

**IDENTIFICATION OF ESSENTIAL  
THERAPEUTIC TARGETS ON *Toxoplasma*  
*gondii* PARASITE TO COMBAT  
TOXOPLASMOSIS**

Even Narrative Reviews must be structured in Introduction, Aim, Material and Methods, Results and Discussion and Conclusion. In the same manner the Abstract.

Under Material and Methods, you need to outline the manner you searched to obtain relevant literature, including citing the search-engine(s) used. In what languages were the selected-literature. What factors (criteria) were taken in account in selecting the articles (literature)?

Try to have not just one (mostly) or two references in what you cite. Try to annotate additional references in each – including contrasting views. Such would differentiate an essay from a Narrative Review.

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**ABSTRACT**

The status of toxoplasmosis management is challenged by drug intolerance, compromised efficacy and potential development of drug resistance. However, currently, there are very limited targets on *T. gondii* that serve as the site of action of current medications. This review aimed to explore more potential targets that are essential to parasite survival and are absent in humans. Many unexplored targets on *T. gondii* exist and their specificities to the parasite make them ideal drug targets. The present review had searched relevant English databases for relevant literature on the therapeutic target of *T. gondii*. Many of the enzyme systems in several pathways are essentially palatable as drug targets. Establishing compounds that can target these enzymes on *T. gondii* will greatly be beneficial in treating acute and chronic

## toxoplasmosis in humans.

*Keywords: toxoplasmosis; T. gondii; essential; drug; targets; enzymes.*

### 1. INTRODUCTION

Toxoplasmosis is distributed worldwide and affects more than a third of the world population [1]. The prevalence of this infection differs between regions, countries and even within a country largely due to climatic factors, feeding habits of the populace, availability of the intermediate host (Which is the intermediate host?) and anthropogenic factors [2]. Human transmission is through consumption of infected meat and contaminated water, fruits, and vegetables following soil contamination with the sporulated oocyst shed by the definitive host, the cats [2]. *T. gondii* has a unique affinity to form a chronic course of infection in the brain, retina, placenta, heart and skeletal muscles cells that can lead to chorioretinitis, encephalitis, focal cerebral lesions and congenital malformation in a fetus infected through the pregnant-mother [1,3]. The severity of these clinical conditions partly depends on the immune status of the individuals and the strain of toxoplasma that caused the infection [1,4].

Generally, the treatment available for toxoplasmosis is not without adverse effect. (First, what is the treatment?) These adverse effects range from hematological and liver function derangement to skin manifestation of hypersensitivity reactions [1]. Reports on treatment failure showed that adverse drug reactions observed in patients were due to the treatment with pyrimethamine in combination with sulfadiazine as first-line therapy ('adverse drug reactions' does not necessarily amount to 'treatment failure'.) [5]. The alternative therapy (what is the 'alternative therapy?'), however, has low efficacy, side effects and can be teratogenic, thus limiting their use as anti-toxoplasma drugs. This problem often leads to treatment failure and may lead to drug resistance (in what manner? Elaborate.).

Though several attempts are ongoing at least to have new drug candidates and/or vaccine against human toxoplasmosis, drug target (define drug-target. Do you mean a structure, or a pathway/reaction, in the parasite?) or a candidate protein (here, you mean specific antigen(s) on the parasite?) for vaccine development, are only suitable if they fulfill certain criteria that; the target must be essential for parasite growth and survival (only these two factors?) and must be specific to the organism with no homology in human (Elaborate. You mean no similar structures or pathway/reaction in humans that could cause serious adverse-reactions?). These criteria, if strictly adhered to, in drug or vaccine development against *T. gondii*, issues related to drug toxicity, immune evasion (The term is not comprehensible. Do you mean autoimmune reactions caused by the drugs? Do you mean vaccine-failure or unwanted immunological-reactions due to immunological factors?), and economic loss (in what manner is economic loss relevant here? Economic-loss, if it happens, is caused by numerous factors, apparent and non-apparent. Best to leave out discussing 'economic loss' at this stage of the article.) will be resolved [6]. In this review, therefore, the potential drug targets that are essential to *T. gondii* survival and specific to the parasite are discussed.

## 2. Drug treatment of toxoplasmosis

In practice today, drug treatment of toxoplasmosis, though may not be required (why is it not required?), is associated with several limitations. Apart from their limited number in use, the drugs are generally nonspecific to *T. gondii*. Most of them (Such as?) act against the replicating tachyzoite stage through inhibition of folic acid biosynthesis. The effect of these drugs and the pressure action by the host immune system facilitate rapid stage conversion from the tachyzoite stage to slowly dividing tissue cyst. Most of these drugs do not act on the tissue cyst and cannot cross the biological barriers (such as? List out). (References relevant to this paragraph?) These factors, favor *T. gondii* intracellular survival in tissues and by extension, favor its possible reactivation and long-term complications.

For decades, the drugs regimen for human toxoplasmosis, include sulfadiazine, pyrimethamine, and atovaquone that have activity and are usually used against Plasmodium parasites. Others are spiramycin, azithromycin, and clindamycin [7]. To avoid the risk of teratogenicity and of development of congenital toxoplasmosis, spiramycin is given to pregnant women that who are seropositive. The drug was assessed to reduced complications (complications after birth, or intra-uterine infection of the fetus?) in infants (use the term fetus, if still in the uterus) [8] and is reliably used in the early phase of toxoplasmosis (in the pregnant-mother?) to prevent fetal transmission [9]. However, a prior report by Habib [10], indicated that more than half of the pregnant women that who received spiramycin treatment still contain the parasite's DNA (where?). Therefore, to prevent obstetric complications (such as? what are the obstetric-complications to the pregnant-mother in toxoplasmosis?) and congenital toxoplasmosis, treatment of seropositive mother becomes necessary using spiramycin [7]. Combination of trimethoprim/sulfamethoxazole is given as prophylaxis (prophylaxis in what? You've already said spiramycin is given to pregnant mothers who are seropositive. Is trimethoprim/sulfamethoxazole second-line treatment then?) to inhibit dihydrofolate reductase, (DHFR) and dihydropteroate synthase (DHPS) respectively. Treatment as prophylaxis for those with HIV infection, depending on their CD4+ counts (<200 cell/ul), prevents sequelae of reactivation of the latent stage [11]. Atovaquone is an anti-plasmodium drug that targets cytochrome bc1 complex which is necessary for mitochondrial electron transport function. It was shown to selectively act on *T. gondii* using cytochrome bc1 as its target. Though it has little or no side effect on the host cell and is effective against acute toxoplasma encephalitis in combination with pyrimethamine or sulfadiazine [12], atovaquone has a very low bioavailability (but, bioavailability sufficient in encephalitis only?) [13]. Atovaquone was also reported to have an effective synergism with sulfadiazine in RH type 1 strain-infected mice [14].

Treatment failure of the available drugs may be due to associated with prolonged and repeated use. However, due to the complex

life cycle of the parasite, possible development of resistance [15], beside treatment failure in toxoplasma chorioretinitis, encephalitis and congenital infections are on the increase and still under investigation [3]. Furthermore, treatment failure may not necessarily only be due to resistance developed by the parasite, but rather, multifactorial causes. Another factor that may lead to the cause treatment failure is malabsorption which leads to poor bioavailability of the drugs. In patients with comorbidities, factors such as drug-drug interaction may contribute to treatment failure. Associated with the treatment failure is the possibility of development of a resistant or low susceptible strains of the parasite when exposed to a long-term prophylactic or maintenance treatment [3,16].

### 3. Drug resistance in *T. gondii* infection

Like any other microorganism, drug resistance in the apicomplexan protozoan has been reported [3,17]. The possibility of drug resistance is suspected to be responsible for certain treatment failures in a patients with acute infections and those with relapse after maintenance therapy [5]. Due to the usually asymptomatic nature of *T. gondii* infection in humans, drug resistance cannot be easily determined because the agent's stages of infection cannot be easily isolated in human [1,5]. However, alteration in genes coding for the common enzyme targets, DHFR, and DHPS was found to be the basis for resistance to common drugs treatment in *Plasmodium falciparum* and *Pneumocystis carinii* [3]. A study by Aspinal et al. [16], described a sulfonamide-resistant isolate of *T. gondii* from clinical cases of human toxoplasmosis. Results from the study showed that sequence alteration in the gene coding for DHPS enzyme confers resistance in *T. gondii* and related parasite *P. falciparum* to sulphonamides. However, *in vitro* model of experiments has been at the forefront in assessing the drug resistance by *T. gondii*. McFadden et al. [18] provided a suitable suggestion that will help researchers to develop new agents through modification of structural arrangement which will make the drug more effective. (Elaborate briefly) Many susceptible, as well as resistant strains of

*T. gondii*, were developed in vitro through chemical mutagenesis to assess the level of resistance (How would this help to develop newer agents?) [16,19]. Furthermore, the susceptibility of different genotypes of the parasite has been evaluated, though, with no difference among them but owing to its high replication rate and virulent nature, the susceptibility of the type I strain was however low in comparison to others (Simplify this sentence in better grammar by breaking it up) [5,20].

#### 4. Potential therapeutic targets in *T. gondii*

The apparent inability of the current drug regimen to act on the tissue cyst containing the slowly replicating bradyzoite in vital tissues, slows the progress of achieving full treatment coverage. To achieve complete treatment of toxoplasmosis, drugs that can be used against different targets, need to be thoroughly explored, identified, and developed. However, the drugs should be specific (why must it be specific to *T. gondii*?) to *T. gondii*, active against different stages of the parasite, with less or no toxicity on the host. Many enzymatic activities of *T. gondii* also need to be taken into consideration in that; certain drug molecules can act to inhibit certain biosynthetic pathways thereby inhibiting parasite growth as exemplified by pyrimethamines and sulphonamides. It is essential ~~and ideal~~ to develop and administer a drug having reliably understood its mechanism of action, specific target site and the consequences of its action on the parasite ~~and by extension,~~ ~~and also~~ the host. This basically calls for screening procedures to assess the effect of the drug on the parasite biology. Such screening procedures (briefly outline a description of such screening procedures) on whole organism or target-based, can be done *in vitro* or *in vivo*. While the latter is associated with ethical procedures and therefore time consuming and expensive, the former is proved to be easy, simple and inexpensive to carry out when handled by a laboratory expert [21].

##### 4.1 Protein kinases



The protein kinases (PKs) in *T. gondii* participate in many biological processes of the parasite life cycle [22, 23]. The serine-threonine kinases present in *T. gondii* have a catalytic domain containing a glycine rich N-terminal ATP-binding pocket in addition to a central conserved aspartic acid residue required for their catalytic activities [23,24]. A report by Piexoto et al. [25] indicated that, *T. gondii* encodes for 108 PKs genes, that have a high tendency of exhibiting catalytic activities and encodes for pseudo kinase genes that have no catalytic domain. Most of these genes identified, have no orthologs (orthologues?) in human, but they are well conserved in apicomplexan parasites like *Plasmodium* species, prokaryotes and plants. This feature makes the PKs suitable to be used as a drug target for drug discovery using those inhibitors that can specifically target the parasite and consequently with little or no side effect on the host [23,26,27]. *T. gondii* possesses a group of kinases some of which are species specific while others are secretory kinases (why are these not species-specific?). Both PKs have several functions that are important for parasite survival such as host cell adherence, gliding motility, the formation of the parasitophorous vacuole (PV), intracellular replication, egress as well as modification of host cell microenvironment [24]. The essential PKs in *T. gondii* that are suitable for drug discovery are herein reviewed.

#### 4.1.1 Calcium dependent protein kinases (CDPK)

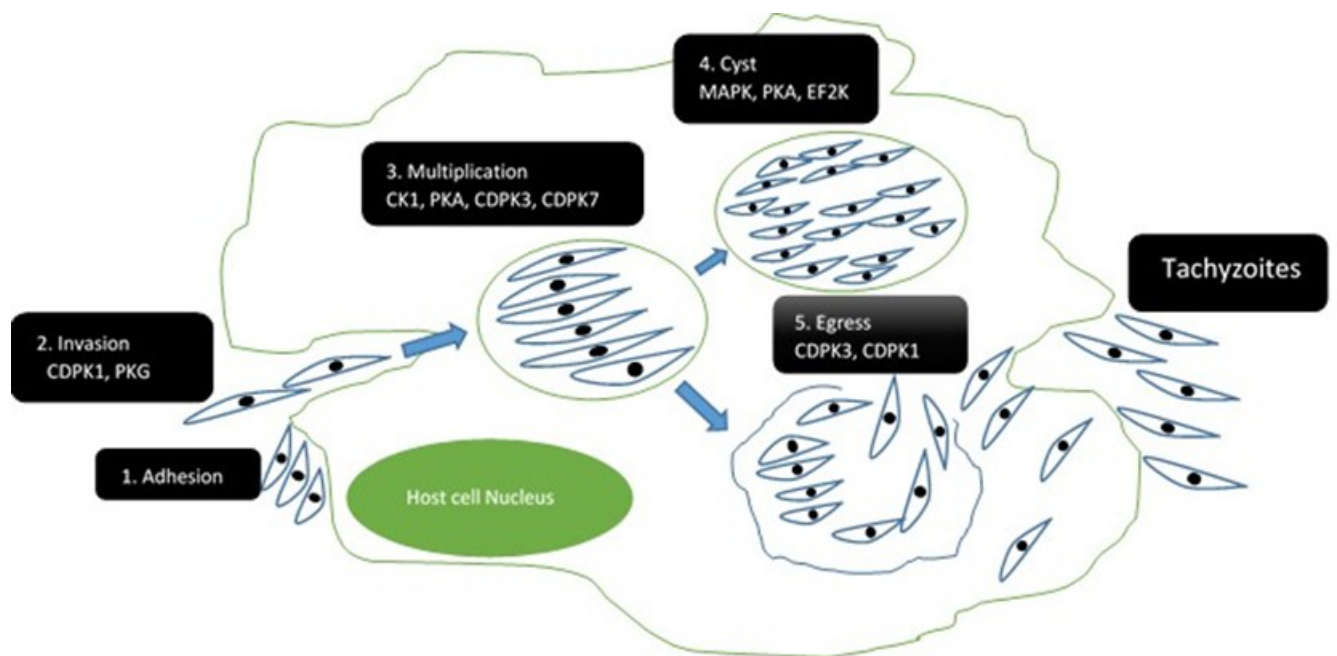
*T. gondii* possesses series of CDPKs (Figure 1) that are essential for various functions that are associated with intracellular Ca<sup>2+</sup> (Ca<sup>2+</sup>?) level [27] that leads to the secretion of microneme proteins essential for host cell invasion, gliding motility and egress. The CDPKs that are conserved among the apicomplexan parasites include CDPK1, CDPK2, CDPK3, CDPK4, CDPK5, CDPK6, and CDPK7 and their physiological role for parasite survival was studied [28]. The CDPK1 plays a critical role in host cell adherence, invasion, and egress as proved by the various knockout study as well as developed chemical inhibitors. Several studies reported that activities of CDPK1 was inhibited following exposure to inhibitors that selectively target it sparing mammalian

host cell. These inhibitors that were used against CDPK1 include bumped kinase inhibitors [26,29], ATP-competitive inhibitor [30] and Benzoylbenzimidazole-based selective inhibitors [31].

The most striking feature that makes it suitable for use as a drug target is that it contains a glycine gatekeeper residue situated adjacent to ATP-binding pocket against which many chemical inhibitors were developed. This feature differentiates it with other CDPKs within the parasite, other apicomplexans and that of the mammals as it was shown to be a target by bumped kinase inhibitors in vivo [26] and conditional knockout of CDPK1 was reported to inhibit microneme secretion [32].

Chen et al. [33] reported that there was the development of humoral and cellular immune response among mice that were challenged with RH tachyzoite after vaccination with plasmid pVAX-CDPK2 vaccine. The mice were also observed to have amylopectin accumulation, CDPK2 is also a potent candidate for vaccine development. A prior study by Zhang et al. [34] indicated that CDPK2 is essential for amylopectin metabolism in that its deficiency leads to accumulation of starch that leads to the death of *T. gondii*. The CDPK3 was reported to be essential in egress of the tachyzoites [35]. Forward genetic experiment reported by Garrison et al. [36] revealed that CDPK3 plays a role in egress and latent stage conversion in mice. Morlon-Guyot et al. [28] reported that CDPK7 is important in cell division and survival. This report followed a knock-down experiment in which deficiencies in tachyzoite division and growth were due to defects on centrosome integrity. This, according to the study causes restriction in cell division within the vacuoles and impairment of endodyogeny. In addition, Wang et al. [24] reported that CDPK7 might be involved in immune evasion in that there was an increase expression of CDPK7 gene in IFN- $\gamma$  knocked out mice with no difference in expression obtained in vitro using HFF cell. A number of these CDPKs in *Plasmodium* parasites has been studied and functions were reported to be matched with their orthologues in *T. gondii* [24].





**Figure 1. Toxoplasma protein kinases in the parasite lytic cycle.** The CDPK1 and PKG1 participate in adhesion and invasion steps where they cause secretion of microneme proteins. CDPK3 and CDPK1 are also reported to act during egress. PKA-C, CDPK3, CDPK7 and CK1 are involved in cell cycle regulation and growth. MAPK1, PKA-C and eukaryotic initiation factor-2 kinase (EIF2K) participate in stress responses and stage conversion from tachyzoite to bradyzoite.

(In case this figure is not original, cite the original owner of the figure, obtain permission for use in your article and thank the original owner under Acknowledgement).

#### 4.1.2 Casein kinase 1 (CK 1)

The CK 1 is an essential enzyme in signaling pathways among the serine-threonine protein kinases in eukaryotes that mediates many biological and physiological processes in the cells [37]. Though, the function of this kinase group has not been ascertained [23] in *T. gondii*, their special function in organisms such as DNA repair, cell cycle regulation as well as cytokinesis, can be exploited as potential drug target in *T. gondii* since their role in another related protozoan (name the protozoan) has been reported [38]. Inhibitors such as purvanolol B and aminopurvanolol A were observed to selectively inhibit the  $\alpha$ -isoform of TgCK1 thereby inhibiting the parasite growth intracellularly while sparing the host cell [39].

#### 4.1.3 Mitogen-activated protein kinase (MAPK)

MAPK is another enzyme that regulates some essential cellular activities in *T. gondii*. These activities include regulation of proliferation, stage differentiation and response to stress by the parasite. ~~and that its activity is~~ Its activities are regulated by intracellular calcium level [23]. Activity of MAPK was reported by Wei et al. [40] to be inhibited by pyridinylimidazole, which that causes morphological changes on the parasite which suggests that the effect of the inhibitor might have caused defects in the parasite's intracellular replication. This inhibitor was also reported to block TgMAPK-1 autophosphorylation [41] thereby reducing its virulence role in toxoplasmosis [42]. For the two isoforms of MAPKs (TgMAPK-1 & TgMAPK-2) present in *T. gondii*, presence of the threonine, aspartic acid and tyrosine motifs that define their essential role as well as uniqueness from mammalian MAPKs make them one of those PKs that can be exploited as good targets for the development of new drugs against the *T. gondii* [42,43].

#### 4.1.4 cGMP-dependent protein kinase (cGMP-PKG)

The cGMP-PKG is one of the PKs involved in microneme secretion that plays a role in host-parasite interaction [23]. It was reported to be an essential protein in *T. gondii* that can be used as a potential drug target against which inhibitors can be developed. Following an in vivo study of a trisubstituted pyrrole also known as compound 1; 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H-pyrrol-3-yl] pyridine, its cytostatic activity on the *T. gondii* tachyzoite was observed [44]. The same activity was also reported against another apicomplexan parasite *Eimeria tenella* [45]. But with the fact that compound 1 is only cytostatic on the parasite, other pathways that regulate microneme secretion may come into play and thus there is still a need to have agents that can rather completely kill, preferably, the parasite stage for in the likes of intracellular parasites equipped with immune evasion mechanism [46].

#### 4.1.5 cAMP-dependent protein kinase (cAMP-PKA)

The cAMP-PKA is one of the important PKs regulating many cellular processes in the eukaryotes [47]. Sugi et al. [48] studied the bidirectional influence (define/explain bidirectional influence) of PKA on asexual stages of *T. gondii*. The study revealed that the cytosolic catalytic (define/explain) subunit of *T. gondii* PKAc3 which is specific to coccidian parasites is responsible for the maintenance of the tachyzoite stage. This was possible despite exposure to certain stress factors that could have triggered differentiation to bradyzoite hence the cystic stage, as demonstrated *in vivo*. Therefore, developing a potent drug against PKAc3, say Sugi et al, will greatly help in restricting tachyzoite to bradyzoite conversion, phenomenon through which the parasite evades the immune system and pressure from other stress factors such as chemotherapeutic agents. Such inhibitors if developed would help combat acute on chronic infection in cases of reactivation if given prophylactically.

#### 4.1.6 Rhoptry kinases (ROP)

The rophtry kinases are secreted from rophtry organelles situated at the neck of *T. gondii*. The ROPs contribute immensely in the pathogenesis of *T. gondii* from the initial stage of moving junction formation, gliding motility, the formation of the PVM (write out in full first, prior to using abbreviation), immune evasion as well as virulence [49]. The ROPs are highly divergent between the various strain types of the parasite while some are well conserved [50]. The diversity in these proteins is due to the differences in amino acid residues required for the catalytic activities. While some of the ROPs (ROP2, ROP4, ROP5, ROP7 and ROP8) lack the glycine loops and the conserved aspartic acid in the catalytic domain that is involved in phosphorylation activities, others such as ROP17, ROP16, and ROP18 were reported to have catalytic activities in vitro [51]. ROP2 families (ROP2, ROP4, ROP5, ROP7, ROP8), are the largest group of rophtry kinases associated with PVM upon secretion. The ROP2 proteins coordinate a lot of activities that include cell invasion, replication, and the association between PVM and host cell organelles, immune evasion and virulence. These ROPs family were reported to share a common C-terminal transmembrane domain which is a PKs like domain and upstream of this PK region, they contain basic amino acids rich N-terminal tail. The ROP2 bring host mitochondria in close association to the PVM. However, a study [52] showed that the recruitment of mitochondria to PVM is not necessarily a sole function of the ROP2 because a knockout study showed no defect in mitochondrial recruitment. ROP5 is a factor in virulence and this was proved by a targeted deletion that was followed by loss of virulence activity whereas restoration of which causes full blown pathogenesis in mice [51]. However, to compliment the activities of ROP18, ROP5 is expressed and this helps the ROP18 to inhibit the accumulation of the interferon gamma induced immunity related GTPases (IRGs) in a murine model. The function of the IRGs is to bind and disrupt the parasite enclosed within the PV under normal circumstances. This function is inhibited by the presence of ROP18, whose activity is enhanced by ROP5 [23] that leads to host cell immune evasion and hence pathogenesis. Moreover, some of the ROPs have no define

**definite** function but were reported to have conserved residue for kinase activity [23,51]. The ROPs targeted host cell nucleus was reported to have kinase domain catalytic ability to phosphorylate host cell molecules and hence modulating cell function and cell cycle [53]. The most studied among them are the ROP16 and ROP18. While the former is expressed in type I and III genotypes, the latter is expressed in type I and type II and their overall function is to regulate host immune system thereby increasing the virulence of the parasite [54]. Regulation of the host immune system was reported to be due to the direct phosphorylation activity of ROP16 on STAT3 and STAT6 leading to down regulation of IL-12, a key cytokine in infection control by the immune system [22,54].

It is also important to note that, the cytokine IFN- $\gamma$ , that is expressed in fighting against *T. gondii*, excess of which is usually checked by IL-10 [54] can be controlled indirectly by ROP16. Direct phosphorylation of STAT3 can make *T. gondii* to mimic IL-10 and this can cause downregulation of the IFN- $\gamma$  thereby enhancing virulence in the host. ROP18, on the other hand, was reported to indirectly inhibit the activities of the IFN- $\gamma$  on immunity related GTPases (IRGs). The IRGs are activated by IFN- $\gamma$  [55] which leads to the destruction of the PV. This action is inhibited by ROP18 which was reported to phosphorylate the IRGs thereby preserving the PV and continuous proliferation of the parasite by evading the immune system [22,54].

In search of a vaccine for toxoplasmosis, *T. gondii* PKs were reported to be promising candidates. Zhang et al. [34] reported that the combination of CDPK6 and ROP18 proteins **in a vaccine** were seen to confer immunity in mice **subsequently** infected with RH tachyzoite. The size of the brain cysts of the infected mice were reduced in comparison to the non-vaccinated control group. Prior to this study, the same group of mice were immunized with pVAX-CDPK3 produced a significant increase in cytokines notably IFN- $\gamma$ , IL-2, IL-12 and IL-23 [56]. The study further reported that the survival rates of the immunized mice were increased associated with reduction in brain cyst formation. For the essential

role played by the CDPKs and ROP kinases in the pathogenesis of *T. gondii*, and because of their absence in mammalian host, drug and or vaccine development to these essential proteins is crucial at this moment to stop the spread of the parasite.

(You appear to be discussing a vaccine against *T. gondii* in the treatment of the disease subsequent to infection and disease in mice rather than in prophylaxis/prevention. I am not aware of vaccines used in this manner in practice in populations in any infectious disease. Discuss this further, citing several studies/authors. In case such use is only experimental on lab-animals presently, say so.)

## 4.2 Apicoplast

Apicoplast is an organelle that is present in apicomplexan (define) protozoa with many metabolic activities. It is a non-photosynthetic organelle originated through endosymbiosis of green algae that is involved in biosynthetic activities of various metabolites and cofactors such as fatty acids, abscisic acid, lipoic acid, isoprenoids and heme [57]. The apicoplast is a good target for drug development owing to its characteristic nature and biosynthetic activities that resemble that of prokaryotes [7,21]. Many metabolic activities in this organelle are different from that of human as such it may be used as a drug target against important parasitic apicomplexan. The potential drug targets in apicoplast are discussed herein:

### 4.2.1 Fatty acid metabolism

Fatty acids are essential to every living cell because of the immense need for the synthesis of many intermediate products that are essential needed for many cellular processes. The life cycle of *T. gondii*, that demands for high supply of lipids to enable intracellular survival. This is evident by the formation of the parasitophorous vacuole that houses the intracellular stage (where? In the parasite or the host?). The vacuole is surrounded by three layered-membranes, the essential organelles



concentrated at the apical end of the parasite (micronemes, rophtry and dense granules) and the membrane-bound apicoplast. (references for this paragraph?)

The presence of type II fatty acid synthase II (FASII) in the apicoplast of *T. gondii* that is involved in fatty acid biosynthesis as found in prokaryotes, is essential for parasite survival and its absence in human, makes it a target for drug development [58]. Inhibition of enzymes that mediate important steps in type II fatty acid synthesis and its elongation in the apicoplast was shown to inhibit parasite growth [59,60]. More especially the acetyl CoA carboxylase (ACC) that catalyzes the formation of Malonyl-CoA, the  $\beta$ -ketoacyl ACP synthase III (FabH), in the first condensation step and the enoyl ACP reductase (FabI) that catalyzes the final step, are very important drug targets. Several compounds such as clodinafop, thiolactomycin and triclosan (Table 2) were shown to have inhibitory activities on these enzymes and block fatty acid synthesis that results to the death of parasite [61]. However, because of And, their absence in human, targeting these enzymes in *T. gondii* will prove beneficial in combating toxoplasmosis.

**Table 1: Potential therapeutic targets in apicoplast**

Enzymes	Inhibitor	Organism	IC <sub>50</sub>	Reference
Enoyl-ACP reductase	Triclosan	<i>T.gondii</i>	62 ng/mL	[65]
		<i>P. falciparum</i>	<2000 ng/mL	
$\beta$ -Ketoacyl-ACP (FabH)	Thiolactomy cin	<i>P. falciparum</i>	50 $\mu$ M	[66]

AcetylCoA carboxylase	Clodinafop	<i>T. gondii</i>	10-100 μM	[67]
DXP reductoisomerase	Fosmidomyc in	<i>P.</i> <i>falciparum</i>	350 nM >500 nM	[62] [63,64]
DNA gyrase	Ciprofloxaci n	<i>T. gondii</i>	30 μM	[68]
		<i>P.</i>	50 μM	[69]
		<i>falciparum</i>	80 μg/mL	[70]
		<i>C. parvum</i>		

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IC<sub>50</sub>: 50% inhibitory concentration

#### 4.2.2 Isoprenoid synthesis

Isoprenoid is important in cell signaling for the control of many cellular activities, protein prenylation, and as a precursor of sterols and ubiquinone. A non-mevalonate pathway, 1-deoxy-D-xylulose-5-phosphate (DOXP) was reported to be present in the apicoplast. The DOXP, was reported [62] to be responsible for isoprenoid biosynthesis in *P. falciparum*. The pathway differs from that obtained in the cytosol of other eukaryotes in the initial substrate for the synthesis of isoprenoid precursors; isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP). The IPP and DMAPP are formed through condensation of glyceraldehyde 3-phosphate and pyruvate in DOXP pathway while in mevalonate pathway in eukaryotes and archaeobacteria, the synthesis of isoprenoids is through condensation of acetyl CoA and acetoacetyl CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Interestingly however, the two key enzymes that featured in the DOXP pathway i.e. DXP reductoisomerase and

DXP synthase found in *P. falciparum* [62] were also reported to be present in *T. gondii* and very essential for the parasite's survival. The absence of these two enzymes in human made makes them a good drug target against which inhibitors can be designed [61]. Fosmidomycin antibiotic that inhibits the activities of DXP reductoisomerase and DXP synthase was reported to inhibit the growth (in what manner?) of *Plasmodium vinckei* in mice and *P. falciparum* [62]. For the fact that Since *T. gondii* and related apicomplexan share the same enzymes in the DOXP pathway, sensitivity to fosmidomycin is anticipated. In contrast But, the activity of this antibiotic on *T. gondii* was rather resisted [63,64] except at higher concentrations (Table 2). That is attributed to a decrease uptake of the drugs by the infected cells [61], the nature of the cell as well as and possible efflux of the antibiotic from the parasite [64] hence significantly affecting its bioavailability. Therefore, at a higher concentration, the fosmidomycin antibiotic can suppress the growth of *T. gondii*.

#### 4.2.3 DNA replication

DNA replication in the apicoplast of *T. gondii* follows sequential order as obtained seen in prokaryotes. The apicoplast contains a circular DNA which is 35-kb in size and therefore unwinds before the replication process as in bacteria. The sequence of events regarding the unwinding of the circular DNA before replication and its subsequent supercoiling are mediated by DNA topoisomerase [71]. DNA gyrase found in bacteria, [72] is a type II DNA topoisomerase that is also present in apicomplexan. DNA gyrase is responsible for DNA replication in apicoplast [71,73]. Several quinolone antibiotics were are reported to inhibit DNA gyrase and that affect parasite growth as observed in bacteria. A notable member of such class, ciprofloxacin has been reported by several studies (not just one study. Additional references required) to inhibit the growth of *P. falciparum* [69], *T. gondii* [68] and *Cryptosporidium parvum* [70]. It was also reported by McFadden and Roos [74] that a fluoroquinolone, trovafloxacin inhibits *T. gondii* growth in vitro at a dose range of 0.77-0.98 µg/mL. Ciprofloxacin, ofloxacin and temafloxacin were shown additionally

effective. The findings from ~~their~~ the *in vivo* study indicated significant parasite clearance in a dose-dependent manner. The quinolones were are reported to inhibit DNA gyrase, hence inhibiting proper DNA replication which results in the death of the parasite [74]. ~~For the fact that~~ Since DNA gyrase is of prokaryotic origin, and hence absent in human, design and synthesis of an inhibitor, potentially less not so toxic to human, can be arrived at carefully done to bypass the possibility of resistance as seen with ciprofloxacin in bacteria [75]. (You could use ciprofloxacin against *T. gondii* in a dose-dependent manner. But, how could such design and synthesis preclude subsequent development of resistance to the antibiotic). (Since ciprofloxacin is presently commonly in use, would you then recommend a clinical-trial in the use of the antibiotic against *T. gondii*?).

#### 4.2.4 Protein synthesis

Protein synthesis in the apicoplast occurs independently from that of the parasite's nucleus. This is because the apicoplast genome encodes only for components of gene expression and protein synthesis [76]. It encodes for a complete set of enzymes and RNAs that are indispensable for ribosome assembly. Like in *Plasmodium* parasite, these set of enzymes and RNAs include the rRNA (small and large subunit), all tRNAs, and translation elongation factor –Tu [75,77]. It is important to note that, antibiotics used for the treatment of toxoplasmosis such as clindamycin, azithromycin and spiramycin were proven to have activities on protein synthesis in prokaryotic organisms by binding to ribosomal subunits. These antibiotics were reported to have *in vitro* and *in vivo* anti-parasitic activities. The study by Pfefferkorn et al. (year) [78] reported that clindamycin has parasitocidal activity on *T. gondii* at a very low concentration despite its delayed onset of action, and that this effect increases with increase in concentration. Though the study did not investigate the mechanism through which it caused its effect, the authors suggest that the antibiotic might have had the same mechanism of action like in bacteria by interfering with protein synthesis. ~~For the fact~~

that Since the protein synthesis in apicoplast is of prokaryotic type, all its components may be suitable for specific drug development. (What is the advantage of having an antibiotic specific only against one microorganism compared with broad-spectrum against many?)

#### 4.2.5 Heme metabolism

Heme is an essential cofactor of several enzymes such as the catalase and the cytochromes. Heme biosynthesis occurs in *T. gondii* as in other apicomplexans, prokaryotes, plants and animals. But a striking difference is that in *T. gondii*, this activity is split between the apicoplast, the mitochondrion and the cytosol [61]. This depends on the location of enzymes in the biosynthetic pathways. In those organisms that are equipped with machineries for the *de novo* heme biosynthesis such as *Plasmodium spp*, *T. gondii*, fungi, plants and animals [79,80], the initial committed precursor, aminolevulinic acid (ALA) is the same but synthesized through different substrate utilization [79]. The ALA is the first precursor of heme, synthesized in the mitochondrion from glycine and succinyl-CoA which must go through different enzymatic steps in both photosynthetic and non-photosynthetic eukaryotes. The first catalytic conversion of ALA to porphobilinogen is mediated by porphobilinogen synthase (PBGS). However, because the PBGS is of plant origin (Why is PBGS of plant-origin when *T.gondii* is a protozoa?) makes it a good drug target (and, why does being of plant-origin make it a good drug target?) against *T. gondii* [78] and its inhibition can lead to the death of the parasite.

#### 5.0 Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) are well conserved in microorganisms and contribute in host cell invasion, growth,

survival and stage conversion from tachyzoite to bradyzoite stage in *T. gondii* infection [81]. During *T. gondii* infection, HSPs (Hsp100, Hsp90, Hsp70, Hsp60, Hsp40) expression is induced by stress factors that favor formation of bradyzoite. The HSPs play an important role in maintaining protein homeostasis which enhances intracellular survival of *T. gondii* [82]. A gene knockout study [81] reported that the Hsp90 knockout parasites were unable to establish infection in Vero cells and that the parasites had a short life span in mice. This indicates that the absence of this protein seriously affects the ability of the parasite to invade the Vero cells and cannot run a chronic bradyzoite stage *in vivo* and hence **has** decreased virulence. (**How is this fact on *in vivo* and virulence established?**) Hsp70 and Hsp60 are nuclear encoded proteins and their role in the post-translational assembly of several proteins make them a good target for drug development. A study [83] reported that Hsp60 is responsible for stage specific induction of respiratory pathway whereas Hsp70 is involved in stage differentiation and virulence. As part of its **virulent–virulence** feature, Hsp70 was reported to protect the parasite from the immune system of the host. Dobbin et al. [84] conducted a study in which quercetin and oligonucleotide (**are these antibiotics?**) were used to treat virulent (RH, ENT) and avirulent (ME49) *T. gondii* strains. The study revealed a reduction in expression of Hsp70 in virulent strain and their proliferation was reduced in mice. However, the result from the *in vitro* study showed that the tachyzoite rapidly converts to bradyzoite. The study further reported that the inducible nitric oxide synthase (iNOS) was increased in RAW 264.7 cells after infection with Hsp70 reduced-virulent parasite. This finding confirmed that the Hsp70 protein altered production of Nitric oxide (NO) which takes part in immune control of toxoplasmosis. Therefore, any drug that can inhibit the activity of Hsp70 will prevent immune evasion by the parasite. (**What about that the “tachyzoite rapidly converts to bradyzoite” above?**)

## **6.0 Metabolic enzymes of *T. gondii***



Study on the similarities between the human host and *T. gondii* with respect to their metabolic pathways was reported by Gautam et al. [6]. According to the study, 11 potential enzymes that can be used as drug targets were identified in *T. gondii* but which were absent in human. Some of the enzymes appear to play a significant role in more than one pathway. Among the 11 enzymes, is glutamate dehydrogenase enzyme (GLDH) which appears to catalyse many metabolic reactions such as amino acid metabolism and oxidation reaction. GLDH catalyses the formation of NADPH which is needed to reduce the oxidised forms of glutathione and thioredoxin in the presence of glutathione reductase (GR) and thioredoxin reductase (TrxR) respectively. The GLDH is unique only in *T. gondii* (and Plasmodium) as such it is suitable to be a drug target as in *Plasmodium* parasite [85]. Other important enzymes in glucose, amino acid and purine metabolisms were identified to be essential in the parasite and but absent in human. Metabolic differences between the various stages of the parasite can serve as a drug target. Differences in metabolism exist between the actively dividing tachyzoite and the slowly replicating bradyzoite within a cyst [86]. In both stages of the infection, lactate is produced as a source of energy in the glycolytic pathway but differ in types of lactate dehydrogenase (LDH) activity. The two isoforms LDH1 in tachyzoite and LDH2 in bradyzoite catalyze the conversion of pyruvate to lactate in anaerobic condition. The activity of LDH is reported to be higher in bradyzoite [87]. In previous study [88], the LDH genes knockdown *T. gondii* were shown to have impaired growth in vitro. The study further showed that, the tachyzoite stage of the parasite was unable to establish chronic infection *in vivo*. Furthermore, the mice infected with LDH knockdown tachyzoite were observed to tolerate (subsequent?) infection when challenged with a maximum infective dose of  $10^4$  tachyzoites. This study shows that developing a live LDH attenuated tachyzoites can be used as a potential vaccine candidate against toxoplasmosis. This finding was recently supported by a study [89] which stressed that LDH is involved in virulence and bradyzoite differentiation of the parasite and that LDH mutant parasite can be used for vaccine development.

Glucose – phosphate-isomerase (GPI) present in the bradyzoite is important in glucose metabolism and appears to be a good target because it differs from that of the humans [21]. Another important enzyme in the parasite purine pathway, adenosine kinase, was also reported to be inhibited by analogues of 7-deaza-6-benzylthioinosine with significant anti parasitic activities [90].

Blume et al [91] reported another important gluconeogenic enzyme, fructose 1,6-bisphosphatase, which exists in two isoforms; TgFBP1 and TgFBP2. Although the enzyme shares properties with that of prokaryotes, but unlike the prokaryotes and other eukaryotes that express this enzyme in glucose depletion state, the enzyme is constitutively expressed in *T. gondii*. The two isoforms are essential in either glucose depleted or repleted situations and they are required for replication in vitro and for virulence in vivo [91]. This finding was a result of a conditional knockdown of TgFBP2 (do you mean reduction in the activity of the enzyme? Say so.) which resulted in the loss of intracellular replication and acute virulence in mice. With this, it was concluded that this enzyme could be a potential drug target.

For intracellular replication and membrane biogenesis, *T. gondii* acquires cholesterol from host cell through a low-density lipoprotein pathway (LDLP) and convert it to cholesterol esters (CE) that are stored in lipid bodies [92]. In fact, a typical organelle with a very high membrane cholesterol level is rhoptry which secretes proteins that facilitates cell invasion [58]. Previous report [93] showed that statins have an anti-proliferative effect on *T. gondii*. The ability of this parasite to convert host cell derived cholesterol and other lipids to CE and stored in lipid bodies is achieved due to activities of certain enzymes from the endoplasmic reticulum. These enzymes are acyl-CoA: cholesterol acyltransferase (ACAT: ACAT1 & ACAT2) whose susceptibility to inhibitors were found to be different from that of mammals [57]. In addition, the most abundant phospholipids in *T. gondii* is phosphatidylcholine [94]. The parasite takes choline, serine and ethanolamine from the surrounding, as precursors for other phospholipids. However, interference with choline metabolism has been shown to inhibit the growth of *T. gondii* [94] and *P. falciparum* [95]. Therefore, while undergoing treatment for

hyperlipidemia, the activity of statins may retard *T. gondii* infection progression in human host (has this been observed among such patients?) and thus, inhibition of choline uptake appears to be a good target for drug discovery. Enoyl reductase involved in type II fatty acid biosynthesis was also identified to be inhibited by some compounds [96] and most importantly the enzyme is lacking in animals. Since triclosan was successfully used to inhibit enoyl reductase [97], a derivative of this compound with low toxicity can be used for the treatment of *T. gondii* infection.

## 8. Conclusion

Treatment of intracellular pathogens can be so challenging because the active ingredient may have to penetrate tissue layers to reach its targets. *T. gondii* is one of those intracellular parasites that survive only within the cell in a confined non-fusogenic vacuole that prevents the action of lysozymes against the parasite. In addition, because of the characteristic features of the different stages of its life cycle, *T. gondii* is potentially difficult to eliminate. The three infective stages (the infective stages are only two: tissue cyst and oocyst) of the parasite can be targeted at a different site if only their physiological characteristic and biological interaction with host agents are well studied. Therefore, drug development should take into consideration, the essentiality and specificity of the targets, to solve the problems of adverse drug reaction and clearance of the parasite at each stage.

(Having discussed thus far, from the point of practicability, what do you think are the best one or two targets in drug development, and the best one or two in vaccine development?)

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## Declaration of interest

The authors declare that there is no conflict of interest.

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