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The potential of quinoline derivatives for the treatment of *Toxoplasma gondii* infection

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HIGHLIGHTS

- Quinoline derivatives are effective at inhibiting the growth of *Toxoplasma*.
- Some quinolones cause the disappearance of the apicoplast.
- 8-Hydroxyquinoline is very effective to inhibit the growth of *Toxoplasma*.

GRAPHICAL ABSTRACT

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ABSTRACT

Here we reported our investigation, as part of our drug repositioning effort, on anti-*Toxoplasma* properties of newly synthesized quinoline compounds. A collection of 4-aminoquinoline and 4-piperazinylquinoline analogs have recently been synthesized for use in cancer chemotherapy. Some analogs were able to outperform chloroquine, a quinoline derivative drug which is commonly used in the treatment of malaria and other parasitic infections. Herein 58 compounds containing one or two quinoline rings were examined for their effectiveness as potential anti-*Toxoplasma* compounds. Of these 58 compounds, 32 were efficient at inhibiting *Toxoplasma* growth ($IC_{50} < 100 \mu M$). Five compounds with single and simple quinoline rings exhibited similar cLogP values of ~ 2 and IC_{50} values between 5 and 6 μM , with one exception of 8-hydroxyquinoline whose IC_{50} value was 213 nM. The addition of one hydroxyl group at position 8 caused a 40-fold increase in the inhibitory effect of quinoline. A significant improvement in anti-*Toxoplasma* effect among quinoline derivatives was detected in **B11**, **B12**, **B23**, and **B24**, whose structures carry two quinoline rings, and their resultant cLogP values are ≥ 7 . Among these compounds, **B23** was the most effective compound with IC_{50} value of 425 ± 35 nM, and TI value of 4.9. It was also noted that compounds with at least one quinoline ring, displaying anti-*Toxoplasma* effects were capable of causing the disappearance of the apicoplast, a plastid-like organelle. When treated with quinoline, 8-hydroxyquinoline or **B23**, 40–45% of the parasites lost their apicoplasts. Our findings recapitulate the properties of quinoline derivatives in diminishing apicoplast. This could aid further investigations of anti-parasitic treatments specific to Apicomplexan. More importantly, **B12** and **B23** which harbor superior anti-cancer properties than chloroquine, have effective anti-*Toxoplasma* activity. These compounds therefore have significant potential for future development of chemotherapeutic agents for patients suffering from breast cancers and parasitic infection.

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1. Introduction

Quinoline alkaloids, heterocyclic aromatic organic (C_9H_7N) natural compounds, are known to harbor various medicinal properties and are present in a number of national pharmacopoeias. Their synthetic quinoline counterparts also carry important biological activities. 8-Hydroxyquinoline has growth inhibitory effects against gram positive bacteria and some lower level eukaryotes (Shen et al., 1999), and has been used in the veterinary sciences as an antimicrobial agent for topical treatment (Gershon et al., 1985). The mode of action of 8-hydroxyquinoline is a result of its chemical property as a metal chelator and thought to sequester metals necessary for important enzymes in micro-organisms (Shen et al., 1999). 8-Nitroquinoline has been successfully exploited in the treatment of topical pathogenic parasitic *Leishmania* infection (Paloque et al., 2012). As early as the 17th century, quinine [(R)-(6-Methoxyquinolin-4-yl)((2S,4S,8R)-8-vinylquinuclidin-2-yl)methanol], an active constituent from the barks of cinchona tree, was used both as a muscle relaxant to halt shivering due to the infection caused by *Plasmodium* spp. and as an anti-malaria agent. Malaria was and remains a serious and sometimes fatal disease. Due to an overwhelming demand for quinine and limited supplies of cinchona barks, the need for readily available and alternative sources of quinine-equivalent compounds gave rise to the discovery of the first synthetic acridine derivative, quina-crine [(RS)-N'-(6-chloro-2-methoxy-acridin-9-yl)-N,N-diethyl-pentane-1,4-diamine], around the time of the First World War (Butler et al., 2010). Soon after the discovery of quina-crine, a more potent quinoline derivative, chloroquine [(RS)-N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine], was synthesized (Butler et al., 2010; Solomon et al., 2009). Quinine, quina-crine, and chloroquine are still currently used as anti-malaria agents (Butler et al., 2010). It was hypothesized that these compounds prevent the formation of hemozoin by *Plasmodium* parasites (Foley and Tilley, 1998). Hemozoin detoxifies free heme intracellularly, as free heme and heme-drug complexes destroy the parasite by creating oxidative stress (Slater, 1992). More detailed structural and functional studies showed that 7-halo substituted compounds are the most active compound. Chloroquine, a 4-aminoquinoline carrying a chlorine moiety at position 7, is an effective anti-malaria agent currently used in the treatment and prevention of malaria (Butler et al., 2010), and as a reference compound to evaluate the ability of chemicals to inhibit hemozoin formation (Foley and Tilley, 1998). Despite their effectiveness, emerging resistance of *Plasmodium* spp. has stimulated further drug discovery and development (Butler et al., 2010).

A simple backbone quinoline and 2-methyl-3-carbethoxyquinoline have also been shown to inhibit the growth of *Toxoplasma gondii*, a *Plasmodium*-related parasite. When treated with either simple quinoline or 2-methyl-3-carbethoxyquinoline, 52–57% of *Toxoplasma* lost their unique organelle called apicoplast (Smith et al., 2007). Similar activity was previously reported with ciprofloxacin, a fluoroquinolone derivative (Fichera and Roos, 1997). It was hypothesized that the crucial pharmacophore conferring this biological activity is the quinoline ring structure (Smith et al., 2007). Interestingly, the presence of an apicoplast is generally used in grouping *Plasmodium* and *Toxoplasma* parasites as pathogenic members of the Apicomplexa phylum. The apicoplast is a non-photosynthetic plastid organelle. In *Plasmodium* and *Toxoplasma* parasites, the apicoplast is crucial for three essential metabolic functions: the synthesis of heme, type II fatty acids and isoprenoid precursors (Sheiner et al., 2013), and thus has long been considered an ideal target for treating infection caused by Apicomplexan (Fichera and Roos, 1997). Pathogenic Apicomplexan, besides *Plasmodium* (malaria in humans) and *Toxoplasma* (toxoplasmosis in

humans and animals), are the following. *Cryptosporidium* can cause cryptosporidiosis whose major symptom in humans is diarrhea (Tzipori and Ward, 2002). *Eimeria* is the major cause of coccidiosis in agriculturally important animals, including poultry, sheep, and cattle (Shirley and Harvey, 2000). *Babesia* parasites can cause a hemolytic disease known as babesiosis in humans and occasionally in domestic animals (Vannier and Krause, 2009). *Neospora* is an important protozoan pathogen in cattle and dogs, while *Theileria* are important cattle parasites (Katzer et al., 2011).

Drug repositioning can be described as the investigation of new medicinal uses and purposes for previously known and tested drugs (Solomon et al., 2010a, 2010b). Such practices could help overcome high costs and time often associated with the development of new drugs because the known and tested drugs have previously or generally been evaluated or recognized for their pharmacokinetics properties (Chong and Sullivan, 2007). The repositioning studies of chloroquine derivatives (Solomon et al., 2010a, 2010b) have been very valuable in generating a large collection of quinoline derivatives. By combining two important pharmacophores, 4-piperazinylquinolones and isatin structure, novel compounds were created with versatile biological effects. For example, **B12** and **B23** with their hybrid pharmacophore exhibit cancer-specific and are more effective than chloroquine (Solomon et al., 2010b). Due to the presence of multiple structural determinants of the hybrid pharmacophores, these newly generated compounds harbor great potentials to target different sites or molecules, which might result in the possibility to disrupt multiple cellular mechanisms simultaneously and/or to overcome drug resistance (Meunier, 2007).

Here we reported our evaluation for anti-*Toxoplasma* activity of a wide array of quinoline derivatives, ranging from simple molecules with null or one substitution to multiple substitutions (Tables 1–3). *Toxoplasma* was chosen as our model organism because (1) it can be easily cultivated in vitro using human fibroblast monolayers (HFF); (2) its doubling time is relatively short (~7 h) (Seeber and Boothroyd, 1996); (3) the availability of its 2F strain to express beta-galactosidase reporter system offers an accurate and effective growth evaluation (Seeber and Boothroyd, 1996), and (4) protocols for growth inhibition and toxicity assays are well documented (Seeber and Boothroyd, 1996; Jones-Brando et al., 2003). In general, *Toxoplasma* infection is quite common, affecting a quarter of the world's population, although not all infections lead to lethal toxoplasmosis. *Toxoplasma* infection can cause very mild or no symptom in healthy individuals, but becomes severe in immunocompromised individuals (Montoya and Rosso, 2005). The infection occurs via ingestion of contaminated food or water. If the infection takes place during pregnancy, the parasite could be transmitted to a fetus and could cause blindness and mental retardation. Current treatments for *Toxoplasma* infection include pyrimethamine in combination with sulfadiazine and/or clindamycin (Montoya and Rosso, 2005). Pyrimethamine and sulfadiazine inhibit the enzyme dihydrofolate reductase of the tetrahydrofolic acid synthesis, whereas clindamycin inhibits bacterial protein synthesis by inhibiting ribosomal translocation. These current treatments could have severe side effects such as, the inhibition of bone marrow formation and the teratogenic nature of pyrimethamine as a folate inhibitor, precluding it from being used in pregnant women during their first trimester. Sulfadiazine could cause allergic reactions in immunocompromised individuals (Montoya and Rosso, 2005; Peters et al., 2007). Because of these limitations associated with current treatments, it is of great importance to evaluate and find alternative safe treatments. Evaluation of the biological properties of quinoline compounds for their ability to inhibit the growth of *Toxoplasma* could reap benefits for the ongoing search for effective treatments against the devastating effects of apicomplexan infection.

Table 1
Simple quinolines.

Compound name	Structure	cLogP	IC ₅₀ (μM)	TD ₅₀ (μM)	TI
Quinoline		2.03	8.00 ± 2.83	12.5 ± 0.707	1.56
8-Hydroxyquinoline		2.08	0.213 ± 0.194	0.500 ± 0.0707	2.35
2-Chloroquinoline		2.83	5.75 ± 1.06	8.50 ± 2.12	1.48
5-Nitroquinoline		1.91	7.25 ± 0.354	11.3 ± 2.47	1.56
Quinoline N-oxide hydrate		1.42	6.40 ± 2.26	7.50 ± 2.12	1.17

2. Materials and methods

2.1. Chemicals

Compounds in Table 1 were obtained from Sigma-Aldrich (Canada), while others in Tables 2 and 3 were synthesized and purified according to Solomon et al. (2010a, 2010b). Stock solutions (10 mM) were prepared in DMSO or water, kept at -20 °C and diluted to desirable concentrations when needed.

2.2. Cell and parasite cultures

HFFs were grown into confluent monolayers for cytotoxicity and parasite's growth inhibition assays using DMEM (Invitrogen, Canada) which was supplemented with 10% cosmic calf serum (Hyclone, Utah), and 0.5x Antibiotic-Antimycotic (Invitrogen, Canada). *Toxoplasma* strain 2F was obtained from ATCC and proliferated using HFF in MEM containing L-glutamine (Invitrogen, Canada) which was supplemented with 1% dialyzed fetal bovine serum and 0.5x Antibiotic-Antimycotic and 20 μg/mL gentamycin (Gibco, Canada).

2.3. Parasite growth inhibition and cytotoxicity assays

Protocols described by McFadden et al. (1997) and Jones-Brando et al. (2003) were adapted for our study. Briefly

HFF monolayers were grown in 96-well plates and used for evaluation of cellular cytotoxicity of the compounds and their ability to inhibit the growth of *Toxoplasma*. Immediately prior to the evaluations, their regular culturing media were rinsed twice and replaced with 150 μl culturing media [1% dialyzed fetal bovine serum (Hyclone, Logan, Utah), and 0.5% Antibiotic-Antimycotic supplemented MEM lacking phenol red, cat #M3024-10X1L, Sigma-Aldrich, Canada]. Tested compounds were added and serially diluted into 11 columns of the plates. Generally the compounds were diluted to achieve concentrations ranging from 10 μM to 0.01 μM. However, for some compounds a higher range (100 μM to 0.1 μM) was needed. Into the six rows (6/8) of the plates, approximately 50 tachyzoites of 2F parasites were added and incubated for 4 days at 37 °C and 5% CO₂. On day 4, chlorophenol Red-β-D-galactoside (CPRG, Sigma-Aldrich, Canada) was added to the wells containing infected monolayers to a final concentration of 100 μM. On the following day, into two (2/8) rows without the parasite additions, cytotoxicity testing reagent called MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) (Sigma, Canada) was added to a final concentration of 5 mg/ml. The plates were incubated for 4 h to allow for tetrazolium salt to form as an indicator of mitochondrial function (Mosmann, 1983). The culture media in the row containing MTT solution was replaced with 100 μl of N-N-dimethylformamide containing 5% SDS. The products of beta-galactosidase and cytotoxicity assays were purple allowing the absorbance measurement at 560 nm using a Victor3

Table 2
4-Piperazinylquinoline derived compounds.

Compound name	Structure	X	R	cLogP	IC ₅₀ (μM)	TD ₅₀ (μM)	TI
A2		Cl	4-Br	5.03	4.25 ± 0.353	9.97 ± 2.05	2.35
A3		Cl	6-Cl	4.95	2.40 ± 1.41	11.7 ± 2.88	4.88
A4		Cl	6-Br	5.03	3.50 ± 1.414	9.50 ± 0.707	2.71
A5		Cl	H	3.99	2.75 ± 0.354	8.00 ± 0.707	2.91
A6		CF ₃	4-Cl	5.10	4.00 ± 2.83	7.95 ± 0.070	1.99
A7		CF ₃	4-Br	5.25	7.5 ± 2.12	14.0 ± 1.41	1.87
A11		Cl	4-Cl	5.18	0.895 ± 0.418	5.00 ± 0.707	5.59
A12		Cl	4-Br	5.33	2.45 ± 0.777	8.00 ± 1.41	3.27
A14		Cl	6-Br	5.33	0.800 ± 0.282	2.80 ± 0.283	3.50
A15		CF ₃	4-Cl	5.40	2.10 ± 0.141	8.00 ± 1.414	3.81
A16		CF ₃	4-Br	5.55	1.75 ± 0.354	5.75 ± 0.353	3.29
A17		CF ₃	6-Cl	5.40	1.05 ± 0.354	4.75 ± 0.354	4.52
A18		CF ₃	6-Br	5.55	0.725 ± 0.176	3.15 ± 0.495	4.34
A24		–	H	1.26	3.00 ± 0.707	9.00 ± 1.41	3.00

microplate reader (Perkin Elmer Life and Analytical Sciences, Woodbridge, ON, Canada). The median inhibitory and cytotoxic doses were used for the calculation, where experimental data were not constrained to 0% and 100%. The Reed–Muench formula (Reed and Muench, 1938) was applied to obtain the concentration at which 50% of parasite multiplication (IC₅₀) was effectively inhibited, and the concentration at which 50% of the monolayers exhibited cytotoxic effect (TD₅₀). Therapeutic index (TI) was calculated as TI = TD₅₀/IC₅₀.

2.4. Microscopic determination

HFFs were grown on coverslips and infected with 2F for 24 h. The media was replaced with chilled methanol for fixing. Hoechst solution (10 μM) was used to stain DNA containing organelles to allow for microscopic determination of apicoplasts. For each test,

at least 50 independent fields of view were examined for whether or not the parasites had retained their apicoplasts (Leica fluorescence microscope). Average percentage of the numbers of the parasites with apicoplasts was calculated from each test and for each compound. Three experimental replicates were performed, from which a final average for each compound was obtained. A one-way ANOVA with corrections for multiple comparisons using the Bonferroni method was performed, with alpha set as 0.05/5 to account for different treatment groups (McDonald, 2008). Furthermore, a two tailed student's *t*-test was performed to test the null hypothesis, that there was no statistical difference between the treatment of parasites with DMSO and the tested compounds. The value of alpha, which is associated with the confidence level of the experiments, was set at 0.05 (1–0.95) for results with 95% level of confidence. The results were tabulated and used in comparing the effects among the treatments.

3. Results and discussion

3.1. *Toxoplasma* growth inhibition and cytotoxicity

Fifty-eight compounds were divided into three groups; (i) five quinolines with single substituents, (ii) twenty-four analogs of 4-piperazinylquinoline (group A), and (iii) twenty-nine 4-aminoquinoline derivatives (groups B), for which chloroquine was used

as reference compound. Notably, DMSO at tested concentrations yielded no adverse effect to HFF and 2F. From the initial round of evaluation, eighteen compounds which did not exhibit a growth inhibition curve were eliminated from the list. Among the remaining forty compounds, eight compounds, which exhibited $IC_{50} > 100 \mu M$, were deemed less probable for further testing due to their limited potency. The remaining thirty-two compounds were subjected to at least two independent assays of which each assay

Table 3
Chloroquine derived compounds.

Compound number	Structure	cLogP	$IC_{50}(\mu M)$	$TD_{50}(\mu M)$	TI
Chloroquine		5.06	2.25 ± 0.354	9.00 ± 1.41	4.00
B4		3.54	1.35 ± 0.212	5.67 ± 1.15	4.20
B11		7.00	0.875 ± 0.177	1.43 ± 0.513	1.63
B12		7.13	0.750 ± 0.141	1.27 ± 0.550	1.69
B15		5.06	0.95 ± 0.071	5.25 ± 0.354	5.53

(continued on next page)

Table 3 (continued)

Compound number	Structure	cLogP	IC ₅₀ (μ M)	TD ₅₀ (μ M)	TI
B16		5.21	1.13 $\pm$ 0.671	3.75 $\pm$ 0.354	3.32
B18		4.70	5.25 $\pm$ 0.354	10.0 $\pm$ 1.41	1.90
B22		6.03	1.13 $\pm$ 0.247	3.00 $\pm$ 1.41	2.65
B23		7.25	0.425 $\pm$ 0.035	2.1 $\pm$ 0.566	4.94
B24		7.46	0.450 $\pm$ 0.071	1.75 $\pm$ 0.354	3.89
B26		5.30	4.75 $\pm$ 1.06	8.25 $\pm$ 1.77	1.74

Table 3 (continued)

Compound number	Structure	cLogP	IC ₅₀ (μM)	TD ₅₀ (μM)	TI
B27		5.45	2.00 ± 0.01	11.0 ± 1.41	5.50
B29		5.45	1.20 ± 1.13	4.75 ± 0.354	3.96

generated at least 8 data points for each test. Their IC₅₀ and TD₅₀ were calculated and analyzed in comparison to their cLogP values.

Simple quinolines (Table 1) were tested to verify the hypothesis made by Smith et al. (2007) that the quinoline rings is the active moiety causing growth inhibition. All compounds of this group displayed IC₅₀ at <10 μM. Quinoline and 5-nitroquinoline, have cLogP values; 2.03 and 1.91 respectively. The values of cLogP are commonly used to correlate the ability of a compound to cross the cell membranes; a low value indicates that the compound is relatively hydrophilic and less likely to cross the cell membrane. Both compounds have similar cLogP values, which could explain the similarities in their IC₅₀ values at 8.00 ± 2.83 μM and 7.25 ± 0.354 μM, and their TD₅₀ values at 12.5 ± 0.707 μM and 11.3 ± 2.47 μM, respectively. This suggested that the 5-nitro substitution did not have a significant effect. 2-Chloroquinoline has a slightly higher cLogP value of 2.83, while showing the IC₅₀ value of 5.75 ± 1.06 μM and TD₅₀ of 8.50 ± 2.12 μM. Within this group, quinoline-*N*-oxide hydrate has the lowest cLogP value at ~1.42, with the IC₅₀ of 6.40 ± 2.26 μM and TD₅₀ of 7.50 ± 2.12 μM, giving this compound the lowest therapeutic index among the series. Interestingly, 8-hydroxyquinoline, with a cLogP value 2.08, which is close to those of quinoline and 5-nitroquinoline, displayed IC₅₀ of 0.213 ± 0.194 μM and TD₅₀ of 0.500 ± 0.0707 μM, resulted in more than a 2-fold difference in both IC₅₀ and TD₅₀, when compared to the other compounds displaying similar cLogP values. This thus indicated that the 8-substitution of a quinoline ring has the strongest influence on the tested activities. It was not surprising because 8-hydroxyquinoline is known to have antiseptic and antifungal properties against gram positive bacteria (Shen et al., 1999) and used as a topical treatment for skin infections (Gershon et al., 1985). Studies in *Plasmodium* spp. (Scheibel and Adler, 1982) showed that 8-hydroxyquinoline could act as a chelating agent, and in *Cryptosporidium parvum* and related species including *T. gondii* (Bessoff et al., 2014) also suggested promising uses of 8-hydroxyquinoline and related quinoline derivatives as anti-parasitic agents. It should be noted that despite a low cLogP value (~2), 8-hydroxyquinoline is the most effective, suggesting that it might

be up taken by other means than passively traversing the cell membranes.

Compounds of group A (Table 2) were derived from a 4-piperazinylquinoline backbone. These compounds are an example of hybrid pharmacophores, including an isatin ring, a quinoline ring and/or a thiosemicarbazone moiety. These moieties have been reported as important pharmacophores conferring various biological actions and effectiveness in the treatment of cancer (Brockman et al., 1956; Solomon et al., 2009; Hu et al., 2010). The compounds were here presented and compared based on their substituents to the 4-piperazinylquinoline ring system and the nature of the group on the 7-position of the 4-piperazinylquinoline ring. In order to evaluate which of the moieties within these hybrid pharmacophores was eliciting the highest bioactivity, a group of thiosemicarbazone derived compounds with isatin ring systems and not containing a quinoline ring system were tested. The isatin rings were either chloro substituted (A20, A22), bromo substituted (A21, A23), or non-substituted (A24). Of these five compounds, only A24 proved to elicit inhibitory effects with an IC₅₀ of 3.00 ± 0.707 μM and a TD₅₀ of 9.00 ± 1.41 μM. This compound does not contain a quinoline ring and displays an inhibition effect suggesting that the isatin ring system or the thiosemicarbazone, are as active as suggested by (Krivogorsky et al., 2008; Tenório et al., 2005). Compared to simple quinolines described earlier, this group was less effective and showed less potential in *Toxoplasma* inhibition.

A2–A5 are derived from a 7-chloro-4-piperazinylquinoline ring system with a substituted isatin ring substituted with a 4-bromo (A2), a 6-chloro (A3), a 6-bromo (A4), or a non-substituted isatin ring (A5). All of these compounds proved to have similar abilities at inhibiting *Toxoplasma*, with the exception of A3, with an IC₅₀ of 2.40 ± 1.41 μM and a TD₅₀ of 11.7 ± 2.88 μM. Interestingly, A3 had a similar cLogP value to A2 (IC₅₀ = 4.25 ± 0.353 μM, TD₅₀ = 9.97 ± 2.05 μM), and A4 (IC₅₀ = 3.50 ± 1.41 μM, TD₅₀ = 9.50 ± 0.707 μM). A5, which displayed the lowest cLogP value among the 4 compounds, inhibited the growth of *Toxoplasma* with an IC₅₀ of 2.75 ± 0.354 μM and a TD₅₀ of 8.00 ± 0.707 μM, and was comparable in activity to A4. A6, A7 were derived from a 7-trifluoromethyl-4-

piperazinyl ring system substituted with a 4-chloro (**A6**), 4-bromo (**A7**) isatin ring. **A6** displayed an IC_{50} value of $4.00 \pm 2.83 \mu M$ and a TD_{50} value of $7.95 \pm 0.07 \mu M$. Within this group, **A7** had relatively high TD_{50} of $14.0 \pm 1.41 \mu M$ and IC_{50} of $7.5 \pm 2.12 \mu M$. The two bioisoteric groups, with their main chains differing only by the group substituted on the 7-position of the 4-piperazinylquinoline lateral side chain (either CF_3 or Cl group), generally, did not differ much in their biological effects, with the exception of **A3** displaying the greatest TI.

The next analyses were carried out for those compounds which were derived from a 7-chlorosubstituted 4-piperazinylquinoline ring system attached to a substituted isatin-3-thiosemicarbazone ring system to carry either a 4-chloro (**A11**), 4-bromo (**A12**), or a 6-bromo (**A14**) substitution. Among these three compounds, **A11** proved to be the most effective displaying an IC_{50} of $0.895 \pm 0.418 \mu M$ and a TD_{50} of $5.00 \pm 0.707 \mu M$, with **A14** just behind it in terms of effectiveness, the IC_{50} value of **A14** was $0.800 \pm 0.282 \mu M$ and its TD_{50} value was $2.80 \pm 0.283 \mu M$, making **A14** slightly more cytotoxic to HFF than **A11**. Of the three compounds, **A12** proved to be the least effective with an IC_{50} of $2.45 \pm 0.777 \mu M$ and a TD_{50} value of $8.00 \pm 1.41 \mu M$. Their bioisoteric counterparts, which contained a 7-trifluoromethyl-4-piperazinylquinoline ring system attached a substituted isatin-3-thiosemicarbazone ring system with either a 4-chloro (**A15**), 4-bromo (**A16**), 6-chloro (**A17**), or 6-bromo (**A18**) group, were generally similar in their ability to inhibit *Toxoplasma*, with **A15** displaying an IC_{50} value $2.10 \pm 0.141 \mu M$ and a TD_{50} of $8.00 \pm 1.41 \mu M$, **A16** with an IC_{50} value of $1.75 \pm 0.354 \mu M$ and a TD_{50} value of $5.75 \pm 0.353 \mu M$, with **A17** displaying an IC_{50} value of $1.05 \pm 0.354 \mu M$ and a TD_{50} of $4.75 \pm 0.354 \mu M$, and **A18** displaying an IC_{50} value of $0.725 \pm 0.176 \mu M$, and a TD_{50} of $3.15 \pm 0.495 \mu M$. When comparing the two bioisoteric groups, 7-chloro vs. 7-trifluoromethyl on the 4-piperazinyl quinoline ring, those with the 7-chloro group proved to be slightly more effective. Noted, compounds with a chloro substituted isatin ring were generally the most effective compounds displaying the greatest TI. Taken together, compounds containing the thiosemicarbazone moiety attached to the isatin ring were generally more effective than the compounds containing simply an isatin ring. This could be due the ability of quinoline, isatin, and thiosemicarbazone to inhibit *Toxoplasma* independently. Additive inhibitory effects on *Toxoplasma* could result in a more potent pharmacophore. This is similar to the effects observed by Solomon et al. (2010a).

The final set of 29 compounds (group B) was derived from a 4-amino quinoline backbone of chloroquine. As a benchmark compound for comparison, chloroquine has a cLogP value of 5.06, IC_{50} of $2.25 \pm 0.354 \mu M$ and TD_{50} of $9.00 \pm 1.41 \mu M$. Only twelve compounds of this set showed inhibitory effects (Table 3) and were compared based on their substituent attached to the 4-aminoquinoline ring system and the nature of the group on the 7-position of the 4-aminoquinoline ring. **B11** and **B12** were derived from a 7-chloro-4-aminoquinoline side chain substituted with a 4-quinolonyl-piperazinyl chain. **B11** has a 4-(7-Chloroquinolin-4-yl)piperazin-1-yl group attached the lateral side chain, while **B12** has a 4-(7-trifluoromethylquinolin-4-yl)piperazin-1-yl attached to the lateral side chain, giving these two compounds differ only by one substitution either a CF_3 or a Cl at the 7-position of the 4-quinolonyl-piperazinyl chain. Both do not differ significantly with the IC_{50} values of $0.875 \pm 0.177 \mu M$ and $0.750 \pm 0.141 \mu M$, and their TD_{50} values of $1.43 \pm 0.513 \mu M$ and $1.27 \pm 0.550 \mu M$, with their cLogP values at 7.00 and 7.13 for **B11** and **B12**, respectively. Correspondingly **B23** and **B24** were derived from the bioisoteric substitution of the 7-chloro group with a 7-trifluoromethyl group on the 4-aminoquinoline ring, with **B23** being the bioisoteric counterpart of **B11**, and **B24** of **B12**. When compared to one another, the activities dis-

played by **B23** and **B24** did not differ significantly in their activities: **B23** with an IC_{50} of $0.425 \pm 0.035 \mu M$ and TD_{50} of $2.10 \pm 0.566 \mu M$, and **B24** with an IC_{50} of $0.450 \pm 0.071 \mu M$ and a TD_{50} of $1.75 \pm 0.0354 \mu M$. Also, their cLogP values were similar; **B23**, 7.25, and **B24**, 7.46. When compared to their bioisoteric counterparts, no significant difference was observed, however **B23** and **B24** were active at slightly lower concentrations, which could be due to their greater cLogP values. A substitution of a 4-(7-trifluoromethylquinolin-4-yl)piperazin-1-yl ring system on a 4-aminoquinoline lateral side chain proved to be more effective when there is a CF_3 group at the seventh position of the ring, but not significantly so, as evidenced by the IC_{50} values of **B23** and **B24** vs. those of **B11** and **B12**. Notably, the nature of the substituent at the 7-position of the ring, either Cl or CF_3 does not seem to have a significant effect on the parasitic growth inhibition. This is different from what was observed in the proliferation of cancer cells, where a Cl group substitution at the seventh position of the ring was the more effective agent at inhibiting cancer cell proliferation.

B15 and **B16** contain a halogenated isatin ring system on a 7-chloro-4-aminoquinoline lateral side chain. **B15** containing a 6-chloroisatin ring, and **B16** a 6-bromoisatin ring. In terms of the inhibitory activity, there was no significant difference. **B15** with an IC_{50} $0.950 \pm 0.071 \mu M$ and a TD_{50} of $5.25 \pm 0.354 \mu M$, while **B16** had an IC_{50} $1.13 \pm 0.671 \mu M$ and TD_{50} of $3.75 \pm 0.354 \mu M$. When **B26**, **B27**, and **B29**, derived from a bioisoteric replacement of the 7-chloro group with a 7-trifluoromethyl group on the 4-aminoquinoline ring, were compared, **B26** displayed an IC_{50} of $4.75 \pm 1.06 \mu M$ and a TD_{50} $8.25 \pm 1.77 \mu M$; **B27** an IC_{50} of $2.00 \pm 0.01 \mu M$ and a TD_{50} of $11.0 \pm 1.41 \mu M$, and **B29** an IC_{50} of $1.20 \pm 1.13 \mu M$ and a TD_{50} $4.75 \pm 0.354 \mu M$. The difference in the identity of the group on the isatin substituent, **B26** with a 4-Cl, **B27** with a 4-Br, and **B29** with a 6-Br group yielded slightly different cLogP values: 5.30 for **B26**, 5.45 for both **B27** and **B29**. Interestingly **B27** proved to be more potent and displayed a TI at least two times greater than that of **B26** and slightly greater than that of **B29**.

It should be noted that the compounds containing substituted quinolin-4-yl-4-piperazinyl chains on a 4-aminoquinoline side chain (**B11**, **B12**, **B23**, **B24**) were more effective in inhibiting *Toxoplasma* than the compounds containing halo-substituted isatin rings attached to a 4-aminoquinoline lateral side chain (**B15**, **B16**, **B26**, **B27**, **B29**). The former group has cLogP values ranging from 7.00 to 7.46, whereas the latter has cLogP values ranging from 5.06 to 5.45. The former group contains two quinoline rings, which may have an additive effect in terms of causing inhibition as opposed to a hybrid pharmacophore containing a quinoline and an isatin ring, perhaps further hinting to the potency and efficiency of the quinoline moiety against the growth of *Toxoplasma*. **B4**, containing a 7-trifluoromethylaminoquinoline lateral side chain with a methanesulfonate substitution, was more effective than chloroquine with an IC_{50} value of $1.35 \pm 0.212 \mu M$, and a TD_{50} value of $5.67 \pm 1.15 \mu M$. When compared to the other compounds, which contain at least two ring systems, it was comparable to those compounds containing an isatin ring system substituted on 7-chloro-4-aminoquinoline lateral side chain (**B15**, **B16**). The cLogP value of **B4** which is 3.54, this is approximately half of the cLogP values observed in **B15** and **B16**.

When **B18**, and **B22**, derived from the 7-trifluoromethylaminoquinoline lateral side chain, were compared for **B18** with a pyrrolidinyl group vs. **B22** with a phenylpiperazinyl ring substitution. **B18** had a relatively high IC_{50} and TD_{50} and proved to be less effective than chloroquine. Among chloroquine-derived compounds, **B4**, **B15**, **B23**, and **B27** displayed TI values greater than that of chloroquine. **B23** was the most potent of this group displaying the lowest IC_{50} value. Interestingly, **B23** was also reported by Solomon et al. (2010a) to be more potent than chloroquine as a cancer

chemotherapeutic agent. This indicates that this compound may have the potential to be used for multiple purposes. Surprisingly, the most potent compound proved to be 8-hydroxyquinoline among all compounds tested. 4-Aminoquinoline analogs were slightly more potent than 4-piperazinyl quinoline derivatives, particularly **B23** and **B24** which displayed IC_{50} ranging from 425 to 585 nM.

3.2. Effect on apicoplast

Smith et al., (2007) showed that quinoline has an apicoplast-disrupting effect. To evaluate the effect of quinoline derivatives tested here on apicoplast maintenance, quinoline (IC_{50} 8.0 $\pm$ 2.8 μ M), 8-hydroxyquinoline (IC_{50} 213 $\pm$ 194 nM), and **B23** (IC_{50} 425 $\pm$ 35 nM) were selected and tested in comparison to **B25** which was an inactive quinoline derivative with IC_{50} > 100 μ M. To account for various levels of heterogeneity of *Toxoplasma* cultures, three independent experiments were performed where equal numbers of newly released parasites ($\sim 1 \times 10^6$ parasites) were used in each experiment. As shown in Fig. 1, due to their growth heterogeneity not all untreated parasites harbored their apicoplasts (73.2 $\pm$ 6.4%). The treatments with quinoline, 8-hydroxyquinoline, or **B23** resulted in the loss of apicoplasts, which can be seen by the smaller proportion of parasites maintaining their apicoplasts (43.2 $\pm$ 3.8%, 43.7 $\pm$ 2.8%, and 39.7 $\pm$ 4.3%, respectively); these data were accounted for 40–45% loss of apicoplasts, when *Toxoplasma* were exposed to the drugs. On the other hand **B25** did not cause apicoplast loss, in comparison to untreated parasites (74.9 $\pm$ 6.5%). The one-way ANOVA analyses yielded $F_{4,10} = 36.7$, $P = 5.96 \times 10^{-6}$, and F_{crit} was 3.48. Since the F value generated from ANOVA analyses was greater than F_{crit} value, it thus indicated significant differences among tested groups. When the Bonferroni correction was applied, the P values obtained was less than the

corrected alpha value (0.01), further confirmed the significant difference among the tested groups. To determine which compounds exhibit a statistically significant effect on apicoplast disappearance, a two tailed student's t -test was performed, and P values < 0.05 were obtained. The analysis confirmed the effect of quinoline, 8-hydroxyquinoline and **B23** on apicoplast, while **B25** showed no effect (Fig. 2).

Although a precise mechanism of disturbance is unknown, our observation is in an agreement with the observations made by Smith et al. (2007). The apicoplast disrupting effect detected for single and two quinoline ring structures suggested stereo-specificity where the single quinoline should be maintained. As seen in the structure of inactive **B25**, the substitutions differing from that of another quinoline ring did not elicit any biological effect. Further structural elucidation will be required to determine an underlying mechanism by which these compounds target the apicoplast biogenesis, and whether they could be further developed for the treatment of infection caused by apicomplexan. Such an activity was not observed, when pyrimethamine or clindamycin was used in the treatment of infected monolayers (data not shown). Indirectly, it suggested that the effect of quinoline derivatives on apicoplast was specific to the organelle functions, but not due to their general toxicity. However, it is remained to be investigated whether and how quinoline derivatives could affect metabolic functions of apicoplast in the synthesis of heme, type II fatty acids or isoprenoid precursors (Sheiner et al., 2013). In conclusion, **B23** has the greatest effect in causing the disappearance of the apicoplast in comparison to the other compounds tested and would be the best candidate for a future study. Undoubtedly, quinoline compounds should be further explored for their effectiveness and selectivity as possible anti-parasitic agents for humans as well as animals.

Fig. 1. Effects of quinoline derivatives on *Toxoplasma*. Approximately 1×10^6 parasites were used to infect confluent HFF monolayers. At the time of infection, quinoline derivatives were added in the culturing media to obtain the final concentration of 1 or 2 times of IC_{50} , when no cytotoxicity effect was observed. The final concentrations of quinoline were at 8 μ M; 8-hydroxyquinoline at 426 nM; **B23**, 850 nM; **B25** 0.8–8 μ M. Twenty-four hours post infection and drug treatments, the parasites were examined as described in Materials and Methods. Double daggers (‡) indicate P -values < 0.05. Asterisks in the micrograph are for the locations of the apicoplasts.

Fig. 2. Structural comparisons. The structures of representative compounds exhibiting apicoplast disrupting effects were shown along with their cLogP values and their effect. In parenthesis, the disruption effect was calculated from the proportion of parasite without apicoplast in each test group in comparison to those treated with DMSO solvent.

4. Uncited reference

Solomon and Lee (2009).

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