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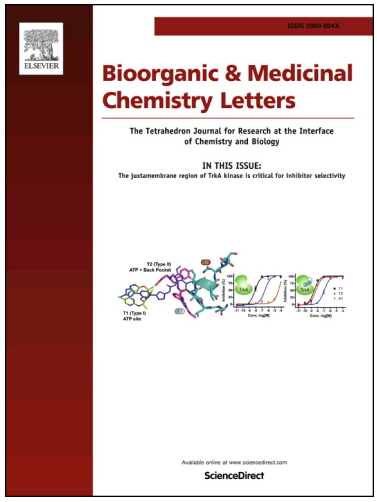

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PII: S0960-894X(17)30226-3
DOI: http://dx.doi.org/10.1016/j.bmcl.2017.03.005
Reference: BMCL 24757

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 30 September 2016
Revised Date: 27 February 2017
Accepted Date: 2 March 2017

Please cite this article as: Vijayaraghavan, S., Mahajan, S., Docking, synthesis and antimalarial activity of novel 4-anilinoquinoline derivatives, *Bioorganic & Medicinal Chemistry Letters* (2017), doi: http://dx.doi.org/10.1016/j.bmcl.2017.03.005

Docking, synthesis and antimalarial activity of novel 4-anilinoquinoline derivatives

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Abstract

A series of 4-anilinoquinoline triazine derivatives were designed, synthesized and screened for *in vivo* antimalarial activity against a chloroquine-sensitive strain of *Plasmodium berghei*. The compounds were further subjected to *in vitro* antimalarial activity against chloroquine-resistant W2 strain of *Plasmodium falciparum* and β-haematin inhibition studies. All the compounds exhibited *in vivo* antimalarial activity better than that shown by the standard drug, chloroquine. Twelve out of fifteen compounds showed better inhibition than that of chloroquine against chloroquine-resistant W2 strain of *Plasmodium falciparum*. Ten compounds showed β-haematin inhibition, better than that of chloroquine, with IC_{50} values in the range of 18 – 25 μM. One compound, **3k**, was found to be better than artemisinin against W2 strain of *Plasmodium falciparum* and also displayed the best β-haematin inhibitory activity, thereby becoming eligible to be explored as a potential lead for antimalarial chemotherapy.

Keywords: Malaria, *Plasmodium falciparum*, falcipain-2, cysteine protease, 4-anilinoquinoline triazine, β-haematin.

Malaria still remains one of the major threats in the developing countries. Globally, an estimated 3.2 billion people in 97 countries and territories are at risk of being infected with malaria and 1.2 billion are at high risk (>1 in 1000 chance of getting malaria in a year). According to the latest estimates by WHO, released in September 2015 and updated on October 2015 (WHO World Malaria Report, 2014), 214 million cases of malaria occurred globally in 2014 and the disease led to 4,80,000 deaths. Approximately, 78% deaths were mostly in children younger than five years old. In India, around 4,00,000 malarial deaths were reported in 2013.

Malaria caused by *Plasmodium falciparum* (*P. falciparum*) is more prevalent than the other less virulent Plasmodium species, which include *Plasmodium vivax* (*P. vivax*), *Plasmodium malariae* (*P. malariae*) and *Plasmodium ovale* (*P. ovale*).[1] The WHO has advocated the use of artemisinin and its analogues in combination with 4-aminoquinoline antimalarials like lumefantrine and mefloquine.[2, 3] However, artemisinin based combination therapy (ACT) possesses limitations due to some serious issues like higher cost of treatment, safety in pregnancy and imbalance in demand over supply.[4, 5] Other conventional methods of malaria treatment and control have been hampered by increasing resistance to all antimalarial drugs and mosquito insecticides.[6, 7] In some parts of the world, ACT is used indiscriminately for self treatment of suspected uncomplicated malaria, which ultimately has lead to the emergence of resistance to artemisinin derivatives in some of the Southeast Asian countries namely, Laos, Cambodia, Myanmar, Vietnam and Thailand.[8] This resistance has the potential to continue to develop and spread, subsequently making malaria chemotherapy more difficult. With the absence of an effective vaccine[9] and also, in view of the widespread of resistant strains, there is an urgent need for development of new, cost-effective and efficacious antimalarial agents with low potential of triggering resistance.

Since the discovery of natural product quinine, structural modifications of its quinoline pharmacophore gave rise to the most effective antimalarial agents namely, chloroquine (CQ) [**Figure 1 (a)**], amodiaquine (AQ) [**Figure 1(b)**] and mefloquine [**Figure 1 (c)**].[10, 11] After chloroquine, compounds such as aminoquinoline derivatives, sulphadoxine, pyrimethamine, cycloguanil, etc. were developed for the treatment of malaria.[12, 13] Although the resistance to chloroquine and related 4-aminoquinoline antimalarial drugs has already evolved and spread; designing novel antimalarials based on the quinoline pharmacophore has discrete benefits due to the exclusive pharmacological effect of 4-aminoquinoline drugs.[14] The 1, 3, 5-triazine derivatives are also quite attractive scaffolds for the production of antimalarial drugs, e.g., cycloguanil, chlorcycloguanil, clociguanil and WRA99210.[15] Therefore, it was planned to synthesize the compounds possessing the combination of both 1, 3, 5-triazine and 4-anilinoquinoline moieties with a view to expect synergistic antimalarial activity and improved efficacy compared to the traditional marketed antimalarial drugs.

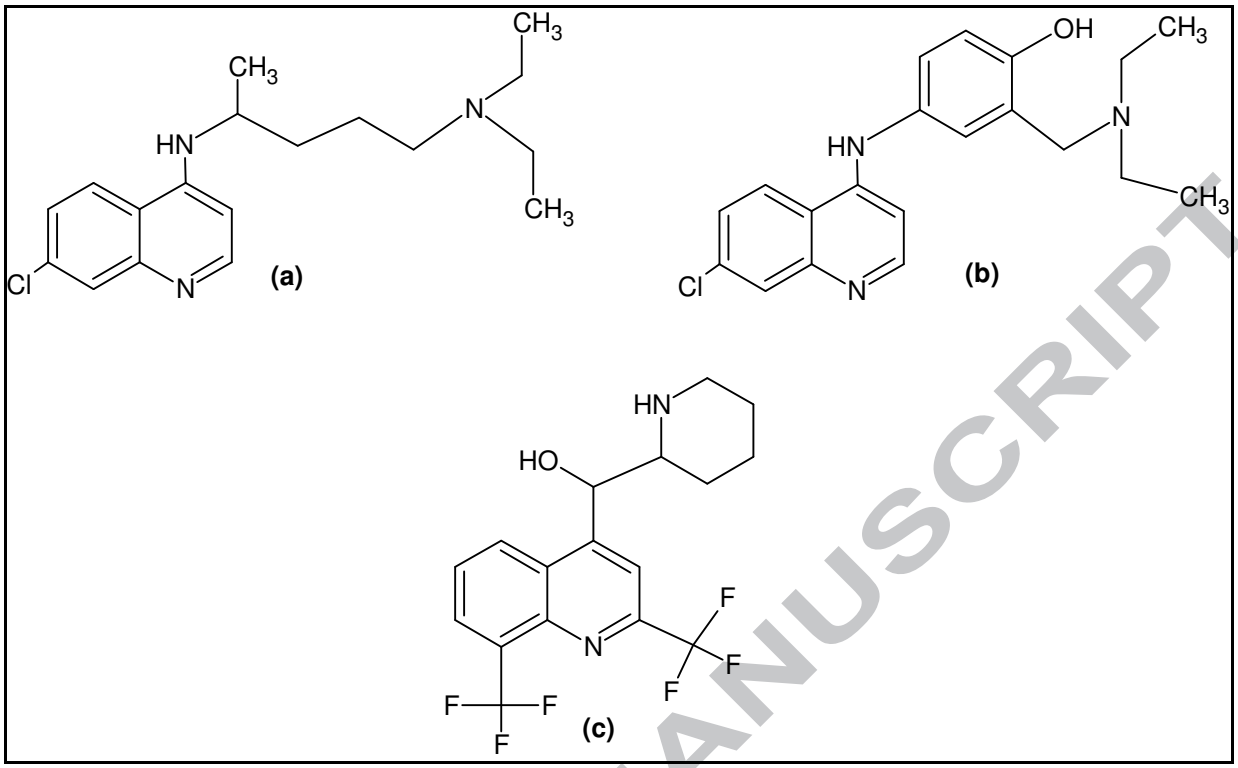


Figure 1. Structures of (a) chloroquine, (b) amodiaquine and (c) mefloquine.

Even if the novel compounds do exhibit antimalarial activity, it is also essential to determine their mechanism of action. Chloroquine and other aminoquinoline drugs act by inhibiting β-haematin, which converts toxic haem into haemozoin in the food vacuole of the parasite, thereby leading to substantial accumulation of toxic haem, causing the death of the parasite.[16, 17, 18] Therefore, the novel synthesized compounds were evaluated for β-haematin inhibition activity. Another target, which is new and promising, is malarial parasitic cysteine protease enzyme. Falcipain-2 (FP-2) is a major cysteine protease that plays a key role in parasite food assimilation by its ability to degrade haemoglobin. Falcipain-2 has been known to cleave denatured and native haemoglobin in the acidic food vacuole of the parasite to produce globin and haem.[19] Globin is then degraded to various proteins and subsequently to amino acids, which are a major requirement for parasite protein synthesis. Inhibition of falcipain-2 will thereby block the degradation of haemoglobin and consequently, parasite growth and development [20]. Falcipain-2 inhibitors have proved to block parasite development *in vitro*[21, 22] and also in murine models.[23, 24] However, all these inhibitors are derived from peptide analogues[25] and show side effects, display susceptibility to host proteases, have poor pharmacological profiles and low absorption rates through cell membranes and therefore, have not been further explored and developed into marketed preparations. Moreover, covalent interactions are formed between the electrophilic groups, such as aldehydes, nitriles, vinyl sulfones and epoxides with thiolate of the catalytic cysteine amino acid, thereby leading to increased toxicity of the molecules. Therefore, it was planned to target the falcipain-2 enzyme by designing non-peptidic molecules in such a way so that they exhibit antimalarial activity by inhibiting the enzyme through non-covalent binding, in order to minimize toxicity while retaining the potential for high *in vivo* and *in vitro* activity and selectivity. A library of structures of 4-anilinoquinoline triazine were docked on the X-ray

crystallized structure of falcipain-2 enzyme before their synthesis and only those compounds showing good interactions with the enzyme were taken up for the synthesis.

Docking studies of the compounds on falcipain-2 enzyme[26] and the validation of the docking protocol[27] were done prior to their synthesis with a view to study the *in silico* interaction of the ligands with the enzyme, giving way to the synthesis of only those best fit lead compounds, which are proposed to exhibit improved antimalarial activity by the mechanism of falcipain-2 inhibition. The procedures for docking and validation have been mentioned in the Supplementary data section. The docking results were expressed in terms of G-Score. **Figures 2** and **3** display the images of E-64 and compound **3a**, respectively, interacting with the enzyme falcipain-2.

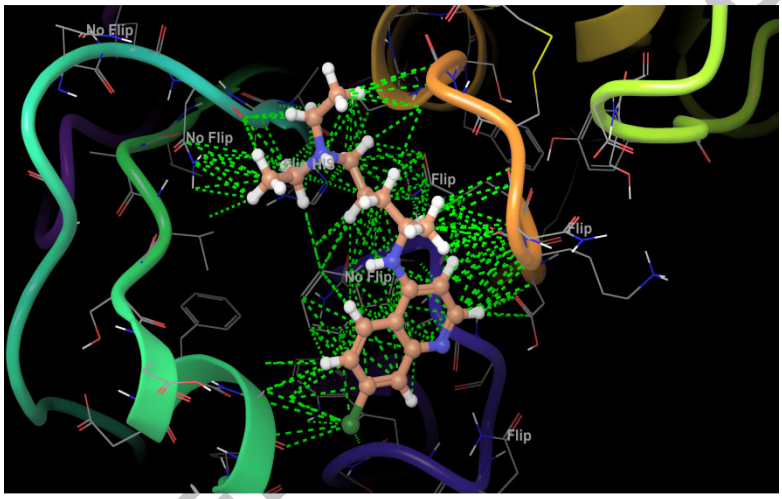


Figure 2. Chloroquine docked on falcipain-2 enzyme.

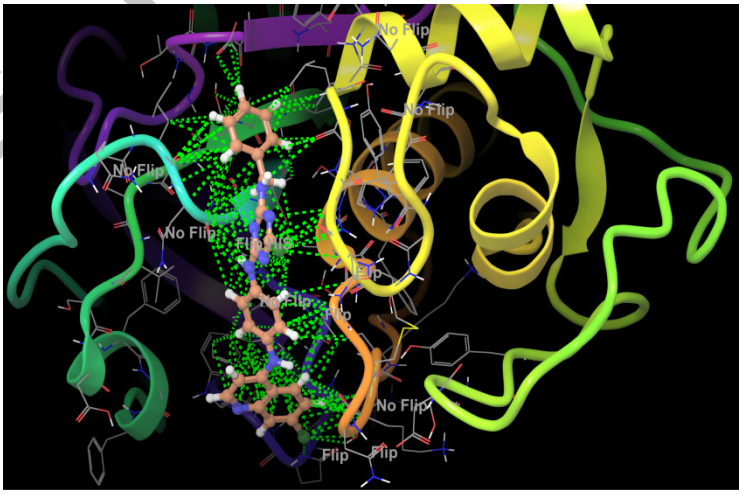


Figure 3. Compound 3a docked on falcipain-2 enzyme.

After docking a series of 4-anilinoquinoline triazine derivatives on falcipain-2 enzyme, those compounds displaying G-score more than that shown by E-64 were successfully synthesized using inexpensive reactants and were produced with good yields of more than 60%. The structures of the compounds were characterized and confirmed by IR, ^{1}H NMR and mass spectral analysis (see Supplementary data). **Table 2** displays the R and R^{1} substituents of the compounds **3a-3n**.

Converting the acidic pH of the food vacuole to the basic pH would disrupt the process of haemoglobin degradation and thereby, improve the activity of the compounds. Therefore, compounds were synthesized in such a way that the basicity of the increased number of nitrogen atoms in the derivatives would help in their accumulation within the acidic food vacuole of the parasite. Linking both the quinoline and 1, 3, 5-triazine moieties with an anilino group at the 4th position of the quinoline ring improved the hydrophobicity of the molecules, thereby displaying good hydrophobic interactions with the required amino acid residues of the falcipain-2 enzyme. Increasing the side chain of the anilino group with aromatic groups and adding secondary amines to the 1, 3, 5-triazine moiety also showed improvement in the G-score and in the *in vivo* and *in vitro* antimalarial activity.

The fifteen derivatives were synthesized in three steps using nucleophilic aromatic substitution reaction as in **Figure 4**. In the first step, first chlorine of cyanuric chloride was substituted with aryl and alkyl amines by keeping the reaction mixture at 0 ºC for the first 20 min and then, overnight at room temperature, to yield monosubstituted triazines.[28] The second step involved substitution of chlorine of 4, 7 - dichloroquinoline with paraphenylene diamine at reflux. The third step included the reflux of the first and second step products to give 4 – anilinoquinoline triazine derivatives. The physical data are included in the Supplementary data.

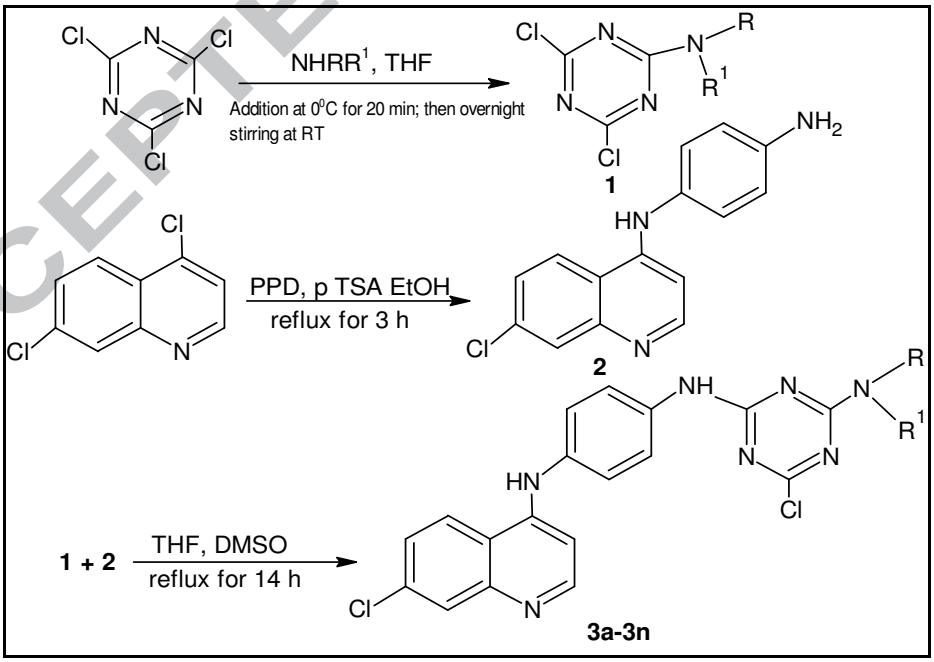
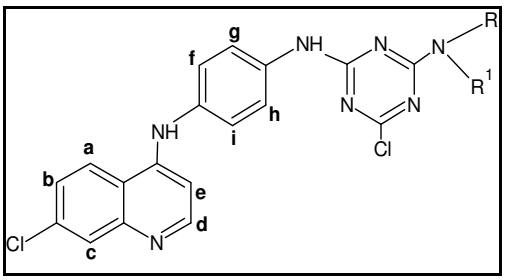
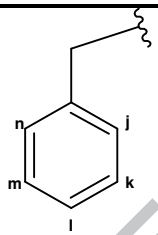
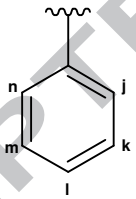
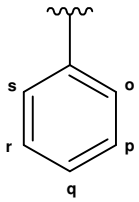
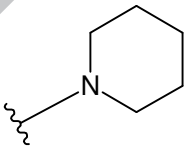
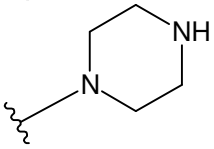
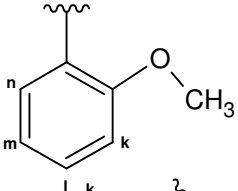
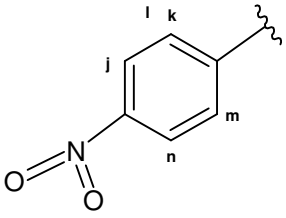
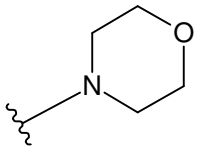
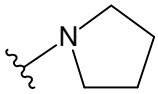
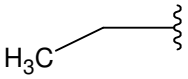
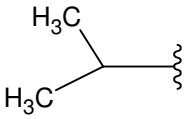
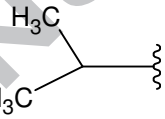
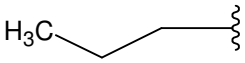
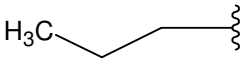
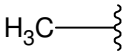
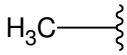
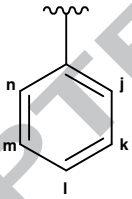
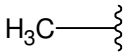
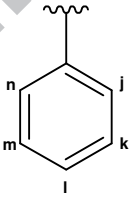
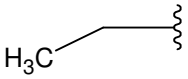


Figure 4. Procedure for the synthesis of 4-anilinoquinoline triazine derivatives.

Table 1: R and R^1 substituents of the titled compounds 3a-3n



Compound	R	R^1
3a		H
3b		
3c		-
3d		-
3e		H
3f		H

3g		-
3h		-
3i		
3j		
3k		
3l		
3m		
3n		

Note: The sign ≈ shows the position of attachment of the R and R^1 groups to the "N" atom of the triazine ring. Compounds 3c, 3d, 3g and 3h already have the "N" atom in their rings, which is the the "N" atom of the triazine moiety.

Before subjecting the synthesized compounds to *in vivo* antimalarial evaluation, they were observed for their toxic effects by performing acute toxicity studies as per the Organization for Economic Co-operation and Development (OECD) guidelines for the testing of chemicals, Test No. 423, 2008. This method helped to determine the lethal dose and whether the synthesized

compounds were toxic or not. From this information, the dose of oral administration of compounds was determined.

The *in vivo* antimalarial testing of compounds was performed by Peter's four days test[29] on Swiss albino mice using chloroquine-sensitive *Plasmodium berghei* strain. It is a highly reliable method as it involves inducing parasitemia in mice, treating them with a single oral dosage of compounds, continuously for 4 days and observing their blood smears. The per cent inhibition of parasitemia was calculated in terms of per cent chemosuppression. The results were subjected to the statistical analysis, calculating mean and standard error of mean (SEM).

In order to determine the IC_{50} values of inhibition of malaria, the compounds were further screened for *in vitro* antimalarial activity against chloroquine resistant W2 strain of *P. falciparum* using human erythrocytes[30] and β-haematin inhibition[31] as per the procedures reported. The IC_{50} values obtained were well corroborated with the G-scores and the data obtained in *in vivo* studies and this helped to determine the most active compounds displaying good therapeutic efficacy. It is observed that better the G-scores, better is the *in vivo* and the *in vitro* activities of the compounds.

In the docking study, the derivatives showed good van der Waal (vdw) interaction with Cys 42, Trp 43, Leu 84, Ile 85, Val 152, Leu 172, Asn 173, His 174 and Ala 175 and formed hydrogen bond with Trp 206. The docking results revealed that the G-scores of all the 15 compounds, as revealed in **Table 2**, were found to be more than -6.73 compared to the score of -6.65 of E-64, -5.78 of chloroquine and -6.70 of artemisinin, which proved that all the synthesized compounds interacted with falcipain-2 enzyme better than the standard drug *in silico*.

Table 2: G- score, % chemosuppression and IC_{50} values of the titled compounds 3a-3n against W2 resistant strain and β-haematin

Compound	G-score	% Chemosuppression (Mean±SEM)	W2 *in vitro* IC_{50} (µM)	β-haematin inhibition IC_{50} (µM)
2	-6.74	85.92 ± 0.3700	0.807	32.5 ± ND
3a	-9.84	97.03 ± 0.3700	0.123	21.8 ± 2.3
3b	-9.60	97.4 ± 0.3700	0.255	24.9 ± 4.3
3c	-9.11	94.07 ± 0.3700	0.118	20.6 ± 4.5
3d	-9.22	94.81 ± 0.3700	0.167	23.7 ± 5.6
3e	-9.23	94.81 ± 0.3700	0.145	21.0 ± 3.0
3f	-9.54	97.4 ± 0.3700	0.145	23.0 ± 5.7
3g	-8.43	97.03 ± 0.3700	0.138	21.1 ± 2.4
3h	-9.20	97.03 ± 0.3700	0.119	28.1 ± 4.6
3i	-9.33	95.55 ± 0.6409	0.268	21.6 ± 3.1
3j	-9.05	95.55 ± 0.6409	0.902	> 500
3k	-9.52	91.48 ± 0.3700	0.008	18.6 ± 2.0
3l	-9.06	95.18 ± 0.3700	0.380	29.7 ± 1.1
3m	-9.60	91.11 ± 0.6409	0.269	23.7 ± 4.2
3n	-9.40	97.03 ± 0.3700	0.220	29.2 ± 4.7
CQ	-5.78	82.96 ± 0.3700	0.282	27.2 ± 3.0
Artemisinin	-6.20	-	0.015	-

E-64	-6.65	-	-

From the toxicity studies, the data revealed that all the synthesized compounds were confirmed to be non-toxic up to 2000 mg/kg dose level and were well tolerated by the experimental animals. Therefore, the dose selected and fixed for pharmacological evaluation was $1/10^{th}$ of 2000 mg/kg i. e. 200 mg/kg.

All the 15 compounds were tested *in vivo* at 200 mg/kg dose and the results were compared with that of chloroquine as the standard drug. The *in vivo* antimalarial activity results revealed that all the compounds displayed % chemosuppression more than 85, which was better than that shown by the standard drug, chloroquine. Compounds **3a-3g**, **3i-3j**, **3l** and **3n** exhibited more than 94% chemosuppression, as shown in **Table 1**.

As displayed in **Table 1**, the *in vitro* antimalarial activity of all the 15 compounds on chloroquine-resistant *Plasmodium falciparum* W2 strain revealed that compounds **3a-3i** and **3m-3n** showed inhibition against the resistant parasite better compared to chloroquine and moderate compared to artemisinin. Compound **3k** (IC_{50} value = 0.008 µM) displayed the highest level of inhibition compared to that of the other derivatives, as well as chloroquine and artemisinin. The IC_{50} values of all the compounds were found to be less than 1 µM.

In β-haematin inhibition, as revealed in **Table 1**, amongst all the compounds, ten compounds **3a-3g**, **3i**, **3k** and **3m** showed β-haematin inhibitory activity with the IC_{50} values in the range of 18 – 25 µM, better than that shown by chloroquine (IC_{50} value 27.2 µM). Compound **3k** displayed the best β-haematin inhibitory activity, with the IC_{50} value of 18.6 µM. These results showed that using heterocyclic and aromatic amines enhanced the β-haematin inhibitory activity of 4 – anilinoquinoline derivatives. Attaching single chain aliphatic amines to cyanuric chloride did not help much in improving the β-haematin inhibitory activity of the derivatives. However, attachment of a branched chain aliphatic amine to the same yielded the best activity.

In conclusion, 4-anilinoquinoline derivatives were successfully designed, synthesized and evaluated for both *in vivo* and *in vitro* antimalarial activity including β-haematin inhibitory activity. These derivatives have displayed good antimalarial activity *in vivo* as well as *in vitro* by the mechanism of β-haematin inhibition. One compound, **3k**, was found to be better than artemisinin against W2 strain of *Plasmodium falciparum* and also displayed the best β-haematin inhibitory activity, thereby becoming eligible to be explored as a potential lead for antimalarial chemotherapy. As the compounds exhibited good *in silico* interaction with falcipain-2 enzyme, they may also act by inhibition of falcipain-2 enzyme in *Plasmodium falciparum*. Study on falcipain-2 inhibition of these derivatives is underway and shall be published in due course of time.

Acknowledgements

The authors wish to thank the Indian Council of Medical Research (ICMR), New Delhi, India, for providing funds for the research work. The authors are also grateful to Dr. Jiri Gut and Philip Rosenthal from the Department of Medicine, San Francisco General Hospital, University

of California San Francisco, USA, for carrying out the *in vitro* antimalarial activity against W2 strain of *P. falciparum* and falcipain-2 enzyme inhibition study.

Supplementary data

Supplementary data include the docking and validation procedures, physical and spectral data of 4 – anilinoquinoline derivatives.

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Highlights

- Falcipain-2 and β-haematin are the promising targets for antimalarial activity.
- All compounds showed better G-score and *in vivo* activity compared to chloroquine.
- Twelve compounds exhibited *in vitro* inhibition better than that of chloroquine.
- Ten compounds exhibited β-haematin inhibition better than that of chloroquine.
- Compound **3k** showed excellent *in vitro* and β-haematin inhibitory activity.