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Review Article

INSIGHTS ON DRUG TARGETING OF *TOXOPLASMA GONDII* HOST INVASION PROTEINS: A REVIEW

INDHUJA THIRUMUDI[1], UMASHANKAR VETRIVEL[1]*, MAHALAKSHMI B[2], LILY THERESE K[2], MADHAVAN HN[2]

[1]Centre for Bioinformatics, Vision Research Foundation, Sankara Nethralaya, Chennai - 600 006, Tamil Nadu, India.
[2]L & T Microbiology Research Centre, Vision Research Foundation, Sankara Nethralaya, Chennai - 600 006, Tamil Nadu, India.
Email: vumashankar@gmail.com

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ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite that infects homoeothermic animals. It is also the major cause of retinochoroiditis in humans. Drugs targeting *T. gondii* proteins involved in the establishment of host-pathogen interactions is well documented to be an efficient way to combat the infections. Basically, parasitic invasion of *T. gondii* occurs by the sequential secretion of apical membrane antigen 1 and rhoptry neck proteins on the parasite and host cell surfaces, respectively. These proteins operate synergistically and form the moving junction (MJ) complex, thereby, enabling attachment and penetration of the parasite into the host cell. Better understanding of molecular interactions of these proteins is essential to develop highly efficient therapeutic modalities. Hence, by this review it is intended to update the current status of rhoptry and other MJ complex proteins as ideal candidates for targeting *T. gondii*.

Keywords: *Toxoplasma gondii*, Rhoptry proteins, Moving junction complex, Toxoplasmosis.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite, which causes toxoplasmosis in homeothermic animals. It is grouped under the apicomplexan parasites, which comprises of various other pathogenic organisms such as *Plasmodium* species, *Babesia* species, *Cryptosporidium* species, *Theileria* species, *Eimeria* species and *Neospora caninum* [1]. *T. gondii* is extensively disseminated in the surroundings and it is reported to be the major cause of retinochoroiditis in humans worldwide [2].

LIFE CYCLE AND PATHOGENESIS OF *T. GONDII*

Infection and transmission of *T. gondii* can arise due to intake of undercooked meat embedded with *T. gondii* cysts, and also when ingesting contaminated water, vegetables, or any other food contaminated with feces of infected feline, which harbors the oocysts. It can also be transmitted maternally to offspring during gestation [3]. The *T. gondii* lifecycle comprises of two stages, which includes sexual reproduction that occurs only within felines (definitive hosts) and asexual reproduction that occurs within other homeothermic animals like humans, cattle, and birds (intermediate hosts) (Fig. 1) [2].

The various forms of *T. gondii* include merozoites, tachyzoites, bradyzoites (established in tissue cysts), and sporozoites (established in oocysts). When a feline is infected with *T. gondii,* the parasite survives and passes through the stomach, during which it penetrates and infects the epithelial cells of feline's small intestine. The sexual reproduction and development of the parasites occurs in the intestinal tract, wherein, it gets multiplied into numerous thick-walled zygote described as oocyst (parasite residing stage-sporozoite) [2]. These immature oocysts undergo sporulation leading to the formation of mature oocysts and are shed in the feline feces and spread through water, soil and contaminated foods [4].

When an oocyst or tissue cyst of the parasite is ingested by the homeothermic animals, the cyst wall gets dissolved due to the action of intestinal proteolytic enzymes present in the host, thereby exposing parasites to the host intestinal cells [5]. During this process, these parasites invade the intestinal epithelium and eventually multiply into tachyzoites, which are the motile and active reproducing stage in the host cell. Further, these tachyzoites proliferate inside the parasitophorous vacuoles (PV) formed during the initial invasion of the host cell. Further, this leads to the rupture of host cells, releasing free tachyzoites into the blood circulation lead to the spread of infection to all other organs and tissues through blood stream [6,7]. Subsequently, tachyzoites gets transformed to bradyzoites (tissue cysts), due to the host immune response. This is a semi-latent, resting and slow dividing stage of the parasite [2]. The crucial means of infection in humans is due to the consumption of meat containing the tissue cysts [2].

LABORATORY DIAGNOSIS

Various manifestations of *T. gondii* infection in humans include acute infections in immunocompetent individuals, infection in immunocompromised individuals, primary infection during pregnancy, infections acquired congenitally and ocular toxoplasmosis [8]. *T. gondii* infection in all these cases are generally diagnosed by the measure of specific immunoglobulin G (IgG) and IgM antibodies levels in host. Moreover, there are other specific diagnostic methods like polymerase chain reaction (PCR) amplification, histologic determination of parasite/antigen, observation in stained tissue sections from a biopsy or blood/body fluids (cerebrospinal fluid and ocular fluids - aqueous and vitreous fluids), and isolation of the parasite are also practiced [8,9].

As discussed earlier the diagnosis varies based on the individual and the type of infection acquired.

Acute infections in immunocompetent individuals

Most of the *T. gondii* infections are asymptomatic [10]. The major symptomatic infection of the parasite is the lymphadenopathy. IgG and IgM antibody level screenings are the preferred primary testing methods. Moreover, biopsy test of skeletal muscle or endomyocardial biopsy is also performed to screen the infection [11]. However, *Toxoplasma* serological profile of the serum is the most preferred in these cases [12,13].

Infection in immunocompromised individuals

Immunocompromised individuals - those with AIDS, hematologic malignancies and bone marrow or organ transplants are usually prone

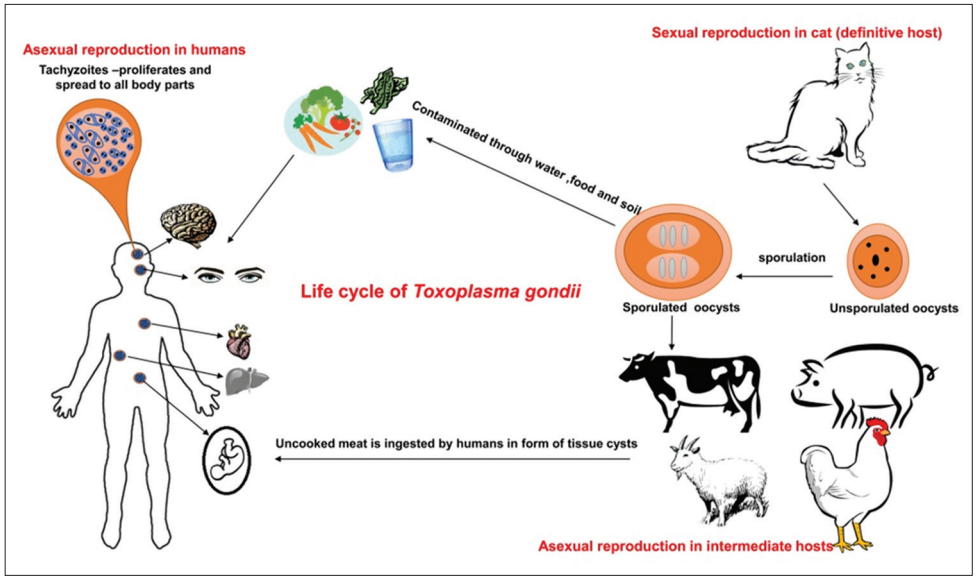

Fig. 1: Schematic representation of *Toxoplasma gondii* life cycle

to life threatening *T. gondii* infections [14]. Especially in case of HIV patients, it is manifested as encephalitis causing focal central nervous system (CNS) lesions [15]. Tests useful to establish toxoplasmosis are PCR amplification of *T. gondii* DNA in blood or body fluids suspected of being infected, isolation of the parasite in blood or body fluids that may contain the parasite, and histological examination of available tissues with *T. gondii* specific stains such as immunoperoxidase [8]. In special cases, if there are any clinical signs indicative of CNS/spinal cord, computed tomography or magnetic resonance imaging also becomes necessary [8].

Primary infections during pregnancy

Primary infection of *T. gondii* in pregnant women is of major concern, as this could lead to congenital toxoplasmosis. In these cases, IgG and IgM antibody levels in blood are constantly monitored during the entire gestation period. Other serologic tests - IgG avidity, IgG/IgM antibody level using formalin-fixed tachyzoites (HS)/acetone or methanol-fixed tachyzoites (AC) are also be performed for confirming the infection [16].

Infections acquired congenitally

Congenital infection of *T. gondii* leads to abnormal fetal development. Hence, ultrasonography is preferred as a primary method for screening the abnormality. Furthermore, amniocentesis is also preferred as a primary screening method in the later stages of gestation, wherein, the amniotic fluid is collected and screened for *T. gondii* specific DNA through PCR amplification. This is usually performed from 18 weeks of gestation [17]. Moreover, if infants are suspected for congenital toxoplasmosis, ophthalmological examination, an ultrasound scan of the brain or computed tomography is also recommended, as it will be helpful to rule out hydrocephalus and calcification states [18].

Ocular toxoplasmosis

T. gondii mediated chorioretinitis occur as congenital or acquired infection [19-21]. The diagnosis of ocular toxoplasmosis is usually based on a characteristic lesion on fundus examination [22,23]. Currently, the clinical diagnosis of ocular toxoplasmosis is based on the observation of a typical necrotizing lesion on the fundus, response to treatment and serological diagnosis [24,25]. Further, laboratory tests include PCR amplification of *T. gondii* DNA in aqueous and vitreous fluids [26-28].

Treatment

Presently practiced drugs for the treatment of *T. gondii* infections mainly target on tachyzoites [29-31]. In general, pyrimethamine in combination with sulfadiazine or with other drugs such as trisulfapyrimidine, sulfonamides, quinine, anti-malarials, and antibodies are used to treat *T. gondii* infections [9]. In a study by Zangerle and Allerberger (1991) [32], it was shown that a combination of trimethoprim and sulfamethoxazole to be significant against *T. gondii* infection. Moreover, new potential drugs have been identified to treat toxoplasmosis, namely, atovaquone, which targets the cytochrome B of mitochondrial electron transport in *T. gondii* [33-35]. Clindamycin which targets the prokaryotic translation machinery is also used as the second line drug to treat *T. gondii* infections [36,37]. However, the dosage of these drugs may change based on the host immunological and gestational status [9].

Necessity for new targets

Presently, the seroprevalence rate of toxoplasmosis in the general population in India is 22.4% and serological IgM positivity (indicating new infections) is 1.43% [38]. Though various drugs as discussed above are available, its usage is limited due to the undesirable side-effects [39,40]. Hence, biologically safer drugs need to be designed to combat toxoplasmosis with minimal off-target effects on the host.

Among the different drug targets for *T. gondii*, the rhoptry proteins which form the moving junction (MJ) complex during host invasion are considered as most potential targets [41-44]. Better understanding of structure-function relationship of these proteins will aid in development of efficient therapeutic and diagnostic modalities. Hence, by this review it is intended to provide insight on rhoptry proteins and MJ complex as ideal candidates for targeting *T. gondii*.

RHOPTRY PROTEINS, MJ COMPLEX AND HOST INVASION

Host cell invasion is the specialized feature of apicomplexan parasites. During the invasion process, these parasites form a tight apposition between the parasites and host cell membrane known as the MJ complex. This helps the pathogen to reorient its apical end to the host cell membrane.

Proteomic analysis has shown 38 novel proteins are involved in parasitic invasion by *T. gondii*. This includes different apical organelles, rhoptry neck (RON2/4/5/8) and rhoptry bulb proteins (ROP) which are secreted by the neck and bulb regions of rhoptry organelle, respectively, while, apical membrane antigen 1 (AMA1) is secreted by the micronemes. Among these RON2/4/5 and AMA1 were shown to be orthologous to *Plasmodium*, *Toxoplasma*, *Neospora*, *Eimeria*, *Theileria* and *Babesia* species, while RON8 seems to be confined to *Toxoplasma*,

Neospora and *Eimeria* species [1,45]. RON's are required for the initial step of host invasion while ROP's take part in the later stages of invasion and parasite establishment [45]. Once these proteins are secreted, subsequent penetration and invasion of parasite is completed in the PV (Figs. 2 and 3) [1,41]. The localization of rhoptry proteins was studied by transmission electron microscopy studies in *Plasmodium falciparum* and *T. gondii*, wherein, it was shown that few sets of specific antigens to be localized in the rhoptry in a site specific manner, which inferred the key role of these proteins in MJ complex formation [46,47]. Proteomics analysis of *T. gondii* rhoptries also showed that only few of its RON proteins to overlap with that of *P. falciparum* and this can be attributed to functional conservation between these apicomplexans [45,48]. Antibody co-localization studies have revealed RON1, RON2, RON4 and RON5 to have common functions in all the apicomplexans [45].

CO-OPERATIVE ASSEMBLY OF RON PROTEINS DURING HOST INVASION

All the rhoptry proteins function co-operatively during the parasitic invasion of apicomplexans on the host cell. Through differential permeabilization and loading of RON specific antibodies targeting the RON complex in the parasite invaded host cell, it was shown that RON4/5/8 continues to be linked with the cytoplasmic region of host [42]. Further, by immunofluorescence analysis, it was shown that RON4 to be co-localized with RON8 in the intracellular region of the host. RON4 and RON8 are basically cytosolic proteins [42], while, RON5 is shown to have N-terminal transmembrane region and rest exposed to the cytosol [42]. Recent studies also demonstrate C-terminal (727-860) of RON4 to interact with C-terminal of human-beta tubulin (315-445) inferred through co-immuno precipitation studies [49].

RON2 and AMA1 complex

RON2 of *T. gondii* comprises of intracellular, extracellular and transmembrane regions. The extracellular region of RON2 mediates anchoring of the parasite to the host cell through its direct interactions with AMA1 protein [50]. The structural complex depicting these interactions are already crystallized (PDB ID 2Y8T) (Fig. 4a and b) [51]. In this, the C-terminal of RON2 was found to interact with AMA1 and the N-terminal of RON2 is predicted to be exposed to the cytoplasmic region of the host cell [50,52]. Moreover, metabolic labeling and co-immuno precipitation has also shown that RON2 and AMA1 were able to interact independently even in the absence of other MJ proteins [42]. In *P. falciparum,* it was also experimentally shown that inhibition of AMA1-RON complex interactions with R1 peptide which is derived based on the interacting interfaces of RON2-AMA1 complex leads to the prevention of parasitic host invasion [52-54]. Immunofluorescent studies with hemagglutinin specific antibodies revealed that the cytoplasmic regions of RON2 also to get co-localized with RON4 protein in the host, during MJ formation [41,50]. Hence, RON2 is considered as the most potential drug target as its function is crucial at the initial level of host-pathogen interactions [42,52,55].

RON8 and host cytoskeleton interactions

RON8 is another crucial MJ protein identified in *T. gondii* and *Neospora* species. This protein is exposed to the cytoplasmic face of the host cell. Biochemical studies have identified RON8 as an additional member of the MJ complex [42,56]. RON8 forms a firm grip when the parasite invades the host cell by its interactions with the cortical cytoskeleton of the host cell. The knockdown of RON8 in *T. gondii* has shown a deficiency in terms of both attachment and entry leading to the loss of firm engagement with the host cell (Fig. 5). RON8 interacts with the actin and microtubule network of the host during the parasitic invasion process [57]. However, the interacting interfaces of RON8 and host cytoskeleton proteins are yet to be elucidated.

RON4 and it interactions with other rhoptry and membrane proteins

RON4 is a cytosolic protein that contributes to MJ formation in apicomplexans [58]. It is found to have a key role during merozoite and sporozoite invasion of the host cells. It is highly conserved in *Plasmodium vivax* and was also found to be homologous to *P. falciparum* RON4 and *Plasmodium knowlesi* RON4. Indirect immunofluroscense of *P. vivax* RON4 at the apical end of schizonts has shown it to be co-localized with *P. vivax* RON2 [48]. The immunoprecipitation assays of *Plasmodium yoelii* merozoite proteins also confirm RON4-AMA1 interactions. However, no evidence for RON2 and RON5 proteins is reported [48,59]. In case of *T. gondii*, *in vitro* studies have demonstrated the probable interactions of AMA1 with RON4. However, it is yet to be elucidated in *in vivo* conditions [42,58,60].

RON5 and its key role in host invasion

RON5 being a cytosolic protein is found to be critical for host invasion by *T. gondii*. Conditional knockout of RON5 protein has shown degradation of RON2 and also mistargeting of RON4, which signifies the loss of RON5 to have an impact on the parasitic invasion of the host cells [44]. Proteolytic separation studies have also shown that C-terminal of RON5 to be essential for the stabilization of RON2 during the invasion process [44]. During RON5 knockdown studies, RON8 was found to be unaffected in contrast to RON4 and RON2. However, the parasites were unable to attack new host cells due to the failure in the release of rhoptries into the host cytosol [44]. All these findings greatly reinforce the significant role of RON5 in host invasion.

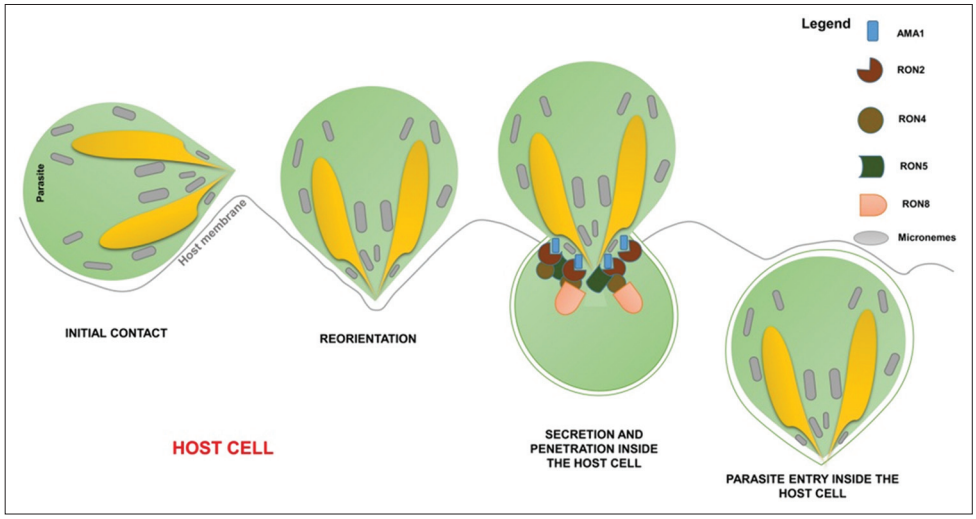

Fig. 2: *Toxoplasma gondii* host invasion mechanism and moving junction formation

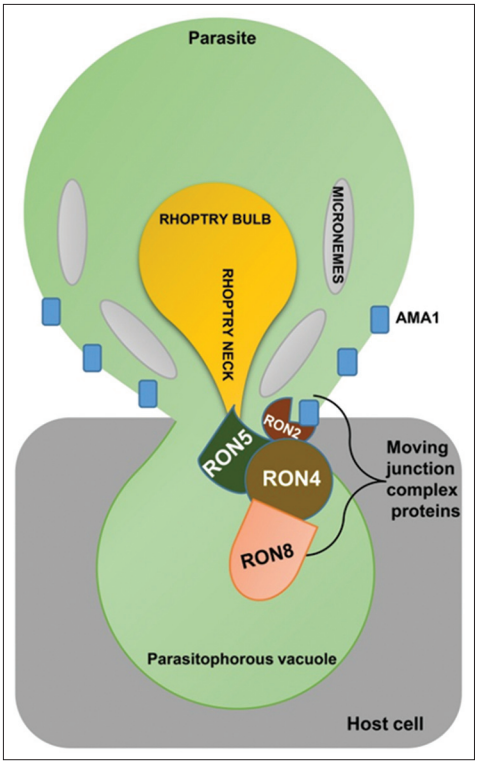

Fig. 3: Detailed representation of the moving junction complex

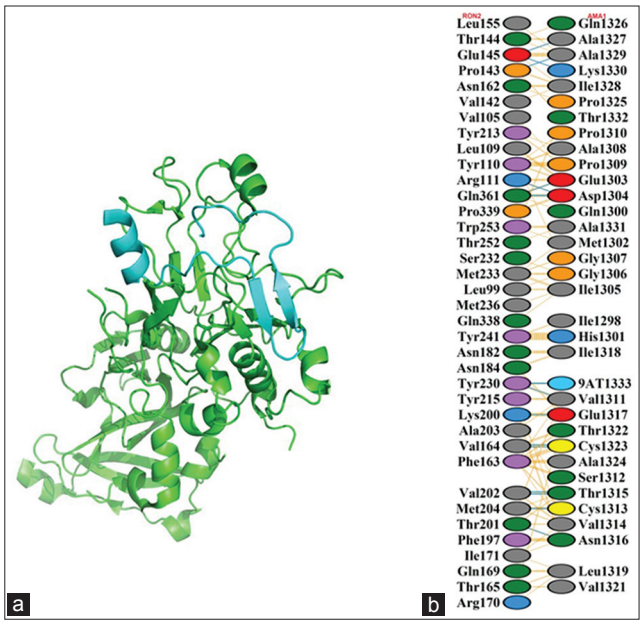

Fig. 4: Molecular interactions of *Toxoplasma gondii* rhoptry neck (RON2) with apical membrane antigen 1 (AMA1) (a) Cartoon representation of RON2-AMA1 complex (RON2-cyan and AMA1-green) (PDB-ID 2Y8T), (b) Ligplot representation of inter-residue contacts between RON2 and AMA1

Role of other rhoptry proteins

RON9 and RON10 are other rhoptry proteins specific to *T. gondii* and yet to be proven as a component in host invasion and MJ complex formation. These proteins were also found to be conserved in *Coccidia* and *Cryptosporidia* species and reported to form a highly stable heteromeric-complex through strong disulfide bonds as inferred through co-immunoprecipitation studies. Moreover, there was no significant change observed in the morphology of these proteins during

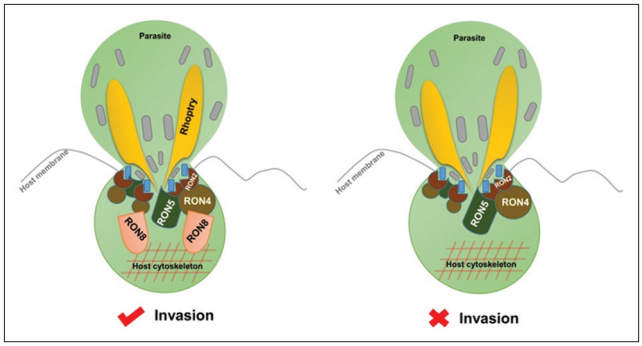

Fig. 5: Schematic representation of rhoptry neck8 and its functional interactions with host cytoskeleton [43]

host invasion process [61]. RON3 is another merozoite rhoptry protein reported in *P. falciparum.* In case of *P. falciparum* infection; this protein is shown to play an important role in the merozoite invasion of the erythrocytes [62]. The gold particle labeling studies also speculate the localization of this protein at rhoptry bulb in *P. falciparum* [62,63]. This protein was also shown to contribute in MJ complex formation during merozoite invasion of host erythrocytes in conjunction with *P. falciparum* RON2 and *P. falciparum* RON4 proteins. Moreover, targeting *P. falciparum* RON3 with antibodies also resulted in the inhibition of parasite invasion on the erythrocytes [62]. *P. falciparum* RON3 protein has three transmembrane regions at its N-terminus. This protein is also found to be highly conserved in the genus of *Plasmodium*, *Eimeria tenella* and *T. gondii.* However, in case of *T. gondii* the role of RON3 and its interactions with other MJ protein is yet to be reported [45,62,64].

RON1 is another addition to *T. gondii* rhoptry proteins [64]. It is reported to be a membrane-associated protein in which the C-terminal contains a glycosylphosphatidylinositol anchor sequences [45]. Another interesting protein of *T. gondii* is Ras-related protein (Rab11) and is suggested to be rhoptry protein inferred through specific antibody targeting studies. Moreover, it was also shown to co-localize with ROP2 [45]. *T. gondii* Rab11 was also shown to be associated with the cytoplasmic face of the rhoptry membrane established by its C-terminal lipid modification [65,66]. Furthermore, Rab11 is also shown to be engaged in regulating exocytosis, trafficking of recycling endosomes and in cholesterol homeostasis [67-69]. Based on all these features Rab11 is suggested to be involved in the regulation of proteins and lipids trafficking into the rhoptries [45].

ROP proteins

ROPs are secreted by the bulb region of rhoptry organelle of apicomplexans during the host invasion. These proteins are sequentially secreted after the RON proteins during the invasion process [64]. The ROP proteins namely, ROP16 and protein phosphatase 2C (PP2C) gets injected to the nucleus of the host cell during parasitic invasion [70,71] whilst, ROP proteins like toxofilin and ROP13 are injected into host cytosol [72,73]. The other ROP proteins, ROP18 and ROP5 belonging to ROP2 family are injected into parasitiphorous vacuole membrane [74-76], whereas the function of other soluble proteins like ROP1 and ROP9 is still unknown [45,77,78]. Furthermore, ROP2, ROP4 and ROP8 are reported to be transmembrane associated proteins of *T. gondii*, among which ROP2 is mainly required for the recruitment of mitochondria in host cell, targeting the exterior part of the PV [45,74,79-81]. Toxofilin is another ROP protein, which is secreted inside the host cytoplasm and was found to interact with parasite actin and PP2C [45,82,83]. Its localization at the apical cytoplasmic regions and presence of a signal peptide suggests that rhoptries are the candidate location for toxofilin [82]. The secretion of ROP proteins is reported to modulate the immune response in the host cell [61]. ROP2 family of proteins is found to have structural conservation of a protein kinase fold [84]. ROP16 and ROP18 are shown to be actively secreted kinases, which highlights these proteins as key virulence factors [61,76,85,86].

CONCLUSION

Current drug targeting studies on *T. gondii* focuses on the inhibition of RON proteins during MJ complex formation. As reported earlier, the initial host-parasite interactions of *T. gondii* is established by the secretion of AMA1 and its interaction with RON2 leading to the formation of tight junction between host and the parasite. Moreover, targeting of AMA1-RON2 interactions is well documented to be a potential strategy to combat *T. gondii* infections. Targeting the interactions of other MJ proteins is also documented to be a potential strategy for therapeutic intervention of *T. gondii* infections. Other studies also speculate RON4 to be a potent target, as it is shown to be a more likely interacting partner of RON2 and RON5. Recent studies also reinforce the high potential of RON5 as a holistic drug target, as it is reported to be the major interacting partner of other key MJ proteins -RON2 and RON4. Among MJ proteins, RON2, RON4 and RON8 are proposed as potential targets, as these proteins play key roles in the initiation of host-pathogen interactions and in the stabilization of MJ complex. Furthermore, as *P. falciparum* RON3 is proven to be a potential drug target, it possess three transmembrane regions at the N-terminal and is also found to be highly conserved in *T. gondii*. This insight opens up opportunities for exploring the possibility of novel MJ complex formation in *T. gondii* as similar to the one demonstrated in *P. falciparum*, thereby, leading to potential target prioritization.

ROP proteins also could be viable targets, as these proteins modulate host immune response and also exhibit kinase activity, which is essential for parasitic virulence. Hence, to combat the *Toxoplasmosis* and other apicomplexan infections, novel drug/peptide inhibitors can be designed to target the molecular interactions of MJ complex proteins as discussed in this review, which shall lead to novel therapeutic approaches toward modulation of host invasion by *T. gondii*.

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