antimycin, myxothiazol, and adenosine arabinoside | and 150 nM/0.1, 1, and 5 µg/ml/20, and 100 µM | | | | | | |
| 34 | 6-Thioxanthine                | 20, 40, and 360 µg/m | In vitro         | Acute         | HFF | RH | Thx$^{-1}$ | The mechanisms of resistance | Incorporation of $[^{[3]}$H]thymidine | The lack of the hypoxanthine-guanine phosphoribosyltransferase is the basis for the resistance of Thx$^{-}$-1-6-thioxanthine | The lack of the enzyme to 6-thioxanthine | Pfefferkorn and Borotz, 1994a |

$^a$Reverse transcription polymerase chain reaction.
$^b$Sulfadiazine.
$^c$Enzyme-linked immunosorbent assay.
$^d$1-deoxy-d-xylulose-5-phosphate.
$^e$Fluorodeoxyuridine.
$^f$Adenine arabinoside.
$^g$Hydroxyurea.
$^h$Sinefungin.
$^i$1-hydroxy-2-dodecyl-4(1) quinolone.
$^j$T. gondii MutS homolog.
$^k$Pyrimethamine.
$^l$Dihydrofolate reductase.
$^{m}$RMS-1995-ABE, TRS-2004-REV, TOU-1998-TRI, RMS-2005-HAQ, GRE-1995-MAE, PSP-2005-MUP, GRE-1998-TRA, RMS-2003-TOU, NED, RMS-1994-LEF, RMS-2003-DJO, RMS-2001-MAU, GUY-2003-MEL.
$^n$Dihydrofolate reductase-thymidylate synthase.
$^o$Difference-gel electrophoresis.
$^p$Intraperitoneally.
enzymes (Pfefferkorn and Pfefferkorn, 1977, 1978; Pfefferkorn and Borotz, 1994b).

Reynolds et al. (2001) reported that using in vitro mutagenesis, single-point mutations in T. gondii DHFR-TS (dihydrofolate reductase-thymidylate synthase) (e.g., W25R, L198S, and L134H) can produce drug resistance in RH strain parasites compared with type II and type III strains. In addition, using site directed mutagenesis and transgenic experiments several mutations were induced in the DHFR-TS gene related to resistance to PYR. The T83N mutation was found to confer resistance to PYR (Donald and Roos, 1993). Resistance is even increased when T83N mutation is associated with mutation of S36R and F245S (Reynolds et al., 2001). Meneceur et al. reported variability in the susceptibilities of T. gondii strains to PYR, with no clear evidence of drug resistance and no relationship with strain genotype or defined mutations in drug target genes (Meneceur et al., 2008).

Given that PYR resistance is differently marked among various strains of T. gondii; it can provide new insights into potential sources of treatment failures and possible drug resistance mechanisms.

**SULFONAMIDES RESISTANCE**

Sulfonamides, in conjunction with PYR, are a mainstay of toxoplasmosis treatment, although AIDS patients are unable to tolerate this treatment. The first experimentally induced drug-resistance was resistance to sulfamethoxazole, when the parasite was exposed to sub-lethal doses of the drug for long periods (Sander and Midtvedt, 1971; Luft and Remington, 1992; Reynolds and Roos, 1998). In a study by Pfefferkorn et al. (1992a), researchers induced resistance in RH strain using chemical mutagenesis and generating parasites in environments with gradually increased SDZ concentrations. Sulfamethoxazole-resistant strain (R-SulR) appeared to be more resistant than the parental RH strain. Further study on R-SulR confirmed previous findings that this strain is sulfonamide resistant with an IC50 value near 5 mM like another SDZ resistant strain Swa-20 which was isolated from patients with clinical toxoplasmosis (Aspinall et al., 2002). In Aspinall et al. (Aspinall et al., 2002) study, the presence of one mutation at positions 407 of DHPS was associated with sulfonamides resistance by direct sequencing of PCR products (Aspinall et al., 2002). This mutation was also retrieved in laboratory induced R-SulR (Pfefferkorn et al., 1992a). As, mutation 407 was not identified in five T. gondii Brazilian isolates obtained from newborns with congenital toxoplasmosis (Silva et al., 2017), a larger number of atypical isolates of T. gondii must be evaluated to confirm these results.

Meneceur et al. (Meneceur et al., 2008) isolated three strains from clinical cases containing: TgA 103001, previously described as B1 (Type I strain), TgH 32006, previously described as RMS-1995-ABE (Type II strain), and TgH 32045, previously described as RMS-2001-MAU (Type II variant strain) which were detected as resistant to SDZ. Doliwa et al. (2013c) found 44% over-expressed proteins in resistant T. gondii strains. The virulence-associated rhoptry protein, ROP2A, was found in greater abundance in both naturally resistant Type II strains TgH 32006 and TgH 32045. Totally, 31 proteins were identified which are differentially modulated between SDZ resistant and sensitive strains of T. gondii according to their genotype using proteomics approach. Recently, two SDZ resistant strains were developed called RH-RSDZ and ME-49-RSDZ in vitro (Doliwa et al., 2013b). Also, other studies analyzed genotypic and/or phenotypic markers of resistance in T. gondii (Doliwa et al., 2013a).

In Oliveira et al. (Oliveira et al., 2016) study, Ck3 and Pgl T. gondii isolates showed SDZ resistance in samples collected from livestock intended for human consumption. Monitoring the presence of resistant parasites, particularly in food products, would seem a prudent public health measure (Sims, 2009).

Silva et al. (Silva et al., 2017) have confirmed the existence of a Brazilian T. gondii isolate, TgCTBr11, isolated from newborns infected with congenital toxoplasmosis, which is resistant to SDZ. Despite the large number of polymorphisms identified in the DHPS gene, no association was found between the profile of susceptibility to SDZ and the virulence-phenotype and genotype of the parasite. However, the mutation in the DHPS gene is known to confer resistance in T. gondii and has demonstrated cross-resistance to several sulfonamides including SDZ and sulfamethoxazole. Until now, T. gondii SDZ resistance has not been related to genetic mutations in DHPS in all clinical isolates. Based on these findings, the range of resistance to sulfonamide is greater than PYR or atovaquone.

**ATOVAQUONE RESISTANCE**

Atovaquone is a substituted hydroxynaphthoquinone compound that is being used clinically for the treatment of T. gondii infections against chronic bradyzoite stage via mitochondrial electron transport chain inhibition (Kovacs, 1992; Tomavo and Boothroyd, 1995); however, atovaquone prophylaxis and treatment failure was reported in hematopoietic cell transplant recipients and AIDS patients (Chirgwin et al., 2002; Gajurel et al., 2016).

Cytochrome b clothing (Cyt b) is a membrane-bound enzyme of the respiratory electron transfer chain located in the inner mitochondrial membrane. It is a successful drug target for combatting diseases, including T. gondii, Plasmodium falciparum, and Babesia microti (Winter et al., 2008; Doggett et al., 2012; Lawres et al., 2016). Cyt b reduces cytochrome c and generates an electrochemical gradient by transferring protons to the intermembrane space. It also creates ubiquinone for pyrimidine biosynthesis. Cyt b has two active sites, the bc1 Qo site (oxidizes ubiquinol) and the bc1 Qi site (reduces ubiquinone) (Crofts, 2004).

The genetic evidence revealed that atovaquone, targets T. gondii Cyt b by binding to Qo domain of cytochrome b confers resistance to atovaquone (McFadden et al., 1997, 2001). M129L and I254L mutations have been identified to be related to atovaquone resistance in T. gondii (31, 32).

Of course, the investigation by Meneceur et al. (Meneceur et al., 2008) did not show any of these mutations, thus further studies will help a better understanding of resistance mechanisms.
Interestingly, Endochin-like quinolones (ELQs) have been shown to be active against atovaquone-resistant Plasmodium and Babesia (Winter et al., 2008; Lawres et al., 2016). Also, treatment with 4(1H)-pyridone compounds, GW844520, and GSK932121, showed anti-malarial activity in vivo (Capper et al., 2015). These anti-parasitic agents have a similar mechanism of action with atovaquone but by inhibiting the Qi site of CYT b6. Furthermore, ELQ-271 and ELQ-316 showed remarkable effects against acute and latent toxoplasmosis at low doses (Doggett et al., 2012). It is likely that ELQs act at the T. gondii cytochrome b Qi site. Therefore, ELQs and 4(1H)-pyridone compounds are promising candidates for the treatment against atovaquone-resistant Toxoplasma.

**MUTANTS OF T. gondii RESISTANT TO 1-HYDROXYQUINOLONES**

1-Hydroxyquinolones are effective inhibitors of T. gondii replication. Using a drug resistant strain, Hegewald et al. (2013) described that the enzyme dihydroorotate dehydrogenase (TgDHODH) of T. gondii is a relevant target for 1-Hydroxy-2-dodecyl-4(1H) quinolone (HDQ) and compound B (1-Hydroxyquinolones derivatives). Thus, drug resistant mutants are approved tools for the identification of drug targets for future to select new anti-Toxoplasma drugs.

**MUTANTS OF T. gondii RESISTANT TO CLINDAMYCIN, SPIRAMYCIN, AND AZITHROMYCIN**

Antibiotics such as clindamycin, spiramycin, and azithromycin are known to be active against T. gondii. However, mutant ClnR-2 (RH) was cross-resistant to clindamycin, azithromycin, and spiramycin antibiotics (Pfefferkorn et al., 1992b; Pfefferkorn and Borotz, 1994a). Interestingly, resistance to these drugs is encoded in the rRNA genes of the 35-kb genome in T. gondii and the apicoplast protein synthesis is known as target of these antibiotics action against T. gondii (Pfefferkorn and Borotz, 1994a; McFadden et al., 2001).

**MUTANTS OF T. gondii RESISTANT TO ARTEMISININ (ART)**

ART is a natural product that is produced by Artemisia annua plant. This important compound plays an indispensable role for combating malaria (Cui et al., 2015). ART is also effective against Toxoplasma in vitro and in vivo (Schultz et al., 2014), although it is not generally used in the treatment of toxoplasmosis. Recent concerns about the development of ART resistance have lead to the exploration of its mechanisms of action. Berens et al. (1998) characterized five clonal isolates that showed cross-resistance to the ART derivatives, dihydroartemisinin and artemether in laboratory studies. In a subsequent study, Nagamune et al. (2007) generated chemically derived T. gondii mutants that were resistant to growth inhibition by ART in vitro. Three ART-resistant mutants were resistant to the induction of protein secretion from micronemes, a calcium-dependent process that is triggered by artemisinin. Based on these findings, calcium homeostasis is involved in the mechanism of ART action against T. gondii and other apicomplexan parasites.

**MUTANTS OF T. gondii RESISTANT TO 1NM-PP1**

T. gondii CDPK1 (TgCDPK1) was found to be the target of 1NM-PP1, which is a bumped kinase inhibitor (BKIs). CDPK1 contains an atypically small glycine gatekeeper residue, which allows entry of BKIs into the ATP binding domain. Most mammalian kinases have larger gatekeeper residues, e.g., methionine. CDPK1 is involved in microneme secretion and host cell invasion and egress. When TgCDPK1 was mutated at position 128 from glycine to methionine, parasites became BKI resistant (Sugi et al., 2010). Resistance to 1NM-PP1 can also be acquired via a mutation in T. gondii mitogen-activated protein kinase like 1, which indicates that this kinase could also be a target (Sugi et al., 2013, 2015). However, CDPK1 has become an important drug target for more recently developed and largely improved BKIs in a variety of apicomplexans beside T. gondii (Van Voorhis et al., 2017).

**MUTANTS OF T. gondii RESISTANT TO DINITROANILINES**

T. gondii is sensitive to dinitroaniline compounds, which disrupt microtubules without affecting host cells. T. gondii containing alpha-tubulin point mutations are dinitroaniline resistant. Ma et al. (2008) identified T. gondii lines that have suppressed microtuble defects in G142S or F52Y mutations. In addition, secondary resistant mutations were isolated that corrects fitness defects in the T. gondii parasite. Based on the current findings, targeting parasite microtubules can be a viable strategy for developing new anti-parasitic therapies.

**MUTANTS OF T. gondii RESISTANT TO ANTI-COCCIDIAL DRUGS**

Anticoecidial agents were assessed in T. gondii mutants for development of resistance in vitro. Mutants had 20- to 50-fold-reduced susceptibility to decoquinate, arprinocid-N-oxide, and CP-25,415. In addition, ionophore-resistant T. gondii mutants were explored in vitro; however, resistance to all of the mutants except ionophores occurs in coccidia in vivo. The availability of a T. gondii mutant resistant to a different drug could aid for assessing the risk of developing resistance in Eimeria species (Ricketts and Pfefferkorn, 1993).

Diclazuril, an anticoecidial compound, is a safe and effective drug that inhibits tachyzoite production of RH strain in T. gondii by >97% at therapeutic dose levels (Oz, 2014). Lindsay et al. (1995) have shown that formation of T. gondii tissue cysts was not prevented by treatment with diclazuril, in vitro. They also showed that GT-1, WTD-3 strains, and a mutant RH strain of T. gondii were resistant to 1.0 µg/ml of diclazuril.

Monensin is a polyether anti-coccidial antibiotic that has been effective against T. gondii. However, within 3 years of...
the drug introduction, monensin-resistant Eimeria maxima were noted. Thus, *T. gondii* was used for studying the monensin’s mechanisms of resistance. The investigators have shown that resistance phenotype is caused by the disruption of *T. gondii* homologs MSH-1 (a homolog of the DNA repair enzyme, MutS). Interestingly, this enzyme localizes to the *T. gondii* parasite mitochondrion (Garrison and Arrizabalaga, 2009). Subsequent studies showed that the disruption of the autophagy pathway could result in drug resistance. Autophagy pathway is a potentially important model of cell death of *T. gondii* in response to monensin (Lavine and Arrizabalaga, 2012).

**MODE OF DRUG ACTION AND MECHANISM OF DRUG RESISTANCE IN *T. gondii***

Several targets were identified against *T. gondii* including folate synthesis pathway, mitochondrial electron transport chain, calcium dependent ATPases, protein synthesis, mitogen-activated protein kinase 1, enzyme TgDHODH, and microtubules for PYR and SDZ, atovaquone, ART, clindamycin, spiramycin and azithromycin, INM-PP1 and 1-hydroxyquinolones HDQ, and compound B, respectively. Thus, drug resistant mutants are approved tools for the characterization of drug targets for future to select new anti-*Toxoplasma* drugs with specific activity against the parasite.

Also, mechanisms of drug resistance in *T. gondii* have been described. Interestingly, analogous amino acid substitutions in the *Toxoplasma* enzyme have been identified to confer PYR resistance in transfected parasites (Donald and Roos, 1993). Moreover, resistance to clindamycin, spiramycin and azithromycin is encoded in the rRNA genes of the 35-kb genome in *T. gondii*.

There are numerous reports with a focus on identifying SDZ resistance mechanisms. However, *T. gondii* SDZ resistance mechanism has not been proved so far. As a consequence, understanding mechanisms of drug resistance in *T. gondii* is essential for controlling the disease particularly among immunocompromised patients. Also, it helps identify targets that are crucial to the parasite and predicts which combinations of drugs should act synergistically (McFadden et al., 2001).

**RECENT TRENDS IN DRUG RESISTANCE IN *T. gondii***

Studies in the past 10 years indicated that drug resistance to SDZ is actually increased. Most resistant strains were found in clinical cases between 2013 and 2017. However, a possible resistance was reported in three strains of *T. gondii* in 2008. Also, six strains resistant to SDZ were found in clinical cases between 2013 and 2017.

The only worrying trend was a very slight recent increase in SDZ resistance to Brazilian *T. gondii* strains obtained from livestock and humans newborns with congenital toxoplasmosis between 2016 and 2017 where *T. gondii* prevalence in Brazil is high (77.5%) (Pappas et al., 2009). Thus, establishing a more effective therapeutic scheme in the treatment of toxoplasmosis is critically needed.

**CONCLUSIONS***

Recent experimental studies in clinical cases have clearly shown that drug resistance in *Toxoplasma* is ongoing. The emergence of *T. gondii* strains resistant to current drugs reviewed here represents a concern not only for treatment failure but also for increased clinical severity in immunocompromised patients. Thus, understanding mechanisms of drug resistance is essential for controlling the disease and it helps identify targets that are crucial to the parasite and predicts which combinations of drugs should act synergistically. Also, establishing a more effective therapeutic scheme in the treatment of toxoplasmosis, particularly among high-risk individuals is critically needed. Additionally, monitoring the presence of resistant parasites, particularly in food products, would thus seem a prudent public health measure. Further development of a greater understanding of exact mechanisms of drug resistance in *T. gondii* is needed to improve the therapeutic outcomes in patients.

**AUTHOR CONTRIBUTIONS**

AD conceived the study. AD and MS designed the study protocol. MM, SS, and AT searched the databases. MM wrote the manuscript. SM and SA critically revised the manuscript. MM, SS, and AT searched the databases. MM wrote the manuscript. SM and SA critically revised the manuscript. All authors read and approved the final manuscript for publication.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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