Rapid discovery of inhibitors of *Toxoplasma gondii* using hybrid structure-based computational approach

Sandhya Kortagere · Ernest Mui · Rima McLeod · William J. Welsh

Abstract *Toxoplasma (T.) gondii*, the causative agent of toxoplasmosis, is a ubiquitous opportunistic pathogen that infects individuals worldwide, and is a leading cause of severe congenital neurologic and ocular disease in humans. No vaccine to protect humans is available, and hypersensitivity and toxicity limit the use of the few available medicines. Therefore, safer and more effective medicines to treat toxoplasmosis are urgently needed. Using the Hybrid Structure Based (HSB) method, we have previously identified small molecule inhibitors of *P. falciparum* that seem to target a novel protein–protein interaction between the Myosin tail interacting protein and myosin light chain. This pathway has been hypothesized to be involved in invasion of host erythrocytes by the parasite and is broadly conserved among the apicomplexans. Guided by similar computational drug design approaches, we investigated this series of small molecules as potential inhibitors of *T. gondii*. Compound C3-21, identified as the most active inhibitor in this series, exhibited an IC$_{50}$ value $\sim$500 nM against *T. gondii*. Among the 16 structural analogs of C3-21 tested thus far, nine additional compounds were identified with IC$_{50}$ values <10.0 μM. In vitro assays have revealed that C3-21 markedly limits intracellular growth of *T. gondii* tachyzoites, but has no effect on host cell human foreskin fibroblasts (HFF) at concentrations more than a log greater than the concentration that inhibits the parasites.

Keywords Hybrid structure based method · *Toxoplasma gondii* · Cytotoxicity · Drug design

Introduction

Toxoplasmosis is a clinical problem in persons with immuno-compromising illnesses. Patient classes who are at risk for reactivation of the disease due to *T. gondii* include those undergoing treatment for cancer and auto-immune diseases, and recipients of tissue or organ transplants [1, 2]. Toxoplasmosis also ranks high among diseases that lead to morbidity and death in HIV/AIDS patients, particularly in the developing world. Re-emergence of toxoplasmosis as a life-threatening disease in HIV/AIDS patients is anticipated in the wake of emerging multi-drug resistant strains [3]. *T. gondii* is designated as an NIAID Category B biowarfare (priority) pathogen [4], in large part due to its danger to both humans and livestock, its persistence in the environment, and the ease with which it disseminates. Taken together, these factors affirm the need for novel, improved medicines to treat and cure toxoplasmosis.

There are only a few medicines that will contain the growth of the tachyzoite stage of the parasite [5–11], and use of these medicines is associated with significant incidences of hypersensitivity (25% of adults) [9] and toxicity [12, 13]. No medicines are available that eliminate encysted, latent bradyzoites [14], which are present in $\sim$30% of the population ($\sim$2 billion people) worldwide [15].

S. Kortagere · W. J. Welsh (✉)
University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA
e-mail: welshwj@umdnj.edu

E. Mui · R. McLeod
The University of Chicago, 5841 South Maryland Avenue, MC 2114, Room S-206, Chicago, IL 60637, USA

Present Address:
S. Kortagere
Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA 19129, USA

Combined administration of pyrimethamine with either sulfadiazine or clindamycin is currently the treatment of choice for toxoplasmosis. These medicines are active against the tachyzoite form of *T. gondii*, but neither can access, inhibit or kill the bradyzoite form of the parasite present in tissue cysts [16]. *T. gondii,* the causative agent of toxoplasmosis in humans and other warm blooded animals is an obligate intracellular protozoan parasite belonging to the Apicomplexan family [17]. *T. gondii* exists as sporozoites in oocysts, as bradyzoites in cysts and as tachyzoites. Upon contact with a host cell, sporozoites, tachyzoites and bradyzoites can invade within seconds in a process dependent on actin and myosin driven by parasite motility [18]. The actin-myosin machinery is required for invasion and gliding motility among all the Apicomplexan parasites [19]. Previous studies have shown that the actin-myosin complex is localized at the parasite's inner membrane complex (IMC) with other accessory proteins such as GAP45 and GAP50 [20, 21]. Invasion of the epithelial cells appears to be mediated via the conoid, a cone shaped structure on the tachyzoite, where the IMC is interrupted [22, 23]. At this junction the parasite membrane fuses with the plasma membrane of the host cell creating a pathway for entry [23]. Given the absence of this actin—myosin machinery in the human host and its remote homology to host myosin heavy chains, the myosin tail interacting protein (MTIP) in complex with the myosin light chain tail (called Myosin-A) is an attractive drug target [24–27]. Thus we have previously designed small molecule inhibitors against this protein–protein interaction in *P. falciparum* using the hybrid structure based method [28]. Since the myosin motor is well conserved among apicomplexans, it could be also hypothesized to be a unique drug target in *T. gondii*.

The Myosin Light Chain 1, or TgMLC1 [29–31] is the ortholog of MTIP in *T. gondii*. Using the crystal structure of MTIP-MyoA complex in *P. falciparum*, we constructed a hypothetical structure model of the MTIP-MyoA complex in *T. gondii*. This complex was used an input to the HSB method to screen for small molecule inhibitors of *T. gondii*. In addition, the proposed study also estimated the effect of cytotoxicity of these compounds and hence their validity as lead compounds against *T. gondii* and possibly other apicomplexans.

Materials and methods

Hybrid structure based method

The HSB protocol, described in detail elsewhere [32], was employed for the virtual screening of nearly 3 million compounds to identify small-molecule inhibitors of

T. gondii. The HSB procedure involved 4 major steps, namely: (1) construct a structural model of the *T. gondii* MTIP; (2) design the 3D pharmacophore based on the key molecular interactions between MyoA and MTIP in the complex; (3) screen the in-house molecular libraries to identify "hits"; and (4) re-rank the hits using customized molecular docking and scoring schemes. Subsequent in vitro biological assays including in vitro toxicity studies were conducted on the top hits emanating from this process.

Construction of a structural model of *T. gondii* MTIP and MyoA

In the absence of the X-ray crystal structure for the *T. gondii* MTIP-MyoA complex, homology modeling techniques were employed to build a structural model of the *T. gondii* MTIP-MyoA_tail complex using the published X-ray crystal structure of *Plasmodium* MTIP-MyoA_tail as the template (PDB Entry 2AUC) [21]. This procedure started by aligning the relevant sequences of the corresponding MTIP and MyoA_tail regions of the two proteins. These sequences were aligned using the multiple sequence alignment program CLUSTALW [33]. A three-dimensional model of the *T. gondii* MTIP-MyoA_tail complex was built based upon the high degree of sequence homology between *T. gondii* and *Plasmodium* in these regions (Fig. 1), using the published X-ray crystal structure 2AUC [21] with the homology modeling program Modeller (version 9.0) [34]. Similarly, the MyoA_tail region of *T. gondii* was generated from the structure of MyoA in *P. yoelii* in the MTIP-MyoA crystal structure. The modeled MyoA_tail was manually docked, guided by the pose of the MyoA_tail in the *Plasmodium* MTIP-MyoA crystal structure. Finally, the side chains of the MTIP-MyoA complex of *T. gondii* were energy minimized until convergence was achieved using the default parameterization in AMBER force field (version 9) [35].

Generation of a 3D pharmacophore

The MTIP-MyoA_tail binding region was explored to identify the specific points of interaction between corresponding chemical groups in the MTIP pocket and the MyoA helix (Fig. 2). This procedure yielded a 4-point pharmacophore, which defines the specific 3D geometric arrangement of structural features (chemical atoms/groups) that are responsible for the tight binding between MTIP and MyoA. This analysis was extremely useful, since we utilized the resulting pharmacophore as a pattern to computationally screen an in-house chemical library containing over three million commercially available compounds to identify hits that match these constraints. The combined pharmacophore

Fig. 1 **a** Sequence comparison of the MTIP regions of *P. knowlesi* and MLC1 from *T. gondii*. Sequence information was taken from the Apicomplexan Database (http://apidb.org/apidb/). Residues colored in *red* represent those amino acids that form the binding pocket for the MyoA_tail helix in *P. knowlesi* (PDB entry 2AUC) [21]. Symbols: *asterisk* means identity; *colon* means high similarity; and *dot* means similarity. **b** Sequence comparison of the corresponding MyoA_tail regions of *P. yoelii* and *T. gondii*. This 13-residue sequence was obtained from the *Plasmodium* crystal structure (PDB entry 2AUC) [21]. Residues colored in *red* were used for the generation of the 4-point pharmacophore
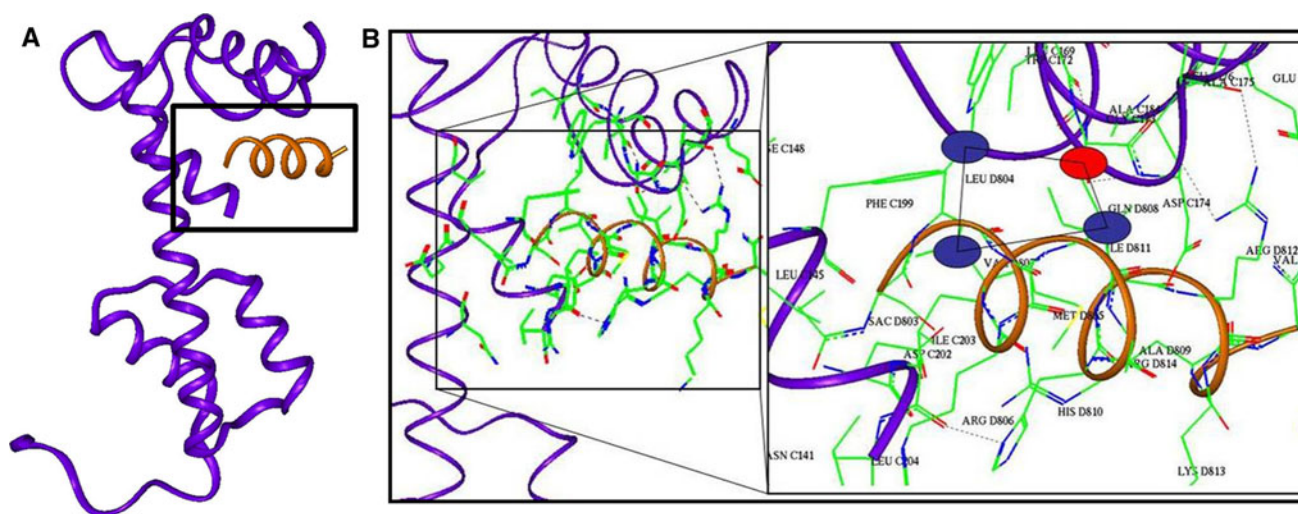

Fig. 2 **a** MTIP-MyoA_tail interaction in *T. gondii* model depicts the helical MyoA_tail (*orange ribbons*) binding within a deep groove of MTIP (*blue ribbons*). **b** Image depicting interaction region for *T. gondii* MTIP (*blue* backbone, *green* sidechains) and the MyoA helix (*amber helix*). Inset at far *right* shows more detailed, close-up image of the binding pocket and key interactions between MTIP and MyoA. Our 4-point pharmacophore comprises residues Gln808 (*red* oval, hydrophilic), Leu804, Val807 and Ile811 (*blue* ovals, hydrophobic)

was used to screen the molecular libraries using UNITY module of Sybyl (version 7.1, Tripos Inc) to identify hits.

The resulting 150 hits were then docked in the MTIP binding groove using the "docking & scoring" program GOLD (version 3.1) [36], which were then ranked using GoldScore, Chemscore and a customized scoring scheme to identify 60 best ranking hits [28, 32]. The customized scoring scheme was developed based on the interactions of

the ligands with MTIP. A complete description of the method is available from Kortagere and Welsh [32]. Briefly, the method consists of deriving the information about all the interacting pairs of atoms between the ligand and the protein binding site residues that are within a specified cutoff distance. A contact score weight w_i was assigned to each of the ligand *i* docked into the protein such that:

$$\sum_{i=1}^{N} w_i = 1 \qquad (1)$$

the customized docking score of an active compound j with i conformations was derived as.

$$S_{i,j} = w_i s_{ij} \qquad (2)$$

where s_{ij} was the Goldscore for the compound j in its ith conformation. Further, similar calculations were performed by replacing Goldscore with Chemscore. Finally, the consensus score for compound j was computed as:

$$S_{\max,j} = \max(S_{i,jgoldscore}, S_{i,jchemscore}) \qquad (3)$$

The 60 best ranking compounds ranked by the consensus scoring scheme were filtered with respect to LogP, MW, and other *Lipinski Rule of 5* criteria (ChemAxon®) to yield a final ranked subset of 25 compounds which were purchased and tested in inhibition assays against *T. gondii* to assess their in vitro inhibitory activity.

Parasite inhibition assays

Four-day old confluent cultures of human foreskin fibroblasts (HFF), cultured in 96-well plates, were infected with 2.0×10^3 tachyzoites of RH strain of *T. gondii* per well and cultured for 1 h to allow parasite invasion. Inhibitors were added and cells cultured for 3 days. They were then supplemented with ³H uracil and cultured overnight, allowing for uracil incorporation into cells and thus parasite growth to be assessed by liquid scintillation counting [37]. Absence of toxicity for mammalian host cells was demonstrated first by visual inspection of the monolayer and in separate ³H thymidine incorporation assays by non-confluent cell monolayers.

Results and discussion

Modeling the MTIP-MyoA interactions and inhibitor discovery

The parasite *T. gondii* exists in three forms: oocysts, bradyzoites and tachyzoites and may remain dormant until it comes into contact with the right type of host cell. Upon contact with a host cell, these parasites can invade within seconds in a process dependent on actin and myosin driven by parasite motility [18]. Uniquely, the invasive machinery employed by *T. gondii* and other apicomplexan parasites for entering and leaving all host cells (insect, animal, human) involves a critical interaction between two proteins: the MyoA_tail, and MTIP. Our design strategy was

to develop a small-molecule competitive inhibitor that disrupts the formation of the protein–protein complex between MTIP and MyoA. Specifically, the inhibitors would block the binding of the α-helix of the MyoA_tail in the MTIP binding pocket of *T. gondii*. The published X-ray crystal structure of the MTIP-MyoA complex in *Plasmodium* (PDB code: 2AUC) [21] enabled us to implement our HSB strategy [32] that integrates ligand-based and structure-based drug design approaches. Since the crystal structure for the *T. gondii* MTIP-MyoA complex was not available during the present study, homology modeling techniques were used to build a structural model of the *T. gondii* MTIP-MyoA_tail complex. This was made possible because sequence comparison between *P. knowlesi* and *T. gondii* for the MTIP protein revealed a high degree of conservation (identity is 80% overall and 85% in the MTIP binding groove) between the two species (Fig. 1a), while sequence alignment of the corresponding MyoA_tail regions revealed 54% identify and 100% similarity (Fig. 1b). However, it is worth noting that the published X-ray crystal structure of the Plasmodium MTIP-MyoA complex [2AUC] is composed of two highly similar, but distinct, *Plasmodium* species: MTIP from *P. knowlesi*, and MyoA from *P. yoelii* [21]. Superposition of the backbone atoms of our *T. gondii* structural model and the *Plasmodium* crystal structure showed a modest root mean squared deviation (rmsd) of 1.27 Å.

The 17 residues of *Plasmodium* MTIP interacting with the MyoA_tail were highly conserved, including Phe-149, Leu-169, Leu-176, and Phe-199 which were totally identical in the sequenced *Plasmodium* and *Toxoplasma* MTIPs. Using this structural information for MTIP and MyoA, a four point pharmacophore was developed based on the interaction of the modeled MyoA tail with modeled MTIP structure of *T. gondii*. One hundred and fifty molecules were initially identified by screening the Shape Signatures databases using the 4-point pharmacophore as a query to the UNITY program (Fig. 2). These hits were docked into the binding site of MTIP of *T. gondii* and the docked complexes were ranked using consensus scoring function to identify the 60 best ranking compounds. Further, these compounds were then filtered through ADMET filters to identify the 25 best ranking drug-like compounds. The in silico screening, docking and scoring of 150 compounds takes about 2 hours on a quad core dual linux processor with an operating system of Red Hat enterprise linux (ver 5), hence justifying the rapidness of the approach.

Results from the in vitro inhibition assay in which tachyzoites of *T. gondii* type 1 strain were incubated before and after challenge with 10 compounds are shown in Fig. 3a. Each compound was tested in triplicate. Comparison of the rank order of the 10 test compounds between
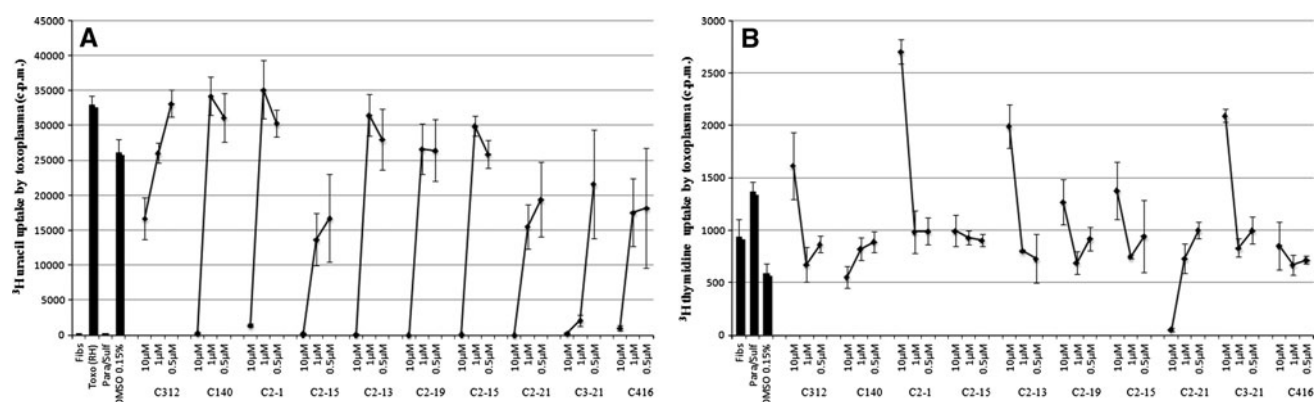
Fig. 3 **a** Inhibition of *T. gondii* tachyzoites by various compounds (shown along *X*-axis with # compound number), measured as diminished uptake of tritiated uracil c.p.m*10^3 (*Y*-axis). Results from three different concentrations (10, 1 and 0.5 μM) of the compounds are reported. **b** Results from cytotoxicity assays for the primary set of 10 compounds are shown. No compounds, except 10 μM of compound 16, were toxic to the host cell using a ^{3}H thymidine assay

their experimental values and computational predictions (Table 1) revealed good agreement (correlation coefficient $r^2 = 0.84$). Among the 10 structural analogs of C3-21 tested, we have found IC$_{50}$ values < 10.0 μM for 9 compounds and < 5.0 μM for 4 compounds (Fig. 4a).

Insights from predicted C3-21 interactions with MTIP

Inspection of C3-21 docked inside the MTIP ligand binding pocket (Fig. 5) revealed several probable key interactions shared by the *T. gondii* MyoA_tail. C3-21 was oriented such that R1 was largely solvent exposed; R2 and R4 blocked the opening of the binding pocket; and R3 extended deep inside (8–9 Å) the binding cleft to engage in specific hydrophobic and hydrophilic contacts. These insights provided valuable clues in making appropriate choices for substituents R1, R2, R3, and R4 to identify the second generation of C3-21 like compounds. Specifically, we chose sterically compact hydrophilic groups at R1 (e.g., –C(O)–OCH$_3$, –CH$_2$OH, –OCH$_3$, –CH$_2$NH$_2$, and –CH$_2$N(Me)$_2$); and aromatic groups at R2 and R4 substituted with hydrophobes –Me, –Et, –Pr, –t–Bu, –F, –Cl, and –Br alone or in combination at the ortho and para positions. Finally, R3 was chosen to mimic MyoA's hydrophobic tail (Leu804, Val807, Ile811) and strongly basic Arg812 that together filled the binding cleft. Based on this choice of functional groups, six candidate molecules were synthesized and screened using the inhibition assay described above. The results are shown in Fig. 4a. In this set of compounds, C3-20 performed significantly better than the parent compound in the parasite inhibition assay against *T. gondii*.

Further, in vitro cytotoxicity studies of C3-21 and its analogs were performed using host human foreskin fibroblasts (HFF). The results from the study showed no evidence of toxicity in these cells despite exposure and incubation with the compounds for 4 days with a concentration at least 1.5 logs greater than the IC$_{50}$ (see Figs. 3b, 4b).

Comparison of activities of compounds between *T. gondii* and *P. falciparum*

All the compounds described in this study were initially assessed against *P. falciparum* [28]. The MTIP and MyoA complex was first discovered in *P. falciparum* and was shown to be a key component of the parasite invasion machinery. The components of the invasion machinery have been hypothesized to be conserved among all members of the Apicomplexan family [20, 22]. Interestingly, C3-21 has an IC$_{50}$ value of 500 nM against *T. gondii* and 385 nM against *P. falciparum*. A similar trend was observed with C3-20 that has an IC$_{50}$ of ∼400 nM against *T. gondii* and 300 nM against *P. falciparum*. Previously, we have shown that C3-21 inhibited the gliding motility of *P. berghei* sporozoites in a dose dependent manner [28]. Given the similar levels of efficacy in both *T. gondii* and *P. falciparum*, we hypothesized that both C3-20 and C3-21 must be inhibiting the parasites through a similar mechanism of action involving MTIP. Among the other compounds, C416 and C2-1 were potent inhibitors of *P. falciparum* with IC$_{50}$ values of 145 and 47 nM respectively. However, both these compounds inhibited *T. gondii* with IC$_{50}$ values between 4 and 10 μM, suggesting they probably work through other targets.

Conclusions

The present study has led to the discovery of C3-21 as a lead compound, with an IC$_{50}$ < 500 nM against *T. gondii*. Among the 16 structural analogs of C3-21 tested thus far, we have found 4 compounds with IC$_{50}$ < 5.0 μM and 9

Table 1 Predicted ranking of compounds using HSB method and their corresponding IC_{90} and IC_{50} values are reported
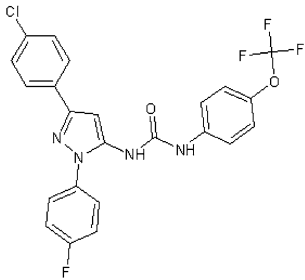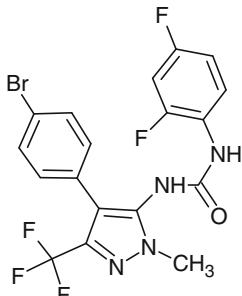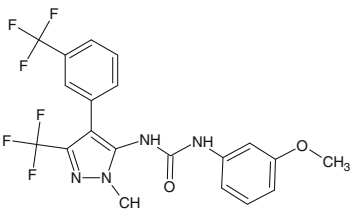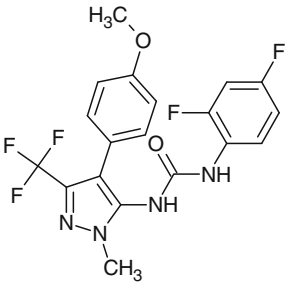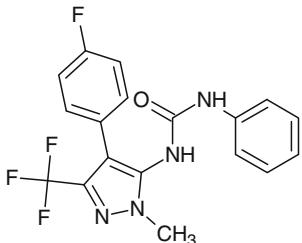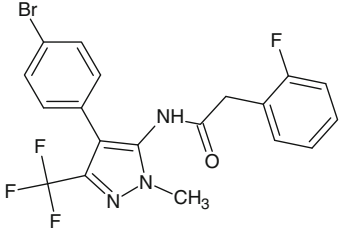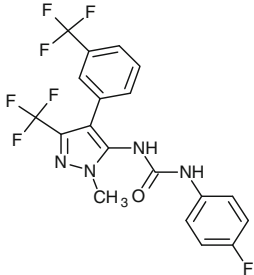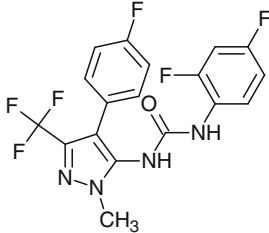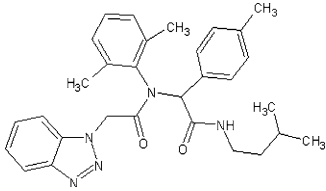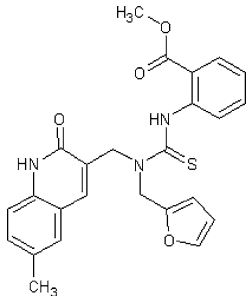
Compound	HSB ranking	Expt. ranking	IC90 (μM)	IC50 (μM)	Toxicity[a]
C3-21	1	1	≤0.75	<0.5	>10
C2-12	2	3	<10[b]	<10	<10
C2-15	3	2	<5	~1	>10
C416	4	4	10 > n > 1 [+]	10 > n > 1	>10
C2-19	5	5	10 > n > 1	10 > n > 1	>10

Table 1 continued

Compound	HSB ranking	Expt. ranking	IC90 (μM)	IC50 (μM)	Toxicity[a]
C2-1	6	9	10 > n > 1	10 > n > 1	>10
C2-21	7	6	10 > n > 1	10 > n > 1	>10
C2-13	8	7	10 > n > 1	10 > n > 1	>10
C140	9	8	10 > n > 1	10 > n > 1	>10
C312	10	10	>10	≥10	>10

[+] 10 > n > 1 denotes the value to be between 10 and 1 μM, less than 10 but greater than 1; 10 and 1 μM were the concentrations tested

[a] Toxicity determined using highest tested concentration of compound (10 μM) on host cells compared with 0.15% DMSO (v/v) control

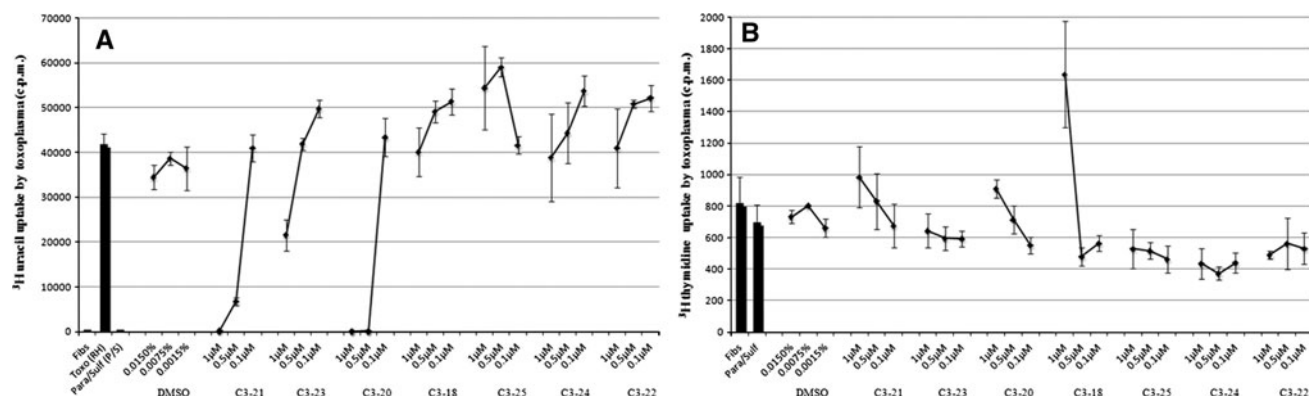[b] Toxic to host cells at this concentration

Fig. 4 a Inhibition of *T. gondii* tachyzoites by analogs of compound C3-21 measured as diminished uptake of tritiated uracil (*Y*-axis). Results from three different concentrations (1, 0.5 and 0.1 μM) of the compounds are reported. **b** All analogs of compound C3-21 (shown along *X*-axis with compound number and measured in 3 different concentrations namely 1, 0.5 and 0.1 μM) showed no evidence of toxicity on human HFF host cells, measured as uptake of tritiated thymidine c.p.m*10^3(*Y*-axis)

Fig. 5 a C3-21 computationally docked inside the MTIP binding pocket. C3-21 is rendered in orange space-filling mode, while MTIP is shown as blue ribbons. **b** Close-up view of C3-21(in *orange* space fill mode) in the binding site of MTIP. Interacting residues from MTIP are shown in stick mode and colored atom type (C-*green*, O-*red*, S-*yellow*, N-*blue*) **c** Drawing of C3-21 core structure and substitution pattern (R1–R4) which is optimized for tight binding within the MTIP binding pocket. X1 and X2 can be C, N, O, S
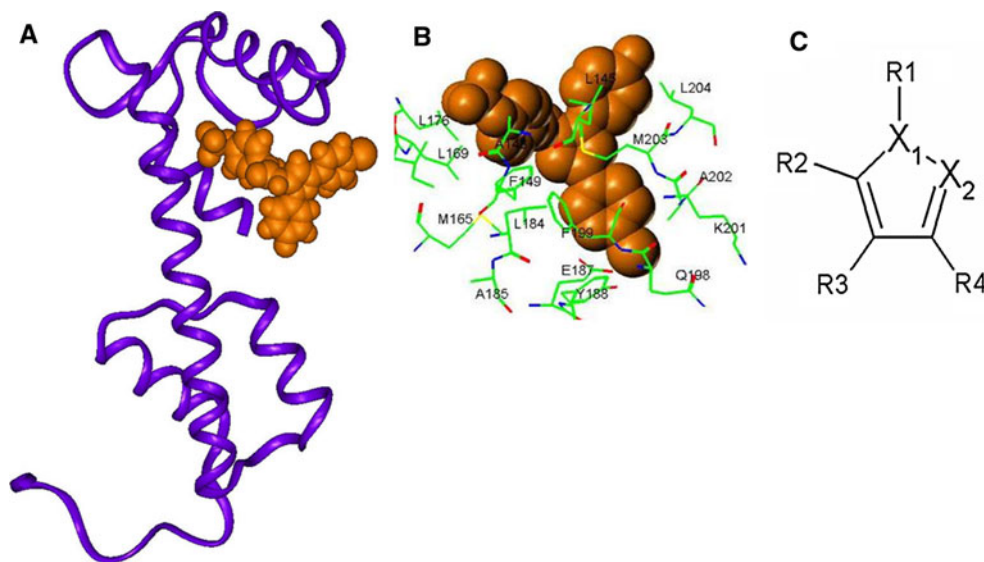
compounds with $IC_{50} < 10.0$ μM. C3-21 and its analogs were selected to target the MTIP binding pocket, thereby acting as competitive inhibitors of the MTIP MyoA_tail interaction. In vitro assays have revealed that C3-21 markedly limits invasion and thus subsequent intracellular growth of *T. gondii* tachyzoites, but has no effect on host cell human foreskin fibroblasts (HFF) at concentrations more than a log greater than the concentration that inhibits the parasites. Interestingly, C3-20 and C3-21 have nearly similar IC50 values against *T. gondii* and *P. falciparum* and may be working through similar mechanism that warrant consideration as broad spectrum inhibitors of apicomplexan parasites. Further studies are planned to validate their target and mechanism of action as starting points for the development of therapeutic inhibitors of *T. gondii*.

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