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Antiplasmodial activity of novel keto-enamine chalcone-chloroquine based hybrid pharmacophores ☆

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ABSTRACT

A series of novel keto-enamine chalcone-chloroquine based hybrids were synthesized following new methodology developed in our laboratory. The synthesized compounds were screened against chloroquine sensitive strain (3D7) of *Plasmodium falciparum* in an in vitro model. Some of the compounds were showing comparable antimalarial activity at par with chloroquine. Compounds with significant in vitro antimalarial activity were then evaluated for their in vivo efficacy in Swiss mice against *Plasmodium yoelii* (chloroquine resistant N-67 strain), wherein compounds **25** and **27** each showed an in vivo suppression of 99.9% parasitaemia on day 4. Biochemical studies reveal that inhibition of hemozoin formation is the primary mechanism of action of these analogues.

1. Introduction

The problem of widespread malaria continues unabated worldwide, as the disease is present in over 100 countries and threatens half of the world's population. Even with decades of research and the victorious progress of combination therapy, malaria remains one of the most serious health problem worldwide, especially in developing countries where it has huge economic and social costs.[1] The resistance of *Plasmodium falciparum* to chloroquine and other antimalarial drugs and the adverse effects of some of the available antimalarial drugs have created an urgent need for new drugs that are safe and effective for the prophylaxis and treatment of malaria.[2,3]

Chloroquine (CQ) and other quinoline antimalarials have been mainstays of antimalarial chemotherapy for more than past 40 years. The achievement of these drugs was based on excellent clinical efficacy, limited host toxicity, ease of use, and simple, cost effective synthesis.[4] Figure 1 shows chemical structure of some clinically useful quinoline based antimalarial agents. Although chloroquine is most widely used clinical agent, but overcoming drug resistance[5] has helped fuel a strong increase in occurrence and consequences of malaria internationally.[6] The biochemical studies on CQ suggested that accumulation of the chemotherapeutic agents in the parasite vacuole is critical for their antimalarial activity.[7] CQ resistance had largely been recognized to mutations in the CQ resistance transporter (*pfcrt*) gene that encodes for the protein believed to mediate efflux of the drug from the digestive vacuole of the parasite, leading to sub-optimal drug concentrations.[8]

To overcome the challenges of multi-drug resistance in *P. falciparum,* many approaches currently being adopted contain optimisation of treatment with available drugs including combination therapy, developing analogues of the existing drugs and evaluation of drug resistance reversers (chemo sensitizers) as well as exploring new chemotherapeutic targets.[9] Among these approaches, the medicinal chemistry hybridization strategy, involves the rational design of new chemical entities by the fusion (usually via a covalent linker) of two drugs, both active compounds and/or pharmacophoric units recognized and derived from known bioactive molecules. In the context of attempting to circumvent antimalarial drug resistance, hybridization is quite an attractive strategy, particularly when the pharmacophores being merged possess independent modes of antimalarial action. Thus the hybrid molecules are chemical entities with two or more structural domains having different biological functions and dual activity.[10] Despite the worldwide distribution of resistance of *Plasmodium falciparum* to chloroquine (CQ), the 4-aminoquinoline analogues continue to

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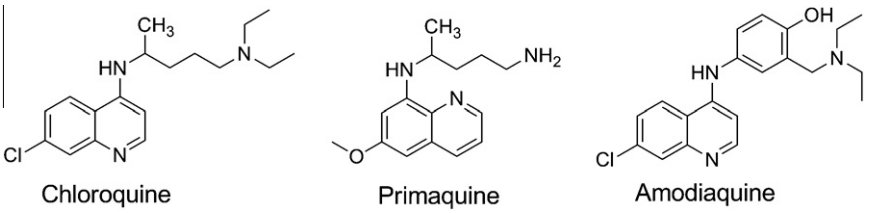


Figure 1. Chemical structures of some clinically useful antimalarials.

attract interest because the resistance seems to be compound specific and not related to changes in the structure of the drug target. Structure–activity relationship studies on CQ-Hematin binding reveals the importance of 7-chloro-4-aminoquinoline scaffold for activity.[11] Based on these facts several groups have reported hybrid molecules by coupling 7-chloro-4-aminoquinolines (lateral side chain modification) with different bioactive molecules like: peroxide based trioxaquine derivatives,[12] aminoquinoline–imipramine hybrid,[13] ferrocene–chloroquine analogs,[14] chloroquine-isatin derivatives,[15] chloroquine–astemizole hybrids,[16] etc.

On the other hand antimalarial activity of chalcones was first noted when licochalcone A was reported to exhibit potent antimalarial activity.[17] Subsequently, several synthetic chalcone derivatives were reported as alternatives in antimalarial chaemotherapy.[18] Recently, Chibale et al. have reported chalcone-chloroquinoline hybrid analogues wherein, these hybrids have shown significant antimalarial activity and biochemical studies reveal that inhibition of hemozoin formation is the primary mechanism of action of these analogues.[19] Based on these observations, and also considering that free heme within the food vacuole of the parasite is still an attractive target, we wanted to design and develop a unique class of antimalarials that interact with free heme on one hand and other part of the molecule inhibit cysteine protease, also this pharmacophore because of extended conjugation is also envisaged to reverse chloroquine resistance (act as chemosensitizer) in *Plasmodium falciparum*. We envisaged that this strategy would result in compounds that could overcome *Plasmodium falciparum* resistance to chloroquine (CQ) and offer a viable strategy for the discovery of new antimalarial therapies.[20]

Thus, we were interested in pursuing a bio inspired hybridization strategy that combines the core portions of the two structurally distinct moieties of CQ and ketoenamine chalcone, each of which possesses significant antiplasmodial activities, via an appropriate linker. It is also possible that hybrid molecules will possess superior bioavailability and/or different mode of action from that of individual drugs in combination. The structure of the designed novel prototype is shown in Figure 2. In this communication, we

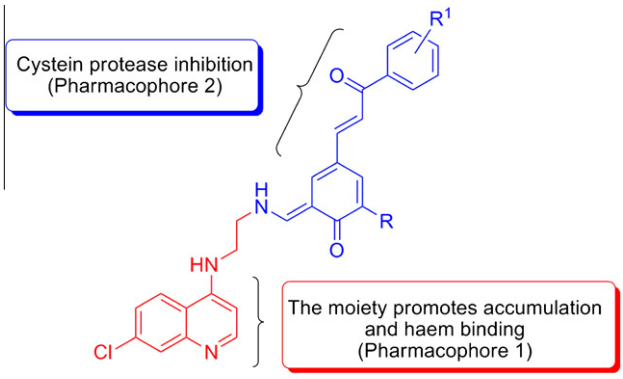


Figure 2. Designing of keto-enamine chalcone-chloroquine hybrids.

wish to report the synthesis and biological evaluation of novel keto-enamine chalcone-chloroquine hybrid analogues.

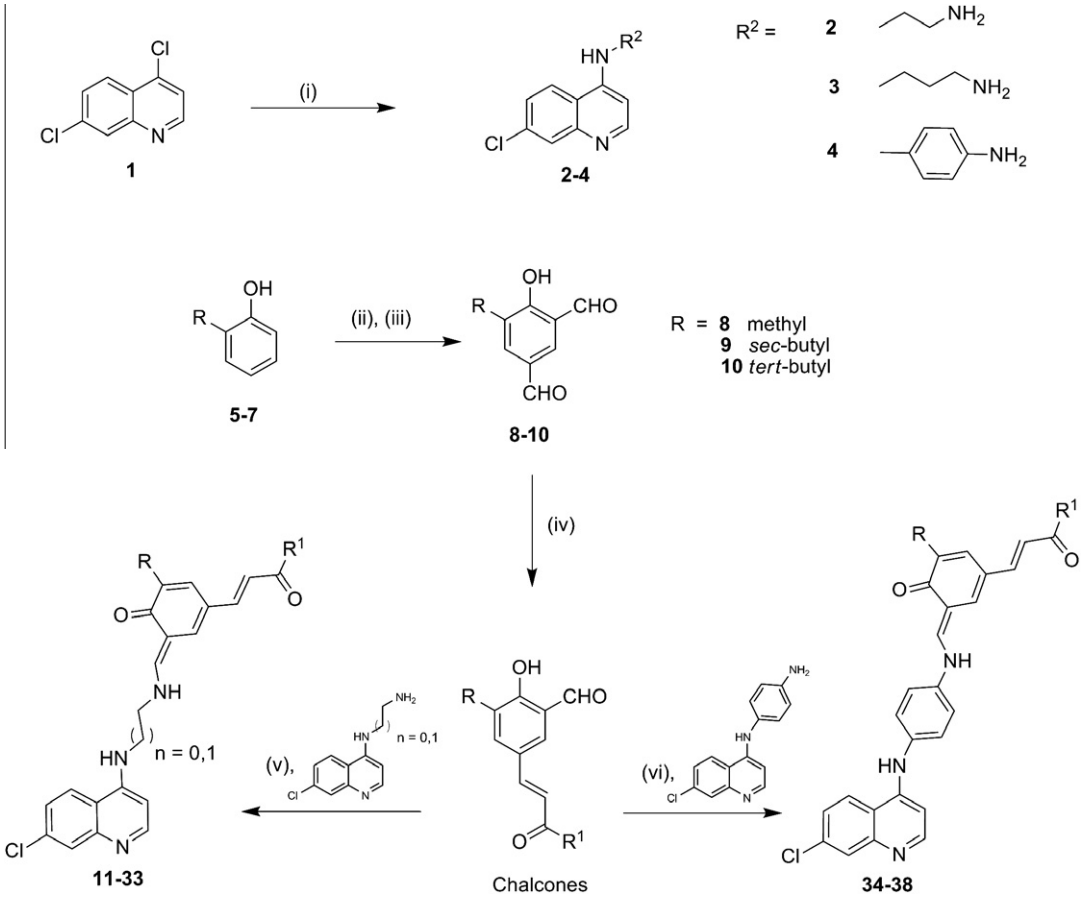
2. Synthesis

The synthesis of target and intermediate compounds were performed as outlined in Scheme 1. Synthesis of compounds **2–4** was achieved by the nucleophilic substitution at the 4th position of 4,7-dichloroquinoline (**1**) with aliphatic and aromatic amines. On the other hand regio selective chalcones were synthesized by utilizing our previously reported protocol.[21] The Duff reaction on *ortho* alkyl substituted phenols (**5–7**) in the presence of hexamethylenetetraamine (HMTA) and TFA at 120 °C gave aromatic dicarbaldehydes (**8–10**), which on coupling with appropriate acetophenones in refluxing dioxane, in the presence of a catalytic amount of concd HCl afforded regio selective *para*-condensed chalcones in excellent yields. Furthermore, these synthesized chalcones were condensed with 4-aminoquinolines (**2–4**) in presence of ethanol as a solvent to furnish final compounds (**11–38**) in which 4-aminoquinoline and chalcones were attached by a keto-enamine linker. The synthetic methodology has been perfected by us in which the reactions were regio selective and the products exists in the keto-enamine form.[22]

3. Results and discussion

All the synthesized hybrid compounds having chalcone and quinoline scaffolds in one frame (**11–38**) were evaluated for their antimalarial activity against CQ sensitive 3D7 strain of *P. falciparum* in vitro according to the procedure reported in the literature. The IC_{50} values are calculated from experiments carried out in triplicate. Some of the selected compounds, which have shown activity comparable to CQ, were also evaluated against the N-67 strain of *P. yoelli* in Swiss mice.

Among the 28 compounds tested, compounds **12** and **27** exhibited antiplasmodial activity comparable to chloroquine with IC_{50} 3.63 and 4.64 ng/mL, respectively. Additionally, 5 compounds (**11**, **18**, **23**, **24** and **25**) showed IC_{50} lower than 10 ng/mL. The difference in the IC_{50} values can be attributed to factors such as number of carbon atoms in the side chain, ring size, and the substitutions on the chalcone ring. The significant activity of the above mentioned compounds underscores the importance of the two carbon linker between the two pharmacophores, as the compounds having aromatic linker between quinoline and chalcones (**34–38**) showed drastic decrease in activity. While on the other hand the compounds with propyl linker (**28–33**) exhibited only mild inhibition in comparison to standard drug. The activity data (Table 1) suggest that two-carbon atoms in the side chain linker are appropriate for the antimalarial activity of compounds with five or six-membered chalcone rings (like **24** and **12**), as an increase in carbon chain length results in reduced activity. To elucidate the role of keto-enamine based chalcones in antimalarial activity we have synthesized compounds (**1b**–**1d**) without quinoline pharmacophore, which is outlined in Scheme 2. However, the synthesized compounds showed moderate in vitro activity (IC_{50} >100 ng/ml) which



Scheme 1. Synthesis of keto-enamine chalcone-chloroquine hybrids. Reagents and conditions: (i) diamines, 110 °C, neat or ethanol; (ii) hexamethylenetramine/TFA, 120 °C, 3 h; (iii) 10% H_2SO_4, 90–100 °C, 2 h; (iv) concd HCl, *p*-R_1COCH_3, dioxane, 80–90 °C, 1.0–1.5 h; (v) **2**/**3**, ethanol, rt, 10 min; (vi) **4**, ethanol, rt, 10 min.

underscores the role of the quinoline pharmacophore for antimalarial activity. The idea of introducing an aryl linker was not without reason, it was envisaged that since these molecules are fluorescent (due to extended conjugation) they would enhance the antimalarial activity due to their conjugation because many dyes with extended conjugation like xanthenes, azines, oxazines, thiazines, etc. are reported in literature for their antimalarial potential.[23] However, on evaluation these compounds (**34–38**) they were found to be less active than compounds with aliphatic linker. The reason for the higher IC_{50} values for these compounds is probably due to less accumulation in the parasite food vacuole by pH trappings for higher pK_a values. Furthermore, in vivo results strongly suggests that halogen atom at *para* position of the chalcone aryl ring improves the in vivo antimalarial activity as compound **25** with chloro and compound **27** with bromo group showed 99.9% parasite inhibition on day four while compound **14** with fluoro group and **16** with bromo group illustrated 99.5% and 99.78% inhibition. Introduction of heteroaryl group like furan and thiophene (compound **18**, **23** and **24**) also increased the in vivo activity.

Furthermore, all compounds were tested for their cytotoxicity against VERO cell line and they were devoid of cytotoxicity as these compounds have high SI values, particularly compounds **12** with a SI value of 27548.21 which was around threefold more as compared to chloroquine while rest of compounds showed SI values ranging between 300 and 3755.

Ten compounds with noteworthy activity in vitro were evaluated for in vivo efficacy in Swiss mice against *Plasmodium yoelii* (chloroquine resistant N-67 strain). Under these conditions, the survival time of untreated animals (control) was 12 days. Treated animals were orally dosed daily for 4 days (do to d3 post infection) at a dose of 100 mg/kg/d. On day 4, parasitaemia was below detectable levels. This result indicates a rapid parasite clearance. However, the number of cured mice at 30 days was 0/5. The mean survival time of these animals was around 18 days, with recrudescence between 17 and 21 days. The synthesized compounds showed significant activity against *P. yoelii* infections in mice, particularly the compounds **25** and **27** exhibited 99.9% suppression on day four compared to 99% suppression displayed by CQ (at 20 mg/kg/d dose), while compounds **11**, **17**, and **18** suppressed 99.78% parasitaemia (Table 3). Additionally compound **25** and **27** showed 97.0% and 96.7% suppression on day 6. These data show that the synthesized derivatives are highly active and able to clear parasitemia below detectable levels in mice orally at 100 mg/kg. However, a curative effect (no recrudescence, cured mice) was not obtained at the tested dose.

As the quinoline based antimalarials like chloroquine act through the inhibition of hemozoin formation, therefore inhibition of β-hematin formation was demonstrated in target hybrids. Most of the synthesized compounds showed β-hematin inhibitory activities (IC_{50} <3.5 μg/ml) better than the reference drug chloroquine (IC_{50} 3.65 μg/ml) (Table 2). Although most of the compounds illustrated good hemozoin inhibitory activities yet there was no steady correlation in comparison to their in vitro antimalarial potency. This difference may occur due to factors like degree of accumulation in parasite food vacuole which is structure and pH dependent. In spite of a major modification in the side chain, this series of

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

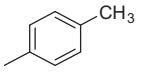
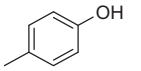
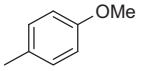
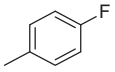
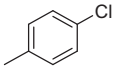
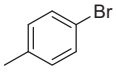
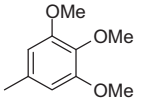
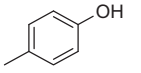
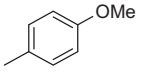
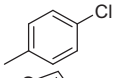
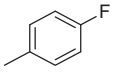
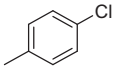
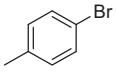
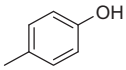
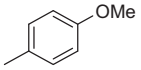
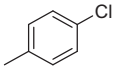
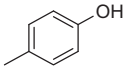
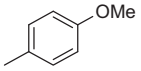
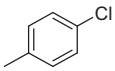
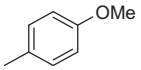
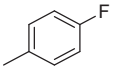
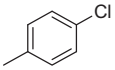
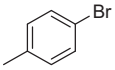
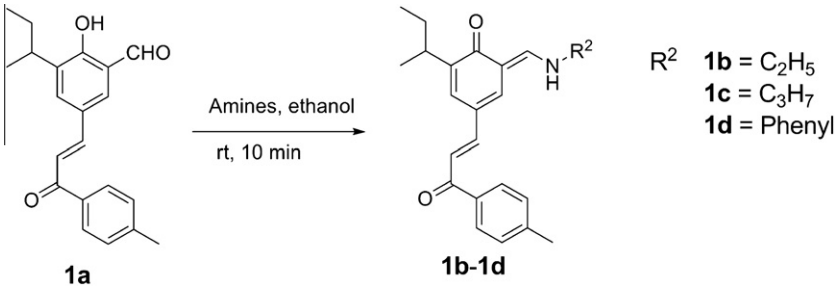
Compound	(Linker)	R	R^1	IC_{50} (ng/ml)	SI	Log*P*
11	1,2-Ethylenediamine	Methyl		9.75	1034.23	4.15
12	1,2-Ethylenediamine	Methyl		3.63	27548.21	3.28
13	1,2-Ethylenediamine	Methyl		16.67	1137.97	3.54
14	1,2-Ethylenediamine	Methyl		10.85	1034.23	3.82
15	1,2-Ethylenediamine	Methyl		22.21	3755.43	4.22
16	1,2-Ethylenediamine	Methyl		18.85	1114.59	4.49
17	1,2-Ethylenediamine	Methyl		10.68	1392.22	3.29
18	1,2-Ethylenediamine	Methyl		9.75	1414.59	3.65
19	1,2-Ethylenediamine	*sec* Butyl		13.63	498.54	4.83
20	1,2-Ethylenediamine	*sec* Butyl		22.03	1071.27	4.44
21	1,2-Ethylenediamine	*sec* Butyl		18.26	1058.05	4.70
22	1,2-Ethylenediamine	*sec* Butyl		17.91	1014.51	5.39
23	1,2-Ethylenediamine	*sec* butyl		7.09	1138.22	3.45
24	1,2-Ethylenediamine	*sec* Butyl		9.1	1373.63	4.81
25	1,2-Ethylenediamine	*tert* Butyl		7.19	1499.3	5.04
26	1,2-Ethylenediamine	*tert* Butyl		28.17	868.3	5.44
27	1,2-Ethylenediamine	*tert* Butyl		4.64	1883.62	5.71
28	1,3-Propanediamine	Methyl		35.12	505.98	3.38
29	1,3-Propanediamine	Methyl		38.76	295.66	3.68
30	1,3-Propanediamine	Methyl		42.86	513.99	4.33
31	1,3-Propanediamine	*sec* Butyl		42.44	486.57	4.55
32	1,3-Propanediamine	*sec* Butyl		41.84	549.12	4.81
33	1,3-Propanediamine	*sec* Butyl		41.93	491.77	5.49
34	1,4-Diaminobenzene	Methyl		200.55	315.33	5.38

Table 1 (*continued*)

Compound	(Linker)	R	R^1	IC_{50} (ng/ml)	SI	Log*P*
35	1,4-Diaminobenzene	Methyl		160.3	407.52	5.67
36	1,4-Diaminobenzene	*sec* Butyl		80.01	678.11	4.12
37	1,4-Diaminobenzene	*tert* Butyl		379.82	196.70	7.28
38	1,4-Diaminobenzene	*tert* Butyl		>100	ND	7.55
CQ				2.45 ± 1.08		

IC_{50}: Concentration corresponding to 50% growth inhibition of the parasite.
SI: IC_{50} values of cytotoxic activity/IC_{50} values of antimalarial activity.
Log*P*: Calculated by ChemDraw Ultra software.
ND: Not done.



Scheme 2. Synthesis of keto-enamine based chalcones.

compounds exhibits antimalarial activity by the same mode of action as that of CQ, namely, inhibition of hemozoin formation.

4. Conclusion

In conclusion, we have synthesized keto-enamine based quinoline-chalcone hybrids using an uncommon method established in our laboratory. Interestingly, this class of compounds exhibits acceptable selectivity against the malaria parasite and shows antimalarial activity in vivo against the MDR rodent malaria parasite *P. yoelii*. This study has identified new class of compounds showing good antimalarial activity in both in vitro and in vivo models. Furthermore, mechanistic studies reveal that said compound act through heme polymerization target. Further structural optimization of these analogues may lead to the development of the more potent molecules.

5. Experimental section

5.1. General procedure for the synthesis of compounds 2, 3 and 4

A mixture of 4,7-dichloroquinoline (1 equiv) and 1,2-diaminoethane/1,3-diaminopropane/*p*-phenylenediamine (1.5–2 equiv) in ethanol/neat were heated for 8 h with continued stirring to drive the reaction to completion. After completion of the reaction the excess solvent was removed under vacuum. The reaction mixture was then poured into ice cold water and precipitate was filtered and washed with ethyl acetate to furnish compound **2**, **3** and **4** in quantitative yields.

5.2. General procedure for the synthesis of compounds 8–10 and chalcones

Synthesis of dicarbaldehyde substrates and regioselective *para* condensed chalcones were achieved by our previously reported protocol.[21]

5.3. General procedure for the synthesis of compounds 11–38

A mixture of **2–4** (1.0 equiv) and appropriate *para* condensed chalcones (1 equiv) in ethanol were stirred for 10 min at room temperature. The solvent was evaporated under vacuum and the solid was purified directly with column chromatography to obtain the respective compounds **11–38** in excellent yields.

5.3.1. (*E*)-6-((2-(7-Chloroquinolin-4-ylamino) ethylamino) methylene)-2-methyl-4-((*E*)-3-oxo-3-p-tolylprop-1-enyl)cyclohexa-2,4-dienone (11)

Yellow solid, yield: 87%; mp 235–236 °C; IR (KBr): 3213, 2841, 1711, 1625, 1599, 1015 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.32 (s, 1H), 8.51 (s, 1H), 8.42 (d, J = 5.4 Hz, 1H), 8.25 (d, J = 9.1 Hz, 1H), 8.02 (d, J = 8.1 Hz, 2H), 7.85 (s, 1H), 7.79 (d, J = 2.0 Hz, 1H), 7.73–7.59 (m, 3H), 7.53–7.44 (m, 2H), 7.35 (d, J = 8.1 Hz, 2H), 6.63 (d, J = 5.4 Hz, 1H), 3.92 (t, J = 5.3 Hz, 2H), 3.66 (d, J = 5.5 Hz, 2H), 2.39 (s, 3H), 2.19 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 188.7, 167.2, 166.3, 152.4, 150.4, 149.5, 144.2, 143.7, 135.9, 134.0, 133.2, 129.7, 128.9, 128.0, 124.7, 124.5, 123.8, 118.7, 117.9, 117.1, 99.5, 54.9, 43.3, 21.6, 15.9; ESI-MS: (*m*/*z*): 484 $(M+H)^+$; HRMS *m*/*z* calcd for $C_{29}H_{26}ClN_3O_2$ $(M+H)^+$ 484.1793, found 484.1796.

Table 2
Inhibition of hemozoin formation

Compounds	Inhibition of β-hematin formation IC_{50} (μg/mL)
11	2.91
12	2.89
13	3.01
14	2.91
15	2.99
16	7.6
17	9.61
18	3.10
19	3.15
20	2.71
21	12.6
22	11.2
23	2.89
24	2.62
25	13.8
26	8.06
27	21.9
28	3.48
29	5.34
30	2.9
31	2.93
32	6.59
33	6.08
34	4.35
35	3.78
36	3.21
37	7.88
38	13.9
CQ	3.65

Data are the mean of three different experiments in triplicate.
The IC_{50} represents the concentration of compound that inhibit β-hematin formation by 50%.

Table 3
In vivo antimalarial activity against chloroquine resistant of *P. yoelii* strain N-67 in Swiss mice at 100 mg/kg/day dose by oral route

Compounds	% Suppression		Mean survival time (days)
	Day 4	Day 6	
11	99.78	88.74	17.6 ± 0.98
14	99.5	83.5	15.6 ± 2.30
16	99.70	80.60	18.0 ± 1.97
17	99.78	82.06	17.0 ± 1.91
18	99.78	82.31	16.2 ± 2.03
19	99.6	83.2	14.6 ± 2.19
23	99.5	85.8	17.6 ± 1.78
24	99.4	86.7	18.2 ± 1.32
25	99.9	97.0	18.4 ± 1.94
27	99.9	96.7	17.6 ± 0.98
Control			12.4 ± 0.68
CQ (20 mg/kg)	99.0	93.9	>28
(10 mg/kg)	99.0	93.6	13.6 ± 1.72

5.3.2. (*E*)-6-((2-(7-Chloroquinolin-4-ylamino)ethylamino) methylene)-4-((*E*)-3-(4-hydroxyphenyl)-3-oxoprop-1-enyl)-2-methylcyclohexa-2,4-dienone (12)

Yellow solid, yield: 90%; mp 265–266 °C; IR (KBr): 3207, 2854, 1711, 1634, 1589, 1028 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.27 (s, 1H), 10.37–10.29 (m, 1H), 8.51 (s, 1H), 8.42 (d, J = 6 Hz, 1H), 8.30–8.25 (m, 1H), 8.03 (d, J = 9.0 Hz, 2H), 7.81 (d, J = 6.0 Hz, 2H), 7.64 (m, 4H), 7.46 (d, J = 9.0 Hz, 1H), 6.88 (d, J = 4.5 Hz, 2H), 6.64 (d, J = 3 Hz, 1H), 3.92 (s, 2H), 3.39 (s, 2H), 2.19 (s, 3H); ^{13}C (DMSO-d_6, 75 MHz) δ: 187.7, 167.6, 165.8, 162.8, 152.3, 151.1, 149.4, 143.6, 134.6, 133.5, 133.1, 131.7, 130.3, 128.1, 128, 125.3, 124.9, 124.6, 119.3, 118.3, 117.6, 116.1, 99.9, 80.0, 55.6, 43.8, 16.3; ESI-MS: (m/z): 486 $(M+H)^+$; HRMS m/z calcd for $C_{28}H_{24}ClN_3O_3$ $(M+H)^+$ 486.1585, found 486.1591.

5.3.3. (*E*)-6-((2-(7-Chloroquinolin-4-ylamino)ethylamino) methylene)-4-((*E*)-3-(4-methoxyphenyl)-3-oxoprop-1-enyl)-2-methylcyclohexa-2,4-dienone (13)

Yellow solid, yield: 91%; mp 238–239 °C; IR (KBr): 3199, 2851, 1723, 1621, 1588, 1010 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.32 (s, 1H), 8.51 (s, 1H), 8.41 (d, J = 5.4 Hz, 1H), 8.25 (d, J = 9.0 Hz, 1H), 8.13 (d, J = 8.8 Hz, 2H), 7.87 (s, 1H), 7.79 (d, J = 2.1 Hz, 1H), 7.75–7.69 (m, 1H), 7.66 (d, J = 1.8 Hz, 1H), 7.63 (s, 1H), 7.57–7.54 (m, 1H), 7.45 (dd, J = 2.1 Hz and J = 8.9 Hz, 1H), 7.06 (d, J = 8.8 Hz, 2H), 6.63 (d, J = 5.5 Hz, 1H), 3.92 (t, J = 5.5 Hz, 3H), 3.66 (d, J = 5.6 Hz, 2H), 2.19 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) 187.0, 166.5, 164.7, 162.8, 151.6, 149.9, 148.9, 142.9, 133.3, 132.6, 132.0, 130.8, 130.4, 127.3, 127.0, 124.0, 123.8, 118.4, 117.4, 116.7, 113.8, 98.9, 55.4, 54.7, 15.1; ESI-MS: (m/z): 500 $(M+H)^+$; HRMS m/z calcd for $C_{29}H_{26}ClN_3O_3$ $(M+H)^+$ 500.1742, found 500.1776.

5.3.4. (*E*)-6-((2-(7-Chloroquinolin-4-ylamino)ethylamino) methylene)-4-((*E*)-3-(4-fluorophenyl)-3-oxoprop-1-enyl)-2-methylcyclohexa-2,4-dienone (14)

Yellow solid, yield: 89%; mp 235–236 °C; IR (KBr): 3212, 2855, 1724, 1633, 1598, 1014 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.32 (s, 1H), 8.5 (s, 1H), 8.43 (d, J = 5.4 Hz, 1H), 8.28–8.18 (m, 3H), 7.87 (s, 1H), 7.79 (d, J = 2.2 Hz, 1H), 7.73–7.61 (m, 4H), 7.47 (dd, J = 2.1 Hz and J = 6.9 Hz, 1H), 7.37 (t, J = 8.8 Hz, 2H), 6.65 (d, J = 5.5 Hz, 1H), 3.93 (t, J = 5.3 Hz, 2H), 3.68–3.66 (m, 2H), 2.19 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 187.7, 167.2, 166.8, 152.0, 150.7, 149.0, 144.8, 135.1, 134.2, 133.6, 133.2, 131.8, 131.6, 128.1, 127.6, 124.8, 124.5, 123.6, 118.3, 117.9, 117.0, 116.3, 116.0, 99.5, 54.8, 43.3, 15.9; ESI-MS: (m/z): 488 $(M+H)^+$; HRMS m/z calcd for $C_{28}H_{23}ClFN_3O_2$ $(M+H)^+$ 488.1542, found 488.1534.

5.3.5. (*E*)-4-((*E*)-3-(4-Chlorophenyl)-3-oxoprop-1-enyl)-6-((2-(7-chloroquinolin-4-ylamino)ethylamino)methylene)-2-methylcyclohexa-2,4-dienone (15)

Yellow solid, yield: 88%; mp 230–231 °C; IR (KBr): 3211, 2839, 1722, 1634, 1583, 1018 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.33 (s, 1H), 8.49 (s, 1H), 8.42 (d, J = 3 Hz, 1H), 8.32 (s, 1H), 8.24 (d, J = 4.5 Hz, 1H), 8.13 (d, J = 4.5 Hz, 2H), 7.87 (s, 1H), 7.79 (d, J = 1.05 Hz, 1H), 7.68–7.60 (m, 5H), 7.46 (dd, J = 1.5 Hz and J = 4.5 Hz, 1H), 6.63 (d, J = 1.5 Hz, 1H), 3.92 (t, J = 3 Hz, 2H), 3.66 (d, J = 3 Hz, 2H), 2.18 (s, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ: 188.0, 167.1, 152.2, 150.4, 149.4, 145.1, 138.2, 137.1, 134.0, 133.8, 133.2, 130.7, 129.7, 128.3, 124.8, 124.5, 123.5, 118.1, 117.9, 116.8, 99.5, 19.6, 54.6, 43.2, 15.9; ESI-MS: (m/z): 504 $(M+H)^+$; HRMS m/z calcd for $C_{28}H_{23}Cl_2N_3O_2$ $(M+H)^+$ 504.1246, found 504.1234.

5.3.6. (*E*)-4-((*E*)-3-(4-Bromophenyl)-3-oxoprop-1-enyl)-6-((2-(7-chloroquinolin-4-ylamino)ethylamino)methylene)-2-methylcyclohexa-2,4-dienone (16)

Yellow solid, yield: 92%; mp 245–246 °C; IR (KBr): 3201, 2840, 1715, 1621, 1597, 1013 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.32 (s, 1H), 8.49 (s, 1H), 8.43 (d, J = 4.5 Hz 1H), 8.26 (d, J = 9.1 Hz, 1H), 8.05 (d, J = 8.4 Hz, 2H), 7.86 (s, 1H), 7.80–7.74 (m, 3H), 7.66–7.58 (m, 4H), 7.47 (dd, J = 2.1 Hz and 8.9 Hz, 1H), 6.65 (d, J = 5.5 Hz, 1H), 3.93 (t, J = 5.3 Hz 2H), 3.67 (d, J = 5.4 Hz, 2H), 2.18 (s, 3H); ^{13}C (DMSO-d_6, 100 MHz) δ: 188.3, 167.2, 167.0, 152.0, 150.6, 149.1, 145.2, 137.5, 134.1, 133.7, 133.3, 132.2, 130.8, 128.3, 127.7, 127.4, 124.8, 124.5, 123.5, 118.2, 117.9, 116.9, 99.5, 54.7, 15.9; ESI-MS: (m/z): 548 $(M+H)^+$; HRMS m/z calcd for $C_{28}H_{23}BrClN_3O_2$ $(M+H)^+$ 548.0741, found 548.0745.

5.3.7. (*E*)-6-((2-(7-Chloroquinolin-4-ylamino)ethylamino) methylene)-2-methyl-4-((*E*)-3-oxo-3-(3,4,5-trimethoxyphenyl) prop-1-enyl)cyclohexa-2,4-dienone (17)

Yellow solid, yield: 87%; mp 241–242 °C; IR (KBr): 3211, 2856, 1724, 1625, 1596, 1013 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ: 14.32 (s, 1H), 8.49 (s, 1H), 8.40 (d, J = 5.2 Hz 1H), 8.23 (d, J = 8.8 Hz, 1H), 7.83 (s, 1H), 7.78 (d, J = 2 Hz, 1H), 7.69–7.61 (m, 3H), 7.49 (t, J = 5.2 Hz, 1H), 7.43 (dd, J = 2 Hz and J = 8.8 Hz, 1H), 7.37 (s, 2H), 6.61 (d, J = 5.1 Hz, 1H), 3.93–3.88 (m, 9H), 3.75 (s, 2H), 3.65 (t, J = 5.2, 2H), 2.18 (s, 3H); ^{13}C (DMSO-d_6, 100 MHz) δ: 188.0, 167.1, 166.3, 153.3, 152.3, 150.4, 149.5, 144.4, 142.2, 133.95, 133.9, 133.3, 128.0, 124.7, 124.7, 123.8, 118.5, 117.9 117.0, 113.9, 112.4, 106.5, 99.5, 60.7, 56.7, 54.9, 43.3, 15.9; ESI-MS: (m/z): 560 $(M+H)^+$; HRMS m/z calcd for $C_{31}H_{30}ClN_3O_5$ $(M+H)^+$ 560.1953, found 560.1943.

5.3.8. (*E*)-6-((2-(7-Chloroquinolin-4-ylamino)ethylamino) methylene)-2-methyl-4-((*E*)-3-oxo-3-(thiophen-2-yl)prop-1-enyl)cyclohexa-2,4-dienone (18)

Yellow solid, yield: 87%; mp 222–223 °C; IR (KBr): 3201, 2839, 1727, 1635, 1596, 1002 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.32 (s, 1H), 8.50 (s, 1H), 8.43 (d, J = 5.5 Hz, 1H), 8.29–8.23 (m, 2H), 8.01 (d, J = 4.6 Hz, 1H), 7.85 (s, 1H), 7.80 (d, J = 2.1 Hz, 1H), 7.69–7.62 (m, 4H), 7.48 (dd, J = 2 Hz and 6.9 Hz 1H), 7.30 (t, J = 4.5 Hz, 1H), 6.66 (d, J = 4.5 Hz, 1H), 3.93 (t, J = 5.2 Hz, 2H), 3.68 (d, J = 5.4 Hz, 2H), 2.19 (s, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 181.8, 167.2, 166.6, 151.8, 150.8, 148.8, 146.4, 143.7, 135.4, 134.3, 133.5, 133.3, 133.2, 129.2, 128.1, 127.4, 124.9, 124.6, 123.5, 118.5, 117.8, 117.0, 99.5, 54.8, 43.3, 15.9; ESI-MS: (m/z): 476 $(M+H)^+$.

5.3.9. (*E*)-2-*sec*-Butyl-6-((2-(7-chloroquinolin-4-ylamino) ethylamino)methylene)-4-((*E*)-3-oxo-3-phenylprop-1-enyl)cyclohexa-2,4-dienone (19)

Yellow solid, yield: 90%; mp 215–216 °C; IR (KBr): 3210, 2853, 1714, 1622, 1588, 1004 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.42 (s, 1H), 8.51 (s, 1H), 8.41 (d, J = 4.9 Hz, 1H), 8.26 (d, J = 7.1 Hz, 1H), 8.12 (d, J = 8.6 Hz, 2H), 7.80–7.77 (m, 2H), 7.71–7.70 (m, 3H), 7.64–7.62 (m, 1H), 7.58–7.53 (m, 3H), 7.45 (dd, J = 2.1 Hz and 6.8 Hz, 1H), 6.63 (d, J = 5.5 Hz, 1H), 3.92 (t, J = 5.3 Hz, 2H), 3.68 (t, J = 5.3 Hz 2H), 3.1–3.07 (m, 1H), 1.72–1.56 (m, 2H), 1.21 (d, J = 6.9 Hz, 3H), 0.811 (t, J = 7.3 Hz, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 189.3, 167.4, 165.5, 152.3, 150.5, 149.5, 144.9, 138.5, 137.0, 134.0, 133.2, 133.0, 130.4, 129.1, 128.8, 127.9, 124.7, 124.5, 124.0, 118.7, 118.0, 117.5, 99.5, 55.2, 43.3, 33.5, 29.3, 20.5, 12.7; ESI-MS: (m/z): 512 $(M+H)^+$; HRMS m/z calcd for $C_{31}H_{30}ClN_3O_2$ $(M+H)^+$ 512.2106, found 512.2133.

5.3.10. (*E*)-2-*sec*-Butyl-6-((2-(7-chloroquinolin-4-ylamino) ethylamino)methylene)-4-((*E*)-3-(4-hydroxyphenyl)-3-oxoprop-1-enyl)cyclohexa-2,4-dienone (20)

Yellow solid, yield: 91%; mp 255–256 °C; IR (KBr): 3212, 2844, 1711, 1619, 1582, 1021 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.38 (s, 1H), 10.37 (s, 1H), 8.51 (s, 1H), 8.41 (d, J = 5.4 Hz, 1H), 8.31–8.25 (m, 1H), 8.04 (d, J = 8.7 Hz, 2H), 7.80–7.45 (m, 6H), 7.46 (dd, J = 2.1 Hz and J = 11.2 Hz, 1H), 6.89 (d, J = 8.7 Hz, 2H), 6.65 (d, J = 6.0 Hz, 1H), 3.92–3.91 (m, 2H), 3.67 (d, J = 6.1 Hz, 2H), 3.09–3.06 (m, 1H), 1.69–1.56 (m, 2H), 1.20 (d, J = 6.9 Hz, 3H), 0.80 (t, J = 7.3 Hz, 3H); ^{13}C (DMSO-d_6, 300 MHz) δ: 187.8, 167.9, 164.9, 162.8, 152.0, 151.3, 149.0, 143.9, 137.1, 134.7, 132.8, 131.8, 130.7, 130.3, 127.7, 125.3, 125.0, 124.9, 119.3, 118.2, 118.0, 116.1, 99.9, 55.8, 43.8, 33.9, 29.7, 20.9, 13.0; ESI-MS: (m/z): 528 $(M+H)^+$; HRMS m/z calcd for $C_{31}H_{30}ClN_3O_3$ $(M+H)^+$ 528.2055, found 528.2068.

5.3.11. (*E*)-2-*sec*-Butyl-6-((2-(7-chloroquinolin-4-ylamino) ethylamino)methylene)-4-((*E*)-3-(4-methoxyphenyl)-3-oxoprop-1-enyl)cyclohexa-2,4-dienone (21)

Yellow solid, yield: 85%; mp 210–212 °C; IR (KBr): 3201, 2839, 1722, 1623, 1579, 1010 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ:14.31 (s, 1H), 8.51 (s, 1H), 8.42 (d, J = 5.4 Hz, 1H), 8.24 (d, J = 9.1 Hz, 1H), 8.13 (d, J = 8.9 Hz, 2H), 7.80–7.77 (m, 2H), 7.71–7.67 (m, 3H), 7.55 (t, J = 5.3 Hz, 1H), 7.46 (dd, J = 2.2 Hz and J = 6.75 Hz, 1H), 7.06 (d, J = 9.1 Hz, 2H), 6.64 (d, J = 5.4 Hz, 1H), 3.94–3.90 (m, 2H), 3.85 (s, 3H), 3.69–3.65 (m, 2H), 3.09 (q, J = 7.1 Hz, 1H), 1.16–1.59 (m, 2H), 1.21 (d, J = 6.9 Hz, 3H), 0.81 (t, J = 7.3 Hz, 3H); ^{13}C (DMSO-d_6, 300 MHz) δ: 187.6, 167.4, 165.0, 163.4, 152.3, 150.4, 149.5, 144.0, 136.9, 133.9, 132.7, 131.2, 130.3, 128.0, 124.7, 124.5, 124.2, 118.7, 117.9, 117.5, 114.4, 99.5, 56.0, 55.3, 43.3, 33.5, 29.3, 20.6, 12.7; ESI-MS: (m/z): 542 $(M+H)^+$; HRMS m/z calcd for $C_{32}H_{32}ClN_3O_3$ $(M+H)^+$ 542.2211, found 542.2215

5.3.12. (*E*)-2-*sec*-Butyl-4-((*E*)-3-(4-chlorophenyl)-3-oxoprop-1-enyl)-6-((2-(7-chloroquinolin-4-ylamino)ethylamino) methylene)cyclohexa-2,4-dienone (22)

Yellow solid, yield: 87%; mp 220–221 °C; IR (KBr): 3198, 2841, 1707, 1622, 1596, 1008 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.43 (s, 1H), (s, 1H), 8.49 (s, 1H), 8.40 (d, J = 6.0 Hz, 1H), 8.28 (d, J = 9.1 Hz, 1H), 8.16–8.13 (m, 2H), 7.8–7.79 (m, 2H), 7.69–7.68 (m, 3H), 7.63–7.60 (m, 2H), 7.54 (t, J = 5.3 Hz, 1H), 7.45 (dd, J = 2.2 Hz and J = 9.1 Hz, 1H), 6.63 (d, J = 5.4 Hz, 1H), 3.92 (t, J = 5.2 Hz, 2H), 3.67 (t, J = 5.19 Hz, 2H), 3.07 (q, J = 6.9 Hz, 1H), 1.69–1.57 (m, 2H), 1.2 (d, J = 6.7 Hz, 3H), 0.81 (d, J = 7.3 Hz, 3H); ^{13}C (DMSO-d_6, 300 MHz) δ: 188.6, 167.8, 166.5, 152.7, 150.9, 149.9, 145.9, 138.6, 137.7, 137.5, 134.4, 133.8, 131.1, 130.8, 129.6, 128.3, 125.1, 124.9, 124.1, 118.6, 118.3, 117.7, 99.9, 55.3, 43.6, 33.9, 29.7, 20.9, 132.0; ESI-MS: (m/z): 546 $(M+H)^+$; HRMS m/z calcd for $C_{31}H_{29}Cl_2N_3O_2$ $(M+H)^+$ 546.1716, found 546.1742.

5.3.13. (*E*)-2-*sec*-Butyl-6-((2-(7-chloroquinolin-4-ylamino) ethylamino)methylene)-4-((*E*)-3-(furan-2-yl)-3-oxoprop-1-enyl)cyclohexa-2,4-dienone (23)

Yellow solid, yield: 87%; mp 220–221 °C; IR (KBr): 3201, 2836, 1710, 1618, 1595, 1004 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.42 (s, 1H), 8.52 (s, 1H), 8.41 (d, J = 5.4 Hz, 1H), 8.26 (d, J = 8.9 Hz, 1H), 8.03–8.02 (m, 1H), 7.7 (d, J = 2.1 Hz, 1H), 7.72–7.64 (m, 4H), 7.53–7.43 (m, 3H), 6.78–6.76 (m, 1H), 6.63 (d, J = 5.4 Hz, 1H), 3.92 (t, J = 5.4 Hz, 2H), 3.67 (t, J = 5.4 Hz, 2H), 3.09–3.06 (m, 1H), 1.67–1.57 (m, 2H), 1.20 (d, J = 6.1 Hz, 3H), 0.80 (t, J = 7.2 Hz, 3H); ^{13}C (DMSO-d_6, 300 MHz) δ: 177.1, 167.4, 165.8, 153.6, 152.3, 150.4, 149.5, 148.4, 143.6, 137.1, 133.9, 133.0, 130.3, 128.0, 124.7, 124.5, 123.6, 119.1, 118.4, 117.9, 117.4, 113.0, 99.5, 54.9, 43.2, 33.5, 29.2, 20.5, 12.7; ESI-MS: (m/z): 502 $(M+H)^+$; HRMS m/z calcd for $C_{29}H_{28}ClN_3O_3$ $(M+H)^+$ 502.1898, found 502.1904.

5.3.14. (*E*)-2-*sec*-Butyl-6-((2-(7-chloroquinolin-4-ylamino) ethylamino)methylene)-4-((*E*)-3-oxo-3-(thiophen-2-yl)prop-1-enyl)cyclohexa-2,4-dienone (24)

Yellow solid, yield: 91%; mp 226–227 °C; IR (KBr): 3202, 2842, 1714, 1618, 1585, 1014 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.43 (s, 1H), 8.50 (s, 1H), 8.41 (d, J = 5.4 Hz, 1H), 8.28–8.24 (m, 2H), 8.0 (d, J = 4.9 Hz, 1H), 7.80–7.77 (m, 2H), 7.68–7.65 (m, 3H), 7.52 (t, J = 5.4 Hz, 1H), 7.45 (dd, J = 2.1 Hz and J = 8.9 Hz, 1H), 7.29 (t, J = 4.7 Hz, 1H), 6.63 (d, J = 5.4 Hz, 1H), 3.92 (t, J = 5.6 Hz, 2H), 3.67 (t, J = 5.3 Hz, 2H), 3.08 (q, J = 7.1 Hz, 1H), 1.70–1.58 (m, 2H), 1.21 (d, J = 6.9 Hz, 3H), 0.81 (t, J = 7.3 Hz, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 181.9, 167.4, 165.7, 152.4, 150.4, 149.6, 146.4, 144.0, 137.1, 135.3, 133.9, 133.4, 133.1, 130.4, 129.2, 128.0, 124.7, 124.5, 123.8, 118.5, 118.0, 117.4, 99.5, 55.1, 43.3,

33.5, 29.3, 20.5, 12.7; ESI-MS: (*m/z*): 518 $(M+H)^+$; HRMS *m/z* calcd for $C_{29}H_{28}ClN_3O_2S$ $(M+H)^+$ 518.1670, found 518.1658.

5.3.15. (*E*)-2-*tert*-Butyl-6-((2-(7-chloroquinolin-4-ylamino) ethylamino)methylene)-4-((*E*)-3-(4-fluorophenyl)-3-oxoprop-1-enyl)cyclohexa-2,4-dienone (25)

Yellow solid, yield: 89%; mp 210–211 °C; IR (KBr): 3211, 2838, 1714, 1622, 1596, 1012 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.69 (s, 1H), 8.49 (s, 1H), 8.41 (d, *J* = 5.4 Hz, 1H), 8.27–8.16 (m, 3H), 7.79 (d, *J* = 2.1 Hz, 1H), 7.73–7.63 (m, 4H), 7.53 (t, *J* = 5.6 Hz 1H), 7.45 (dd, *J* = 2.1 Hz and *J* = 8.9 Hz, 1H), 7.37 (t, *J* = 8.8 Hz, 2H), 6.65 (d, *J* = 5.5 Hz 1H), 3.92 (t, *J* = 5.5 Hz, 2H), 3.68 (d, *J* = 5.2 Hz 2H), 1.41 (s, 9H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 187.8, 168.3, 167.6, 152.3, 150.4, 149.5, 145.4, 139.6, 135.2, 133.9, 131.8, 131.6, 130.4, 128.0, 124.7, 124.5, 122.8, 118.0, 117.7, 116.3, 99.6, 54.5, 43.2, 35.1, 29.6; ESI-MS: (*m/z*): 530 $(M+H)^+$; HRMS *m/z* calcd for $C_{31}H_{29}ClFN_3O_2$ $(M+H)^+$ 530.2011, found 530.2002.

5.3.16. (*E*)-2-*tert*-Butyl-4-((*E*)-3-(4-chlorophenyl)-3-oxoprop-1-enyl)-6-((2-(7-chloroquinolin-4-ylamino)ethylamino) methylene)cyclohexa-2,4-dienone (26)

Yellow solid, yield: 93%; mp 240–241 °C; IR (KBr): 3202, 2839, 1714, 1622, 1596, 1009 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.68 (s, 1H), 8.48 (s, 1H), 8.41 (d, *J* = 6 Hz, 1H), 8.25 (d, *J* = 9.0 Hz, 1H), 8.11 (d, *J* = 9.0 Hz, 2H), 7.78 (d, *J* = 3.0 Hz, 1H), 7.73 (s, 1H), 7.69–7.67 (m, 2H), 7.62–7.51 (m, 4H), 7.45 (dd, *J* = 3 Hz and *J* = 9 Hz, 1H), 6.65 (d, *J* = 3 Hz, 1H), 3.91 (d, *J* = 6 Hz, 2H), 3.68 (d, *J* = 6 Hz, 2H), 1.41 (s, 9H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 188.2, 168.7, 167.6, 152.3, 150.4, 149.5, 145.8, 139.7, 138.1, 137.2, 134.1, 133.9, 30.7, 130.4, 129.2, 128.0, 124.7, 124.5, 122.7, 117.9, 117.8, 117.7, 99.6, 54.4, 43.1, 35.1, 29.6; ESI-MS: (*m/z*): 546 $(M+H)^+$; Anal. Calcd for $C_{31}H_{29}Cl_2N_3O_2$: C, 68.13; H, 5.35; N, 7.69. Found: C, 68.19; H, 5.31; N, 7.60.

5.3.17. (*E*)-4-((*E*)-3-(4-Bromophenyl)-3-oxoprop-1-enyl)-2-*tert*-butyl-6-((2-(7-chloroquinolin-4-ylamino)ethylamino) methylene)cyclohexa-2,4-dienone (27)

Yellow solid, yield: 88%; mp 260–261 °C; IR (KBr): 3201, 2844, 1715, 1632, 1585, 1018 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.5 (s, 1H), 8.51 (s, 1H), 8.40 (d, *J* = 4 Hz, 1H), 8.22 (d, *J* = 8.8 Hz, 1H), 7.97 (d, *J* = 8.4 Hz, 2H), 7.78–7.64 (m, 6H), 7.51–7.47 (m 1H), 7.4 (dd, *J* = 2.4 Hz and *J* = 6.8 Hz, 1H), 7.30 (s, 1H), 6.62 (d, *J* = 5.4 Hz, 1H), 3.94 (t, *J* = 6 Hz, 2H), 3.7 (t, *J* = 5.3 Hz, 2H), 1.41 (s, 9H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 187.9, 167.7, 167.0, 151.8, 149.9, 149, 145.1, 139.1, 137.0, 133.4, 133.2, 131.6, 130.2, 129.9, 127.4, 126.6, 124.1, 123.9, 122.3, 117.4, 117.2, 116.2, 114.7, 113.3, 99.05, 54.0, 42.6, 34.5, 29; ESI-MS: (*m/z*): 590 $(M+H)^+$; Anal. Calcd for $C_{31}H_{29}BrClN_3O_2$: C, 63.01; H, 4.95; N, 7.11. Found: C, 62.97; H, 4.90; N, 7.21.

5.3.18. (*E*)-6-((3-(7-Chloroquinolin-4-ylamino)propylamino) methylene)-4-((*E*)-3-(4-hydroxyphenyl)-3-oxoprop-1-enyl)-2-methylcyclohexa-2,4-dienone (28)

Yellow solid, yield: 85%; mp 159–160 °C; IR (KBr): 3217, 2841, 1720, 1622, 1585, 1006 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.43 (s, 1H), 10.45–10.44 (m, 1H), 8.55 (s, 1H), 8.39 (d, *J* = 5.5 Hz, 1H), 8.31–8.26 (m, 2H), 8.04 (d, *J* = 8.8 Hz, 2H), 7.84 (s, 1H), 7.77 (d, *J* = 2.2 Hz, 1H), 7.68–7.62 (m, 3H), 7.47–7.42 (m, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.51 (d, *J* = 5.6 Hz, 1H), 3.75 (t, *J* = 6.4 Hz, 2H), 2.18 (s, 3H), 2.08 (t, *J* = 6.5 Hz, 3H); ^{13}C (DMSO-d_6, 300 MHz) δ: 187.8, 166.9, 166.4, 162.8, 152.4, 151.1, 149.4, 143.7, 134.5, 133.4, 133.2, 131.8, 130.4, 128.3, 127.9, 125.0, 124.4, 119.2, 118.3, 117.4, 116.2, 99.6, 80.0, 54.9, 29.6, 16.3; ESI-MS: (*m/z*): 500 $(M+H)^+$; HRMS *m/z* calcd for $C_{29}H_{26}ClN_3O_3$ $(M+H)^+$ 500.1742, found 500.1732.

5.3.19. (*E*)-6-((3-(7-Chloroquinolin-4-ylamino)propylamino) methylene)-4-((*E*)-3-(4-methoxyphenyl)-3-oxoprop-1-enyl)-2-methylcyclohexa-2,4-dienone (29)

Yellow solid, yield: 92%; mp 198–199 °C; IR (KBr): 3212, 2841, 1712, 1618, 1598, 1013 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.45 (s, 1H), 8.55 (s, 1H), 8.39 (d, *J* = 5.4 Hz, 1H), 8.26 (d, *J* = 9.1 Hz, 1H), 8.13 (d, *J* = 8.9 Hz, 2H), 7.86 (s, 1H), 7.78–7.75 (m, 1H), 7.70 (s, 1H), 7.66–7.60 (m, 2H), 7.45–7.37 (m, 2H), 7.07 (d, *J* = 8.9 Hz, 1H), 3.86 (s, 3H), 3.75 (t, *J* = 6.4 Hz, 2H), 2.18 (s, 3H), 2.07 (t, *J* = 6.5 Hz, 2H); ^{13}C (DMSO-d_6, 300 MHz) δ: 187.5, 166.5, 166.4, 163.4, 152.3, 150.5, 149.4, 143.8, 133.9, 133.1, 133.0, 131.3, 131.1, 128.0, 127.9, 124.6, 124.5, 123.8, 118.5, 117.9, 116.9, 114.4, 99.2, 56.0, 54.3, 29.2, 15.9; ESI-MS: (*m/z*): 514 $(M+H)^+$; HRMS *m/z* calcd for $C_{30}H_{28}ClN_3O_3$ $(M+H)^+$ 514.1898, found 514.1889.

5.3.20. (*E*)-4-((*E*)-3-(4-Chlorophenyl)-3-oxoprop-1-enyl)-6-((3-(7-chloroquinolin-4-ylamino)propylamino)methylene)-2-methylcyclohexa-2,4-dienone (30)

Yellow solid, yield: 93%; mp 216–217 °C; IR (KBr): 3201, 2845, 1714, 1621, 1599, 1013 cm^{-1}; ^1H NMR (TFA-*d*, 300 MHz) δ: 8.94 (s, 1H), 8.4 (d, *J* = 4.2 Hz, 1H), 8.24 (d, *J* = 8.3 Hz, 1H), 8.26–7.96 (m, 6H), 7.79–7.63 (m, 4H), 6.96 (d, *J* = 4.9 Hz, 1H), 4.35 (s, 2H), 3.99 (s, 2H), 2.66 (s, 2H), 2.54 (s, 3H); ^{13}C (DMSO-d_6, 300 MHz) δ: 187.7, 166.6, 166.5, 163.6, 152.5, 150.6, 149.6, 143.9, 134.0, 133.3, 133.2, 131.4, 131.3, 128.1, 128.0, 124.7, 124.6, 124.0, 118.7, 118.1, 117.1, 114.5, 99.3, 54.5, 29.3, 16.1; ESI-MS: (*m/z*): 518 $(M+H)^+$; Anal. Calcd for $C_{29}H_{25}Cl_2N_3O_2$: C, 67.19; H, 4.86; N, 8.11. Found: C, 67.15; H, 4.81; N, 8.07.

5.3.21. (*E*)-2-*sec*-Butyl-6-((3-(7-chloroquinolin-4-ylamino) propylamino)methylene)-4-((*E*)-3-(4-hydroxyphenyl)-3-oxoprop-1-enyl)cyclohexa-2,4-dienone (31)

Yellow solid, yield: 88%; mp 158–159 °C; IR (KBr): 3216, 2854, 1722, 1634, 1595, 1013 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.52 (s, 1H), 10.34 (s, 1H), 8.57 (s, 1H), 8.39 (d, *J* = 5.4 Hz, 1H), 8.27 (d, *J* = 9.1 Hz, 1H), 8.05 (d, *J* = 8.7 Hz, 2H), 7.78–7.75 (m, 2H), 7.69–7.60 (m, 3H), 7.45–7.38 (m, 2H), 6.89 (d, *J* = 8.7 Hz, 2H), 6.50 (d, *J* = 5.5 Hz, 1H), 3.76 (t, *J* = 6.4 Hz, 2H), 3.42–3.38 (m, 2H), 3.07 (q, *J* = 7.0 Hz, 1H), 2.10–2.04 (m, 2H), 1.72–1.56 (m, 2H), 1.21 (d, *J* = 6.9 Hz, 3H), 0.82 (t, *J* = 7.3 Hz, 3H); ^{13}C (DMSO-d_6, 300 MHz) δ: 187.7, 166.8, 165.4, 163.5, 152.3, 150.7, 149.4, 144.2, 137.1, 134.0, 132.8, 131.4, 131.3, 130.3, 127.9, 124.7, 124.2, 118.8, 118.0, 117.5, 114.5, 99.3, 54.7, 33.6, 29.4, 20.6, 12.8; ESI-MS: (*m/z*): 542 $(M+H)^+$; HRMS *m/z* calcd for $C_{32}H_{32}ClN_3O_3$ $(M+H)^+$ 542.2211, found 542.2199.

5.3.22. (*E*)-2-*sec*-Butyl-6-((3-(7-chloroquinolin-4-ylamino) propylamino)methylene)-4-((*E*)-3-(4-methoxyphenyl)-3-oxoprop-1-enyl)cyclohexa-2,4-dienone (32)

Yellow solid, yield: 88%; mp 128–129 °C; IR (KBr): 3199, 2843, 1712, 1626, 1596, 1013 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.54 (s, 1H), 8.57 (s, 1H), 8.39 (d, *J* = 4.9 Hz, 1H), 8.26 (d, *J* = 7.1 Hz, 1H), 8.14 (d, *J* = 8.6 Hz, 2H), 7.77–7.68 (m, 5H), 7.45–7.37 (m, 2H), 7.07 (d, *J* = 8.6 Hz, 2H), 6.5 (d, *J* = 5.3 Hz, 1H), 3.86–3.76 (m, 2H), 3.40–3.32 (m, 6H), 3.12–3.05 (m, 1H), 2.09 (t, *J* = 6.1 Hz, 2H), 1.72–1.56 (m, 2H), 1.21 (d, *J* = 6.8 Hz, 3H), 0.81 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 187.1, 166.2, 164.8, 162.9, 151.7, 150.1, 143.6, 136.5, 133.4, 132.2, 130.8, 130.7, 129.7, 127.3, 124.1, 124.0, 123.6, 118.2, 116.9, 113.9, 98.7, 55.5, 54.1, 33.0, 28.8, 28.7, 20.0, 12.2; ESI-MS: (*m/z*): 556 $(M+H)^+$; Anal. Calcd for $C_{33}H_{34}ClN_3O_3$: C, 71.27; H, 6.16; N, 7.56; Found: C, 71.21; H, 6.20; N, 7.61.

5.3.23. (*E*)-2-*sec*-Butyl-4-((*E*)-3-(4-chlorophenyl)-3-oxoprop-1-enyl)-6-((3-(7-chloroquinolin-4-ylamino)propylamino) methylene)cyclohexa-2,4-dienone (33)

Yellow solid, yield: 91%; mp 131–132 °C; IR (KBr): 3212, 2838, 1712, 1621, 1582, 1001 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.57 (s, 1H), 8.55 (s, 1H), 8.39 (s, 1H), 8.27 (t, J = 8.0 Hz, 1H), 8.15 (d, J = 6.3 Hz, 2H), 7.78–7.61 (m, 7H), 7.40 (t, J = 9.0 Hz, 2H), 6.49 (s, 1H), 3.76 (s, 2H), 3.35 (s, 6H), 3.08 (d, J = 5.1 Hz, 1H), 2.08 (s, 2H), 1.68–1.55 (m, 2H), 1.21 (d, J = 3.7 Hz, 3H), 0.81 (s, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 187.7, 166.2, 166, 151.8, 150, 149, 145, 137.7, 136.9, 136.7, 133.4, 132.9, 130.2, 129.8, 128.7, 127.4, 124.1, 124, 123.1, 117.6, 117.5, 116.7, 98.7, 53.7, 33, 28.8, 28.7, 20, 12.2; ESI-MS: (m/z): 560 $(M+H)^+$; Anal. Calcd for $C_{31}H_{31}Cl_2N_3O_2$: C, 68.57; H, 5.57; N, 7.50. Found: C, 68.52; H, 5.62; N, 7.56.

5.3.24. (*E*)-6-((4-(7-Chloroquinolin-4-ylamino)phenylamino) methylene)-4-((*E*)-3-(4-methoxyphenyl)-3-oxoprop-1-enyl)-2-methylcyclohexa-2,4-dienone (34)

Red solid, yield: 93%; mp 165–166 °C; IR (KBr): 3200, 2854, 1725, 1621, 1582, 1003 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.26 (s, 1H), 9.23 (s, 1H), 9.0 (s, 1H), 8.51 (d, J = 6 Hz, 1H), 8.42 (d, J = 9.0 Hz, 1H), 8.14 (d, J = 9.0 Hz, 2H), 7.93–7.91 (m, 3H), 7.84–7.78 (m, 1H), 7.69–7.64 (m, 1H), 7.60–7.53 (m, 3H), 7.46 (d, J = 9.0 Hz, 2H), 7.09–7.03 (m, 3H), 3.86 (s, 3H), 2.29 (s, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ:187.0, 163.0, 161.7, 161.3, 152, 151.8, 149.5, 147.4, 142.3, 139.7, 133.9, 133.3, 132.2, 130.6, 127.6, 126.2, 125.4, 125.0, 124.5, 124.3, 122.7, 122.4, 120.1, 119.3, 118.5, 118.3, 114.5, 113.7, 113.3, 112.5, 109.2, 102.6, 102.2, 101.8, 55.8, 55.3, 55.1, 15.3, 15.1; ESI-MS: (m/z): 548 $(M+H)^+$; HRMS m/z calcd for $C_{33}H_{26}ClN_3O_3$ $(M+H)^+$ 548.1742, found 548.1725.

5.3.25. (*E*)-6-((4-(7-Chloroquinolin-4-ylamino)phenylamino)methylene)-4-((*E*)-3-(4-fluorophenyl)-3-oxoprop-1-enyl)-2-methylcyclohexa-2,4-dienone (35)

Red solid, yield: 91%; mp 150–151 °C; IR (KBr): 3213, 2839, 1718, 1620, 1590, 1017 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.34–14.29 (m, 1H), 9.24 (s, 1H), 9.02 (s, 1H), 8.52 (d, J = 5.4 Hz, 1H), 8.43 (d, J = 9.1 Hz, 1H), 8.26–8.21 (m, 2H), 7.97–7.92 (m, 3H), 7.85–7.68 (m, 2H), 7.61–7.54 (m, 3H), 7.49–7.37 (m, 4H), 7.04 (d, J = 5.3 Hz, 1H), 2.29 (s, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 187.9, 162.2, 152.4, 150.0, 148.0, 144.3, 142.8, 140.2, 134.9, 134.5, 133.9, 133.1, 131.8, 131.7, 128.1, 126.9, 125.7, 125.6, 124.9, 123.3, 123.0, 119.6, 119.0, 118.8, 116.4, 116.1, 102.9, 15.7; ESI-MS: (m/z): 536 $(M+H)^+$; HRMS m/z calcd for $C_{32}H_{23}ClFN_3O_2$ $(M+H)^+$ 536.1542, found 536.1567.

5.3.26. (*E*)-2-*sec*-Butyl-6-((4-(7-chloroquinolin-4-ylamino) phenylamino)methylene)-4-((*E*)-3-(furan-2-yl)-3-oxoprop-1-enyl)cyclohexa-2,4-dienone (36)

Red solid, yield: 92%; mp 170–171 °C; IR (KBr): 3212, 2838, 1711, 1624, 1582, 1015 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.43 (s, 1H), 9.23 (s, 1H), 9.04 (s, 1H), 8.52 (d, J = 5.2 Hz, 1H), 8.43 (d, J = 9.1 Hz, 1H), 8.06 (s, 1H), 7.95–7.92 (m, 2H), 7.85 (s, 1H), 7.77–7.73 (m, 2H), 7.62–7.55 (m, 4H), 7.47 (d, J = 8.7 Hz, 2H), 7.04 (d, J = 5.3 Hz 1H), 6.80–6.79 (m, 1H), 3.16 (q, J = 6.7 Hz, 1H), 1.79–1.59 (m, 2H), 1.21 (d, J = 6.9 Hz, 3H), 0.81 (t, J = 7.3 Hz, 3H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 177.1, 162.6, 161.5, 153.6, 152.4, 150.0, 148.5, 147.9, 143.2, 142.8, 140.2, 136.0, 134.5, 132.5, 131.0, 128.1, 125.7, 125.6, 124.9, 123.3, 123.0, 119.7, 119.4, 119.1, 113.1, 102.9, 33.5, 29.3, 20.6, 12.6; ESI-MS: (m/z): 550 $(M+H)^+$; Anal. Calcd for $C_{33}H_{28}ClN_3O_3$: C, 72.06; H, 5.13; N, 7.64, Found: C, 72.10; H, 5.17; N, 7.68.

5.3.27. (*E*)-2-*tert*-Butyl-4-((*E*)-3-(4-chlorophenyl)-3-oxoprop-1-enyl)-6-((4-(7-chloroquinolin-4-ylamino)phenylamino) methylene)cyclohexa-2,4-dienone (37)

Red solid, yield: 87%; mp 145–146 °C; IR (KBr): 3202, 2841, 1712, 1619, 1592, 1008 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.89 (s, 1H), 9.23 (s, 1H), 9.02 (s, 1H), 8.52 (d, J = 6 Hz, 1H), 8.42 (d, J = 9.1 Hz, 1H), 8.14 (d, J = 9.0 Hz, 2H), 8.05 (s, 1H), 7.91 (d, J = 3.1 Hz, 1H), 7.80–7.76 (m, 3H), 7.64–7.54 (m, 5H), 7.47 (d, J = 9.0 Hz, 2H), 7.04 (d, J = 6.0 Hz, 1H), 1.47 (s, 9H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 193.1, 168.0, 167.6, 157.2, 154.8, 152.6, 149.9, 147.2, 145.0, 143.1, 142.9, 141.7, 139.2, 137.8, 135.9, 135.5, 134.0, 132.9, 130.3, 130.1, 129.7, 128.0, 127.7, 124.3, 124.2, 123.8, 107.7, 39.9, 34.4; ESI-MS: (m/z): 594 $(M+H)^+$; Anal. Calcd for $C_{35}H_{29}Cl_2N_3O_2$: C, 70.71; H, 4.92; N, 7.07. Found: C, 70.66; H, 4.95; N, 7.12.

5.3.28. (*E*)-4-((*E*)-3-(4-Bromophenyl)-3-oxoprop-1-enyl)-2-*tert*-butyl-6-((4-(7-chloroquinolin-4-ylamino)phenylamino) methylene)cyclohexa-2,4-dienone (38)

Red solid, yield: 92%; mp 166–167 °C; IR (KBr): 3214, 2839, 1714, 1623, 1598, 1014 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ: 14.89 (s, 1H), 9.2 (s, 1H), 9.02 (s, 1H), 8.52 (d, J = 5.3 Hz, 1H), 8.43 (d, J = 9.1 Hz, 1H), 8.08–8.04 (m, 3H), 7.91 (d, J = 2.1 Hz, 1H), 7.80–7.74 (m, 5H), 7.60–7.55 (m, 3H), 7.47 (d, J = 8.6 Hz, 2H), 7.04 (d, J = 5.4 Hz 1H), 1.47 (s, 9H); ^{13}C NMR (DMSO-d_6, 300 MHz) δ: 188.6, 163.3, 162.8, 152.4, 150.0, 147.9, 145.2, 142.5, 140.2, 138.2, 137.3, 134.5, 133.0, 132.2, 131.2, 130.9, 128.1, 127.5, 125.6, 125.3, 124.9, 123.2, 122.9, 119.6, 119.4, 119.0, 103.0, 35.2, 29.6; ESI-MS: (m/z): 638 $(M+H)^+$; HRMS m/z calcd for $C_{35}H_{29}BrClN_3O_2$ $(M+H)^+$ 638.1211, found 638.1224.

5.4. General procedure for the synthesis of compounds 1a–1d

Synthesis of **1a** was achieved by our previously reported protocol.[21]

5.4.1. 3-*sec*-Butyl-2-hydroxy-5-(3-oxo-3-*p*-tolyl-propenyl)-benzaldehyde (1a)

White solid: mp 85–86 °C; IR (KBr, cm^{-1}): 3433, 3022, 2846, 1654, 1591; ^1H NMR ($CDCl_3$, 300 MHz): δ 11.60 (s, 1H), 9.94 (s, 1H), 7.94 (d, J = 8.0 Hz, 2H), 7.77 (d, J = 15.7 Hz, 1H), 7.69 (d, J = 2.4 Hz, 1H), 7.45 (d, J = 15.7 Hz, 1H), 7.31 (d, J = 8.1 Hz, 2H), 3.23–3.11 (m, 1H), 2.43 (s, 3H), 1.74–1.61 (m, 2H), 1.28 (d, J = 6.6 Hz, 3H), 0.88 (t, J = 8.0 Hz, 3H); ^{13}C NMR (75 MHz): δ 196.8, 189.9, 161.5, 143.8, 143.3, 137.3, 135.8, 133.8, 132.1, 129.5, 128.8, 127.0, 120.9, 120.4, 33.5, 29.5, 21.8, 20.2, 12.2; ESI-MS: m/z: 323 $(M+H)^+$; Anal. Calcd for $C_{21}H_{22}O_3$: C, 78.23; H, 6.88; O, 14.89. Found: C, 78.21; H, 6.90; O, 14.88.

A mixture of **1a** (1.0 equiv) and appropriate amines (1.0 equiv) in ethanol were stirred for 10 min at room temperature. The solvent was evaporated under vacuum and the solid was purified directly with column chromatography to obtain the respective compounds **1b–1d** in excellent yields.

5.4.2. (*E*)-2-*sec*-Butyl-6-((methylamino)methylene)-4-((*E*)-3-oxo-3-*p*-tolylprop-1-enyl)cyclohexa-2,4-dienone (1b)

Red solid, yield: 92%; mp 91–92 °C; IR (KBr): 3220, 2834, 1711, 1620, 1598, 1014 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ: 14.57–14.54 (m, 1H), 8.36 (s, 1H), 7.94 (d, J = 9.0 Hz, 2H), 7.81–7.76 (m, 1H), 7.53 (d, J = 2.1 Hz, 1H), 7.42–7.30 (m, 4H), 3.67 (q, J = 6.5 Hz, 2H), 3.20 (q, J = 6.9 Hz, 1H), 2.45 (s, 3H), 1.77–1.62 (m, 2H), 1.37 (t, J = 6 Hz, 3H), 1.28 (d, J = 6.9 Hz, 3H), 0.94–0.89 (m, 3H); ESI-MS: (m/z): 350 $(M+H)^+$.

5.4.3. (*E*)-2-*sec*-Butyl-6-((ethylamino)methylene)-4-((*E*)-3-oxo-3-*p*-tolylprop-1-enyl)cyclohexa-2,4-dienone (1c)

Red solid, yield: 91%; mp 86–87 °C; IR (KBr): 3211, 2830, 1705, 1624, 1591, 1011 cm^{-1} ^1H NMR ($CDCl_3$, 300 MHz) δ: 14.62–14.57 (m, 1H), 8.35 (s, 1H), 7.95 (d, J = 9.1 Hz, 2H), 7.81–7.76 (m, 1H), 7.53 (d, J = 2.1 Hz, 1H), 7.42–7.70 (m, 4H), 3.59 (t, J = 6.8 Hz, 2H), 3.20 (q, J = 6.9 Hz, 1H), 2.45 (s, 3H), 1.80–1.59 (m, 4H), 1.28 (d, J = 6 Hz, 3H), 1.02 (t, J = 7.4 Hz, 3H), 0.93 (d, J = 7.3 Hz, 3H); ESI-MS: (m/z): 364 $(M+H)^+$.

5.4.4. (*E*)-2-*sec*-Butyl-4-((*E*)-3-oxo-3-p-tolylprop-1-enyl)-6-((phenylamino)methylene)cyclohexa-2,4-dienone (1d)

Red solid, yield: 88%; mp 95–96 °C; IR (KBr): 3202, 2844, 1712, 1621, 1598, 1002 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ: 14.20–14.16 (m, 1H), 8.70 (s, 1H), 7.96 (d, J = 9.0 Hz, 2H), 7.84–7.79 (m, 1H), 7.60 (d, J = 3 Hz, 1H), 7.55 (d, J = 2 Hz, 1H), 7.49–7.43 (m, 3H), 7.34–7.31 (m, 5H), 3.30–3.21 (m, 1H), 2.45 (s, 3H), 1.82–1.67 (m, 3H), 1.31 (t, J = 6.9 Hz, 3H), 0.96 (d, J = 7.3 Hz, 3H); ESI-MS: (m/z): 398 $(M+H)^+$.

6. Biological materials and methods

6.1. In vitro antimalarial assay

The compounds were dissolved in DMSO at 5 mg/mL. Twofold serial dilutions of test samples were made in 96 well plates and incubated with 1.0% parasitized cell suspension containing 0.8% parasitaemia (asynchronous culture with more than 80% ring stages). The plates were incubated at 37 °C in CO_2 incubator in an atmosphere of 5% CO_2 and air mixture. Later (72 h) 100 μl of lysis buffer containing 1× concentration of SYBR Green-I (Invitrogen) was added to each well and incubated for 1 h at 37 °C. The plates were examined at 485 ± 20 nm of excitation and 530 ± 20 nm of emission for relative fluorescence units (RFUs) per well using the fluorescence plate reader (FLUO star, BMG lab technologies). Data was transferred into a graphic programme (EXCEL) and IC_{50} values were obtained by Logit regression analysis.[24] Chloroquine was used as the standard reference drug.

6.2. Cytotoxicity assay

Cytotoxicity of the compounds was carried out using Vero cell line (C1008; Monkey kidney fibroblast) following the method of Mosmann (1983)[25] with certain modifications. The cells were incubated with different dilutions of test agents for 72 h and MTT was used as reagent for the detection of cytotoxicity. 50% cytotoxic concentration (CC_{50}) was determined using non-linear regression analysis. Selectivity Index (SI) was calculated as: $SI = CC_{50}/IC_{50}$.

6.3. Inhibition of β-hematin formation assay

Male Swiss mice, weighing 15–20 g were inoculated with 1×10^5 *P. yoelii* infected RBCs. Blood of infected animal at~50% parasitemia was collected by cardiac puncture in 2.0% citrate buffer and centrifuged at 3000 rpm for 10 min at 4 °C. The plasma was used in assay of β hematin formation. The assay mixture contained 100 mM sodium acetate buffer pH (5.1), 50 μL plasma, 100 μM hemin as the substrate and 1–10 μg compound/drug in a total volume of 1.0 mL. The control tube contained all reagents except compound. The reaction mixture in triplicate was incubated at 37 °C for 16 h in a rotary shaker. The reaction was stopped by centrifugation at 10,000 rpm for 10 min at 30 °C. The pellet was suspended in 100 mM Tris–HCl buffer pH (7.4) containing 2.5% SDS. The pellet obtained after centrifugation was washed thrice with distilled water (TDW) to remove free hemin attached to β-hematin. The pellet was solubilized in 50 μL of 2 N NaOH and volume was made up to 1.0 mL with TDW. Absorbance was measured at 400 nm.[26]

6.4. In vivo antimalarial assay

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 (24 ± 2 g) were inoculated with 1×10^5 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 100 mg/kg/day by oral route. Parasitaemia levels were recorded from thin blood smears on days 4 and day 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. The animals were followed further till day 28 to record the day of death. Mice treated with CQ served as reference controls.[27]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.03.011.

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