Resistance as a tool in the study of old and new drug targets in Toxoplasma

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Abstract Drug resistance generated in vitro in the protozoan parasite Toxoplasma gondii is described. We focus on drugs that are in use in patients, that show some promise for such use, or that represent lead compounds for further development. No instance has yet been reported where resistance to any of these drugs has arisen in a patient or in the field although different strains do show varying degrees of sensitivity. For many of these drugs, however, resistant lines have been generated in the laboratory and these have proven very useful for elucidating a given drug’s target. These targets range from metabolic pathways in the cytosol to organelar functions encoded in the mitochondrion or plastid. Such information makes predictions about how fast resistance will arise in the field but more importantly, it helps identify targets that are crucial to the parasite and predicts which combinations of drugs should act synergistically.

INTRODUCTION

Toxoplasma gondii is a protozoan parasite within the phylum Apicomplexa and class Sporozoa. Its close cousins include Eimeria, Neospora and Sarcocystis, all important pathogens of animals. It is a more distant relative of Plasmodium, the cause of malaria. Toxoplasma is one of the most successful protozoan parasites on earth with a host range that includes most warm-blooded animals and a geographic range that is nearly world-wide and with a very high prevalence in many regions.

The life cycle of Toxoplasma is complex and includes both sexual and asexual components. The sexual cycle occurs exclusively in the intestinal epithelium of cats and is initiated when an infected bird or rodent is eaten. Following differentiation to gametes and fertilization, environmentally stable oocysts are eventually shed in the cat’s feces. These can be ingested by grazing animals, including birds, rodents and herbivores. Humans that accidentally ingest contaminated soil, for example eating uncooked vegetables, can also become infected. Once in the intermediate host, the oocysts rupture and sporozoites emerge to infect the new host’s gut epithelium. There the sporozoites differentiate to tachyzoites, which disseminate throughout the body of the host, eliciting a potent immune response.

Toxoplasma is an obligate, intracellular parasite and once inside a host is capable of infecting nearly any cell type. The strong immune response is apparently a signal for tachyzoites to differentiate to bradyzoites and encyst within brain tissue and occasionally skeletal or cardiac muscle. These tissue cysts lead to a life-long chronic infection of the host and are infectious to other intermediate hosts if the infected animal is eaten by carnivores or scavengers. If the ingesting animal is a cat, the parasites enter into the sexual cycle. If the ingesting animal is a warm-blooded animal other than a cat, the bradyzoites released from the ingested tissue cysts differentiate back to tachyzoites and another asexual cycle of infection is initiated.

In humans, Toxoplasma infection causes a range of disease depending on the immune status and age of the host. The strain of parasite responsible for the infection may also affect disease outcome. The parasite is a frequent cause of encephalitis in AIDS patients and occasionally in other immunocompromised states. This condition is life threatening if left untreated. In the developing fetus of an otherwise healthy woman, Toxoplasma can cause death or lead to severe ocular and neurological manifestations later in life.

Even in healthy adults, infection can lead to sometimes severe disease, including retinchoroiditis.

In most cases, infection with Toxoplasma can be effectively treated with a range of drugs. The most common treatment (except during pregnancy) is a combination of pyrimethamine and sulfa. When patients become intolerant or allergic to sulfa, clindamycin is often the second choice for treatment. Atovaquone has also been used with some success in people, although its efficacy has so far appeared to be less than what is seen with the pyrimethamine combinations. A number of other drugs have been used experimentally but few have reached the clinic.

Although toxoplasmosis is a zoonosis, control strategies have so far focused on public health measures to educate people about ways to avoid infection and/or screening programs for high-risk patients (e.g. pregnant women in areas where human infection is prevalent). Drug treatment of the animal reservoir, even as a prophylactic measure, has not been used, so selection for resistance in these hosts has not occurred. In humans, where prolonged treatment strategies are sometimes necessary, resistance could in theory arise but such a phenomenon has yet to be documented. Fortunately, given there is no human-to-human transmission, if development of resistance does occasionally happen, the consequences would be restricted to the patient concerned. As a result, drug resistant strains are not now and are unlikely to become a major clinical problem for treatment of this disease.

Development of resistance is only one potential explanation for the occasional failure of infection to respond to chemotherapy. Nevertheless, the study of drug resistance generated in the laboratory is an important topic for it can yield key information about a given drug’s target. This can help in improving a drug’s efficacy through subtle changes in its chemical structure or by combination therapy. Drug resistance strains generated in vitro can also be used to estimate how fast resistance will arise in the field.

Laboratory studies have also shown that different strains of Toxoplasma can differ markedly in their susceptibility to a given drug (up to 100-fold different IC50). The reasons for this are not known but this observation is clinically important to ensure that the optimal dosage is used.
In this review, we will describe results that have been obtained on resistance to several drugs that are currently in clinical use or which could ultimately lead to new drug treatments. They will be described in groups, based on their presumed or known target.

**DRUGS AFFECTING NUCLEOTIDE METABOLISM**

Pyrimethamine and sulfa-containing drugs, usually sulfadiazine, are the standard treatment for acute cases of toxoplasmosis. This combination was among the original chemotherapies identified and remains one of the most effective therapies to date.9

Pyrimethamine and sulfa have a synergistic effect, targeting two different enzymes along the folate synthesis pathway. Sulfa compounds competitively inhibit dihydropteroate synthase (DHPS) while pyrimethamine inhibits the downstream enzyme dihydrofolate reductase (DHFH). In *Toxoplasma*, unlike in humans, DHPS and DHFR activities are parts of bifunctional proteins. The activities of DHPS and hydroxymethyl-dihydropterin pyrophosphokinase, the enzyme acting prior to DHPS in folate synthesis, are present in a single homodimeric protein containing distinct domains for each enzyme (PPPK-DHPS).10, 11 Likewise, DHFR and thymidylate synthase (TS) activities exist as two active domains within another homodimeric protein (DHFR-TS).12 TS acts immediately downstream of DHFR and couples folate reduction to the synthesis of dTMP from dUMP. *Toxoplasma* is unable to salvage pyrimidines other than uracil from their host cell milieu, thus, in the presence of pyrimethamine/sulfa the pyrimidine pools are fatally depleted.

Despite selectivity of pyrimethamine and sulfadiazine for the parasite enzymes, inhibition of the mammalian enzymes does occur. One of the main side effects of treatment is sulfadiazine-induced immune suppression. This can be partially reversed through the co-administration of leucovorin, a folic acid analog salvaged exclusively by mammalian cells. Numerous modifications to pyrimethamine, the less active DHFR inhibitors methotrexate and trimethoprim, and analogs of the DHPS substrate para-aminobenzoic acid (pABA) have been studied.10, 13–15 None so far contain both increased selectivity and increased potency, although several candidates with improvement in a single criterion were identified.

Some of the polymorphisms identified in pyrimethamine-resistant field isolates of *Plasmodium* confer pyrimethamine resistance in *Toxoplasma*.16 By molecularly transforming parasites with DHFR-TS genes containing the polymorphisms, several amino acid residues, either alone or in combination, were shown to be important in development of resistance. All mutations identified are predicted to affect the binding of either the NADPH cofactor (F245S) or the folate substrate (A10V, S36R, T83N/S) when the positions were mapped to a putative *T. gondii* structure, based on the DHFR-TS crystal structure from *Leishmania*. Biochemical assessment of the effect of the mutations on DHFR activity demonstrated that different combinations would dramatically alter the mechanism of pyrimethamine inhibition from uncompetitive mixed inhibition for the drug-sensitive enzyme to non-competitive or competitive inhibition for the drug-resistant enzymes.16 The study of pyrimethamine-resistance in *Toxoplasma* demonstrates the importance of cofactor binding and enzyme kinetics for the identification of novel DHFR inhibitors.

The PPPK-DHPS enzyme of sulfa-resistant *Toxoplasma*, generated by chemical mutagenesis, demonstrated an increase in Km for pABA, the substrate for DHPS, and also dihydropteroate pyrophosphate, the substrate for PPPK.17 The mutation(s) leading to resistance in these parasites are unknown and so it is unclear if alterations affecting both domains are necessary for resistance. The drug-resistance phenotype was reversible by pABA suggesting the binding pocket of the substrate/inhibitor was not grossly altered. Polymorphisms in PPPK-DHPS that lead to sulfadoxine-resistant malaria have yet to be tested in *Toxoplasma*. However, the prediction from the malaria data is that a single mutation in the substrate binding pocket will provide an initial level of drug-resistance followed by the development of other mutations, not necessarily in the binding pocket, which will confer a further increase in the level of drug-resistance.18

A limited number of clinical isolates from patients that failed treatment with pyrimethamine alone or pyrimethamine plus sulfadiazine have been examined for drug-resistance and no evidence of mutations or altered drug-sensitivity were identified.11, 19

The long success of pyrimethamine and sulfadiazine points to the vulnerability of the parasite to inhibition of its nucleotide metabolic pathways. This is further supported by the activity of purine and pyrimidine analogs: 5-Fluorodeoxyuridine (FUDR), adenosine arabinoside (Ara-A), and 6-thioxanthine (6-TX). Each are taken up by the parasite’s salvage pathways and metabolized to inhibitory monophosphate nucleotides by the relevant enzyme (uracil phosphoribosyltransferase, UPRT; adenosine kinase, AK) or to an inhibitory substrate of a metabolic enzyme (hypoxanthine-xanthine-guanine phosphoribosyltransferase, HXGPR).20–23 Resistant strains selected in vitro were viable despite defects in these crucial nucleotide synthesis enzymes suggesting that individual salvage pathways are not essential for parasite viability.20–22 Nonetheless, the parasite is obviously reliant on nucleotides and combination therapy aimed at targeting multiple pathways may prove successful.

FUDR, Ara-A, and 6-TX do show activity against mammalian cells but can be considered lead agents for the development of more parasite-specific inhibitors. Genetic studies and crystal structures for AK, UPRT, and HXGPR have identified differences from the human enzymes, including substrate specificity and catalytic mechanisms, that could be exploited in designing toxic substrate analogs.22–26 In addition, a high capacity, low affinity transporter of adenosine and other purines has been identified and is a viable chemotherapeutic target since adenosine is the predominant purine salvaged.27

**ELECTRON TRANSPORT INHIBITORS**

A novel hydroxynaphthoquinone compound, atovaquone, originally developed for the treatment of malaria, has broad spectrum activity against protozoan parasites (*Plasmodium, Toxoplasma, Theileria* and *Babesia*) and the opportunistic fungal pathogen *Pneumocystis carinii*.28–51 Significantly, it is
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The cytochrome bc$_1$ complex functions as an acceptor of electrons from multiple enzymatic reactions and in turn commits them to energy production by donating those electrons to the cytochrome c oxidase complex. A crucial component of the complex is ubiquinone (coenzyme Q) which undergoes the necessary oxidation-reduction reactions to transfer electrons. Ubiquinone can occupy two sites within the cytochrome bc$_1$ complex: the Q$_i$ site where electrons are accepted and the Q$_o$ site where electrons are donated. Analysis of the cytochrome b gene from atovaquone-resistant mutants of *Toxoplasma*, generated by chemical mutagenesis, demonstrated that either of two different mutations (M129L, I254L) affecting the Q$_o$ domain of the protein are likely to be responsible for drug-resistance. These residues are within the ubiquinone binding area of the Q$_o$ domain and presumably disrupt the binding of atovaquone. Atovaquone-resistance can also be mediated by changes not altering cytochrome b as one mutant under study contained wild type cytochrome b sequence. Parasites resistant to another potential inhibitor of mitochondrial function, decoquinate, also contained a mutation (F260L) within the predicted Q$_o$ domain of the cytochrome b gene. Thus, the resistance data suggest that atovaquone and decoquinate inhibit Q$_o$ domain activity. Mutations identified in atovaquone-resistant strains of *Plasmodium* and *Pneumocystis* also point to the Q$_o$ domain as the target of the drug. Strikingly, the residues mutated in *Toxoplasma* are also among those found altered in atovaquone-resistant *Plasmodium*. The use of atovaquone as monotherapy for malaria led to the rapid development of resistance, and development of resistance is a major limiting factor for antococcidials. Whether rapid atovaquone resistance develops in the clinical setting for *Toxoplasma* remains to be determined. However, such resistance would be confined to the individual and would not lead to widespread development of resistant strains in the environment. Utilizing the available co-crystal structure of the chicken cytochrome bc$_1$ complex with the Q$_i$ inhibitor stigmatellin, a molecular model for atovaquone binding and resistance development has been put forth that may aid in the development of new Q$_i$ inhibitors.

INHIBITORS OF PROKARYOTIC TRANSLATION

Antibiotics such as tetracyclines, lincosamides, and macrolides are receiving increased attention for the treatment of a number of parasitic infections. The lincosamide clindamycin has become the main alternative to the standard pyrimethamine/sulfadiazine combination for the treatment of toxoplasmosis when the standard therapy is not well tolerated (reviewed in refs 2, 44). Macrolides like clarithromycin and azithromycin protect against toxoplasmosis in the murine model, and are being evaluated as alternative therapies as well. Spiramycin, a macrolide antibiotic, is currently the drug of choice for the treatment of toxoplasmosis during pregnancy in Europe. These antibiotics have also proven useful for the clinical management of other diseases caused by apicomplexan parasites, including chloroquine-resistant malaria, *Cryptosporidium* infection in AIDS patients, and *Babesia* infection (reviewed in ref 45).

The antibiotics mentioned above are structurally diverse drugs that inhibit translation in bacteria (reviewed in ref. 46), which suggests they may have a common mechanism of action in protozoa as well. In prokaryotes, lincosamides, macrolides, and chloramphenicol all target the large subunit of the ribosome, and will be referred to as large subunit (LSU) antibiotics. LSU antibiotics share a peculiar phenomenon in *Toxoplasma*: exposure of intracellular parasites for only a few hours causes a dramatic reduction in their long-term viability in vitro, although no effect on parasite morphology or replication is apparent for at least two days. The effect on replication only becomes apparent after the parasites lyse the host cell and invade a new host and is associated with the appearance of multinucleated bodies. A markedly delayed effect for LSU antibiotics and tetracyclines has also been described in *Plasmodium*. The molecular target of inhibitors of prokaryotic translation within the protozoa has been the subject of much speculation. Initially, the suspected target was mitochondrial translation, as this would be prokaryotic in nature. Consistent with this hypothesis, LSU antibiotic and tetracycline activity in *Plasmodium* was shown to be extremely oxygen-dependent, and tetracycline was reported to inhibit mitochondrial protein synthesis and function. In the early 1990s, the sequence form a small extrachromosomal DNA was found to have clear similarity to nonphotosynthetic plastids. This genome was localized to an organelle that had been previously identified in electron-micrographs. Since then, it has become apparent that this plastid, now termed the 'apicoplast' to distinguish it from plant and algal plastids, constitutes a valid target for chemotherapy (reviewed in ref. 56).

Five pieces of evidence, obtained in *Toxoplasma*, now clearly indicate that the apicoplast is the target for LSU antibiotics: (1) Based on bacterial data, only the apicoplast ribosomal sequence, and not the mitochondrial sequence, predicts sensitivity to macrolides and clindamycin; (2) Clindamycin has no apparent effect on mitochondrial protein synthesis or function; (3) Several LSU antibiotics were shown to inhibit plastid DNA replication; (4) Ciprofloxacin, a fluoroquinolone that inhibits plastid DNA gyrase, shows a delayed kinetics of killing similar to LSU antibiotics, suggesting that LSU antibiotics and fluoroquinolones share the apicoplast as a target; (5) A mutation in the apicoplast rRNA was found in association with clindamycin resistance. The ClnR-4 mutant carries a mutation located in close vicinity to the main determinant of antibiotic resistance in bacterial rRNA, in a residue that is protected by clindamycin from ketoxal modification in *E. coli*. ClnR-4 exhibits limited cross-resistance to azithromycin and chloramphenicol, but remains sensitive to two drugs with unrelated targets, pyrimethamine and antimycin A.
This observation is consistent with the hypothesis that most or all LSU antibiotics share a target in *Toxoplasma*. Similarly, studies with an azithromycin-resistant mutant (Azir\(^-\)\(^1\)) and another clindamycin-resistant strain (ClinR\(^\text{R-2}\)) also suggest that these two drugs share the same target.\(^{47, 58}\) ClinR\(^\text{R-2}\) is completely cross-resistant to azithromycin, and partially cross-resistant to spiramicin.\(^{59}\) Azir\(^-\)\(^1\) is cross-resistant to spiramycin but sensitive to clindamycin.\(^{58}\) The phenotype of ClinR\(^\text{R-2}\), with strong cross-resistance between two structurally unrelated compounds, is consistent with a modified target. Azir\(^-\)\(^1\) could also result from ribosomal modification, since no alteration in azithromycin uptake or retention was detected.\(^{57}\) Finding no mutation in domain V for either mutant,\(^{57}\) does not rule out an alteration in the ribosomal RNA as a plausible mechanism of resistance in these parasites. A dominant mutation in domain V, that would be present in 1 of the ∼12 copies of apicoplast rDNA,\(^{50}\) would be difficult to detect by standard sequencing methods (each parasite has about six apicoplast DNA circles, each of which has two genes for the rRNA). The mutation might also be present elsewhere in the apicoplast ribosomal RNA. Deletions in domain II, for example, have been associated with erythromycin resistance.\(^{60}\) Alternatively, clindamycin resistance in these latter mutants might be the result of an RNA-modifying activity.

In sum, these data indicate that LSU antibiotics generally target the apicoplast while SSU antibiotics, or at least tetracycline, target the mitochondrion. Until resistance mutations are identified for the latter class of drugs, however, their exact target will not be certain. Some circumstantial data suggest that the apicoplast may also be inhibited by a SSU antibiotic, doxycycline: this drug shows a delayed effect that is comparable to that of clindamycin,\(^{49}\) and the ClinR\(^\text{R-4}\) mutant is slightly hypersensitive to doxycycline.\(^{49}\)

The doxycycline hypersensitivity phenotype could result from a conformational alteration induced by the clindamycin resistance mutation in the LSU. Even though the main binding site for tetracyclines is in the 30S subunit, they bind across the interface between subunits to the central loop of domain V in a manner similar to chloramphenicol.\(^{61}\) It is conceivable, then, that a structural change in the LSU may affect doxycycline sensitivity. These data are not necessarily in conflict with the evidence supporting a mitochondrial target for tetracycline in apicomplexans.\(^{48, 52, 53}\) Unlike LSU antibiotics, the specificity of tetracyclines is determined by the permeability properties of the target membrane rather than by the target rRNA sequence. Thus, depending on their chemical structure, different tetracyclines might vary in their specificity for the mitochondrion versus the apicoplast, and, at a high enough concentration, it is conceivable that they may target both organelles.

**OTHER ANTICOCCIDIALS**

*Eimeria* parasites are pathogens that are closely related to *Toxoplasma*. They cause coccidiosis, a disease that leads to important economic loss in poultry and, to a lesser degree, in ruminants and rabbits. Given the strong biological similarities between *Eimeria* and *Toxoplasma*, anticoccidials are potential sources of or at least leads for alternative anti-*Toxoplasma* agents. Many of the anticoccidials are active against *Toxoplasma* in vitro,\(^{62}\) and two of these agents, arprinocid and diclazuril, have shown a strong protective effect in the mouse model of acute toxoplasmosis.\(^{63, 64}\)

Mutants resistant to anticoccidials provide insights into the mechanism of action of these drugs. As mentioned above, a mutation associated with decoquinate resistance located in the cytochrome b gene showed that mitochondrial electron transport is the target of decoquinate.\(^{35}\) R-Ano\(^\text{R-1}\), a mutant that is 20-fold resistant to arprinocid-N-oxide (Ano), a metabolite of arprinocid, is another case in point.\(^{59}\) While R-Ano\(^\text{R-1}\) was sensitive to arprinocid in vitro, in vivo an arprinocid treatment that is effective against a wild-type infection failed to provide protection against R-Ano\(^\text{R-1}\). This implies that a metabolite of arprinocid, presumably Ano, and not arprinocid itself, is the therapeutically-active compound. Strains that are resistant to diclazuril (a triazine) and artemisinin (an antimalarial sesquiterpene lactone) have also been characterized.\(^{66, 67}\) These strains were obtained by serial passage in increasing concentrations of drug and may contain multiple mutations or amplifications that contribute to the phenotype. The molecular basis of resistance in these later mutants has not yet been determined.

The usefulness of anticoccidial drugs has been severely limited by the development of resistance.\(^{51–45}\) *Toxoplasma* strains were selected for resistance to various anticoccidials to establish the potential for resistance development.\(^{62}\) The strongest level of drug-resistance was only produced by selection with decoquinate, arprinocid-N-oxide, and CP-25, 415 (20–50-fold more resistant than the parental strain), whereas no resistant mutants were obtained from the selections with clopidol, toltrazuril, and robenidine. These results are in agreement with the field experience for *Eimeria tenella*, validating the generation of anticoccidial-resistant mutants in *Toxoplasma* in vitro as a model system for evaluation of the potential of anticoccidials for development of resistance.

**CONCLUDING REMARKS**

Although drug-resistance in *Toxoplasma* has yet to prove of clinical importance, the studies reported here have contributed greatly to our understanding of how antiparasitic agents work. Such information will likely soon have a significant impact on treatment of toxoplasmosis itself as well as other diseases caused by apicomplexan parasites.
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34. Peflerkorn ER, Borotz SE, Nothnagel RF. Mutants of Toxoplasma gondii resistant to atovaquone (566C80) or decoquinate. J Parasitol 1999; 75: 559–564.


36. Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q. Mutations in plasmodium falciparum cytochrome b that are
associated with atovaquone resistance are located at a putative drug-binding site [In Process Citation]. Antimicrob Agents Chemotherapy 2000; 44: 2100–2108.


