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Preliminary communication

Discovery of highly selective 7-chloroquinoline-thiohydantoins with potent antimalarial activity

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ABSTRACT

A series of C-3 thiourea functionalized β-lactams, β-lactam-7-chloroquinoline conjugates and 7-chloroquinoline-thiohydantoin derivatives were prepared with the aim of probing antimalarial structure–activity relationships. 7-Chlorquinoline-thiohydantoin derivatives were found to be potent inhibitors of cultured *Plasmodium falciparum*, with the most potent and non-cytotoxic compound exhibiting an IC_{50} of 39.8 nM. Studies of β-hematin formation suggested that inhibition of haemozoin formation could be primary mechanism of action, with IC_{50} values comparable to those of chloroquine. Evaluation of cytotoxicity against HeLa cells demonstrated high selective indices.

1. Introduction

Malaria is considered to be the most prevalent human parasitic disease and remains as one of the world's greatest health challenges. About 2.2 billion people live in malaria endemic areas, and the World Health Organization (WHO) estimated that 207 million cases and 627,000 deaths occurred in 2012, mostly in young children [1]. This disease is caused by mosquito-borne protozoa of the genus *Plasmodium*. Among the Plasmodial species, *Plasmodium falciparum* is the most problematic, due to its high prevalence, virulence and drug resistance and is responsible for most malaria-related deaths [2]. Antimalarial drugs with a quinoline scaffold were extensively utilized for the treatment of malaria [3]. However, the emergence of resistance to chloroquine (CQ) and other drugs has limited their utility and are no longer administered in most countries [4]. CQ resistance is mainly attributed to mutations in the *P. falciparum* chloroquine resistance transporter gene (PfCRT), with the mutant protein mediating the export of the drug out of digestive vacuole of the parasite and hence away from its site of action [5]. The newer drugs of choice for the treatment of infection with *P. falciparum* are now artemisinin-based combination therapies (ACTs). ACTs contain derivatives of the natural endoperoxide compound artemisinin (artemether, artesunate and dihydroartemisinin), which are potent and fast acting antimalarials. These are combined with longer acting partner drugs that kill parasites that are not cleared by artemisinins and help to prevent selection of drug resistant parasites [6]. However, recent reports of delayed parasite clearance after treatment with ACTs in southeast Asia are disconcerting, and have provided the impetus for the discovery of new antimalarials to feed the preclinical pipeline [7]. Among existing pharmacophore templates, chloroquinoline class of therapeutics remain successful for combating malaria even after several decades of drug development efforts due to its excellent clinical efficacy, ease of administration, low toxicity, and low cost synthesis [8].

Historically, a constrained β-lactam ring is a feature of an important class of antibiotics, after the discovery of naturally occurring penicillins and cephalosporins [9]. A broad spectrum of biological properties has also been associated with β-lactams, including inhibition of HIV-1 protease [10], inhibition of cholesterol

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absorption [11], antiviral [12], antitumor [13] and antimalarial activity [14]. Recent studies have also identified the β-lactam moiety as a versatile synthon for the preparation of diversely functionalized heterocycles [15,16].

The introduction of urea, oxalamide, and thiourea functionalities in 4-aminoquinolines has been shown to enhance antimalarial activity, which may be attributed to their ability to form hydrogen bonds. In addition to their inhibition of β-hematin formation, these derivatives have also been identified as potent antimalarials targeting the dihydrofolate reductase (DHFR) enzyme, a well defined and widely exploited target in antimalarial chemotherapy [17,18]. Recent contributions from our group have shown the potential of β-lactam-4-aminoquinoline conjugates as antimalarial agents. A diverse range of linkers *viz.* amide [19] and non-ionizable covalent bonds [19], urea [20] and oxalamide [20] functionalities have been introduced in such hybrids along with well modulated alkyl chain length between the two amino functionality at the C-4 position of the quinoline ring. From these studies, the most potent and non-cytotoxic conjugate, with an optimum combination of *N*-cyclohexyl substituent at *N*-1, alkyl chain length ($n = 6$) and oxalamide functionality exhibited an IC_{50} of 39.8 nM. Encouraged by these results, we describe the synthesis and antimalarial evaluation of thiourea tethered 7-chloroquinoline-β-lactam conjugates and 7-chloroquinoline-thiohydantoin analogs, based on the established antimalarial potential of the thiohydantoin structural motif [21].

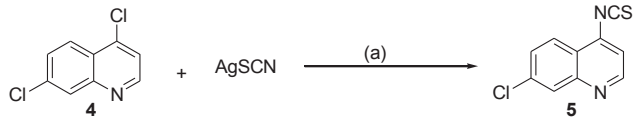
2. Result and discussions

2.1. Synthetic chemistry

The treatment of precursor 3-isothiocyanato-2-azetidinones **1**, synthesized *via* Staudinger reaction of 3-azido-β-lactam with triphenylphosphine and carbon disulfide, with primary aliphatic/aromatic amines resulted in the isolation of corresponding thioureas **2**. Sodium methoxide promoted tandem intramolecular amidolysis-β-elimination led to the formation of corresponding thiohydantoins **3** in good to excellent yields (Scheme 1).

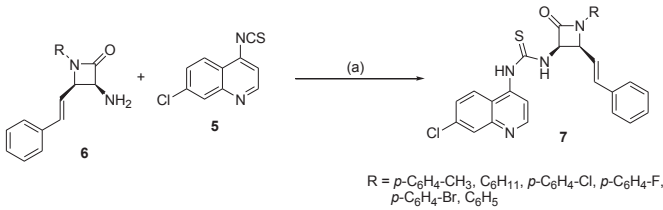
For the synthesis of thiourea-tethered 7-chloroquinoline-β-lactam conjugates, the precursor **5** was synthesized by refluxing of 4,7-dichloroquinoline **4** with silver thiocyanate in anhydrous toluene at 120 °C for 18 h (Scheme 2) [22]. The synthesized 7-chloroquinoline isothiocyanate **5**, upon treatment with variedly substituted 3-amino-2-azetidinones **6**, prepared *via* Staudinger reaction of *N*-substituted 1-azadiene with azidoketene generated *in situ* from azido acetic acid and *p*-toluene sulphonyl chloride in the presence of triethylamine with subsequent reduction using Zn/NH_4Cl in EtOH: H_2O (9.5:0.5) mixture, in dry acetone at room temperature, gave the desired thiourea-tethered 7-chloroquinoline-β-lactam hybrids **7** in excellent yields (Scheme 3).

We also considered synthesizing analogs of 7-chloroquinoline-β-lactam thioureas having varied alkyl chain length at the C-4 position of the quinoline ring, since the introduction of well



Reagents and conditions: (a) Anhydrous toluene, 120 °C, 18 h

Scheme 2. Synthesis of 7-chloroquinoline based isothiocyanate **5.**



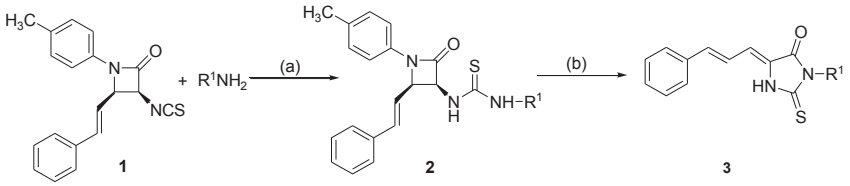
Reagents and conditions: (a) Dry acetone, rt, 10 min.

Scheme 3. Synthesis of thiourea tethered 7-chloroquinoline-β-lactam conjugates **7a**–**7f.**

modulated alkyl chain length has been shown to influence antimalarial efficacy [23]. Interestingly, room temperature stirring of **1** with **8** in $CHCl_3$:MeOH (9:1) mixture resulted in the isolation of corresponding 7-chloroquinoline-thiohydantoin derivatives **10**, instead of the expected thiourea-tethered β-lactam-7-chloroquinoline conjugates **9** (Scheme 4).

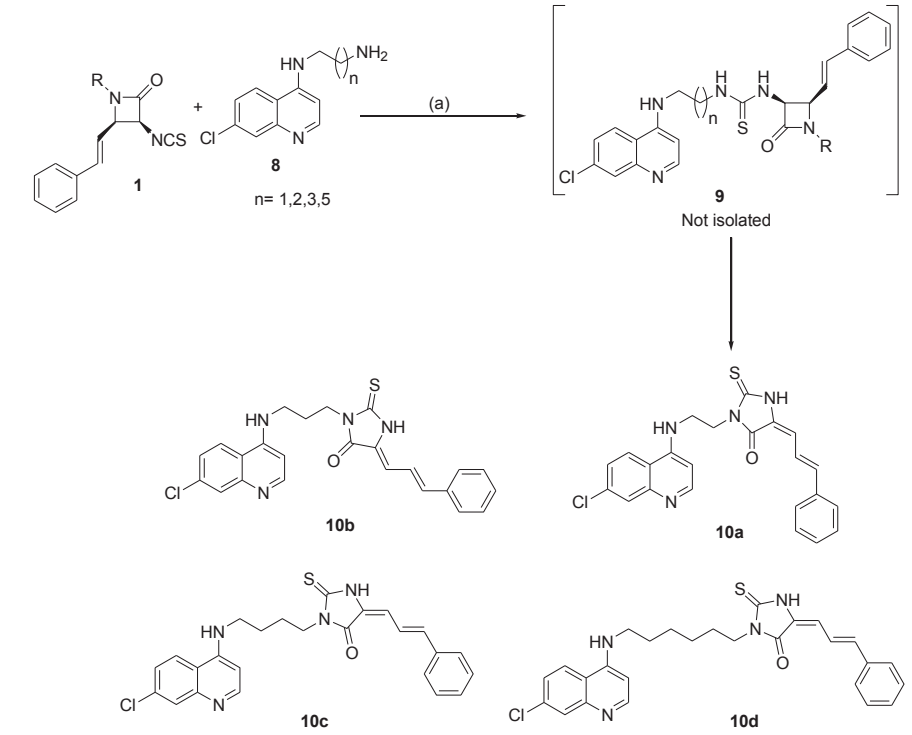
The structures of the synthesized hybrids were assigned based on spectral and analytical data described in detail in the experimental section. Some salient features are discussed here. The compound **10b**, for example, showed a molecular ion peak $[M+H]^+$ at 449.1134 in its mass spectrum. Its ^1H NMR spectrum exhibited the presence of multiplets at δ 1.76, 3.34 and 3.82 corresponding to methylene protons, a doublet at δ 7.12 with coupling constant of 15.3 Hz corresponding to H^1, along with the characteristic quinoline ring protons. The assigned s-*trans* configuration of the synthesized 7-chloroquinoline-thiohydantoins is based on the observed coupling constant, $J = 12$ Hz between H^2 and H^3. The presence of characteristic absorption at δ 176.2 corresponding to the thiocarbonyl carbon of the thiohydantoin ring along with the requisite number of carbons in its ^{13}C NMR spectrum further confirmed the assigned structure.

The plausible mechanism for the formation of **10** in a single pot synthesis is depicted in Scheme 5 and may involve an initial formation of thiourea **9** with subsequent proton abstraction by free amine from the α-carbon of the β-lactam ring to generate the ketene intermediate **11**. The ketene intermediate **11** formed then underwent tandem intramolecular cyclization-enolization-β-elimination to result in the formation of corresponding 3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one **10a**. The above mechanism was further



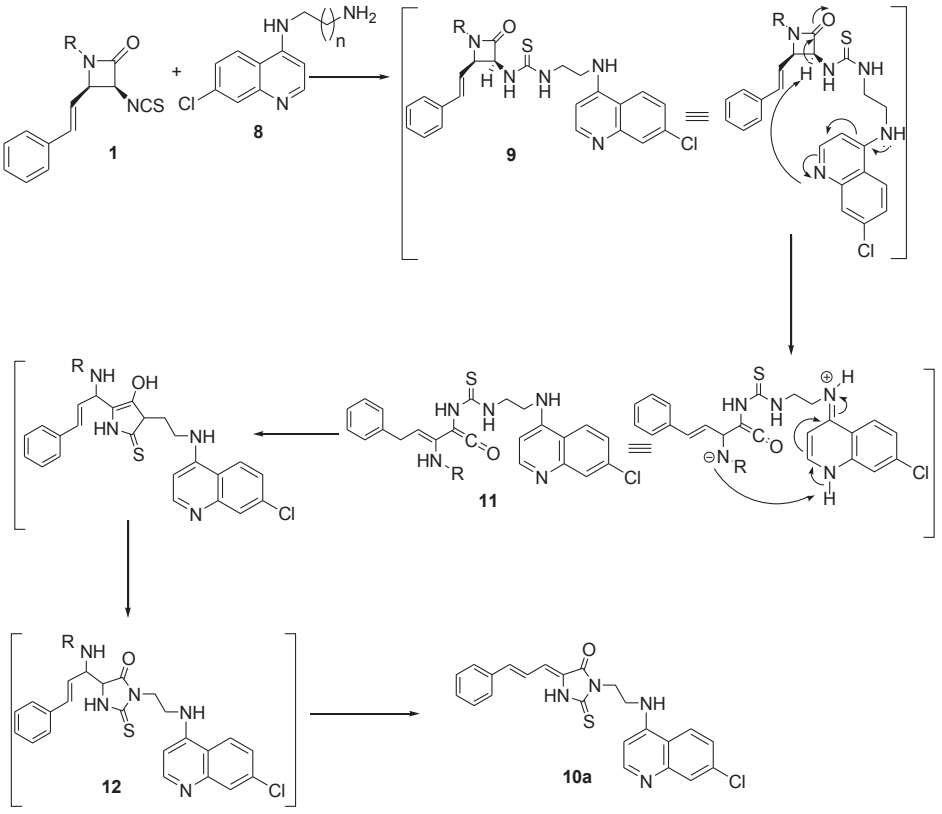
Reagents and conditions: (a) Dry acetone, rt, 5-10 min.; (b) NaOMe, dry MeOH, rt, 50-60 min.

Scheme 1. Synthesis of thiourea-based β-lactams **2a**–**2e** and their corresponding thiohydantoins **3a**–**3e**.

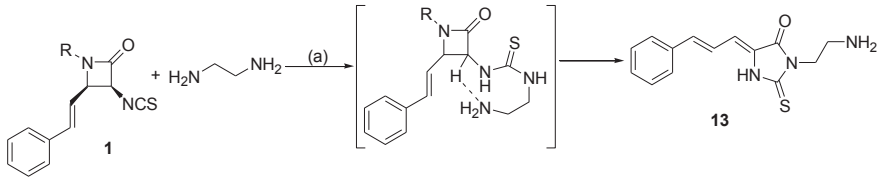


Reagents and conditions: (a) Dry $CHCl_3$:MeOH (9:1), rt, 10 min.

Scheme 4. Synthesis of 7-chloroquinoline linked thiohydantoins **10a**–**10d**.



Scheme 5. Mechanistic pathway for the formation of 3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one **10a**.



Reagents and conditions: (a) Dry acetone, rt, 10 min.

Scheme 6. Synthesis of thioimidazolidine-4-one with diamine **13**.

validated by reacting **1** with diamino-ethane, leading to the synthesis of the corresponding thiohydantoin *via* an eight membered transition state, as depicted in Scheme 6.

2.2. In vitro anti-plasmodial, inhibition of β-haemozoin formation and cytotoxic evaluation

Synthesized compounds were evaluated for their *in vitro* antiplasmodial profiles against CQ-resistant (W2 strain) *P. falciparum*. The C-3 thiourea tethered β-lactams **2** and their corresponding thiohydantoins **3** and **13** did not demonstrate any antimalarial activity (Table 1). The introduction of a 7-chloroquinoline ring in thiourea-tethered β-lactam-7-chloroquinoline conjugates **7** failed to improve their antimalarial activity. However, 7-chloroquinoline based thiohydantoins **10** showed good antimalarial activities, with IC_{50} values ranging from 39 to 386 nM. Structure–activity relationships in these conjugates showed clear dependence upon the length of the alkyl chain between two amino groups present at the C-4 position of the quinoline ring. The compounds with shorter alkyl chains showed good antimalarial efficacy; the activity decreased 10-fold with $n = 5$ (**10d**).

Three of the most potent of the test compounds *viz.* **10a**–**10c** were examined for inhibitory activity against β-hematin formation. These conjugates inhibited β-hematin formation with IC_{50} values comparable to that of chloroquine (Table 2). A statistically significant linear correlation between the length of the chain (C atoms between the thioxoimidazolidinone and the quinoline) and the IC_{50} for inhibition of β-hematin formation was observed (Fig. 1). A possible explanation for this observation could be due to a decrease in the π–π stacking ability of heme with the quinoline moiety when there is an increase in molecular flexibility. The molecule with lowest number of carbon atoms has the least degrees of rotational freedom and is more conformationally restrained. Since π–π stacking is an interaction between planar molecules, restriction in rotation leads to lower entropy loss when this interaction takes place, resulting in better inhibitory activity. The data suggest that inhibition of haemozoin formation could be the primary mechanism of action of these compounds against *P. falciparum*.

Cytotoxicity of three most potent compounds *viz.* **10a**–**10c** was assessed against mammalian HeLa cells. As depicted in Table 3, compounds were non-cytotoxic against mammalian cells and therefore had selectivity for inhibition of *P. falciparum*.

In conclusion, we describe herein the β-lactam-synthon interceded synthesis and characterization of highly selective and potent 7-chloroquinoline-thiohydantoin derivatives. Some of these compounds demonstrated nanomolar antimalarial activity, with evidence strongly supporting the mechanism of action *via* inhibition of β-hematin formation with minimal toxicity to mammalian cells.

Table 1
Antimalarial activity results of test compounds.

Compound	R	R^1	*n*	*P. falciparum* W2 (CQ-R) strain[a] IC_{50} (nM)
2a	–	*n*-C_4H_9	–	>10,000
2b	–	*i*-C_4H_9	–	>10,000
2c	–	*p*-C_6H_4–CH_3	–	>10,000
2d	–	C_6H_{11}	–	>10,000
2e	–	C_6H_5	–	>10,000
3a	–	n-C_4H_9	–	>10,000
3b	–	*i*-C_4H_9	–	>10,000
3c	–	*p*-C_6H_4–CH_3	–	>10,000
3d	–	C_6H_{11}	–	>10,000
3e	–	C_6H_5	–	>10,000
7a	*p*-C_6H_4–CH_3	–	–	>10,000
7b	C_6H_{11}	–	–	6134
7c	*p*-C_6H_4–Cl	–	–	8752
7d	*p*-C_6H_4–F	–	–	>10,000
7e	C_6H_5	–	–	>10,000
7f	*p*-C_6H_4–Br	–	–	5723
10a	–	–	1	39.84
10b	–	–	2	42.54
10c	–	–	3	57.21
10d	–	–	5	386.9
13	–	–	–	>10,000
Chloroquine (CQ)				99.0
Artemisinin (ART)				14.0

[a] CQ-R: Chloroquine resistant strain.

3. Experimental section

Melting points were determined by open capillary using a Veego Precision Digital Melting Point apparatus (MP-D) and are uncorrected. IR spectra were recorded on a Shimadzu D-8001 spectrophotometer. ^1H NMR spectra were recorded in deuterochloroform and dimethylsulfoxide-d_6 with a Jeol 300 (300 MHz) spectrometer using TMS as an internal standard. Chemical shift values are expressed as parts per million downfield from TMS and *J* values are in hertz. Splitting patterns are indicated as s: singlet, d: doublet, t: triplet, m: multiplet, dd: double doublet, ddd: doublet of a doublet of a doublet, and br: broad peak. ^{13}C NMR spectra were recorded on Jeol 300 (75 MHz) spectrometers in dimethylsulfoxide using TMS as internal standard. High resolution mass spectra were recorded on Bruker-micrOTOF-Q II spectrometer.

Table 2
IC_{50} for inhibition of β-hematin formation with standard errors.

Compound	*n*	IC_{50} ± standard error (β-haemozoin inhibition) in μM
10a	1	13.86 ± 0.58
10b	2	19.85 ± 0.52
10c	3	28.35 ± 0.89
CQ	–	20.06 ± 1.85

3.1. General procedure for the synthesis of thiourea-based β-lactams (**2a**–**2e**)

To a stirred solution of 2-isothiocyanate-azetidin-2-ones, **1** (1 mmol) was added a solution of aryl/alkyl amine (1 mmol) in dry acetone (20 mL). The reaction mixture was allowed to stir at room temperature for 15–20 min and the progress was monitored by using TLC. The solvent was removed under vacuum resulted in a crude solid which was further recrystallized using diethyl ether to yield the desired product **2**.

3.1.1. 1-Butyl-3-(2-oxo-4-styryl-1-p-tolyl-azetidin-3-yl)-thiourea (**2a**)

White Solid; Yield: 92%; m.p. 162–163 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 0.74 (t, J = 7.2 Hz, 3H, $-CH_3$), 1.17–1.22 (m, 2H, $-CH_2$), 1.42–1.54 (m, 2H, $-CH_2$), 2.35 (s, 3H, CH_3), 3.39–3.51 (m, 2H, $-N-CH_2-$), 4.62 (dd, J = 4.5, 6.3 Hz, 1H, H^2), 4.92 (dd, J = 4.5, 7.2 Hz, 1H, H^1), 6.09 (dd, J = 6.3, 15.0 Hz, 1H, H^3), 6.64 (d, J = 15.0 Hz, 1H, H^4), 6.94 (d, J = 8.4 Hz, 2H, ArH), 7.12 (d, J = 8.4 Hz, 2H, ArH), 7.28–7.40 (m, 5H, ArH), 7.84 (d, J = 7.2 Hz, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 12.4, 18.7, 20.4, 28.6, 31.7, 59.4, 63.1, 126.3, 127.2, 127.5, 128.4, 128.8, 129.6, 133.4, 134.7, 137.7, 139.2, 171.4, 179.6; HRMS Calculated for $C_{25}H_{29}N_3OS$ $[M+H]^+$ 394.1875 found 394.1869; Anal. Calcd (%) for: C, 70.19; H, 6.92; N, 10.68, found: C, 70.25; H, 6.83; N, 10.79.

3.1.2. 1-Isobutyl-3-(2-oxo-4-styryl-1-p-tolyl-azetidin-3-yl)-thiourea (**2b**)

White Solid; Yield: 90%; m.p. 159–160 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.07 (d, J = 7.2 Hz, 6H, 2×$-CH_3$), 2.36 (s, 3H, $-CH_3$), 2.52–2.57 (m, 1H, −CH), 3.28–3.35 (m, 2H, $-N-CH_2$), 4.64 (dd, J = 4.8, 6.3 Hz, 1H, H^2), 4.94 (dd, J = 4.8, 7.5 Hz, 1H, H^1), 6.06 (dd, J = 6.3, 15.6 Hz, 1H, H^3), 6.62 (d, J = 15.6 Hz, 1H, H^4), 6.90 (d, J = 8.1 Hz, 2H, ArH), 7.10 (d, J = 8.1 Hz, 2H, ArH), 7.26–7.39 (m, 5H, ArH), 7.86 (d, J = 7.5 Hz, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 19.5, 20.5, 33.5, 42.4, 59.5, 63.2, 126.4, 127.1, 127.8, 128.2, 128.8, 129.7, 133.5, 134.6, 137.9, 139.3, 171.5, 179.4; HRMS Calculated for $C_{25}H_{29}N_3OS$ $[M+H]^+$ 394.1875 found 394.1883; Anal. Calcd (%) for: C, 70.19; H, 6.92; N, 10.68 found: C, 70.31; H, 6.97; N, 10.72.

3.1.3. 1-(2-Oxo-4-styryl-1-p-tolyl-azetidin-3-yl)-3-p-tolyl-thiourea (**2c**)

White Solid; Yield: 94%; m.p. 151–152 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.32 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 4.60 (dd, J = 4.5, 6.3 Hz, 1H, H^2), 4.92 (dd, J = 4.5, 7.2 Hz, 1H, H^1), 6.04 (dd, J = 6.3, 15.3 Hz, 1H, H^3), 6.64 (d, J = 15.3 Hz, 1H, H^4), 6.75 (d, J = 8.4 Hz, 2H, ArH), 6.80–6.98 (m, 4H, ArH), 7.12 (d, J = 8.4 Hz, 2H, ArH), 7.18–7.32 (m, 5H, ArH), 7.82 (d, J = 7.2 Hz, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 20.4, 20.9, 59.5, 63.1, 120.6, 123.4, 124.5, 125.8, 126.7, 127.6, 127.8, 128.5, 128.9, 129.4, 133.7, 134.3, 137.2, 139.9, 171.2, 179.6; HRMS Calculated for $C_{26}H_{25}N_3OS$ $[M+H]^+$ 428.1718 found 428.1725; Anal. Calcd (%) for: C, 73.04; H, 5.89; N, 9.83 found: C, 72.96; H, 5.96; N, 9.76.

3.1.4. 1-Cyclohexyl-3-(2-oxo-4-styryl-1-p-tolyl-azetidin-3-yl)-thiourea (**2d**)

White Solid; Yield: 91%; m.p. 148–149 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.19–2.36 (m, 10H, cyclohexyl-H), 2.38 (s, 3H, CH_3), 4.50–4.60 (m, 1H, cyclohexyl-H), 4.64 (dd, J = 4.8, 6.0 Hz, 1H, H^2), 4.92 (dd, J = 4.8, 7.2 Hz, 1H, H^1), 6.06 (dd, J = 6.0, 15.3 Hz, 1H, H^3), 6.62 (d, J = 15.3 Hz, 1H, H^4), 6.90 (d, 2H, ArH), 7.10 (d, J = 8.1 Hz, 2H, ArH), 7.20–7.32 (m, 5H, ArH), 7.84 (d, J = 7.2 Hz, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 20.7, 24.5, 25.6, 28.9, 56.6, 59.9, 63.6, 126.6, 127.2, 127.8, 128.3, 128.9, 129.7, 133.4, 134.7, 137.3, 139.4, 171.4, 179.6; HRMS Calculated for $C_{25}H_{29}N_3OS$ $[M+H]^+$ 420.2031 found 420.2026; Anal. Calcd (%) for: C, 71.56; H, 6.97; N, 10.01 found: C, 71.44; H, 6.90; N, 10.10.

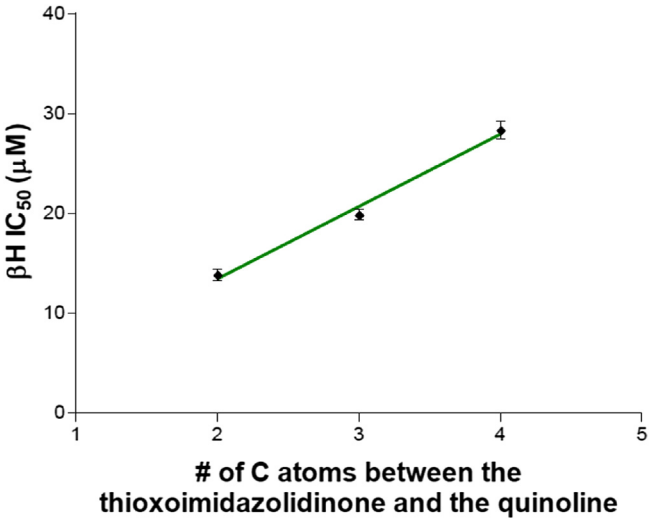


Fig. 1. Linear correlation between the length of the chain and βHi IC_{50} value.

3.1.5. 1-(4-Fluoro-phenyl)-3-(2-oxo-4-styryl-1-p-tolyl-azetidin-3-yl)-thiourea (**2e**)

White Solid; Yield: 86%; m.p. 158–159 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.34 (s, 3H, CH_3), 4.62 (dd, J = 4.8, 6.3 Hz, 1H, H^2), 4.94 (dd, J = 4.8, 7.2 Hz, 1H, H^1), 6.02 (dd, J = 6.3, 15.6 Hz, 1H, H^3), 6.70 (d, J = 15.6 Hz, 1H, H^4), 6.85–6.94 (m, 4H, ArH), 7.02 (d, J = 8.4 Hz, 2H, ArH), 7.15 (d, J = 8.1 Hz, 2H, ArH), 7.20–7.32 (m, 5H, ArH), 7.84 (d, J = 7.2 Hz, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 20.7, 59.8, 63.6, 120.7, 123.1, 124.7, 125.6, 126.4, 127.4, 127.9, 128.5, 128.8, 129.9, 133.6, 134.8, 137.5, 139.6, 171.6, 179.7; HRMS Calculated for $C_{25}H_{22}N_3OSF$ $[M+H]^+$ 432.1461 found 432.1472; Anal. Calcd (%) for: C, 69.58; H, 5.14; N 9.74 found: C, 69.50; H, 5.17; N, 9.68.

3.2. General procedure for the synthesis of β-lactam based thiohydantoins (**3a**–**3e**)

To the stirred solution of thiourea **2** (1 mmol) in dry methanol was added a solution of sodium methoxide (1 mmol) in dry methanol. The reaction mixture was allowed to stir at room temperature for 50–60 min and the progress was monitored by using TLC. On completion, the crude product was precipitated out, filtered and then recrystallized to yield 3-aryl/alkyl-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one **3** as yellow crystals using a mixture of ethylacetate:hexane (6:4).

3.2.1. 3-Butyl-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**3a**)

Yellow Solid; Yield: 89%; m.p. >220 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 0.74 (t, J = 7.5 Hz, 3H, $-CH_3$), 1.17–1.22 (m, 2H, $-CH_2$), 1.43–1.50 (m, 2H, $-CH_2$), 3.40–3.54 (m, 2H, $-N-CH_2$), 6.54 (d, J = 11.4 Hz, 1H, H^3), 7.08 (dd, J = 11.4, 15.6 Hz, 1H, H^2), 7.19 (d, J = 15.6 Hz, 1H, H^1), 7.34–7.52 (m, 5H, ArH), 12.60 (s, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 12.4, 18.7, 28.7, 31.8, 115.2, 121.6, 125.6, 127.3, 128.9, 129.2, 134.4, 142.3, 166.6, 178.2; HRMS Calculated for $C_{16}H_{18}N_2OS$ $[M+H]^+$ 287.1140 found 287.1130; Anal. Calcd (%) for: C, 67.10; H, 6.33; N, 9.78 found: C, 67.19; H, 6.44; N, 9.85.

Table 3
Cytotoxicity and selective index of conjugates **10a**–**10c**.

Compound	Cytotoxicity[a]	*P. falciparum*	SI[c]
	IC_{50} ± standard error (μM)	W2 (CQ-R) strain[b] IC_{50} (nM)	
10a	72 ± 6.22	39.84	1807
10b	91 ± 8.12	42.54	2139
10c	>100	57.21	>1747

[a] CQ-R: Chloroquine resistant strain.
[b] Cytotoxicity against HeLa cell line.
[c] SI: Selective index is ratio of IC_{50} of HeLa cell line to that of W2-resistant strain.

3.2.2. 3-Isobutyl-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**3b**)

Yellow Solid; Yield: 84%; m.p. >220 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.03 (d, J = 7.5 Hz, 6H, 2×−CH_3), 2.52 (m, 1H, −CH), 3.28 (m, 2H, −N−CH_2), 6.50 (d, J = 11.7 Hz, 1H, H^3), 7.06 (dd, J = 11.7, 15.3 Hz, 1H, H^2), 7.18 (d, J = 15.3 Hz, 1H, H^1), 7.32–7.50 (m, 5H, ArH), 12.61 (s, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 19.3, 33.6, 42.7, 115.3, 121.6, 125.8, 127.4, 128.9, 129.3, 134.6, 142.1, 166.4, 178.1; HRMS Calculated for $C_{16}H_{18}N_2OS$ $[M+H]^+$ 287.1140 found 287.1135; Anal. Calcd (%) for: C, 67.10; H, 6.33; N, 9.78 found: C, 67.22; H, 6.43; N, 9.64.

3.2.3. 5-(3-Phenyl-allylidene)-2-thioxo-3-p-tolyl-imidazolidin-4-one (**3c**)

Yellow Solid; Yield: 88%; m.p. >220 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.35 (s, 3H, CH_3), 6.56 (d, J = 11.7 Hz, 1H, H^3), 7.04 (dd, J = 11.7, 15.0 Hz, 1H, H^2), 7.14 (d, J = 15.0 Hz, 1H, H^1), 7.26–7.80 (m, 9H, ArH), 12.58 (s, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 20.5, 112.6, 113.9, 114.4, 120.7, 125.7, 125.8, 127.2, 127.6, 128.5, 129.1, 134.6, 138.4, 164.3, 175.3; HRMS Calculated for $C_{19}H_{16}N_2OS$ $[M+H]^+$ 321.0983 found 321.0990; Anal. Calcd (%) for: C, 71.22; H, 5.03; N, 8.74 found: C, 71.13; H, 5.10; N, 8.66.

3.2.4. 3-Cyclohexyl-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**3d**)

Yellow Solid; Yield: 90%; m.p. >220 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.19–2.39 (m, 10H, cyclohexyl-H), 4.51–4.62 (m, 1H, cyclohexyl-H), 6.52 (d, J = 11.7 Hz, 1H, H^3), 7.02 (dd, J = 11.7, 15.0 Hz, 1H, H^2), 7.16 (d, J = 15.0 Hz, 1H, H^1), 7.30–7.75 (m, 5H, ArH), 12.62 (s, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 24.7, 25.9, 28.7, 56.6, 115.4, 121.5, 125.8, 127.51, 128.9, 129.5, 134.7, 142.0, 166.5, 178.2; HRMS Calculated for $C_{18}H_{20}N_2OS$ $[M+H]^+$ 313.1296 found 313.1291; Anal. Calcd (%) for: C, 69.20; H, 6.45; N, 8.97 found: C, 69.27; H, 6.55; N, 8.90.

3.2.5. 3-(4-Fluoro-phenyl)-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**3e**)

Yellow Solid; Yield: 87%; m.p. >220 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 6.55 (d, J = 12.0 Hz, 1H, H^3), 7.05 (d, J = 15.0 Hz, 1H, H^1), 7.22–7.69 (m, 10H, H^2 + ArH), 12.50 (s, 1H, −NH-exchangeable with D_2O); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 112.5, 113.9, 114.2, 120.8, 125.6, 125.9, 127.2, 127.4, 128.9, 129.0, 134.8, 138.6, 161.3, 174.5; HRMS Calculated for $C_{18}H_{13}N_2OFS$ $[M+H]^+$ 325.0733 found 325.0742; Anal. Calcd (%) for: C, 66.65; H, 4.04; N, 8.64 found: C, 66.71; H, 4.16; N, 8.50.

3.3. General procedure for the synthesis of thiourea tethered 7-chloroquinoline-β-lactam conjugates (**7a**–**7f**)

To a stirred solution of compound **5** (1 mmol) in dry acetone, 3-amino-2-azetidinone **6** (1 mmol) was added. The reaction mixture was stirred at room temperature for 10 min, after which a yellow colored solid precipitated out in reaction mixture. This crude product was filtered and purified by recrystallization in $CHCl_3$:Hexane (9:1) to afford pure compound **7**.

3.3.1. 1-(7-Chloro-quinolin-4-yl)-3-(2-oxo-4-styryl-1-p-tolyl-azetidin-3-yl)-thiourea (**7a**)

Yellow Solid; Yield: 89%; m.p. 151–152 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.23 (s, 3H, −CH_3); 5.01 (t, J = 5.7 Hz, 1H, H^3); 5.90 (s, 1H, H^4); 6.24 (dd, J = 6.9, 16.2 Hz, 1H, H^2); 6.68 (d, J = 15.9 Hz, 1H, H^1); 6.94 (d, J = 8.1 Hz, 2H, ArH); 7.07–7.33 (m, 10H, −NH-exchangeable with D_2O + H^6 + H^8 + 7ArH); 7.68 (d, J = 9.0 Hz, 1H, H^7); 7.82 (s, 1H, −NH-exchangeable with D_2O); 7.94 (s, 1H, H^9); 8.56 (d, J = 4.2 Hz, 1H, H^5); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 20.9, 60.0, 63.7, 100.1, 117.3, 121.6, 122.0, 123.0, 123.7, 124.9, 126.7, 128.2, 128.7, 128.9, 129.6, 134.2, 134.7, 135.5, 136.2, 149.1, 150.8, 152.3, 163.3, 174.9; HRMS Calculated for $C_{28}H_{23}ClN_4OS$ $[M+H]^+$ 499.1281 found 499.1288; Anal. Calcd (%) for: C, 67.39; H, 4.65; N, 11.23, found: C, 67.45; H, 4.54; N, 11.32.

3.3.2. 1-(7-Chloro-quinolin-4-yl)-3-(1-cyclohexyl-2-oxo-4-styryl-azetidin-3-yl)-thiourea (**7b**)

Yellow Solid; Yield: 92%; m.p. 164–165 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.05–1.87 (m, 10H, cyclohexyl-H); 3.30–3.38 (m, 1H, Cyclohexyl-H); 4.61 (dd, J = 4.5, 8.1 Hz, 1H, H^3); 5.70 (s, 1H, H^4); 6.10 (dd, J = 8.1, 15.9 Hz, 1H, H^2); 6.71 (d, J = 15.9 Hz, 1H, H^1); 7.17–7.36 (m, 7H, −NH-exchangeable with D_2O + H^8 + 5ArH); 7.67 (d, J = 4.5 Hz, 1H, H^6); 7.74 (d, J = 9.0 Hz, 1H, H^7); 8.02 (d, J = 1.8 Hz, 1H, H^9); 8.13 (s, 1H, −NH-exchangeable with D_2O); 8.67 (d, J = 4.2 Hz, 1H, H^5); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 23.6, 25.2, 29.1, 30.3, 37.7, 50.9, 57.9, 61.9, 100.5, 114.1, 120.4, 122.6, 123.8, 125.3, 125.4, 126.7, 127.3, 129.4, 133.5, 134.9, 147.9, 150.0, 151.8, 163.9, 175.2; HRMS Calculated for $C_{27}H_{27}ClN_4OS$ $[M+H]^+$ 491.1594 found 491.1585; Anal. Calcd (%) for: C, 66.04; H, 5.54; N, 11.41, found: C, 66.16; H, 5.48; N, 11.49.

3.3.3. 1-[1-(4-Chloro-phenyl)-2-oxo-4-styryl-azetidin-3-yl]-3-(7-chloro-quinolin-4-yl)-thiourea (**7c**)

Yellow Solid; Yield: 85%; m.p. 188–189 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 4.75 (dd, J = 4.8, 7.8 Hz, 1H, H^3); 5.80 (s, 1H, H^4); 6.22 (dd, J = 7.8, 15.9 Hz, 1H, H^2); 6.70 (d, J = 15.9 Hz, 1H, H^1); 6.99 (d, J = 8.1 Hz, 2H, ArH); 7.05–7.35 (m, 10H, −NH-exchangeable with D_2O + H^6 + H^8 + 7ArH); 7.76 (d, J = 9.0 Hz, 1H, H^7); 7.92 (s, 1H, −NH-exchangeable with D_2O); 7.96 (s, 1H, H^9); 8.63 (d, J = 4.2 Hz, 1H, H^5); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 59.8, 62.4, 100.3, 116.6, 121.7, 122.1, 122.8, 123.5, 124.6, 126.7, 128.4, 128.7, 128.9, 129.6, 130.1, 134.4, 135.5, 136.3, 149.7, 150.3, 152.4, 163.6, 175.0; HRMS Calculated for $C_{27}H_{20}Cl_2N_4OS$ $[M+H]^+$ 519.0735 found 519.0742; Anal. Calcd (%) for: C, 62.43; H, 3.88; N, 10.79, found: C, 62.33; H, 3.80; N, 10.73.

3.3.4. 1-(7-Chloro-quinolin-4-yl)-3-[1-(4-fluoro-phenyl)-2-oxo-4-styryl-azetidin-3-yl]-thiourea (**7d**)

Yellow Solid; Yield: 93%; m.p. 156–157 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 4.90 (dd, J = 5.1, 7.8 Hz, 1H, H^3); 5.76 (s, 1H, H^4); 6.25 (dd, J = 7.8, 15.9 Hz, 1H, H^2); 6.76 (d, J = 15.9 Hz, 1H, H^1); 7.04 (d, J = 8.1 Hz, 2H, ArH); 7.09–7.36 (m, 10H, −NH-exchangeable with D_2O + H^6 + H^8 + 7ArH); 7.72 (d, J = 9.0 Hz, 1H, H^7); 7.91 (s, 1H, −NH-exchangeable with D_2O); 7.98 (s, 1H, H^9); 8.61 (d, J = 4.2 Hz, 1H, H^5); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 59.6, 62.7, 100.4, 117.3, 121.5, 122.1, 122.9, 123.6, 124.7, 126.8, 128.3, 128.5, 128.8, 129.0, 129.6, 134.8, 135.5, 136.3, 149.6, 151.0, 152.2, 163.5, 175.1; HRMS Calculated for $C_{27}H_{20}ClFN_4OS$ $[M+H]^+$ 503.1030 found 503.1040; Anal. Calcd (%) for: C, 64.47; H, 4.01; N, 11.14, found: C, 64.56; H, 4.06; N, 11.06.

*3.3.5. 1-(7-Chloro-quinolin-4-yl)-3-(2-oxo-1-phenyl-4-styryl-azetidin-3-yl)-thiourea (**7e**)*

Yellow Solid; Yield: 90%; m.p. 172–173 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 4.81 (dd, J = 5.1, 7.8 Hz, 1H, H^3); 5.77 (s, 1H, H^4); 6.23 (dd, J = 7.8, 15.9 Hz, 1H, H^2); 6.72 (d, J = 15.9 Hz, 1H, H^1); 7.02–7.38 (m, 12H, –NH-exchangeable with D_2O + H^6 + H^8 + 9ArH); 7.78 (d, J = 9.0 Hz, 1H, H^7); 8.00 (d, J = 1.8 Hz, 1H, H^9); 8.09 (s, 1H, –NH-exchangeable with D_2O); 8.58 (d, J = 4.5 Hz, 1H, H^5); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 60.1, 62.2, 100.2, 117.5, 121.4, 122.1, 122.7, 123.8, 124.7, 125.4, 126.7, 128.0, 128.5, 128.7, 129.3, 134.8, 135.6, 136.4, 149.5, 151.0, 152.4, 163.5, 174.8; HRMS Calculated for $C_{27}H_{20}Cl_2N_4OS$ $[M+H]^+$ 519.0735 found 519.0727; Anal. Calcd (%) for: C, 62.43; H, 3.88; N, 10.79, found: C, 62.38; H, 3.78; N, 10.86.

*3.3.6. 1-[1-(4-Bromo-phenyl)-2-oxo-4-styryl-azetidin-3-yl]-3-(7-chloro-quinolin-4-yl)-thiourea (**7f**)*

Yellow Solid; Yield: 94%; m.p. 186–187 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 4.82 (dd, J = 5.1, 8.1 Hz, 1H, H^3); 5.71 (s, 1H, H^4); 6.21 (dd, J = 8.1, 15.9 Hz, 1H, H^2); 6.73 (d, J = 15.9 Hz, 1H, H^1); 6.96 (d, J = 8.1 Hz, 2H, ArH); 7.05–7.32 (m, 10H, –NH-exchangeable with D_2O + H^6 + H^8 + 7ArH); 7.69 (d, J = 8.7 Hz, 1H, H^7); 7.87 (s, 1H, –NH-exchangeable with D_2O); 7.99 (s, 1H, H^9); 8.59 (d, J = 4.2 Hz, 1H, H^5); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 60.1, 62.9, 100.3, 117.4, 121.4, 122.1, 122.8, 123.7, 124.7, 126.5, 128.1, 128.3, 128.7, 129.4, 134.3, 134.6, 135.4, 136.4, 149.3, 151.2, 152.6, 163.7, 175.2; HRMS Calculated for $C_{27}H_{20}BrClN_4OS$ $[M+H]^+$ 563.0230 found 563.0220; Anal. Calcd (%) for: C, 57.51; H, 3.57; N, 14.17, found: C, 57.63; H, 3.66; N, 14.23.

*3.4. General procedure for the synthesis of compounds (**10a**–**10d**)*

To a stirred solution of compound **1** (1 mol) in mixture of dry $CHCl_3$ + MeOH (9:1), **8** (1 mmol) was added and stirred the reaction mixture at room temperature for 10 min. The completion of the reaction was monitored by TLC using $CHCl_3$:MeOH (8:2) as the mobile phase. The reaction mixture was filtered and washed with chloroform to generate a crude product which was purified by recrystallization in $CHCl_3$:MeOH (9:1) mixture.

*3.4.1. 3-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**10a**)*

Yellow Solid; Yield: 94%; m.p. 201–202 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 3.35–3.47 (m, 2H, $-CH_2-$); 3.98–4.02 (m, 2H, $-CH_2-$); 6.46 (d, J = 12.0 Hz, 1H, H^3); 6.71 (d, J = 5.4 Hz, 1H, H^5); 7.10 (d, J = 15.6 Hz, 1H, H^1); 7.37–7.61 (m, 8H, –NH-exchangeable with D_2O + H^2 + H^7 + 5ArH); 7.78 (d, J = 2.1 Hz, 1H, H^8); 8.12 (d, J = 9.0 Hz, 1H, H^6); 8.42 (d, J = 5.4 Hz, 1H, H^4); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 39.0, 40.1, 99.0, 114.0, 117.9, 122.9, 124.5, 124.8, 127.3, 127.5, 128.2, 129.4, 129.5, 134.2, 136.9, 140.3, 148.7, 150.9, 151.6, 163.7, 176.7; HRMS Calculated for $C_{23}H_{19}ClN_4OS$ $[M+H]^+$ 435.0960 found 435.0966; Anal. Calcd (%) for: C, 63.51; H, 4.40; N, 12.88, found: C, 63.45; H, 4.32; N, 12.98.

*3.4.2. 3-[3-(7-Chloro-quinolin-4-ylamino)-propyl]-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**10b**)*

Yellow Solid; Yield: 94%; m.p. 186–187 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.66–1.77 (m, 2H, $-CH_2-$); 3.29–3.36 (m, 2H, $-CH_2-$); 3.80–3.84 (m, 2H, $-CH_2-$); 6.47–6.51 (m, 2H, H^3+H^5); 7.12 (d, J = 15.3 Hz, 1H, H^1); 7.34–7.57 (m, 8H, –NH-exchangeable with D_2O + H^2 + H^7 + 5ArH); 7.76 (d, J = 1.8 Hz, 1H, H^8); 8.25 (d, J = 9.0 Hz, 1H, H^6); 8.38 (d, J = 5.4 Hz, 1H, H^4); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 24.9, 42.0, 45.6, 98.6, 99.4, 113.5, 122.4, 124.3, 124.4, 127.0, 127.5, 128.9, 129.0, 129.9, 134.1, 136.4, 139.8, 149.3, 150.1, 151.0, 163.1, 176.2; HRMS Calculated for $C_{24}H_{21}ClN_4OS$ $[M+H]^+$ 449.1125 found 449.1134; Anal. Calcd (%) for: C, 64.20; H, 4.71; N, 12.48, found: C, 64.27; H, 4.65; N, 12.57.

*3.4.3. 3-[4-(7-Chloro-quinolin-4-ylamino)-butyl]-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**10c**)*

Yellow Solid; Yield: 94%; m.p. 205–206 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.58–1.92 (m, 4H, 2×$-CH_2-$); 3.39–3.49 (m, 2H, $-CH_2-$); 3.91–3.95 (m, 2H, $-CH_2-$); 6.47–6.51 (m, 2H, H^3+H^5); 7.13 (d, J = 15.6 Hz, 1H, H^1); 7.34–7.56 (m, 8H, –NH-exchangeable with D_2O + H^2 + H^7 + 5ArH); 7.84 (d, J = 1.8 Hz, 1H, H^8); 8.31 (d, J = 9.0 Hz, 1H, H^6); 8.43 (d, J = 5.4 Hz, 1H, H^4); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 24.4, 25.1, 43.1, 45.8, 98.7, 99.4, 114.0, 122.0, 124.4124.7, 127.2, 127.6, 129.0, 129.1, 129.8, 134.0, 136.7, 140.1, 149.5, 150.2, 151.4, 163.3, 176.4; HRMS Calculated for $C_{25}H_{23}ClN_4OS$ $[M+H]^+$ 463.1281 found 463.1271; Anal. Calcd (%) for: C, 64.85; H, 5.01; N, 12.10, found: C, 64.79; H, 4.92; N, 12.03.

*3.4.4. 3-[6-(7-Chloro-quinolin-4-ylamino)-hexyl]-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**10d**)*

Yellow Solid; Yield: 94%; m.p. 190–191 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.54–1.99 (m, 6H, 3×$-CH_2-$); 3.35–3.44 (m, 2H, $-CH_2-$); 3.89–3.94 (m, 2H, $-CH_2-$); 6.42–6.46 (m, 2H, H^3 + H^5); 7.10 (d, J = 15.3 Hz, 1H, H^1); 7.32–7.57 (m, 8H, –NH-exchangeable with D_2O + H^2 + H^7 + 5ArH); 7.89 (d, J = 1.8 Hz, 1H, H^8); 8.29 (d, J = 9.0 Hz, 1H, H^6); 8.45 (d, J = 5.1 Hz, 1H, H^4); ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm = 24.4, 24.8, 28.9, 30.2, 41.9, 45.6, 98.5, 99.3, 113.8, 122.2, 124.1124.6, 127.4, 127.7, 128.9, 129.3, 129.9, 134.4, 136.6, 139.7, 149.3, 150.0, 151.3, 163.1, 176.0; HRMS Calculated for $C_{27}H_{27}ClN_4OS$ $[M+H]^+$ 491.1594 found 491.1589; Anal. Calcd (%) for: C, 66.04; H, 5.54; N, 11.41, found: C, 66.14; H, 5.61; N, 11.36.

*3.5. General procedure for synthesis of thioimidazolidine-4-one with diamines (**13**)*

To stirred solution of **1** (1 mmol) in dry acetone, add ethylene diamine (1 mmol) in it. The reaction mixture was allowed to stir at room temperature for 10 min and the progress was monitored using thin layer chromatography. After completion, the reaction mixture was quenched with water (10 mL) and extracted with chloroform (2 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate, concentrated under reduced pressure and solid was obtained in RBF which was washed with diethyl ether and dried under vacuum to yield **13** as a yellow solid.

*3.5.1. 3-(2-Amino-ethyl)-5-(3-phenyl-allylidene)-2-thioxo-imidazolidin-4-one (**13a**)*

Yellow solid; Yield: 85%; m.p. 156–158 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.70–2.75 (m, 2H, CH_2), 3.40–3.46 (m, 2H, CH_2), 6.56 (d, J = 12.0 Hz, 1H, H^3), 7.02 (dd, J = 12.0, 15.6 Hz, 1H, H^2), 7.17 (d, J = 15.6 Hz, 1H, H^1), 7.20–7.50 (m, 5H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm = 43.0, 54.1, 112.5, 113.9, 118.2, 120.8, 128.9, 129.0, 134.8, 139.6, 166.3, 178.0; HRMS Calculated for $C_{14}H_{15}N_3OS$ $[M+H]^+$ 273.0936 found 273.0945; Anal. Calcd (%) for: C, 61.51; H, 5.53; N, 15.37; found C, 61.42; H, 5.60; N, 15.28.

3.6. Methods for assessment of antimalarial activity of test compounds

The W2 strain of *P. falciparum* was cultured in RPMI-1640 medium with 10% human serum, following standard methods, and parasites were synchronized with 5% D-sorbitol [24]. Beginning at the ring stage, microwell cultures were incubated with different concentrations of compounds for 48 h. The compounds were added from DMSO stocks; the maximum concentration of DMSO used was 0.1%. Controls without inhibitors included 0.1% DMSO. After 48 h

when control cultures had progressed to new rings, the culture medium was removed, and cultures were incubated for 48 h with 1% formaldehyde in PBS, pH 7.4, at room temperature. Fixed parasites were then transferred to 0.1% Triton X-100 in PBS containing 1 nM YOYO-1 dye (Molecular Probes). Parasitemia was determined from dot plots (forward scatter *vs*. fluorescence) acquired on a FACSort flow cytometer using Cell Quest software (Beckton Dickinson). IC_{50} values for growth inhibition were determined from plots of percent control parasitemia over inhibitor concentration using the Prism 3.0 program, (GraphPad Software), with data from duplicate experiments fitted by non linear regression [25].

3.7. Detergent mediated assay for β-hematin inhibition

The β-hematin formation inhibition assay method described by Carter et al. [26,27] was modified for manual liquid delivery. Samples were dissolved in DMSO to give 20 μM solutions and 20 μL of each were delivered to wells in the last column of a 96-well plate together with distilled water (140 μL) and NP40 detergent (305.5 μM, 70 μL). A solution containing water/NP40 (305.5 μM)/DMSO at a v/v ratio of 70%/20%/10% respectively was prepared and added to all other wells. A serial dilution of each compound (100 μL) from column 12 down to column 2 was carried out. Column 1 served as a blank with 0 μM sample. A 25 μM hematin stock solution was prepared by sonicating hemin in DMSO for one minute and then suspending 178 μL of this in a 1 M acetate buffer (pH 4.8). The homogenous suspension (100 μL) was then added to the wells to give final buffer and hematin concentrations of 0.5 M and 100 μM respectively. The plate was covered and incubated at 37 °C for 5–6 h in a water bath. Analysis was carried out using the pyridine-ferrichrome method developed by Ncokazi and Egan [28]. A solution of 50% (v/v) pyridine, 30% (v/v) H_2O, 20% (v/v) acetone and 0.2 M HEPES buffer (pH 7.4) was prepared and 32 μL added to each well to give a final pyridine concentration of 5% (v/v). Acetone (60 μL) was then added to assist with hematin dispersion. The UV–vis absorbance of the plate wells was read on a SpecrtaMax plate reader. Sigmoidal dose–response curves were fitted to the absorbance data using GraphPad Prism v3.02 to obtain a 50% inhibitory concentration (IC_{50}) for each compound.

3.8. In vitro analysis of cytotoxicity on HeLa cells

HeLa cells were cultured in 60 mm × 15 mm tissue culture dishes containing 5 mL of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin and streptomycin. Compounds were dissolved in DMSO to 100 μM concentrations. Once cell cultures reached 70% confluency, 5 μL of compound was added to the DMEM in the tissue culture dish for a final concentration of 100 μM. Cells were incubated for 24 h in a 37 °C CO_2 incubator. After 24 h incubation, the media was removed from the HeLa cells and the cells were then washed with 5 mL of 1X PBS. The cells were then cleaved off of the bottom of the plate *via* 5-min incubation with 0.5 mL of 0.25% trypsin. Cells were re-suspended in 1 mL of 1X PBS and transferred to a microcentrifuge tube. 100 μL of trypan blue solution were added to the re-suspended cells and allowed to incubate at room temperature for approximately 10 min. Viable and dead cells were visualized and counted with a hemacytometer. IC_{50} values were determined using GraphPad PRISM.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.07.048.

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