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# Synthesis and bioevaluation of hybrid 4-aminoquinoline triazines as a new class of antimalarial agents

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ABSTRACT

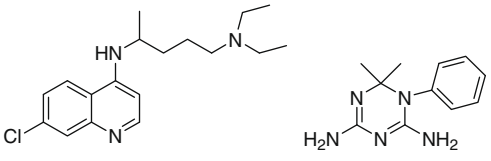
The emergence and rapid spread of chloroquine resistant strains of *Plasmodium falciparum* has dramatically reduced the chemotherapeutic options. Towards this goal, a series of new class of hybrid 4-aminoquinoline triazines were synthesized and screened against CQ sensitive strain 3D7 of *P. falciparum* in an in vitro model. Compounds **65** and **69** exhibited more than 99% suppression on day 4 and on day 6 post treatment, compound **69** showed impressive 99.11% suppression against CQ resistant strain N-67 of *P. yoelii* in an in vivo assay.



Malaria is a major tropical parasitic disease, currently killing more people than any other communicable disease aside from tuberculosis. Today, malaria is transmitted in more than 100 countries. Some 500 million people in Africa, India, Southeast Asia and South America are infected with malaria, with an estimated 2.5 million deaths annually, and about a million of these being children under the age of five.[1] Of the four species, *Plasmodium falciparum* is responsible for the most severe form of malaria, cerebral malaria, and cause the majority of morbidity case.[2] Chloroquine (CQ) has been the drug of choice for antimalarial chemotherapy since its discovery as it was safe, affordable, and effective but the emergence and rapid spread of resistance of *P. falciparum* to CQ and other related antimalarials has dramatically reduced the therapeutic options[3] (Fig. 1). The combination therapy was identified as a strategic and viable option in improving the efficacy, and delaying development and selection of resistant parasite.[4] In this context, development of hybrid antimalarial agents, combining 4-aminoquinoline with other chemical entity having the antimalarial potency, might prove to be an effective approach as 4-aminoquinoline based hybrid molecules can overcome the problem of drug resistance, and have the low potential to induce the resistance to parasite.[5]

CQ and other structurally related antimalarials exert their effect by binding to heme molecules released from the hemoglobin that is digested by malaria parasites as they grow within their host red blood cells. This binding inhibits the formation of hemozoin, resulting the accumulation of heme to toxic level that leads to the death of parasite.[6] CQ resistance in *Plasmodium falciparum* is associated with mutations in the digestive vacuole (DV) trans membrane protein, *P. falciparum* resistance transporter (pfCRT).[7] Excessive export of CQ from its site of action in the DV is thought to result from these mutations. The mechanism of Chloroquine resistance (CQR) in *P. falciparum* probably involves specific structural interactions between Chloroquine and amino acid substitutions in pfCRT that were slow to evolve because of their complexity. Therefore the heme is still an attractive drug target for the development of 4-aminoquinoline based antimalarials which are not easily recognized by CQR mechanism.[8]

The comprehensive structure activity relationship studies on CQ–Hematin binding have been explored to identify the optimum structural requirements for designing the new antimalarial agents. It was established that 7-chloro-4-aminoquinoline is critical for the antimalarial activity.[9–11] To counter the problem of drug resis-




1. Chloroquine 2. Cycloguanil

**Figure 1.** Structure of antimalarial chloroquine and cycloguanil.

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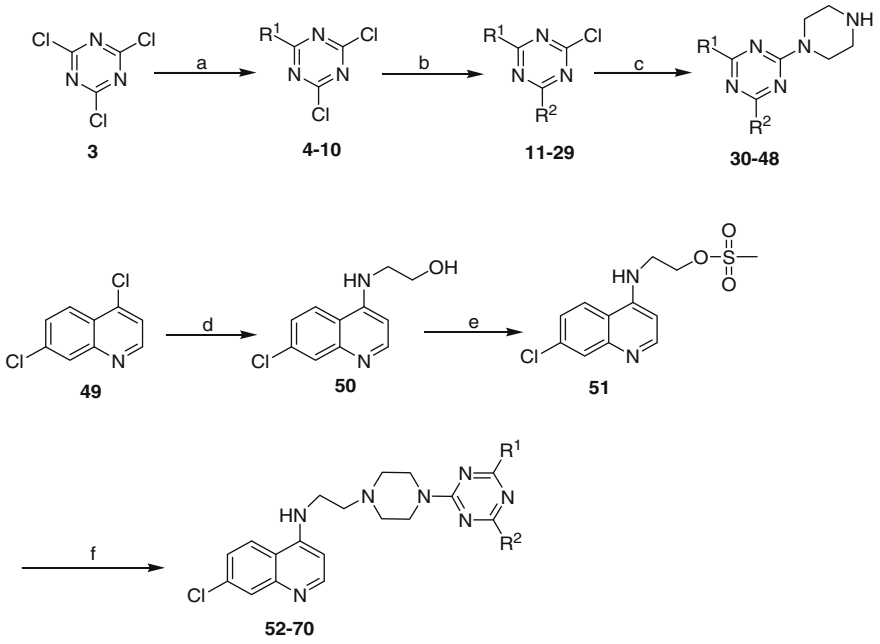
tance, a diverse functionalization of the lateral side chain of chloroquine with other antimalarial moiety led to the identification of new 4-aminoquinoline based antimalarials effective against both Chloroquine sensitive and chloroquine resistant strains of *P. falciparum*. It included the 4-aminoquinoline-based isatin derivatives,[12] the 4-aminoquinoline-based β-carbolines,[13] the peroxide-based trioxaquine derivatives,[14,15] ferrocene–chloroquine analogs,[16] or the 4-aminoquinolines based on inhibitors of a neutral zinc aminopeptidase.[17] Additionally, a new hybrid chloroquine reversal agents have also been developed through coupling of imipramine (reversal agent) with 4-aminoquinoline.[18]

*Plasmodium falciparum* dihydrofolate reductase (pfDHFR) is an essential enzyme in the folate pathway and exists as a bifunctional enzyme linked to thymidylate synthase (TS), and has been an important target for malarial therapy.[19] Pyrimethamine and cycloguanil, the active metabolite of proguanil, are potent DHFR inhibitors and are clinically used for *P. falciparum* malaria treatment. Cyclogunil represents the triazine chemical class (Fig. 1). Our group has previously identified substituted triazines as potential antimalarial agents.[20]

We envisioned that incorporation of triazine moiety in the side chain attached to 4-aminoquinoline pharmacophore would lead to develop the new antimalarial agents active against chloroquine resistance strains of *P. falciparum*. It was well established that basic nature of side chain of the 4-aminoquinoline was crucial for accumulation of the drug within acidic food vacuole of the parasite along the pH gradient.[10] Considering the importance of basicity for antimalarial activity, triazine functionalized with piperazine was linked to 4-aminoquinoline component. Herein, we report the synthesis and evaluation of the antimalarial activity of new hybrid 4-aminoquinoline triazines against both chloroquine sensitive 3D7 and chloroquine resistant N-67 strains of *P. falciparum*.

The target compounds (**52–70**) were synthesized by a synthetic protocol as outlines in Scheme 1. The synthesis of 2,4,6-trisubstituted-[1,3,5]triazines were achieved by consecutive nucleophilic substitution of cyanuric chloride. The sequence of addition of amines to cyanuric chloride depends upon the strength and structure of nucleophile. First chlorine of cyanuric chloride (**3**) was displaced with the moderately nucleophilic aromatic amines at 0 °C for 1 h to give the monosubstituted triazines (**4**–**10**) followed by the treatment with more nucleophilic amine to provide the disubstituted triazines (**11**–**29**). Final chlorine of disubstituted triazines was replaced with excess of piperazine (3 times) at 0 °C to afford the corresponding 2,4,6-trisubstituted triazines (**30**–**48**). Coupling of 4,7-dichloroquinoline (**49**) with excess of 2-amino ethanol gave the 2-(7-chloro-quinolin-4-ylamino)-ethanol (**50**) in a good yield.[21] Chemoselective *o*-mesylation was achieved in pyridine at 0 °C for 5 h to yield the methanesulfonic acid 2-(7-chloro-quinolin-4-ylamino)-ethyl ester (**51**).[22] Chemoselectivity in the *o*-mesyaltion may be attributed to the presence of poor nucleophilic 4-amino (NH) group owing to the conjugation of the lone pair of electrons into the quinoline ring. Compound (**51**) was subjected to nucleophilic substitution with trisubstituted triazines to yield the corresponding targeted compounds (**52**–**70**) under microwave condition. All the synthesized compounds were well characterized by IR, mass, NMR, and elemental analysis.[26]

The antimalarial activity of all the synthesized compounds was evaluated in an in vitro malaria assay against a chloroquine sensitive strain of *P. falciparum* 3D7, according to a described protocol[23] (Table 1). Variation of different substituents on the triazine nucleus around four and six position has been explored to identify the better possible combination of substituents for the improvement of antimalarial potency. Among the 19 evaluated compounds of the series, three compounds showed the antimalarial potency comparable to CQ, six compounds displayed $IC_{50}$ values ranging from 10.49 to 21.27 ng/mL and the rest of the compounds showed $IC_{50}$ values in the range from 37.98 to 255.66 ng/mL. To study the effect of substituents on the antimalarial activity, variation of the $R^2$ substituents at position-6 have been done while keeping the $R^1$ substituent at position-4 fixed. When aniline was placed at position-4, compounds having piperidine (**52**) and cyclohexylamine (**53**) at position-6 displayed almost equal antimalarial potency. Compound with morpholine (**54**) at position-4 showed an $IC_{50}$ 21.27 ng/mL, while placement of *N*-ethyl piperazine (**56**) significantly decreased the inhibitory activity as compared to the **52** and **53**. Putting the aniline at both position (**55**) had the adverse ef-




**Scheme 1.** Reagents and conditions: (a) aromatic amines/secondary amines, 0 °C temp, 1 h, THF; (b) secondary amines/primary amines, room temp, THF, 2 h; (c) piperazine (3 times), 0 °C, 2 h, THF; (d) 1-amino ethanol, *n*-butanol, 90 °C, 8 h; (e) methanesulfonyl chloride, pyridine, 0 °C, 3 h; (f) trisubstituted triazines (1.5 times), MW, 30 s, NMP.

**Table 1**
In vitro antimalarial activity against chloroquine sensitive strain 3D7 of *P. falciparum* and in vitro cytotoxicity of compounds on VERO cell line

| Compound | $R^1$ (4-position) | $R^2$ (6-position) | $IC_{50}$ (ng/mL) | SI |
|---|---|---|---|---|
| **52** | Aniline | Piperidine | 17.82 | 116.72 |
| **53** | Aniline | Cyclohexylamine | 16.23 | 154.18 |
| **54** | Aniline | Morpholine | 21.27 | 469.57 |
| **55** | Aniline | Aniline | 75.64 | 27.76 |
| **56** | Aniline | *N*-Ethylpiperazine | 37.98 | 41.81 |
| **57** | *o*-Toluidine | Morholine | 19.16 | 377.35 |
| **58** | *o*-Toluidine | *o*-Toluidine | 185.88 | 75.32 |
| **59** | *o*-Toluidine | Cyclohexylamine | 194.42 | 28.97 |
| **60** | *p*-Toluidine | *N*-Phenylpiperazine | 64.57 | 52.19 |
| **61** | *p*-Toluidine | Cyclohexylamine | 255.56 | 114.96 |
| **62** | *p*-Toluidine | Morpholine | 43.48 | 512.09 |
| **63** | *p*-Toluidine | *N*-Ethylpiperazine | 19.01 | 41.03 |
| **64** | *p*-Toluidine | *p*-Toluidine | 38.26 | 155.25 |
| **65** | *p*-Fluoroaniline | Piperidine | 7.15 | 328.61 |
| **66** | *p*-Fluoroaniline | Cyclohexylamine | 7.83 | 261.81 |
| **67** | *o*-Fluoroaniline | Piperidine | 159.16 | 14.95 |
| **68** | Piperidine | Piperidine | 44.38 | 53.17 |
| **69** | Piperidine | Cyclohexylamine | 4.43 | 481.48 |
| **70** | Morpholine | *N*-Formylpiperazine | 10.49 | 4105.82 |
| CQ | | | 2.6 | 8983 |

$IC_{50}$: concentration corresponding to 50% growth inhibition of the parasite.
$SI = \frac{IC_{50} \text{values of toxic activity}}{IC_{50} \text{values of antimalarial activity}}$.

**Table 2**
In vivo antimalarial activity against chloroquine resistant strain N-67 of *P. yoelii* in swiss mice at dose 50 mg/kg/day by intraperitoneal route

| Compound | % Suppression on day 4 | % suppression on day 6 |
|---|---|---|
| **52** | 66.67 | 55.36 |
| **53** | 86.43 | 78.73 |
| **65** | 99.9 | 96.51 |
| **66** | 74.81 | 71.75 |
| **69** | 99.9 | 99.11 |
| CQ | 99.9 | 94.26 |

fect on the antimalarial potency. In case of compounds having *o*-toluidine as $R^1$ substituent, interesting activity pattern has been observed, compound (**57**) having morpholine at postion-6 showed an $IC_{50}$ 19.16 ng/mL, whereas the compound (**58**) having the *o*-toluidine at both position exhibited remarkably decreased inhibitory activity with $IC_{50}$ 185.88 ng/mL, compound bearing the cyclohexylamine at position-6 (**59**) exhibited low antimalarial potency as compared to **53**. Compound (**60**) bearing the *p*-toluidine and *N*-phenylpiperazine had an $IC_{50}$ 64.57 ng/mL while compound (**63**) with the *p*-toluidine and *N*-ethylpiperazine showed an $IC_{50}$ 19.01 ng/mL. It seems that bulky phenyl group exerted negative effect on the antimalarial potency. Combination of *p*-toluidine and cyclohexylamine as substituents (**61**) showed an $IC_{50}$ 255.56 ng/mL, while the replacement of cyclohexylamine with morpholine (**62**) led to the increased inhibitory activity having an $IC_{50}$ 43.48 ng/mL. Compound **64** having the *p*-toluidine at both positions exhibited an $IC_{50}$ 38.16 ng/mL. Compound **64** was more active than compound **55** and **58**. This observation indicated that substituting methyl in the aryl ring at *para*-position was favorable for the antimalarial activity while *ortho*-substitution in the aryl ring had the detrimental effect on the antimalarial potency. Introduction of the fluoro group at *para*-position in the phenyl ring (**65** and **66**) increased the activity while fluoro group at *ortho*-position (**67**) in the phenyl ring reduced the activity dramatically. Compound (**66**) was more active than compound (**61**). So it was evident that the nature and position of the substituent in the phenyl ring influence the inhibitory activity. Compound (**68**) bearing piperidine at both positions 4 and 6 had an $IC_{50}$ 44.38 ng/mL while compound (**69**) having piperidine at position-4 and cyclohexylamine at position-6 exhibited lowest $IC_{50}$ 4.43 ng/mL amongst the evaluated compounds. Compound with substituents morpholine and *N*-formylpiperazine (**70**) exhibited an $IC_{50}$ 10.49 ng/mL. Structure–activity relationship studies based on the activity results obtained revealed that combination of piperidine, cyclohexylamine, *p*-fluoroaniline, aniline and morpholine as substituents on triazine nucleus were well tolerated for the antimalarial activity.

All the compounds were also tested for their cytotoxicity on VERO cells using MTT assay.[24] Tested compounds were endowed with selectivity index ranging from 27.76 to 4105.82. Most of the compounds possessing good in vitro inhibitory activity have shown decent selectivity index. Compound (**70**) having an $IC_{50}$ 10.49 ng/mL showed the highest selectivity index amongst the tested compounds and compound (**69**), the most potent compounds of the series displayed the selectivity index 481.48, thus demonstrating good activity profile.

The most active 4-aminoquinoline triazine hybrids based on their in vitro activity were screened in an in vivo model against chloroquine resistant N-67 strain of *P. yoelii* in swiss mice at 50 mg/kg/day for 4 days by intraperitoneal route (ip)[25] (Table 2). The evaluated compounds have shown significant suppression, in particular compounds **65** and **69** exhibited more than 99% suppression on day 4. On day 6 post treatment, compound **69** showed impressive 99.11% suppression but could not provide significant protection to the treated mice in 28 days survival assay. Further structural optimization of 4-aminoquinoline triazine analogs may lead to the development of the more potent molecules.

In conclusion, a series of new class of hybrid 4-aminoquinolinetriazine derivatives have been identified as potential antimalarial agents. Many of the evaluated compounds have shown significant antimalarial potency against CQ sensitive 3D7 strain of *P. falciparum* in an in vitro model. Some of the selected compounds, in particular compound **65** and **69** exhibited a promising antimalarial activity in vivo against *P. yoelii* by ip route at a dose of 50 mg/kg/day. The synthesis of easily accessible and affordable hybrid molecules can be utilized in the development of antimalarial agents.

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23. The compounds were evaluated for antimalarial activity against CQ sensitive 3D7 strain of *P. falciparum* BY SYBER Green I-based fluorescence (MSF) assay1. The compounds were dissolved in DMSO at 5 mg/mL. For the assays, fresh dilutions of all compounds in screening medium were prepared and 50 μl of highest starting concentration (500 ng/mL except for compound nos. **65**, **66**, **69** the highest starting concentration was 100 ng/mL) was dispensed in duplicate wells in row B of 96 well tissue culture plate. The highest concentration for chloroquine was 25 ng/ml. Subsequently two fold serial dilutions were prepared up to row H (seven concentrations). Finally 50 μl of 2.5% parasitized cell suspension containing 0.5% parasitaemia was added to each well except four wells in row A which received non-infected cell suspension. These wells containing non-infected erythrocytes in the absence of drugs served as negative controls, while parasitized erythrocytes in the presence of CQ served as positive control. After 72 h of incubation, 100 μl of lysis buffer [20 mM tris (pH 7.5), 5 mM EDTA, 0.008% (wt/vol) saponin, and 0.08% (vol/vol) Triton X-100] containing 1× concentration of SYBER Green I (Invitrogen) was added to each cell. The plates were re-incubated for 1 h at room temperature and examined for the relative fluorescence units (RFUs) per well using the FLUOstar, BMG lab technologies. The 50% inhibitory concentration ($IC_{50}$) was determined using non-linear regression analysis dose–response curves. Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J. X.; Wilairat, P.; Riscoe, M. *Antimicrob. agents Chemother*. **2004**, *48*, 1803–1806.
24. Cytotoxicity of the compounds was determined against VERO cell lines (C-1008; Monkey kidney fibroblast cells) using MTT assay. A total of $1 \times 10^4$ cells/well were incubated with varying concentrations of compound for 72 h. The highest concentration of compound was 100 μg/mL. The 50% inhibitory concentration ($IC_{50}$) was determined using non-linear regression analysis dose–response curves and represented the concentration of compound required to kill 50% of the fibroblast cells. Mosmann T. *J. Immunol. Methods*. **1983**, *65*, 55.
25. The in vivo drug response was evaluated in Swiss mice infected with *P. yoelii* (N-67 strain) which is innately resistant to CQ. The mice (22 ± 2 g) were inoculated with $1 \times 1$ parasitized RBC on day 0 and treatment was administered to a group of five mice from day 0 to 3, once daily. The aqueous suspensions of compounds were prepared with a few drops of Tween 80. The efficacy of test compounds was evaluated at 50 mg/kg/day and required daily dose was administered in 0.2 mL volume via intraperitoneal route. Parasitaemia levels were recorded from thin blood smears between days 4 and 6. The mean value determined for a group of five mice was used to calculate the percent suppression of parasitaemia with respect to the untreated control group. Mice treated with CQ served as reference controls. Puri, S. K.; Singh, N. *Exp. Parasitol.* **2000**, *94*, 8.
26. Spectroscopic data for **63**: MS: 587 (M+1); IR (KBr): 3408, 2937, 1582, 1369, 1003 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm) 8.54 (d, 1H, $J$ = 5.22 Hz, Ar–H), 7.98 (d, 1H, $J$ = 1.92 Hz, Ar–H), 7.69 (d, 1H, $J$ = 8.94 Hz, Ar–H), 7.41–7.38 (m, 3H, Ar–H), 7.01 (d, 2H, $J$ = 8.31 Hz, Ar–H), 6.58 (br s, 1H, NH), 6.39 (d, 1H, $J$ = 5.22 Hz, Ar–H), 5.94 (br s, 1H, NH), 3.86–3.81 (m, 8H, $NCH_2$), 3.39 (t, 2H, $J$ = 5.59 Hz, NH–$CH_2$), 2.85 (t, 2H, $J$ = 5.52 Hz, $NCH_2$), 2.58 (t, 4H, $J$ = 4.2 Hz, $NCH_2$), 2.48–2.42 (m, 6H, $NCH_2$), 2.32 (s, 3H, $CH_3$), 1.14 (t, 3H, $J$ = 7.11 Hz, $CH_2$–$CH_3$); $^{13}C$ ($CDCl_3$, 50 MHz): 165.62, 165.46, 164.83, 152.48, 150.14, 149.47, 137.32, 135.30, 132.17, 129.61, 129.14, 125.83, 121.49, 120.22, 117.73, 99.71, 56.03, 53.12, 52.98, 52.86, 43.67, 43.51, 39.32, 21.16, 12.35; Anal. Calcd for $C_{31}H_{39}ClN_{10}$: Calcd C 63.41, H 6.69, N 23.85. Found: C 63.58, H 6.72. N 23.98. Compound **69**: MS: 550 (M+1); IR (KBr): 3415, 1591, 1450, 1015, 853 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 300 MHz): $\delta$ (ppm) 8.55 (d, 1H, $J$ = 5.37 Hz, Ar–H), 8.01 (d, 1H, $J$ = 2.01 Hz, Ar–H), 7.41 (dd, 1H, $J$ = 2.01, 8.91 Hz, Ar–H), 7.73 (d, 1H, $J$ = 8.91 Hz, Ar–H), 6.42 (d, 1H, $J$ = 5.37 Hz, Ar–H), 6.12 (br s, 1H, NH), 4.82 (br s, 1H, NH), 3.83 (t, 4H, $J$ = 4.85 Hz, $NCH_2$), 3.71 (t, 4H, $J$ = 5.53 Hz, $NCH_2$), 3.38 (t, 2H, $J$ = 5.45 Hz, NH–$CH_2$), 2.83 (t, 2H, $J$ = 6.09 Hz, $NCH_2$), 2.57 (t, 4H, $J$ = 4.65 Hz, $NCH_2$), 2.02–1.98 (m, 2H, $CH_2$), 1.76–1.72 (m, 2H, $CH_2$), 1.64–1.56 (m, 6H, $CH_2$), 1.39–1.31 (m, 2H, $CH_2$), 1.27–1.18 (m, 4H, $CH_2$); $^{13}C$ ($CDCl_3$, 75 MHz): 165.11, 151.21, 150.23, 148.17, 135.28, 127.94, 125.58, 121.38, 117.17, 99.15, 55.62, 52.65, 49.17, 44.14, 43.19, 39.05, 33.21, 25.80, 24.94; Anal. Calcd for $C_{29}H_{40}ClN_9$: Calcd C 63.31, H 7.33, N 22.91. Found: C 63.42, H 7.21, N 22.79.