Journal of Enzyme Inhibition and Medicinal Chemistry, 2011; 26(4): 569–578

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.3109/14756366.2010.539566

informa
healthcare

ORIGINAL ARTICLE

Novel oxazine skeletons as potential antiplasmodial active ingredients: Synthesis, *in vitro* and *in vivo* biology of some oxazine entities produced via cyclization of novel chalcone intermediates

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Abstract

A novel series of 6-(2-chloroquinolin-3-yl)-4-substituted-phenyl-6H-1,3-oxazin-2-amines were synthesized and evaluated for *in vitro* antimalarial efficacy against chloroquine sensitive (MRC-02) as well as chloroquine resistant (RKL9) strains of *Plasmodium falciparum*. The activity tested was at nanomolar concentration. β-Hematin formation inhibition activity ($BHIA_{50}$) of oxazines were determined and correlated with antimalarial activity. A reasonably good correlation ($r = 0.49$ and 0.51, respectively) was observed between antimalarial activity (IC_{50}) and $BHIA_{50}$. This suggests that antimalarial mode of action of these compounds seems to be similar to that of chloroquine and involves the inhibition of hemozoin formation. Some of the compounds were showing better antimalarial activity than chloroquine against resistant strain of *P. falciparum* and were also found to be active in the *in vivo* experiment.

Keywords: Chalcones, *Plasmodium falciparum*, antimalarial activity, oxazines

Introduction

Frequency of malaria and other diseases is progressively increasing worldwide. New emerging strains of bacterium and resistance to currently available drugs make this field more conscientious and alarming. Malaria is the most common parasitic disease in tropical and subtropical regions and worldwide. Around 40% of the population is living in the malarial endemic area. According to the survey done by World Health Organization, it was found that a quantum of people living in these areas is at the risk of malarial transmission, which is literally alarming. Approximately 1.0–3.0 millions of population died due to the non-availability of proper chemotherapy.[1,2] The situation regarding the control and treatment of malaria has progressively worsened with the spread of insecticide-resistant mosquito vectors and drug-resistant malarial parasites.[3] Therefore, the need for new agents that are active against multi-resistant *Plasmodium* strains, through the identification of new targets is very urgent.[4,5] These points are critical for the disease process or essential for the survival of the parasite.

Plasmodium falciparum and *Plasmodium vivax* are the two major human malarial parasites. *P. falciparum* is responsible for the most deaths and it has developed resistance against almost all the available drugs[6] so far in the market. The search for novel antimalarial drugs against specific parasitic targets is thus an immense priority[7,8] for the medicinal chemists all over the world for the sake of humanity. The potential antimalarial activity of chalcones has generated a great interest.[9–12] Their antimalarial activity was first reported when licochalcone A, a natural product isolated from Chinese liquorice root, exhibited potent antimalarial activity.[9] Subsequently, a synthetic analogue 2,4-dimethoxy-4′-butoxy-chalcone was reported to have antimalarial activity.[10] Some chalcones inhibit falcipain cysteine proteases, but it is

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(Received 30 May 2010; revised 11 October 2010; accepted 09 November 2010)

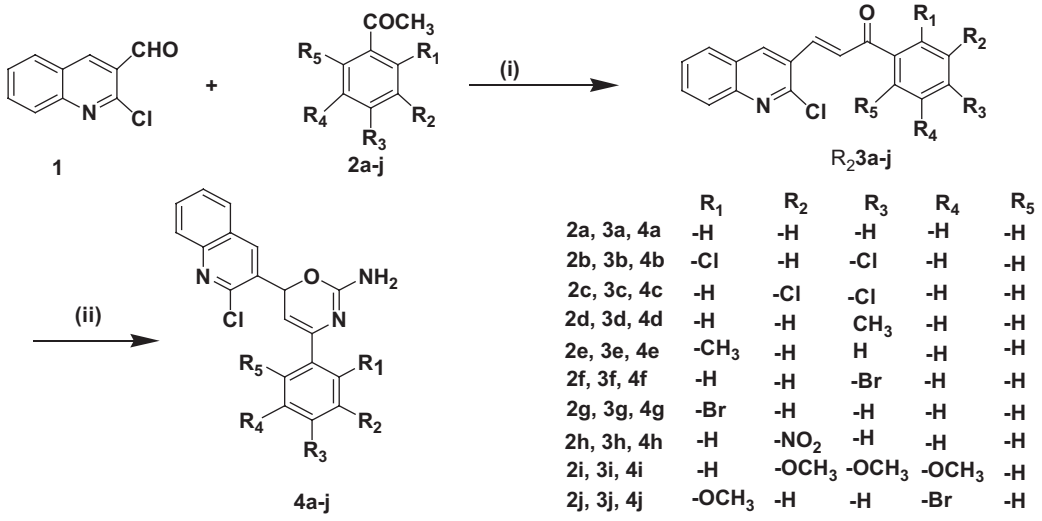
unclear whether the antimalarial activity of this class is primarily due to protease inhibition[11] or others. Several oxygenated chalcones and bis-chalcones were reported to have antimalarial activity.[12] Chalcones have been used as intermediate for the preparations of compounds having multi-therapeutic value. The literature review reveals that chalcone derivatives exhibit diverse pharmacological activities, such as potential cytotoxic agents, antimalarial agents, antimicrobial agents, antiviral, anti-inflammatory, anesthetics, and mydriatics.[13–22]

In this study, we have synthesized and characterized a series of novel quinoline-based chalcones and their oxazine derivatives. In this study, we have also reported the antimalarial activity of substituted oxazines against chloroquine (CQ) sensitive (MRC-02) as well as CQ resistant (RKL9) strains of *P. falciparum*. In this study, only the substitutions on "B" ring of the 6-(2-chloroquinolin-3-yl)-4 substituted phenyl-6H-1,3-oxazin-2-amine moiety was changed by keeping the basic skeleton intact. The compounds were also evaluated for β-hematin formation inhibition activity and correlated with their antiplasmodial activity against CQ sensitive as well as resistant strains of malaria parasite. From the correlations data, the mode of action of this class of compounds was proposed to follow haem detoxification inhibition pathway. Three compounds (**4b**, **4g,** and **4i**) were found most active *in vitro* against CQ-resistant malaria parasite were also tested *in vivo* against CQ-resistant N-67 strain of *Plasmodium yoelli.*

Chemistry

6-(2-Chloroquinolin-3-yl)-4 substituted phenyl-6-H-1-,3-oxazin-2-amines **4(a–j)** were synthesized according to the method as shown in Scheme 1. In the first step, synthesis of chalcones was carried out by the well-known Claisen–Schmidt reaction, and products were isolated in 60–70% yield after recrystallization. In the second step, chalcone and urea with catalytic amount of NaOH were refluxed in ethanol to obtain the desired products **4(a–j)**. Factors such as the structure and position of the substituents have profoundly influenced the rate of reaction. Halogenated chalcones viz. **3b**, **3g**, and **3f** took 10 h for complete conversion into final oxazines, whereas unsubstituted **3a**, methyl substituted (**3e** and **3d**) and alkoxy substituted **3i** chalcones took 12, 14, and 14.5 h, respectively. The general accepted interpretation regarding chemistry of this reaction involves the nucleophilic attack of oxygen on the carbon–carbon double bond at β position. Hence, the electropositive nature of β-carbon may control the overall rate of the reaction. The electropositive nature of β-carbon is controlled by the aromatic ring directly connected to it (ring C as shown in Figure 1). Halogens being electron withdrawing in nature significantly increase the positive character of β-carbon leading to faster reaction while electron donating alkyl and alkoxy groups contributed to execute slower reaction. However, this phenomenon was not observed in the case of nitro group substituents (**compound 4h**). The possible explanation might be the meta-directing nature of nitro group. In compound **4h**, nitro group is placed at meta position of the phenyl ring and by virtue of its meta-directing nature, electron density at β-carbon increases and its electro-positivity decreases leading to longer time for the completion of reaction.

Structures of compounds **4(a–j)** were confirmed by elemental analysis, IR, MS, and NMR techniques. All the substituted oxazine possesses similar basic skeletal structure. Proton NMR signals were assigned by comparing the spectra of the products **4(a–j)** with their corresponding chalcones. The ^{1}H-NMR spectrum of oxazine derivatives were recorded on Bruker Avance II 400 (400 MHz) NMR spectrometer in dimethyl sulphoxide (DMSO)-d_6 using TMS as an internal standard: Peak at 2.31 ppm (singlet) was attributed to (2H) of –NH_2. Doublets were observed at



	R_1	R_2	R_3	R_4	R_5
2a, 3a, 4a	-H	-H	-H	-H	-H
2b, 3b, 4b	-Cl	-H	-Cl	-H	-H
2c, 3c, 4c	-H	-Cl	-Cl	-H	-H
2d, 3d, 4d	-H	-H	CH_3	-H	-H
2e, 3e, 4e	$-CH_3$	-H	H	-H	-H
2f, 3f, 4f	-H	-H	-Br	-H	-H
2g, 3g, 4g	-Br	-H	-H	-H	-H
2h, 3h, 4h	-H	$-NO_2$	-H	-H	-H
2i, 3i, 4i	-H	$-OCH_3$	$-OCH_3$	$-OCH_3$	-H
2j, 3j, 4j	$-OCH_3$	-H	-H	-Br	-H

(i) Methanolic NaOH at r.t, 24hrs, (ii) Urea, Ethanolic NaOH, reflux

Scheme 1.Synthesis of substituted oxazines **4(a–j).**

5.21 and 6.31 ppm for 1H of (6-H) and (5-H), respectively, of oxazine ring in the spectrum. The NMR spectrum also showed multiplets around 7.13–8.30 ppm for aromatic protons. Mass spectral data provided useful information regarding the structural analysis. The molecular ion peak of **4a** was found at 335 Da. There were some guide peaks in the IR spectrum of 4a, which were helping in the structural analysis regarding the different functionalities present in the moieties. Some of the guides peaks were: 3412 (–NH_2), 1394 (Het–C–O), 1570 (C=C, ring skeleton Ar moiety), 1415 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str for oxazine ring), and 853 (C–Cl, for quinoline skeleton).The other signals and peaks of ^{1}H-NMR and IR are in complete agreement with the assigned structures.

Materials and methods

In vitro antimalarial assay

P. falciparum strains MRC-02 (CQ sensitive) and RKL9 (CQ resistant) were obtained from National Institute of Malaria Research, New Delhi, India, and were maintained in a continuous culture using the standard method described by Trager and Jensen.[23] Parasites were cultured in human B (+) erythrocytes in RPMI-1640 media supplemented with 25 mM HEPES buffer, 10% human AB (+) serum, and 0.2% sodium bicarbonate (Sigma). It was maintained at 5% CO_2. Cultures containing predominantly early ring stages were synchronized by addition of 5% D-sorbitol (Sigma) lysis,[24] used for testing. Initial culture was maintained in small vials with 10% haematocrite (i.e. 10 µl erythrocytes containing 1.0% ring stage parasite in 100 µl complete media). The culture volume per vial for the assay was kept at 100 µl. Number of parasites for the assay was adjusted at 1–1.5% by diluting with fresh human B (+) RBC. Assay was done in 96-well microlitre flat-bottomed tissue culture plates, which were incubated at 37°C for 24 h in the presence of twofold serial dilutions of compounds and CQ diphosphate for their effect on schizont maturation. Compounds were dissolved in ethanol and further diluted with RPMI-1640 medium (the final ethanol concentration did not exceed 0.5%, which did not affect parasite growth). CQ diphosphate was dissolved in aqueous medium. Test was done in duplicate vials for each dose of the drugs. Solvent control culture containing the same concentrations of the solvent as present in the test vials was done with RPMI-1640 containing 10% AB (+) serum. Parasite growth was found unaffected at the solvent concentrations. Growth of the parasites from duplicate vials of each concentration was monitored in Giemsa-stained blood smears by counting number of schizont per 100 asexual parasites. Percent schizont maturation inhibition was calculated by the formula: $(1 - N_t/N_c) \times 100$, where N_t and N_c represent the number of schizont in the test and control vial, respectively.

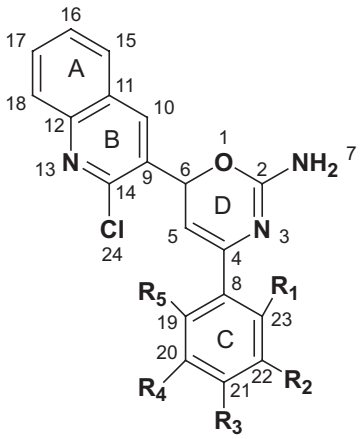


Figure 1. Structure of 6-(2-chloroquinolin-3-yl)-4 substituted phenyl-6H-1,3-oxazin-2-amine.

In vivo antimalarial assay

The *in vivo* efficacy was determined by Peter's 4-day suppressive test[25] by using *P. yoelli* (N-67 strain) and male white Swiss albino mice (18–20 g of body weight). In brief, groups of five mice each were inoculated intraperitoneally with ~30% of *P. yoelli*-infected erythrocytes from a donor mouse. Four hours later, selected test compounds were administered at a dose of 50 and 25 mg/kg/day intraperitoneally. CQ (8 mg/kg/day) was taken as standard for comparison. A total of four doses were given on days 0, 1, 2, and 3. Control groups received the same amount of solvent used to suspend the compounds. The tail blood smears were made on day 4 and day 7 stained with Giemsa and were examined microscopically. The percent parasitaemia and average percent suppression of parasitaemia in comparison with control groups were determined. Mean survival time (MST) was calculated for dead mice during the 28-day observation period.

In vitro cytotoxicity assay

Human cervical epithelial cells were routinely maintained at 37°C in Eagles Minimum Essential Medium (EMEM; Sigma) supplemented with foetal bovine serum (10%), L-glutamine (2 mM), and gentamycine (80 mg/l). Cytotoxicity was assessed using the neutral red (NR) dye uptake assay[26] using 96-well micro titer tissue (MT) culture plates (Greiner, Germany). Briefly, 2×10^3 cells were seeded into each well of a 96-well MT plate and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. All the compounds were dissolved in DMSO and subsequently serial twofold dilutions were prepared in EMEM to give a broad range of concentration. Before assessment of cytotoxicity, the preformed cell monolayer was washed with EMEM and replaced with drug solutions in duplicate keeping cell control including corresponding dilution of DMSO. The plates were incubated at 37°C and observed microscopically on daily routine basis for the appearance of any morphological changes. After 72-h incubation, the cells were fixed with 5% glutaraldehyde solution prepared in phosphate-buffer saline (PBS) kept at room temperature for 45 min. The fixed cells were washed with

PBS, stained with 0.2% NR dye aqueous solution and incubated for 1 h at room temperature. After washing, the stained cells were subjected to acid alcohol lysis by adding 100 µl of 0.5% acetic acid (V/V) in 50% ethanol to each well followed by 10-min incubation. Absorbances of the resulting solutions were read at 550 nm and cell survival was calculated as the absorbance of the treated cells divided by the control (EMEM plus DMSO). Results were expressed in terms of the maximum non toxic dose values, that is, the maximum concentration of drug at which 100% cell survival was observed.

Determination of pK_a values

Acid dissociation constants (pK_a) were determined spectrophotometrically as previously described[27] by using a Unicam UV-300 spectrometer of Thermo Spectronic. Absorbances of each compound (0.025 mg/ml) dissolved in buffers of different pH values ranging from 5.8 to 11.2 as well as in 0.1 M NaOH and 0.1 M HCl were determined. Buffers from pH 5.8 to 8.0 were prepared from 0.1 M solutions of monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) and from pH 8.4 to 11.2 were prepared from 0.1 M glycine and 0.1 M NaOH. Analytical wavelengths (223, 250, 260, and 367 nm) were chosen at which significant difference between the absorbance of molecules in 0.1 M HCl and 0.1 M NaOH was observed. To reduce error, pK_a was determined in duplicate for each compound.

Inhibition of β-hematin formation *in vitro*

The quantitative β-hematin inhibitory activity assay is based on the differential solubility of hematin and β-hematin in DMSO and NaOH solution, respectively[28,29]. The method determines a 50% inhibitory concentration for β-hematin formation inhibition ($BHIA_{50}$) of the compound. In a micro-centrifuge tube, 100 µl of 6.4 mM solution of hematin freshly dissolved in 0.2 N NaOH solution, 50 µl of compound dissolved in ethanol (chloroquine diphosphate was dissolved in water), 200 µl of 3 M solution sodium acetate trihydrate ($C_2H_3NaO_2{\cdot}3H_2O$), and 50 µl of glacial acetic acid were incubated at 37°C for 24 h. Concentration of compounds varied from 6 to 0.3 µM. Water and ethanol were taken as controls for water-soluble and ethanol-soluble compounds, respectively. After incubation, the tubes were centrifuged for 15 min. The supernatant was discarded and the pellet was reconstituted in DMSO and again centrifuged for 15 min at 3300*g*. The supernatant was discarded and β-hematin was obtained as a pellet. After centrifugation, to isolate DMSO insoluble β-hematin, the pellet was dissolved in 0.1 N NaOH and absorbance was recorded at 405 nM to calculate $BHIA_{50}$ as previously described.[29]

Experimental protocols

2-Chloroquinoline-3-carbaldehyde was synthesised starting from *N*-arylacetamides by Vilsmeier Haack cyclization.[30,31] All the aromatic ketones were obtained from Sigma-Aldrich. Melting points were determined by open-tube capillaries method and are uncorrected. Mass spectra were recorded on a Micromass Q-To F high-resolution mass spectrometer equipped with electrospray ionization (ESI) on Masslynx 4.0 data acquisition system. ESI was used in positive ionization mode. Infrared spectra were recorded on a Schimadzu-IR Prestige 21 with a using (KBr) optics. ^{1}H-NMR spectra were acquired on Bruker Avance II 400 (400 MHz) NMR spectrometer in DMSO-d_6 as a solvent. The chemical shifts were expressed in the ppm (δ scale) downfield from TMS. Purity of chalcones and substituted oxazines was checked on thin layer chromatography (TLC; Merck Silica gel 60F254).

General method for synthesis of chalcones (3a–3j)

To the well-stirred solution of (8 mmol, 1.1 g) 2-chloroquinoline-3-carbaldehyde (**1**) in 5 ml of methanol in ice cold condition, freshly prepared 2 N methanolic NaOH solution (30 ml) was added and stirring was continued for further 10 min. To this, 8 mmol of appropriate ketones (**2a**–**j**) was added and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was cooled on an ice bath and neutralized with dilute hydrochloric acid. The precipitate thus formed was separated by filtration and washed three times with 50 ml distilled water to give the crude product. The product so obtained was recrystallized from methanol. The purity of the products was checked on TLC by using mixture of ethyl acetate and hexane as a mobile phase, which have shown single spot of the products in each case. The R_f values are given along with the spectral data below.

3-(2-Chloroquiolin-3-yl)-1-phenylprop-2-en-1-one (3a)

Prepared by the above method from **2** (8 mmol, 1.1 g) and acetophenone (8 mmol, 1.42 g); yield: 1.42 g, 61%; R_f = 0.44 in EtOAc/hexane, 3:7; yellow solid. mp:132–140°C; MS (M^+), *calcd.*: 294.753, *observed:* 294.062; FTIR (cm^{-1}):1732 (C=O), 1639 (CH=CH), 853 (C–Cl); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 7.53 (1H, d, H_α), 8.52 (1H, d, H_β), 7.40–8.31 (m, 10H, aromatic); Anal. Calcd: $C_{18}H_{12}ClNO$: C, 73.54; H, 4.17; N, 4.75. Found: C, 73.60; H, 4.24; N, 4.78.

3-(2-Chloroquinolin-3-yl)-1-(2,4-dichorophenyl)-)prop-2-en-1-one (3b)

Prepared by above method from **2** (8 mmol, 1.1 g) and 2,4-dichloroacetophenone (8 mmol, 0.89 g); yield: 1.21 g, 67%, R_f = 0.65 in EtOAc/hexane, 3:7. Pale yellow solid mp: 135–138°C; MS (M^+):, *calcd.*: 360.682, *observed:* 360.981; FTIR (cm^{-1}): 1732 (C=O), 1639 (CH=CH), 853 (C–Cl); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 7.65 (1H, d, H_α), 8.51 (1H, d, H_β), 7.34–8.32 (m, 8H, aromatic).Anal. Calcd: $C_{18}H_{10}Cl_3NO$: C, 59.60; H, 2.76; N, 3.81. Found: C, 59.65; H, 2.79; N, 3.86.

3-(2-Chloroquinolin-3-yl)-1-(3,4-dichlorophenyl)prop-2-en-1-one (3c)

Prepared by above method from **2** (8 mmol, 1.1 g) and 3,4-dichloroacetophenone (8 mmol, 1.12 g); yield: 1.20g, 63%, R_f = 0.61 in EtOAc/hexane, 3:7; Pale yellow solid.

mp: 145–148°C; MS (M^+): *calcd.*: 360.072, *observed:* 360.214; FTIR (cm^{-1}): 1730 (C=O), 1643 (CH=CH), 853 (C–Cl); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 7.70 (1H, d, H_α), 8.55 (1H, d, H_β), 6.99–8.25 (m, 8H, aromatic)., Anal. Calcd: $C_{18}H_{10}Cl_3NO$: C, 59.40; H, 2.76; N, 3.80. Found: C, 59.45; H, 2.78; N, 3.88.

3-(2-Chloroquinolin-3-yl)-1-p-tolyl prop-2-en-1-one (3d)

Prepared by above method from **2** (8 mmol, 1.1 g) and 4-methyl acetophenone (8 mmol, 1.15 g); yield: 1.14 g, 60%, R_f = 0.56 in EtOAc/hexane, 3:7; yellow crystalline solid. mp: 140–148°C; ESI-MS (M^+): *calcd.*: 307.107, *observed:* 307.184; FTIR (cm^{-1}): 1728 (C=O), 1643 (CH=CH), 853 (C–Cl); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 2.38 (3H, s, CH_3), 7.54 (1H, d, H_α), 8.59 (1H, d, H_β), 7.29–8.33 (m, 9H, aromatic)., Anal. Calcd: $C_{19}H_{14}ClNO$: C, 74.12; H, 4.56; N, 4.49. Found: C, 74.15; H, 4.58; N, 4.18.

3-(2-Chloroqunolin-3-yl)-1-o-tolyl prop-2-en-1-one (3e)

Prepared by above method from **2** (8 mmol, 1.1 g) and 2-methyl acetophenone (8 mmol, 1.45 g); yield: 1.54 g, 65%, R_f = 0.51 in EtOAc /hexane, 3:7; yellow crystalline solid. mp: 132–140°C; ESI-MS (M^+): *calcd.*: 307.128, *observed*: 307.161; FTIR (cm^{-1}):1728 (C=O), 1643 (CH=CH), 853 (C–Cl); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 2.38 (3H, s, CH_3), 7.54 (1H, d, H_α), 8.51 (1H, d, H_β), 7.29–8.27 (m, 9H, aromatic). Anal. Calcd: $C_{19}H_{14}ClNO$: C, 74.11; H, 4.56; N, 4.49. Found: C, 74.15; H, 4.58; N, 4.18.

3-(2-Chloroquinolin-3-yl)-1-(4-bromophenyl) prop-2-en-1-one (3f)

Prepared by above method from **2** (8 mmol, 1.13 g) and 4-bromoacetophenone (8 mmol, 1.1 g); yield: 1.55 g, 64%; R_f = 0.59 in EtOAc/hexane, 3:7; pale yellow crystalline solid. mp: 146–150°C; ESI-MS (M+Na): *calcd.*: 393.132, *observed:* 393.148; FTIR (cm^{-1}): 1728 (C=O), 1643 (CH=CH), 853 (C–Cl), 588 (C–Br);^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 7.54 (1H, d, H_α), 8.53 (1H, d, H_β), 7.43–8.31 (m, 9H, aromatic).Anal. Calcd: $C_{18}H_{11}ClBrNO$: C, 58.01; H, 2.96; N, 3.74. Found: C, 58.04; H, 4.98; N, 3.78.

3-(2-Chloroquinolin-3-yl)-1-(2-bromophenyl) prop-2-en-1-one (3g)

Prepared by above method from **2** (8 mmol, 1 g) and 2-bromoacetophenone (8 mmol, 1.58 g); yield: 1.65 g, 66%, R_f = 0.58 in EtOAc/hexane, 3:7; yellow crystalline solid. mp: 166–170°C; ESI-MS (M^+): *calcd.*: 370.123, *observed:* 370.190. FTIR (cm^{-1}):1728 (C=O), 1643 (CH=CH), 853 (C–Cl), 588 (C–Br); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 7.54 (1H, d, H_α), 7.90 (1H, d, H_β), 7.43–8.32 (m, 9H, aromatic). Anal. Calcd: $C_{18}H_{11}ClBrNO$: C, 58.01; H, 2.96; N, 3.74. Found: C, 58.04; H, 4.98; N, 3.78.

3-(2-Chlroquinlin-3-yl)-1-(3-nitrophenyl) prop-2-en-1-one (3h)

Prepared by above method from **2** (8 mmol, 1.1 g) and 3-nitroacetophenone (8 mmol, 1.75 g); yield: 1.61 g, 60%, R_f = 0.54 in EtOAc/hexane, 4:6; pale yellow solid. mp: 145–150°C; ESI-MS (M^+): *calcd.*: 338.012, *observed:*338.966; FTIR (cm^{-1}): 1732 (C=O), 1645 (CH=CH), 853 (C–Cl), 1589(–NO_2); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 7.53 (1H, d, H_α), 8.51 (1H, d, H_β), 7.23–8.10 (m, 9H, aromatic). Anal. Calcd: $C_{18}H_{11}ClN_2O_3$: C, 68.71; H, 3.26; N, 8.24. Found: C, 63.84; H, 3.28; N, 8.28.

3-(2-Chloroquinolin-3-yl)-1-(3,4,5-trimethoxyphenyl) prop-2-en-1-one (3i)

Prepared by above method from **2** (8 mmol, 1.1 g) and 3,4,5-trimethoxyacetophenone (8 mmol, 1.49 g); yield: 1.61 g, 67%, R_f = 0.48 in EtOAc/hexane, 3:7; yellow crystalline solid. mp: 174–175°C; ESI-MS (M^+): *calcd.*: 383.132, *observed:* 383.401; FTIR (cm^{-1}): 1732 (C=O), 1645 (CH=CH), 853 (C–Cl); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 7.53 (1H, d, H_α), 8.57 (1H, d, H_β), 3.80 (s, 6H, CH_3), 3.64 (s, 3H, CH_3), 7.43-8.28 (m, 7H, aromatic). Anal. Calcd: $C_{21}H_{18}ClNO_4$: C, 65.71; H, 4.71; N, 3.64. Found: C, 65.84; H, 4.78; N, 3.68.

3-(2-Chloroquinolin-3-yl)-1-(5-bromo-2-methoxyphenyl) prop-2-en-1-one (3j)

Prepared by above method from **2** (8 mmol, 1.1 g) and 2-methoxy-5-bromoacetophenone (8 mmol, 1.2 g); yield: 1.31 g, 61%, R_f = 0.63 in EtOAc/hexane, 4:6; yellow solid. mp: 160–168°C; ESI-MS (M^+):, *calcd.*: 400.850, *observed:* 400.991; FTIR (cm^{-1}): 1736 (C=O), 1645 (CH=CH), 853 (C–Cl), 588 (C–Br); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm 7.53 (1H, d, H_α), 8.57 (1H, d, H_β), 3.73 (s, 3H, CH_3), 7.40–8.31 (m, 7H, aromatic). Anal. Calcd: $C_{20}H_{14}ClBrNO_2$: C, 56.61; H, 3.21; N, 3.44. Found: C, 56.64; H, 3.25; N, 3.46.

General method for synthesis of 6-(2-chloroquinolin-3-yl)-4 substituted phenyl-6H-1,3-oxazin-2-amine (4a–4j)

To the well-stirred solution of (4 mmol) appropriate chalcones (**3a**–**3j**) in 10 ml of ethanol, 4 mmol (0.55 g) of urea (**5**) and catalytic amount of NaOH was added and the reaction mixture was refluxed for 8–14 h. Conversion was monitored for every 60-min interval on pre-coated silica TLC plates by using mixture of acetone and petroleum ether (40:60 V/V) as mobile phase. The reaction mixture was cooled and poured into ice-cold water with excess stirring and kept in refrigerator for 1 h. The precipitate was filtered, washed five times with distilled water, and recrystallized with methanol. The purity of the products was checked on TLC by using mixture of ethyl acetate and hexane as mobile phase, which have shown single spot of the products in each case. The R_f values are given along with the spectral data below.

6-(2-chloroquinilin-3-yl)-4-phenyl-6H-1,3-oxazin-2-amine (4a)

Prepared by above method from **4a** (4 mmol, 1.17 g) and **5** (4 mmol, 0.55 g) after 12 h reflux; yield: 1.61 g, 98%; brown amorphous solid. mp: 148–150°C; R_f = 0.48 in EtOAc/hexane, 3:7; ESI-MS (M^+): *calcd.*:335.062, *observed*: 335.080; IR (KBr, cm^{-1}): 3412 (–NH_2), 1394 (Het–C–O), 1570 (C=C, ring skeleton phenyl and heterocyclic

moiety), 1415 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str, ring skeleton oxazine moiety), 853 (C–Cl, heterocyclic moiety); ^{1}H-NMR(DMSO-*d6*, δ): 2.31 (2H, s, –NH_2), 5.21 (1H, d, 6-H), 6.31 (1H, d, H), 7.21-8.08 (m, 10H, aromatic). Anal. Calcd: $C_{19}H_{14}ClN_3O$: C, 67.94; H, 4.21; N, 12.44. Found: C, 67.98; H, 4.25; N, 12.46.

6-(2-Chloroquinolin-3-yl)-4-(2,4-dichlorophenyl)-6H-1,3-oxazin-2-amine (4b)

Prepared by above method from **4b** (4 mmol, 0.89 g) and **5** (4 mmol, 0.55 g) after 10 h reflux; yield: 1.34 g, 98%; brown amorphous solid. mp: 145–150°C; *R*f = 0.56 in EtOAc/hexane, 3:7; ESI-MS (M^+): *caltd.*:403.139, *observed*: 403.312; IR (KBr, cm^{-1}): 3400 (–NH_2), 1396 (Ar–C–O), 1572 (C=C, ring skeleton phenyl and heterocyclic moiety), 1415 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str, ring skeleton oxazine moiety),858 (C–Cl, heterocyclic moiety); ^{1}H-NMR (DMSO-d_6, δ): 2.32 (2H, s, –NH_2), 5.19 (1H, d, 6-H), 6.21 (1H, d, 4H), 7.10–8.08 (m, 8H, aromatic). Anal. Calcd: $C_{19}H_{12}Cl_3N_3O$: C, 56.34; H, 2.92; N, 10.43. Found: C, 56.38; H, 2.99; N, 10.46.

6-(2-Chloroquinolin-3-yl)-4-(3,4-dichlorophenyl)-6H-1,3-oxazin-2-amine (4c)

Prepared by above method from **4c** (4 mmol, 0.95 g) and **5** (4 mmol, 0.55 g) after 10-h reflux; yield: 1.38 g, 97%; brown amorphous solid.mp: 156–158 °C; R_f = 0.58 in EtOAc/hexane, 3:7; ESI-MS (M^+):, *calcd.*: 403.155, *observed*: 403.075; IR (KBr, cm^{-1}): 3398 (–NH_2), 1396 (Ar–C–O), 1572 (C=C, ring skeleton Ar moiety), 1415 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str),858 (C–Cl); ^{1}H-NMR (DMSO-d_6, δ): 2.32 (2H, s, –NH_2), 5.19 (1H, d, 6-H), 6.21 (1H, d, 4-H), 7.12-8.08 (m, 8H, aromatic). Anal. Calcd.: $C_{19}H_{12}Cl_3N_3O$: C, 56.36; H, 2.92; N, 10.46.Found: C, 56.41; H, 2.99; N, 10.48.

6-(2-chloroquinolin-3-yl)-4-p-tolyl-6H-1,3-oxazin-2-amine(4d)

Prepared by above method from **4d** (4 mmol, 0.95 g) and **5** (4 mmol, 0.55 g) after 14-h reflux; yield: 1.38 g, 97%; dark yellow crystalline solid.mp: 160–175°C; R_f = 0.52 in EtOAc/hexane, 3:7; ESI-MS (M^+):, *calcd.*: 349.155, *observed*: 349.075; IR (KBr, cm^{-1}): 3406 (–NH_2), 1393 (Ar–C–O), 1569 (C=C, ring skeleton Ar moiety), 1418 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str), 858 (C–Cl); ^{1}H-NMR (DMSO-*d6*, δ): 2.34 (3H, s, CH_3), 2.32 (2H, s, -NH_2), 5.16 (1H, d, 6-H), 6.23 (1H, d, 4-H), 7.02-8.08 (m, 9H, aromatic).Anal.Calcd: $C_{20}H_{16}ClN_3O$: C, 68.58; H, 4.60; N, 12.02. Found: C, 68.61; H, 4.58; N, 12.01.

6-(2-chloroquinolin-3-yl)-4-o-tolyl-6H-1,3-oxazin-2-amine (4e)

Prepared by above method from **4e** (4 mmol, 1.19 g) and **5** (4 mmol, 0.55 g) after 14-h reflux; yield: 1.57 g, 95%;%; light brown crystalline solid. mp: 173–178°C; R_f = 0.58 in EtOAc/hexane, 3:7; ESI-MS (M^+):, *calcd.*: 349.055, *observed*: 349.075; IR (KBr, cm^{-1}): 3403 (–NH_2), 1393 (Ar–C–O), 1569 (C=C, ring skeleton Ar moiety), 1418 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str),858 (C–Cl); ^{1}H NMR(DMSO-d_6, δ): 2.34 (3H, s, CH_3), 2.32 (2H, s, -NH_2), 5.16 (1H, d, 6-H), 6.23 (1H, d, 4-H), 7.02–8.08 (m, 9H, aromatic). Anal. Calcd.: $C_{20}H_{16}ClN_3O$: C, 68.58; H, 4.60; N, 12.02. Found: C, 68.61; H, 4.58; N, 12.01.

6-(2-chloroquinolin-3-yl)-4-(4-bromophenyl)-6H-1,3-oxazin-2-amine (4f)

Prepared by above method from **4f** (4 mmol, 1.20 g) and **5** (4 mmol, 0.55 g) after 10-h reflux; yield: 1.66 g, 98%; brown solid. mp: 158–160°C. R_f = 0.61 in EtOAc/hexane, 4:6; ESI-MS (M^+):, *calcd.*: 412.945, *observed*: 412.992; IR (KBr, cm^{-1}): 3410 (–NH_2), 1393 (Ar–C–O), 1569 (C=C, ring skeleton Ar moiety), 1418 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str), 858 (C–Cl), 588 (C–Br); ^{1}H-NMR(DMSO-d_6, δ): 2.02 (2H, s, –NH_2), 5.19 (1H, d, 6-H), 6.32 (1H, d, 4-H), 7.19-8.08 (m, 9H, aromatic). Anal. Calcd.: $C_{19}H_{13}ClBrN_3O$: C, 55.08; H, 3.16; N, 10.12. Found: C, 55.16; H, 3.18; N, 10.18.

6-(2-Chloroquinolin-3-yl)-4-(2-bromophenyl)-6H-1,3-oxazin-2-amine (4g)

Prepared by above method from **4g** (4 mmol, 1.25 g) and **5** (4 mmol, 0.54 g) after 10-h reflux; yield: 1.68 g, 97%; dark brown solid. mp: 184–186°C; R_f = 0.68 in EtOAc/hexane, 3:7; ESI-MS (M^+):, *calcd.*: 412.935, *observed*: 412.982; IR (KBr, cm^{-1}): 3418 (–NH_2), 1393 (Ar–C–O), 1569 (C=C, ring skeleton Ar moiety), 1418 (C=C, ring skeleton oxazine moiety), 1246 (C–N, str), 858 (C–Cl), 588 (C-Br); ^{1}H-NMR (DMSO-d_6, δ): 2.02 (2H, s, –NH_2), 5.19 (1H, d, 6-H), 6.32 (1H, d, 4-H), 7.19–8.08 (m, 9H, aromatic). Anal. Calcd: $C_{19}H_{13}ClBrN_3O$: C, 55.03; H, 3.16; N, 10.13. Found: C, 55.16; H, 3.18; N, 10.18.

6-(2-chloroquinolin-3-yl)-4-(3-nitrophenyl)-6H-1,3-oxazin-2-amine (4h)

Prepared by above method from **4h** (4 mmol, 1.32 g) and **5** (4 mmol, 0.54 g) after 16-h reflux. Yield: 1.73 g, 96%; reddish yellow solid. mp: 168–172°C; R_f = 0.58 in EtOAc/hexane, 4:6; ESI-MS (M^+):, *calcd.*: 380.075, *observed*: 380.098; IR (KBr, cm^{-1}): 3407 (–NH_2), 1398 (Ar–C–O), 1565 (C=C, ring skeleton Ar moiety), 1416 (C=C, ring skeleton oxazine moiety), 1256 (C–N, str), 858 (C–Cl), 1589 (–NO_2); ^{1}H-NMR (DMSO-d_6, δ): 2.22 (2H, s, –NH_2), 5.11 (1H, d, 6-H), 6.52 (1H, d, 4-H), 7.47–8.28 (m, 9H, aromatic). Anal. Calcd: $C_{19}H_{13}ClBrN_4O_3$: C, 59.90; H, 3.41; N, 14.73. Found: C, 59.96; H, 3.48; N, 14.78.

6-(2-Chloroquinolin-3-yl)-4-(3,4,5-trimethoxyphenyl)-6H-1,3-oxazin-2-amine (4i)

Prepared by above method from **4i** (4 mmol, 1.21 g) and **5** (4 mmol, 0.55 g) after 8-h reflux; yield: 1.65 g, 98%; brown amorphous solid. mp: 184–186°C; R_f = 0.48 in EtOAc/hexane, 3:7; ESI-MS (M^+):, *calcd.*: 425.175, *observed*: 425.198; IR (KBr, cm^{-1}): 3397 (–NH_2), 1398 (Ar–C–O), 1565 (C=C, ring skeleton Ar moiety), 1416 (C=C, ring skeleton oxazine moiety), 1256 (C–N, str), 858 (C–Cl); ^{1}H-NMR (DMSO-d_6, δ): 2.22 (2H, s, –NH_2), 5.11 (1H, d, 6-H), 6.52

(1H, d, 4-H), 3.73 (s, 6H, CH_3), 3.64 (s, 3H, CH_3), 6.47–8.28 (m, 7H, aromatic). Anal. Calcd: $C_{22}H_{20}ClN_3O_4$: C, 62.03; H, 4.71; N, 9.73.Found: C, 62.09; H, 4.78; N, 9.87.

6-(2-Chloroquinolin-3-yl)-4-(5-bromo-2-methoxyphenyl)-6H-1,3-oxazin-2-amine (4j)

Prepared by above method from **4j** (4 mmol, 1.21 g) and **5** (4 mmol, 0.55 g) after 8-h reflux; yield: 1.65g, 98%; brown crystalline solid. mp: 174–180°C; R_f = 0.57 in EtOAc/hexane, 3:7; ESI-MS (M^+):, *calcd.*: 443.717, *observed*: 443.798; IR (KBr, cm^{-1}): 3412 (–NH_2), 1398 (Ar–C–O), 1565 (C=C, ring skeleton Ar moiety), 1416 (C=C, ring skeleton oxazine moiety), 1256 (C-N, str), 858 (C–Cl), 588 (C–Br); ^{1}H-NMR(DMSO-d_6, δ): 2.12 (2H, s, –NH_2), 5.19 (1H, d, 6-H), 6.12 (1H, d, 4-H), 3.73 (s, 3H, CH_3), 6.61-8.08 (m, 8H, aromatic). Anal. Calcd: $C_{20}H_{15}ClBrN_3O_2$: C, 54.03; H, 3.39; N, 9.43. Found: C, 54.09; H, 3.42; N, 9.84.

Results and discussion

In vitro antimalarial activity

The *In vitro* antimalarial activity (Table 1) of **4a–c** and **4f, 4g, 4i** were found comparable with CQ diphosphate (IC_{50} = 0.021 μM) against CQ sensitive strain (MRC-02) of *P. falciparum.* Compounds **4b, 4c, 4e, 4d, 4g,** and **4i** of this series have shown better activity than CQ diphospahte (IC_{50} = 0.177 μM) against CQ-resistant strain (RKL9) of parasite. Compound **4i** was found to be most potent against resistant (IC_{50} = 0.0435 μM) as well as sensitive (IC_{50} = 0.0265 μM) strains of *P. falciparum*. For compound **4i**, R_2, R_3, and R_4 are methoxy groups. Interestingly, compound **4a**, for which R_1 to R_5 are all hydrogen, also showed antiplasmodial efficacy close to **4i** against CQ-sensitive strain (IC_{50} = 0.0302μM). However, against resistant strain, compound **4a** (IC_{50}= 0.1305 μM) was found to be less active than **4i** (IC_{50} = 0.0435μM). **4b**, **4c**, **4g,** and **4f** are the four halogen containing compounds showed very good activity against both CQ-sensitive and -resistant strains. Methyl group in place of R_1 (**4e**) and R_3 (**4d**) also delivered good activities. **4j** is the compound containing two electron withdrawing groups each but showed moderate to poor *in vitro* antiplasmodial activity.

In vitro cytotoxicity

The maximum concentration of DMSO in any cell was 0.1% and did not affect cell growth. To compare all compounds, we have used the 50% inhibitory concentration IC_{50} and selectivity index (SI), which is defined as the ratio of cytotoxicity over antimalarial activity, each one expressed with IC_{50}. Table 1 summarizes IC_{50} and SI of all the compounds. For all the compounds, the SI values were >1, indicating better selectivity against *P. falciparum* even though a long cell drug exposure time (72 h) was given for cytotoxicity test. SI of two most potent antimalarial compounds **4g** and **4i** were 59.56 and 19.48, respectively, for resistant strain of *P. falciparum*. In case of sensitive strain, those values were 140.14 and 31.24, respectively. The most potent substituted oxazine **4i** was found to be most toxic and second best active **4g** was found to be least toxic among the 10 compounds under study. SI of **4g** was found best for both sensitive and resistant strains of malaria parasite.

In vivo antimalarial activity

The three most active compounds (**4b**, **4g,** and **4i**) found in the *in vitro* antiplasmodial assays were tested *in vivo* activity in Swiss mice against *P. yoelli* (N-67 strain) intraperitoneally (Table 2). Compound **4b** showed 69.93% average suppression at 50 mg/kg/day in comparison with 100% suppression by CQ at 8 mg/kg/day. Similarly, for compounds **4g** and **4i** *in vivo* antimalarial activity followed the same trend and showed a correlation. The MST was found in accordance with inhibition data.

Correlation of antiplasmodial activities with β-hematin formation inhibition

To ascertain the mode of action, vacuolar accumulation ratio (VAR) of all the compounds were theoretically

Table 1. Cytoxicity, *in vitro* antimalarial activity of 6-(2-chloroquinolin-3-yl)-4 substitutedphenyl-6H-1,3-oxazin-2-amine.

Compound	Cytotoxicity[1] (μM)	Activity V/s RKL9 strain[2] (μM)	Selectivity index (S.I)	Activity V/s MRC-02 strain[3] (μM)	Selectivity index (SI)
4a	1.875	0.1305 ± 0.031	14.36	0.0302 ± 0.006	61.67
4b	1.250	0.0680 ± 0.019	18.38	0.0375 ± 0.005	33.33
4c	1.786	0.1213 ± 0.002	13.27	0.0302 ± 0.004	59.47
4d	2.187	0.0887 ± 0.005	24.65	0.0555 ± 0.016	39.40
4e	1.812	0.1019 ± 0.002	17.78	0.6625 ± 0.022	28.99
4f	3.062	0.0786 ± 0.003	38.95	0.0260 ± 0.011	117.76
4g	3.812	0.0640 ± 0.003	59.56	0.0272 ± 0.005	140.14
4h	3.750	0.4235 ± 0.041	8.85	0.1065 ± 0.056	35.21
4i	0.828	0.0435 ± 0.005	19.48	0.0265 ± 0.005	31.24
4j	2.660	0.2420 ± 0.089	10.99	0.0495 ± 0.011	53.73
CQ	63.238	0.1770 ± 0.003	373.18	0.0218 ± 0.004	3122.95

[1]Cytoxicity (maximum nontoxic dose) against HeLa cells.
[2]Antiplasmodial activity against chloroquine resistant (RKL9) strain of *P. falciparum*, IC_{50}, μM ± SD, result of two separate determinations.
[3]Antiplasmodial activity against chloroquine (MRC-02) strain of *P. falciparum*, IC_{50}, μM ± SD, result of two separate determinations.

Table 2. *In vivo* antimalarial activity against Chloroquine resistant N-67 strain of *P. yoelli* in Swiss mice.

Compound	% Suppression on day 4[1]	Mean survival time (MST in days) ± SD[1]	% Suppression on day 4[2]	Mean survival time (MST in days) ± SD[2]	Rt (min)[3]	Purity[3]
4b	69.93	13.24 ± 1.19	60.58	11.56 ± 1.06	12.78	98.76
4g	48.52	10.33 ± 1.98	34.98	9.08 ± 1.07	11.64	98.18
4i	44.72	11.03 ± 0.78	32.78	9.00 ± 1.55	11.87	9.87
CQ	100*	All alive	—	—	—	—
Control	0**	6.44 ± 0.98	—	—	—	—

[1]At 50 mg/kg/day, *a* Percent suppression = $[(C - T)/C] - 100$; where C = parasitaemia in control group and T = parasitaemia in treated group. MST calculated for the mice that died during the 28 day observation period, and the mice that survived beyond 28 days are excluded.
[2]At 25 mg/kg/day.
[3]HPLC.
*At 8 mg/kg/day.
**Without drug.

calculated from experimentally determined p*K*a values (Table 3) using equation 1.[32]

$$VAR = \frac{Q_i}{Q_e} = \frac{\left\{1 + \frac{[H^+]_i}{Ka_2} + \frac{[H+]_i^2}{Ka_1 Ka_2}\right\}}{1 + \frac{[H^+]_e}{Ka_2} + \frac{[H+]_e^2}{Ka_1 Ka_2}} \quad (1)$$

The two p*K*a values of CQ diphosphate were determined experimentally and were found close to the previously reported values.[32] In equation 1, VAR represents the ratio of drug inside and outside of the parasite food vacuole. pH_i = pH inside food vacuole (assumed to be pH 5.3) and pH_e = pH externally (assumed to be pH 7.2). The calculated VAR and the VAR relative to **4a** (α) are shown in Table 3. Compound **4a** was taken as reference molecule to calculate α because this molecule was not substituted at B ring. VAR values for the compounds of this series are calculated in the range of 603 (**4h**) to 2457 (**4g**). For CQ diphosphate, the value was 5890. A significant difference between VAR values were observed between **4g** (VAR = 2457) and **4f** (VAR = 1545) in which bromine is present at ortho and meta positions, respectively. In case of **4j** (VAR = 1551) bromine is present at Meta position and its VAR value was found closer to **4f**. The relative VAR (α) values for all the compounds were calculated in the range 0.48–1.95 and that for CQ diphospahte was 4.69. Due to higher p*K*a values, CQ diphosphate achieved 2.5–9 times better accumulation in the parasite food vacuole in comparison with the substituted oxazine **4(a–j)**. Normalized IC_{50} values ($IC_{50} \times \alpha$) were obtained by multiplying experimentally obtained antimalarial IC_{50} with α. Normalized IC_{50} values are the theoretically expected IC_{50}values, if the compounds accumulate in the vacuole to the same extent as **4a**. Having adjusted for the differential accumulation of the compounds, the normalized antiplasmodial IC_{50} values and the inhibition of β-hematin formation ($BHIA_{50}$) should show a clear correlation. Hawley et al.[33] and Kaschula et al.[32] reported direct proportionality between normalized antiplasmodial activity and inhibition of β-hematin formation of quinoline antimalarials using chloroquine-sensitive strains of *P. falciparum*. In this study, we have taken both CQ-sensitive (MRC-02) and CQ-resistant (RKL9) strains of *P. falciparum*. The quantities of the compounds required to inhibit the formation of β-hematin by 50% ($BHIA_{50}$) were found in the range 1.53 (**4b**) to 2.43 μM (**4e**). Unsubstituted **4a** showed comparable β-hematin polymerization inhibition activity (1.56 μM). Exceptionally, compound **4e** containing a methyl group showed better $BHIA_{50}$ than **4i** (1.72 μM), which contains three methoxy groups. In case of **4g** and **4f**, unlike VAR, the position of bromine did not make any significant difference between their $BHIA_{50}$ values. CQ diphosphate showed a comparable value of 1.87 μM. Although chloroquine showed lower β-hematin inhibition activity, due to better VAR value it was found to be more potent than **4i** against chloroquine sensitive *P. falciparum*. The normalized IC_{50} ($IC_{50} \times \alpha$) values for all the compounds including CQ diphosphate were calculated for both sensitive and resistant strains. A plot of log ($IC_{50} \times \alpha$) against log $BHIA_{50}$ (Figure 2) showed some correlation present between the normalized IC_{50} and the ability of the compounds to inhibit β-hematin formation. The *r* values were found decreased to 0.51 and 0.49 for sensitive and resistant strains respectively when chloroquine was included. This indicates that chloroquine and substituted oxazines **4(a–j)** have similar antimalarial mode of action. These findings further strengthened the hypothesis that haem polymerization inhibition may be a possible mode of action for this series of compounds **4(a–j)**.

Conclusions

We have reported the synthesis and evaluation of substituted oxazines as potential antimalarial agents. The molecules were found active against *in vitro* culture of both CQ-sensitive and CQ-resistant strains of *P. falciparum*. Compound **4i** was found to be most active as well as most cytotoxic. The second best active compound **4g** was found to be least cytotoxic among the 10 compounds. Three compounds (**4b**, **4g**, and **4i**) were tested against *P. yoelli* (N-67 strain) in mouse model found active.

Table 3. Relationship between p*K*a, normalized antiplasmodial IC_{50} values, and inhibition of β-hematin formation for substituted oxazine and CQ diphosphate.

Compound	pKa_1, pKa_2	VAR[1]	α[2]	$IC_{50} \times \alpha$[3]	$IC_{50} \times \alpha$[4]	$BHIA_{50}$[5]
4a	6.78, 8.95	1254.89	1.00	0.1309	0.0302	1.56 ± 0.454
4b	6.66, 9.08	1001.23	0.79	0.0547	0.0297	1.53 ± 0.025
4c	6.36, 8.42	1225.67	0.98	0.1221	0.0298	1.28 ± 0.036
4d	6.80, 9.01	1303.19	1.03	0.0923	0.0579	1.57 ± 0.142
4e	7.08, 8.89	2049.08	1.63	0.1665	0.1023	2.43 ± 0.010
4f	6.90, 9.01	1545.75	1.23	0.0974	0.0323	2.06 ± 0.035
4g	7.20, 9.11	2457.65	1.95	0.1257	0.0532	1.83 ± 0.463
4h	6.38, 8.95	603.30	0.48	0.2059	0.0515	2.07 ± 0.630
4i	6.95, 8.98	1676.66	1.34	0.0570	0.0348	1.72 ± 0.002
4j	6.90, 9.10	1551.03	1.25	0.2993	0.0611	1.87 ± 0.002
CQ	8.49, 9.63	5890.15	4.69	0.8308	0.0986	1.87 ± 0.008

[1]Vacuolar accumulation ratio (VAR) calculated using equation 1 and assuming vaculor pH of 5.5 and external pH 7.4.
[2]Vaculor accumulation ratio relative to **4a**.
[3]Normalized antiplasmodial activity against CQ resistant (RKL9) strain of *P. falciparum*.
[4]Normalized antiplasmodial activity against CQ sensitive (MRC-02) strain of *P. falciparum*.
[5]Activity of β-hematin inhibitory activity, IC_{50} μM ± SD, results of two separate determination of CQ diphosphate.

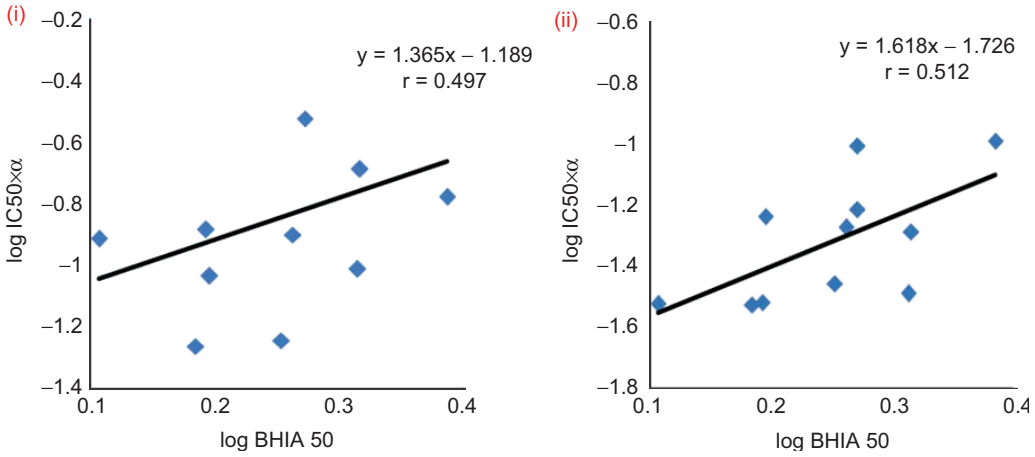


Figure 2. A Plot of the normalized antiplasmodial IC_{50} (IC_{50} X α) versus log of the activity against the inhibition of β-hematin formation. (i) Study involving chloroquine resistant (RKL9) strain with CQ diphosphate. (ii) Study involving chloroquine sensitive (MRC-02) strain with CQ diphosphate.

A good correlation was observed between normalized antimalarial activity ($IC_{50} \times \alpha$) and β-hematin formation inhibition ($BHIA_{50}$) against both sensitive and resistant strains. These observations indicated that the substituted oxazines are antimalarial and their mode of action may be through inhibition of haem detoxification process.

Acknowledgement

The authors thank Head of Department of Chemistry, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur (India), for providing necessary laboratory facilities and Director, SAIF Lucknow (India) for providing necessary spectral and biological data of compounds.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S. Antimalarial drug discovery: efficacy models for compound screening. Nat Rev Drug Discov 2004;3:509–520.
2. Wiesner J, Ortmann R, Jomaa H, Schlitzer M. New antimalarial drugs. Angew Chem Int Ed Engl 2003;42:5274–5293.
3. Winstaley PA, Parasitol. Today. 2000;16:136–153.
4. Bremen J, Trop A J. Med. Hyg. 2001;64:1–11.
5. Domínguez JN. Chemotherapeutic agents against malaria: what next after chloroquine? Curr Top Med Chem 2002;2:1173–1185.
6. White NJ. Drug resistance in malaria. Br Med Bull 1998;54:703–715.
7. Rosenthal PJ, Miller LH, In Antimalarial Chemotherapy: Mechanisms of Action, Resistance, and New Directions in Drug Discovery; P.J., Rosenthal, Ed, Humana Press Inc.: Totowa, NJ, 2001: pp 3–13.
8. Domı´nguez JN, Lo´pez S, Charris J, Iarruso L, Lobo G, Semenov A, Olson JE, Rosenthal PJ. J. Med. Chem. 1997;40:2726–2732.
9. Chen M, Theander TG, Christensen SB, Hviid L, Zhai L, Kharazmi A. Licochalcone A, a new antimalarial agent, inhibits *in vitro* growth of the human malaria parasite Plasmodium falciparum and protects mice from P. yoelii infection. Antimicrob Agents Chemother 1994;38:1470–1475.

10. Chen M, Brøgger Christensen S, Zhai L, Rasmussen MH, Theander TG, Frøkjaer S et al. The novel oxygenated chalcone, 2,4-dimethoxy-4'-butoxychalcone, exhibits potent activity against human malaria parasite Plasmodium falciparum *in vitro* and rodent parasites Plasmodium berghei and Plasmodium yoelii in vivo. J Infect Dis 1997;176:1327–1333.
11. Li R, Kenyon GL, Cohen FE, Chen X, Gong B, Dominguez JN et al. *In vitro* antimalarial activity of chalcones and their derivatives. J Med Chem 1995;38:5031–5037.
12. Liu M, Wilairat P, Croft SL, Tan AL, Go ML. Structure-activity relationships of antileishmanial and antimalarial chalcones. Bioorg Med Chem 2003;11:2729–2738.
13. Domı´nguez JN, Charris JE, Lobo G, Gamboa-Domı´nguez N, Moreno MM, Riggione F, Sa´nchez E, Olson J, Rosenthal PJ. Eur. J. Med. Chem. 2001; 36: 555–560.
14. Vibhute YB and Basser MA. Ind.J. of Chem. 2003;42B:202–205.
15. Bhat BA, Dhar KL, Puri SC, Saxena AK, Shanmugavel M, Qazi GN. Synthesis and biological evaluation of chalcones and their derived pyrazoles as potential cytotoxic agents. Bioorg Med Chem Lett 2005;15:3177–3180.
16. Edwards ML, Stemerick DM, Sunkara PS. Chalcones: a new class of antimitotic agents. J Med Chem 1990;33:1948–1954.
17. Kalirajan R, Palanivelu M, Rajamanickam V, Vinothapooshan G, Anandarajagopal K. Int. J. of Chem. Sci. 2007;5:73–80.
18. Udupi RH, Bhat R, Kumar K. Indian J. Het.Chem. 1998;8:143–146.
19. Pande A and Saxena VK. Ind.J.of Chem. 1987;26B:390–392.
20. Gupta U, Sareen V, Khatri V, Chugh S. Indian J. Het.Chem. 2005;14:265–266.
21. Pandey VK, Gupta VD, Tiwari DN. Indian J. of Het.Chem. 2004;13:399–400.
22. Mishra RM and Wahab A. Indian J. Het.Chem. 2003;13:29–32.
23. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976;193:673–675.
24. Lambros C, Vanderberg JP. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol 1979;65:418–420.
25. Peters W, Portus JH, Robinson BL, Ann. Trop. Med. Parasitol. 1975;69:155–171.
26. Borenfreund E, Puerner JA. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. Toxicol Lett 1985;24:119–124.
27. Albert A, Serjeant EP, The Determination of Ionization Constants. Chapman and Hall Ltd: London 1971: pp 94.
28. Basilico N, Pagani E, Monti D, Olliaro P, Taramelli D. A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs. J Antimicrob Chemother 1998;42:55–60.
29. Baelmans R, Deharo E, Bourdy G, Muñoz V, Quenevo C, Sauvain M et al. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach. Part IV. Is a new haem polymerisation inhibition test pertinent for the detection of antimalarial natural products? J Ethnopharmacol 2000;73: 271–275.
30. Meth-Cohn O, Narine B, Tarnowski B. J chem. Soc., Perkin Trans. 1981;1:1520.
31. Ali MM, Tasneem, Rajanna KC, Sai Prakash PK. Synlett. 2001;2:251.
32. Kaschula CH, Egan TJ, Hunter R, Basilico N, Parapini S, Taramelli D et al. Structure-activity relationships in 4-aminoquinoline antiplasmodials. The role of the group at the 7-position. J Med Chem 2002;45:3531–3539.
33. Hawley SR, Bray PG, Mungthin M, Atkinson JD, O'Neill PM, Ward SA. Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in Plasmodium falciparum in vitro. Antimicrob Agents Chemother 1998;42: 682–686.