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Research Article

Design and Synthesis of a New Class of 4-Aminoquinolinyl- and 9-Anilinoacridinyl Schiff Base Hydrazones as Potent Antimalarial Agents

Moni Sharma[1], Kuldeep Chauhan[1], Rajeev K. Srivastava[2], Shiv V. Singh[3], Kumkum Srivastava[2], Jitendra K. Saxena[3], Sunil K. Puri[2] and Prem M. S. Chauhan[1,*]

[1]Medicinal and Process Chemistry Division, CSIR-Central Drug Research Institute, Lucknow 226031, India
[2]Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow 226031, India
[3]Division of Biochemistry, CSIR-Central Drug Research Institute, Lucknow 226031, India
**Corresponding author: Prem M. S. Chauhan, prem_chauhan_2000@yahoo.com; premsc58@hotmail.com*

A series of novel 4-aminoquinolinyl and 9-anilinoacridinyl Schiff base hydrazones have been synthesized and evaluated for their antimalarial activity. All compounds were evaluated *in vitro* for their antimalarial activity against chloroquine-sensitive strain 3D7 and the chloroquine-resistant K1 strain of *Plasmodium falciparum* and for cytotoxicity toward Vero cells. Compounds 17, 20, and 21 displayed good activity against the 3D7 strain with IC_{50} values ranging from 19.69 to 25.38 nM. Moreover, compounds 16, 17, 21, 24, 32, and 33 exhibited excellent activities (21.64–54.26 nM) against K1 strain and several compounds displayed β-hematin inhibitory activity, suggesting that they act on the heme crystallization process such as CQ. Compounds were also found to be non-toxic with good selectivity index.

Key words: 4-aminoquinoline, 9-anilinoacridine, antimalarial, oxalamide, Schiff base

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Malaria, a devastating infectious disease, caused by five species of *Plasmodium*, responsible for 216 million clinical cases in 2010, 65 5000 deaths in children below the age of 5 years and pregnant women (1). Among the five major species of the malaria parasite *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale,* and *P. knowlesi* that infect the humans, *P. falciparum* is the most fatal one (2,3). The prevalent drug resistance to currently established antimalarials such as aminoquinolines (**1**), anilinoacridines (**2**), antifolates (**3**), and drug combinations on artemisinins (**4**–**6**, Figure 1) (ACTs) (4–8) is driving the rise in global mortality due to malaria. Owing to emergence of growing resistance, the WHO recommends combination therapy as first-line treatment against resistant malaria (9). Catabolism of hemoglobin and subsequent detoxification of released heme by the malaria parasite is one of the most important biochemical target for drug development (10). To fulfill the requirement of amino acids, the parasite can degrade as much as 80% of an infected host's red blood cell hemoglobin during the intraerythrocytic phase of its life cycle and producing free heme ($Fe^{III}PPIX$) which is toxic to the parasite. To prevent the toxic effects of free heme, parasite detoxify it into an insoluble biomineral known as hemozoin (11,12). Several established drugs, for example, chloroquine and other quinoline antimalarials are known to inhibit the formation of hemozoin and, therefore, make it a target of choice for the development of new antimalarial chemotype (13–15).

Schiff base hydrazones are interesting scaffolds, possessing a range of biological activities including antimalarial (16,17), antitumor (18), antibacterial (19,20), antifungal (21), antitubercular (22), anti-inflammatory (23), antimicrobial (24), antiproliferative (25), antiviral (26), and cytotoxic actions (27). Among the reported heterocyclic hydrazones, quinolylhydrazones are known for their excellent antimalarial and antitubercular activities (28–30). Recently, the Schiff bases of N_4O_2 complex were developed as novel antimalarials that inhibit the aggregation of hemozoin (31). Furthermore, oxalamide derivatives also represent important substructures in molecules that show diverse biological activities (32,33).

The contemporary trend of drug discovery is shifted toward the molecule having multiple ligands instead of single pharmacophore that provide structural features to modulate numerous biological targets simultaneously within that molecule. In this context, hybridization approach is recently developed which involves the molecular hybridization of two or more than two different chemical entities in a single molecule by simple fusion (usually via a covalent linker). The rational of this approach depends upon the hybridization of two drugs, both active compounds and pharmacophoric units recognized and derived from known bioactive molecules (34,35). Some hybrid molecules based on

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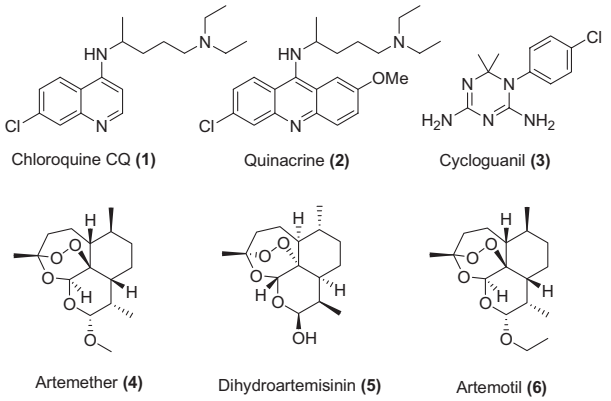


Figure 1: Structure of some traditional (**1**–**3**) as well as artemisinin-based (**4**–**6**) antimalarials.

4-aminoquinoline and 9-anilinoacridine core reported by us and other groups as potent hybrid antimalarial agents (Figure 2) (36–41).

Therefore, in continuation of our ongoing anti-infective research program and in an earlier attempt to introduce the oxalamide linker as flexible tether in aminoquinoline (42–44), here we report that the derivatives of 4-aminoquinolinyl and 9-anilinoacridinyl Schiff base hydrazones having an oxalamide functionality have antimalarial activity (**14**–**26** and **31**–**41**).

Experimental

General chemistry

All reagents were commercial and were used without further purification. Chromatography was carried on silica gel (60–120 and 100–200 mesh). All reactions were monitored by thin-layer chromatography (TLC), and silica gel plates with fluorescence F254 were used. Melting points were taken in open capillaries on Complab melting point apparatus and are presented uncorrected. Infrared spectra were recorded on a Perkin-Elmer AC-1 spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker-Supercon Magnet DRX-300 spectrometer (operating at 200, 300 MHz for ^{1}H and 50, 75 MHz for ^{13}C) using $CDCl_3$, CD_3OD, DMSO-d_6, and TFA as solvents and tetramethylsilane (TMS) as internal standard. Chemical shifts are reported in parts per million. Multiplicities are reported as follows: singlet (s), doublet (d), triplet (t), multiplet (m), and broad singlet (brs). Some signals for NH proton are not presented in the few ^{1}H NMR data, and FAB mass spectra were recorded on JEOL SX 102/DA 6000 mass spectrometer using argon/xenon (6 Kv, 10 mA) as the FAB gas. Chemical analysis was carried out on carlo-Erba-1108 instrument. The melting points were recorded on an electrically heated melting point apparatus and are uncorrected.

Bioevaluation methods

In vitro antimalarial assay

The compounds were evaluated for antimalarial activity against both 3D7 (CQ sensitive) and K1 (CQ resistant) strains of *Plasmodium falciparum* using Malaria SYBR Green I nucleic acid staining dye based fluorescence (MSF) assay as mentioned by Singh *et al.* (45). The stock (5 mg/mL) solution was prepared in DMSO, and test dilutions were prepared in culture medium (RPMI-1640-FBS). Chloroquine was used as reference drug. The compounds

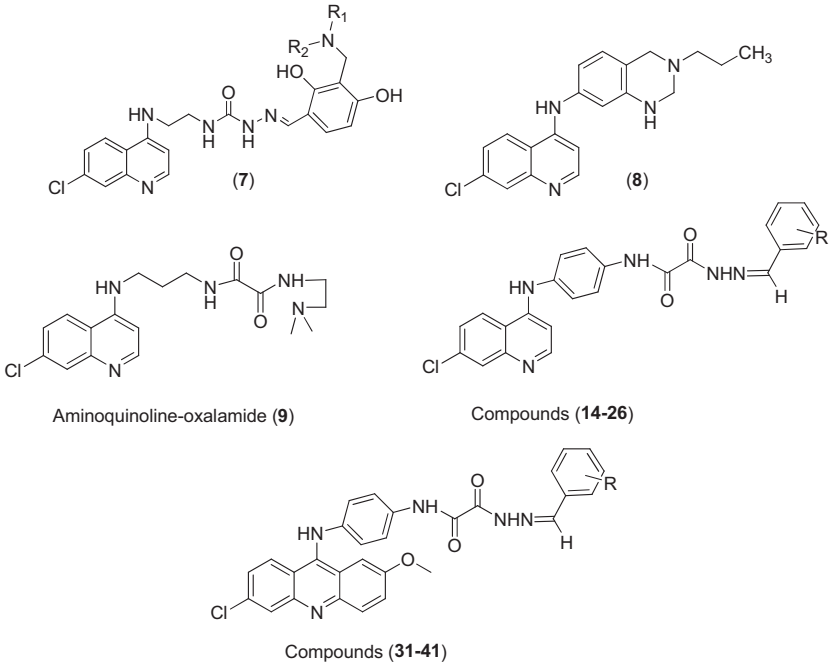


Figure 2: Hybrid antimalarials.

were tested in 96-well plate (in duplicate wells). 1.0% parasitized cell suspension containing 0.8% parasitaemia was used. The plates were incubated at 37 °C in CO_2 incubator in an atmosphere of 5% CO_2 and air mixture. After 72 h, 100 μL of lysis buffer containing 1 × concentration of SYBR Green I (Invitrogen) was added to each well and incubated for another 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 were transferred into a graphic program (EXCEL), and IC_{50} values were obtained by logit regression analysis using preprogrammed Excel spreadsheet.

In vitro cytotoxicity evaluation assay

Cytotoxicity of the compounds was carried out using Vero cell line (C1008; Monkey kidney fibroblast) following the method of Mosmann (46) with certain modifications. The cells were incubated with compound dilutions for 72 h, and MTT was used as reagent for the detection of cytotoxicity. Fifty percent cytotoxic concentration (CC_{50}) was determined using nonlinear regression analysis using preprogrammed Excel spreadsheet. Selectivity index was calculated as SI = CC_{50}/IC_{50}.

β-Hematin inhibitory (BHIA) 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 1844 × *g* 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 1–20 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 7378 × *g* 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 L of 2 N NaOH, and volume was made up to 1.0 mL with TDW. Absorbance was measured at 400 nm^3. The 50% inhibitory concentration (IC_{50}) was determined using nonlinear regression analysis dose–response curves (47).

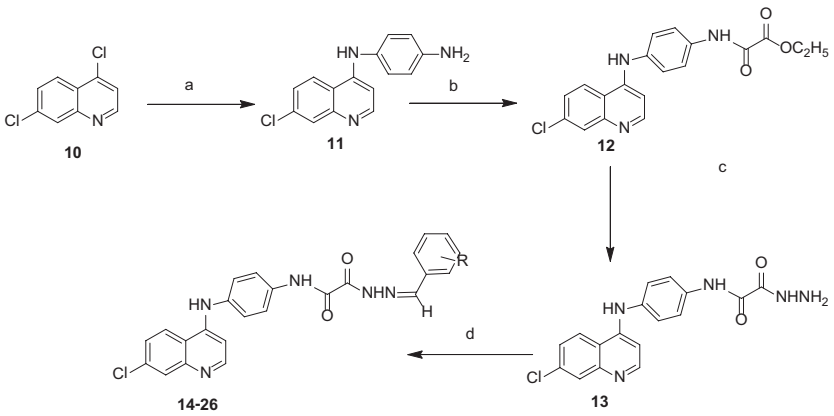
Results and Discussion

Chemistry

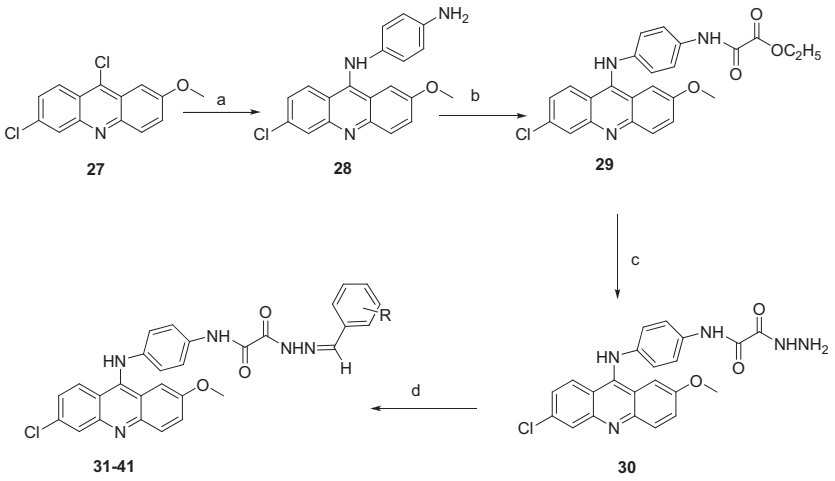
Proposed prototypes were prepared in economical way using inexpensive starting materials. A relatively straightforward synthetic approach (Schemes 1 and 2) was followed for the synthesis of target *N*-(4′-(7-chloroquinolin-4-ylamino)aryl)-2″-(benzylidene)hydrazinyl)-2″-oxoacetamide (**14**–**26**) and *N*-(4′-(6-chloro-2-methoxy-acridine-9-ylamino)phenyl)-2″-(benzylidene)hydrazinyl)-2″-oxoacetamide (**31**–**41**). The chemistry for the synthesis of compounds **11** and **28** was described previously (36,39). The so-formed diamines (**11**) and (**28**) on reaction with ethyl 2-chloro-2-oxoacetate give the corresponding oxoacetate, namely ethyl 2-(4-(7-chloroquinolin-4-ylamino)phenylamino)-2-oxoacetate (**12**) and ethyl 2-(4-(6-chloro-2-methoxyacridin-9-ylamino)phenylamino)-2-oxoacetate (**29**) in quantitative yield. These intermediates with hydrazine hydrate provide the corresponding hydrazinyl oxoacetamide, *N*-(4-(7-chloroquinolin-4-ylamino) phenyl)-2-hydrazinyl-2-oxoacetamide (**13**) and *N*-(4-(6-chloro-2-methoxyacridin-9-ylamino)phenyl)-2-hydrazinyl-2-oxoacetamide (**30**) in quantitative yield. Finally, this hydrazinyl oxoacetamide on condensation with various aldehydes led to the formation of desired prototypes (**14**–**26** and **31**–**41**) as given in Table 1.

In vitro *antimalarial evaluation*

Analogues **14**–**26** and **31**–**41**, based on 4-aminoquinoline and 9-anilinoacridine nuclei, were designed and synthesized for the study of SARs (structure–activity relationship). The *in vitro* antiplasmodial SAR initially involved in the

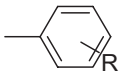


Scheme 1: Reagents and conditions: (a) *p*-phenylenediamine, *p*-TSA, EtOH, 3 h., (b) ethyl chlorooxoacetate, DMF, Et_3N, rt., (c) hydrazine hydrate 80%, EtOH, rt., (d) different aldehydes, EtOH, HCl, rt.



Scheme 2: Reagents and conditions: (a) *p*-phenylenediamine, *p*-TSA, EtOH, 3 h., (b) ethyl chlorooxoacetate, DMF, Et_3N, rt., (c) hydrazine hydrate 80%, EtOH, rt., (d) different aldehydes, EtOH, HCl, rt.

Table 1: *In vitro* antimalarial activity of compounds against 3D7 and K1 strains of *Plasmodium falciparum* and their cytotoxicity against Vero cell line

Compound no.	Linker (L)		IC_{50} (nM)[a]		SI[b]
			3D7	K1	
14	*p*-Phenylene diamine	4-Ethylphenyl	38.34	168.74	55.37
15	*p*-Phenylene diamine	4-Propylphenyl	37.07	110.18	131.81
16	*p*-Phenylene diamine	4-Isopropylphenyl	40.88	21.64	65.55
17	*p*-Phenylene diamine	4-*t*-butylphenyl	19.69	51.96	229.91
18	*p*-Phenylene diamine	2-Fluorophenyl	261.06	ND	15.87
19	*p*-Phenylene diamine	4-Fluorophenyl	43.94	155.03	146.10
20	*p*-Phenylene diamine	4-Bromophenyl	20.78	85.68	316.71
21	*p*-Phenylene diamine	4-Trifluoromethylphenyl	25.38	22.52	185.81
22	*p*-Phenylene diamine	4-Methoxyphenyl	91.75	185.83	39.63
23	*p*-Phenylene diamine	3,4,5-Trimethoxyphenyl	47.74	422.47	968.60
24	*p*-Phenylene diamine	4-Thiomethylphenyl	43.81	34.10	274.88
25	*p*-Phenylene diamine	3-Pyridyl	152.35	431.57	155.16
26	*p*-Phenylene diamine	2-Fluoro, 3-Pyridyl	81.47	293.67	155.68
31	*p*-Phenylene diamine	Phenyl	43.30	201.98	1569.97
32	*p*-Phenylene diamine	4-Isopropylphenyl	94.84	54.26	1866.02
33	*p*-Phenylene diamine	4-*t*-butylphenyl	42.43	53.05	2668.70
34	*p*-Phenylene diamine	2-Fluorophenyl	211.16	510.09	875.35
35	*p*-Phenylene diamine	4-Fluorophenyl	665.15	ND	43.87
36	*p*-Phenylene diamine	4-Bromophenyl	105.89	406.54	578.35
37	*p*-Phenylene diamine	4-Methoxyphenyl	NA	ND	ND
38	*p*-Phenylene diamine	3,4,5-Trimethoxyphenyl	627.83	ND	259.41
39	*p*-Phenylene diamine	4-Thiomethylphenyl	51.22	677.31	1065.71
40	*p*-Phenylene diamine	2-Fluoro, 3-Pyridyl	44.92	200.55	4106.77
41	*p*-Phenylene diamine	3-Indolyl	71.49	131.56	398.25
	CQ (mean ± SD)		4.16 ± 2.09	495.94 ± 127.1	8983.00

[a]IC_{50} (nM): concentration corresponding to 50% growth inhibition of the parasite.
[b]SI: (IC_{50} values of cytotoxic activity/IC_{50} values of antimalarial activity).

evaluation of analogues against the CQ-sensitive 3D7 strain and CQ-resistant K1 strain (MRA-159, MR4, ATCC, Manassas, Virginia) of *P. falciparum* using a standardized inexpensive assay based on SYBR Green I nucleic acid staining dye based fluorescence (MSF) assay as mentioned by Singh *et al.* (45). The *in vitro* antiplasmodial activities as indicated by their IC_{50} values are shown in Table 1. The test compounds showed superior or comparable activity to the control drug (CQ) against K1 strain of *P. falciparum*. However, compounds (**17**, **20,** and **21**) showed a good antimalarial activity with IC_{50}s ranging from 19.69 to 25.38 nM against the 3D7 strain. These results encourage us to prepare a series of compounds to investigate the antimalarial effect of various substituents of arylidene and heterocycles. Previously, we have reported that lipophilicity appears to be an important factor influencing

in vitro activity and selectivity (36). Furthermore, for the study of SAR, we synthesized the 9-anilinoacridine derivatives (**31**–**41**); all showed a good selectivity index except compounds **35** and **37** and were found to be non-toxic.

The 4-aminoaryl quinoline analogue **17**, having *t*-butyl group at the 4-position of arylidene moiety, showed the best potency among all the synthesized molecules having IC_{50} 19.69 nM against the CQ-sensitive strain. 4-bromo- and 4-trifluoromethyl-substituted analogues have shown the comparable potency (**20**, IC_{50} = 20.78 nM; **21**, IC_{50} = 25.38 nM). Alkyl groups such as ethyl, propyl, and isopropyl at the para position of arylidene equally affect the antimalarial potency of their corresponding analogues **14**, **15**, and **16** (IC_{50} = 38.34 nM; IC_{50} = 37.07 nM; IC_{50} = 40.88 nM). Para-fluoro-substituted compound **19** showed moderate activity (IC_{50} = 43.94 nM), whereas ortho-fluoro-substituted analogue **18** found to be inactive (IC_{50} = 261.06 nM). In the quinoline series (**14**–**26**), the arylidene substituent gave rise to compounds generally more active than the heterocyclic counterparts. In particular, the introduction of a 3-pyridyl ring (**25**) led to a twofold drop in potency (IC_{50} = 152.35 nM) compared with the compound bearing 2-fluoro-3-pyridyl moiety, IC_{50} = 81.47 nM (**26**, Table 1).

To validate our hypothesis, we exploited the effect of *p*-phenylenediamine linker on antimalarial potency of acridinyl Schiff base system (**31**–**41**). Compound **31**, having unsubstituted arylidene moiety, showed the IC_{50} = 43.30 nM. Introduction of electron releasing groups such as *t*-butyl at the para position of arylidene moiety **33** showed the reasonable activity (IC_{50} = 42.43 nM), whereas an isopropyl group reduced the antiplasmodial activity of molecule **32** (IC_{50} = 94.84 nM). In contrast, a thiomethyl group on the arylidene increased the activity of the analogue **39**, IC_{50} = 51.22 nM as compared to the **32**. Compound **40** bearing the 2-fluoro-3-pyridyl as part of R showed better antimalarial activity (IC_{50} = 44.92 nM) than the compound **26** with the same substituent (IC_{50} = 81.47 nM). Replacement of 2-fluoro-3-pyridyl with 3-Indolyl, significantly lowered the activity of analogue **41**, IC_{50} = 71.49 nM. Analogously, introduction of the 4-Br, 2-F, and 4-F substituents in **36**, **34**, and **35** dropped down the activity against the strains tested.

On further screening of molecules against the K1 strain of *P. falciparum*, we found several molecules showed greater potency than the standard (CQ). Among these, analogues **16**, **17**, **21**, **24**, **32**, and **33** showed many fold better activity with IC_{50} ranging from 21.64 to 54.26 nM, as compared to CQ (IC_{50} = 495.94 nM) (Table 1). Compounds **16** and **21** having the isopropyl and trifluoromethyl substituents at the 4-position of arylidene moiety were the most prominent in growth inhibition with IC_{50} value of 21.64 and 22.52 nM. Whereas, compounds **32** and **33** with the same substituents showed moderate activity (IC_{50} = 54.26 nM and 53.05 nM, respectively). Compounds **17** and **24**, which contained the 4-*t*-butyl and 4-thiomethyl substituents as part of R, exhibited good potency, IC_{50} = 51.96 and 34.10 nM, respectively. Therefore, SAR studies showed that substitution at the para position is essential for antiplasmodial activity.

Table 2: β-hematin inhibitory activity of selected molecules[a]

Compound no.	IC_{50} (μg/mL)	Compound no.	IC_{50} (μg/mL)
14	3.43	**26**	3.48
15	3.92	**31**	5.25
16	2.80	**33**	4.71
17	2.70	**39**	10.2
19	6.90	**40**	6.10
20	5.98	**41**	2.68
21	3.35	**CQ**	3.80
22	5.41		
23	3.14		
24	2.96		

[a]The IC_{50} represents the concentration of compound that inhibit β-hematin formation by 50%.

The cytotoxicity of all the synthesized hybrid molecules (**14**–**26**) and (**31**–**41**) was carried out using Vero cell line (C1008; monkey kidney fibroblast) following the method of Mosmann (46) with certain modifications. The cells were incubated with compound dilutions for 72 h, and MTT was used as reagent for the detection of cytotoxicity. The 50% cytotoxic concentration (CC_{50}) was determined using nonlinear regression analysis using preprogrammed Excel spreadsheet. Most of the compounds have low cytotoxicity with fairly high selectivity index. Eight compounds **23**, **31**–**34**, **36**, **39**, and **40** exhibited good selectivity having SI value ranging from 578.35 to 4106.77. Compound **40** showed the highest selectivity index (SI = 4106.77) among the tested compounds. Therefore, these compounds demonstrated the promising safe and good activity profile for further lead optimization (Table 1).

β-hematin inhibitory activity

Moreover, to find out the mechanism of inhibition, several molecules found to be active in primary screening were further evaluated for β-hematin inhibitory activity using the β-hematin inhibitory (BHIA) assay as previously described (47). The 50% inhibitory concentration (IC_{50}) was determined using nonlinear regression analysis dose–response curves. Eight compounds **14**, **16**, **17**, **21**, **23**, **24**, **26**, and **41** showed a better dose dependent inhibition in the BHIA assay (IC_{50} ranging from 2.68 to 3.72 μg/mL) than CQ (IC_{50} = 3.80 μg/mL) in inhibiting hemozoin formation as depicted in Table 2.

Conclusion

In summary, a novel series of 4-aminoquinolinyl and 9-anilinoacridinyl Schiff base hydrazones with oxalamide functionality have been identified. *In vitro* inhibitory effects

of compounds proved their better effectiveness against the K1 strain of *P. falciparum*. Moreover, compounds were able to maintain safe selectivity profile and excellent β-hematin inhibitory activity. We believe that these derivatives might be an innovative avenue for the treatment of multi-drug-resistant malaria and open up possibilities for further derivatization in future.

Acknowledgments

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Conflict of Interest

The authors declare no competing financial interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. General procedure for the synthesis of compounds **12**, **29**, **13**, **30**, **14**–**26** and **31**–**41** and the characterization of compounds ^{1}H, ^{13}C and CHN data for **14**–**26** and **31**–**41**.