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Research Letter

7-Chloroquinoline–isatin Conjugates: Antimalarial, Antitubercular, and Cytotoxic Evaluation

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A series of twenty piperazine-tethered 7-chloroquinoline–isatin hybrids have been synthesized *via* either direct nucleophilic substitution or Cu()Cl-mediated Mannich reaction. These new conjugates were evaluated for their antimalarial and antitubercular efficacy against a chloroquine-resistant strain of *Plasmodium falciparum* and *Mycobacterium tuberculosis*, respectively, while the cytotoxic profiles were evaluated against 3T6 cell line, a permanent mouse embryonic fibroblast cell line. The most potent of the test compound with IC_{50} of 0.22 μM against W2 strain of *P. falciparum* and 31.62 μM against the embryonic fibroblast cell line (cytotoxicity) displayed a high selective index of 143.73.

Key words: 7-chloroquinoline–isatin scaffolds, antimalarial activity, antitubercular activity, cytotoxicity, structure activity relationship

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Malaria and tuberculosis (TB) are infectious diseases that continue to be global health problems with devastating impact on mankind. According to World Health Organization (WHO), they are responsible for one million and 1.4 million deaths, each year, respectively[a,b]. The statistics showed an estimated 219 million cases of malaria with an estimated 660 000 deaths in 2010. About 80% of the estimated cases of malaria occur in sub-Saharan Africa and 86% of deaths occur in children < 5 years of age. According to the world malaria report 2012, resistance of parasite to artemisinin (ART, **1** in Figure 1) has now been detected in four countries of the Greater Mekong subregion *viz.* Cambodia, Myanmar, Thailand, and Viet Nam[a].

In conjunction with malaria, TB ranks as the second leading cause of death from a contagious disease after AIDS[b]. One-third of the world's population asymptomatically harbor a dormant or latent form of *M. tuberculosis* with a lifelong risk of disease reactivation (1). HIV-positive people are prone to be infected with TB rendering latent TB to an active condition, often with fatal consequences (2,3). The use of the first-line antitubercular drugs isoniazid (INH) and rifampicin (RIF) is considered inadequate due to the development of multidrug-resistant strain of TB (MDR-TB), requiring administration of more expensive second-line agents (3,4). The problem is further exacerbated by the development of extensively drug-resistant TB (XDR-TB) following previous treatment with second-line antitubercular drugs contributing to the increased morbidity and mortality (5). The widespread emergence of MDR strains of *P. falciparum* and *M. tuberculosis* to clinically available drugs has generated a constant demand to develop novel, safe, highly effective, and fast-acting antimalarial and anti-TB agents.

Quinolines are considered as important and established scaffolds for antimalarial drug discovery. Among the quinoline derivatives, 4-aminoquinoline especially chloroquine (CQ, **2** in Figure 1) was considered as prime therapy for malaria treatment due to its clinical efficacy, low toxicity, cheap synthesis, and ease of administration (6). CQ acts by the inhibition of hemozoin formation within the acidic food vacuole of parasite (7,8), but the development of resistant strains of *P. falciparum* to CQ has limited its use (9). Despite the development of resistance, literary rationale has revealed a number of reports appearing on the synthesis of a number of new 4-aminoquinoline analogs with enhanced activity against chloroquine-resistant (CQR) strains developed *via* synthetic modifications of the CQ side chain (10–14). Chibale *et al.* in a recent communication explored the synthesis of a series of triazole-tethered chalcone and dienone hybrid compounds containing aminoquinoline and

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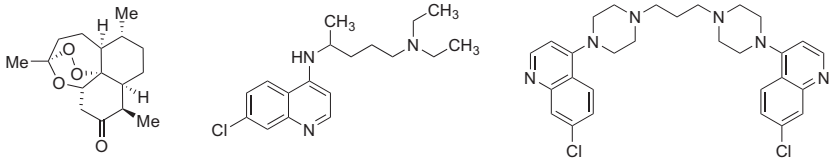


Figure 1: Antimalarials: Artemisinin (ART) (**1**), chloroquine (CQ) (**2**), piperaquine (**3**).

azidothymidine (AZT) leading to the identification of highly active hybrids having submicromolar IC_{50} values against D10, Dd2, and W2 strains of *P. falciparum* (15). Further, a series of urea-/oxalamide-tethered β-lactam-7-chloroquinoline hybrids as potential antimalarial agents has been reported by our group. Their antiplasmodial profiles against CQ-resistant W2 strain revealed the dependency of activity profile on the *N*-1 substituent of the β-lactam ring, the nature of the linker as well as the length of the alkyl chain (16). The exploration of novel 4-aminoquinoline analogs thus has emerged as a promising strategy to construct the molecules with enhanced activity against drug-resistant *P. falciparum.* Apart from its significance as an antimalarial agent, quinoline nucleus has also shown its potential against TB, which can be deduced from the synthesis of diarylquinoline TMC 207 (ex R207910) (**4** in Figure 2). This analog was shown to possess a new mechanism of action based on interaction with the mycobacterial adenosine triphosphate (ATP) synthase (17).

Another privileged heterocyclic scaffold, isatin (1*H*-Indole-2,3-dione), has many promising biological activities such as anti-HIV (18), antiviral (19), antitumor (20–22), antifungal (23,24), antiangiogenic (25), anticonvulsants (26), anti-Parkinson's disease therapeutic (27), antimalarial (28,29), along with good tolerance in humans and its ability to bind with the hydrophobic sites of the target. Recently, Chibale and co-workers have described the synthesis of thiolactone–isatin conjugates and their tetracyclic by-products which exhibited superior antiplasmodial activity. The most potent of the synthesized tetracyclic conjugate from the series displayed an IC_{50} of 6.92 μM against the chloroquine-resistant (W2) strain of *P. falciparum* (30).

Recent report from our laboratory has described the synthesis of 1*H*-1,2,3-triazole-tethered isatin-7-chloroquinoline hybrids along with their antimalarial profile. The activity profiles showed dependence on the substituents at the C-5 position of isatin as well as the length of the alkyl chain, with the most potent compound having an IC_{50} value of 1.21 μM against cultured chloroquine-resistant W2 strain of *P. falciparum* (31). With our efforts in designing molecular scaffolds using hybridization protocols (32–35), we report herein the extension of the above approach comprising the synthesis of 7-chloroquinoline–isatin conjugates tethered *via* piperazine functionality and non-hydrolyzable alkyl chain (**5** and **6** in Figure 3) along with their *in vitro* antimalarial and anti-TB evaluation. The piperazine core has been introduced because of its well-established profile in improving the biological activities as evidenced by good antimalarial profiles of piperaquine (**3** in Figure 1) and anti-TB profiles of piperazine-tethered benzamidines (36–39). Interestingly, piperazine nucleus also constitutes an integral part of rifampicin, the first-line anti-TB drug (40). It was further envisaged that a non-hydrolyzable alkane spacer (linker) would augment the lipophilicity of the target compounds, a property considered important for antitubercular activity because of its contribution toward improving the membrane permeability (41–46).

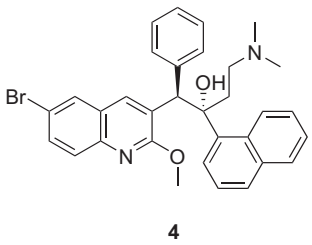


Figure 2: Antitubercular TMC 207 (**4**).

Experimental Section

Materials and methods

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

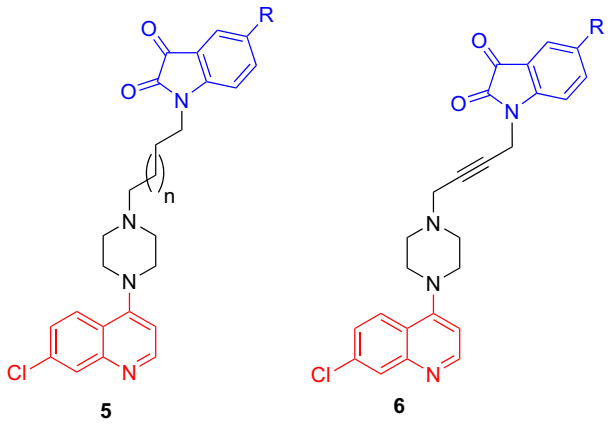


Figure 3: General structure of the target hybrids **5** and **6**.

Shimadzu D-8001 spectrophotometer. ^{1}H NMR spectra were recorded in deuterochloroform 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) and BRUKER AVANCE II (400 MHz) spectrometers in deuterochloroform using TMS as internal standard. High-resolution mass spectra were recorded on Bruker-micrOTOF-Q II spectrometer.

General procedure for the synthesis of conjugates, 5a–5p

To a stirred solution of 7-chloro-4-piperazin-1-yl-quinoline **9** (1 mmol) in 15 mL DMF, K_2CO_3 (3 mmol) and catalytic amount of KI was added and the resulting solution was stirred for 10 min; this was followed by the addition of *N*-alkylated isatin **11** (1 mmol) and the stirring was continued for 10–12 h at 85 °C. Progress of the reaction was monitored by TLC, and on completion, the solvent was evaporated and water (15 mL) was added to it. The reaction mixture was then extracted with ethyl acetate (3 × 50 mL), and the oragnic layer was dried over anhydrous sodium sulfate. The organic layer was then filtered and concentrated under reduced pressure resulting into the crude product which was purified by column chromatography using chloroform/methanol (95:5) mixture.

General procedure for the synthesis of Mannich adducts, 6a–6d

A mixture of *N*-propargylated isatin **12** (1 mmol), paraformaldehyde (2.5 mmol), 7-chloro-4-piperazin-1-yl-quinoline **9** (1 mmol), and anhydrous cupric chloride (0.5 mmol) in dry 1,4-dioxane was heated at 100 °C for 3 h. After completion of reaction as evidenced by TLC, the reaction mixture was filtered and the filtrate was treated with brine solution and extracted with ethyl acetate (3 × 60 mL). Combined organic layers were dried over Na_2SO_4, and the solvent was evaporated under reduced pressure resulting in a crude product which was purified by column chromatography using a chloroform/methanol (95:5) mixture.

Biological evaluations

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 (47). 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 versus fluorescence) acquired on a FACSort flow cytometer using Cell Quest software (Beckton Dickinson, San Jose, CA, USA). 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, San Diego, CA, USA), with data from duplicate experiments fitted by nonlinear regression (48).

Methods for assessment of *In vitro* antitubercular activity of test compounds

Bacterial strains and growth conditions. *Mycobacterium tuberculosis* mc^{2}6230 was grown at 37 °C in Sauton's medium supplemented with 24 μg/mL of pantothenic acid.

Drug susceptibility testing. The susceptibility of *M. tuberculosis* to the various compounds was determined as reported previously (49). In brief, Middlebrook 7H10 solid medium containing oleic-albumin-dextrose-catalase enrichment (OADC) and 24 μg/mL of pantothenic acid was supplemented with increasing concentrations of the chemical analogs. Serial 10-fold dilutions of each actively growing culture were plated and incubated at 37 °C for 2–3 weeks. The MIC was defined as the minimum concentration required to inhibit 99% of the growth.

Method for assessment of *in vitro* cytotoxicity of test compounds

The cytotoxicity of compounds was evaluated using 3T6 fibroblast cells (kindly provided by the Laboratory of Biology and Embryology of the University of Mons). These cells are a permanent mouse embryonic fibroblast cell line. They were cultured in DMEM supplemented with 10% heat-inactivated FBS (InvitroGen, Merelbeke, Belgium), in a CO_2 incubator (37 °C, 5% CO_2). 200 μL of 5 × 10^3 trypsinized 3T6 cells was seeded in each well (except for the wells on the edge) of a 96-well plate. Under sterile conditions, 8 successive dilutions of each compound were realized (starting at 100 μM to reach 0.05 μM). After 24 h of incubation, the culture medium was eliminated and replaced by 198 μL of culture medium and 2 μL of each dilution. Also 200 μL of culture medium was added to the cell growth control rows, and the solvent control wells received 200 μL of a mixture of solvent (DMSO; Sigma Aldrich, Bornem, Belgium) and culture medium. For the blanks, the same mixture was added to the wells without cells. After 48 h of incubation, the cells were carefully washed with PBS, and 100 μL of fresh culture medium was added into each well.

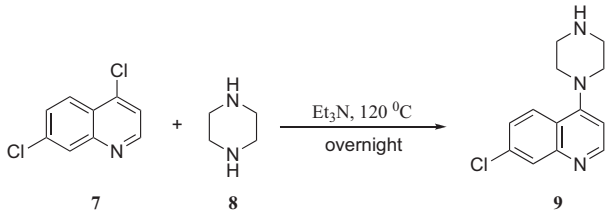
10 μL of MTT (*In vitro* toxicology assay kit, MTT based; Sigma Aldrich) was then added to all wells and incubated for 3 h. The MTT solubilization reagent was also added into each well to solubilize formazan crystals. The plate was incubated overnight at 37 °C. With a microplate reader, the optical density of each well was measured at 540 nm against the background absorbance whose reference filter was set at 690 nm. Eight concentrations of each test compound (100–0.05 μM) were verified in triplicate. Blank controls (culture medium), free-drug controls, and solvent (DMSO) controls were included in each assay.

The % inhibition (% IC) of cell proliferation was calculated using the formula:% IC = 100 – [corrected mean OD sample × 100/corrected mean OD solvent controls] where corrected mean OD sample/solvent = mean $OD_{540–690}$ of samples/controls – mean $OD_{540–690}$ of blanks. The concentration inhibiting 50% of cell proliferation (IC50) of each compound was obtained by plotting the% inhibition of activity against the compound concentration scale.

Result and Discussions

Chemistry

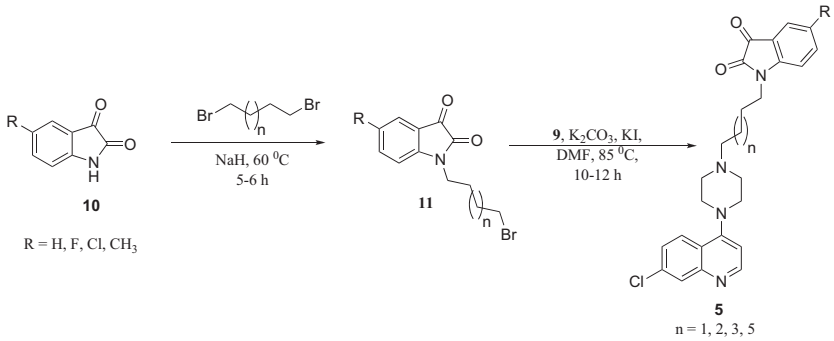
7-Chloro-4-piperazin-1-yl-quinoline **9**, required for the synthesis of desired scaffolds, was synthesized by heating overnight 4,7-dichloroquinoline **7** with an excess of piperazine **8** and triethylamine at 120 °C (Scheme 1). The other precursor, *N*-alkylated isatins **11**, was prepared by reacting a solution of isatin in DMF with sodium hydride with subsequent treatment with dibromoalkanes. The target conjugates **5a**–**5p** were synthesized by initial stirring of a solution of K_2CO_3 and KI with 7-chloro-4-piperazin-1-yl-quinoline **9** in dry DMF for 5–10 min, followed by subsequent addition of *N*-alkylated isatin **11** (Scheme 2). The reaction mixture was stirred at 85 °C for 10–12 h, and the progress of reactions was monitored by TLC. After usual workup on the completion of reaction, solvent was evaporated and the desired product was obtained after purification *via* column chromatography using a chloroform/methanol (95:5) mixture.



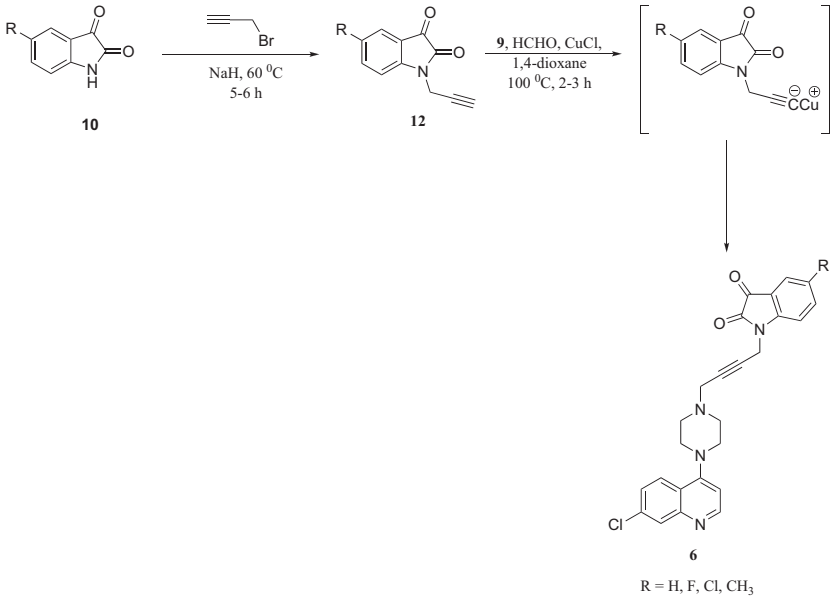
Scheme 1: Synthesis of 7-chloro-4-piperazin-1-yl-quinoline **9**.

The structure to **5** was assigned on the basis of spectral data and analytical evidence. For example, **5b**, showed a molecular ion peak $[M]^+$ at 466.1580, along with characteristic peaks in ^1H and ^{13}C NMR spectra. The ^1H NMR spectrum displayed peaks at δ 1.57, 1.72, 2.46, and 3.72 corresponding to methylene protons of the alkyl chain along with the characteristic protons of 7-chloroquinoline ring. The requisite number of carbons in the ^{13}C NMR spectrum along with the two characteristic peaks at δ 157.8 and 182.8 assigned to isatin ring carbonyls further attested the assigned structure.

The precursor for 7-chloroquinoline–isatin Mannich adducts *viz. N*-propargylated isatin **12** was prepared by an initial treatment of isatin with sodium hydride with subsequent addition of propargyl bromide at 60 °C. The *N*-propargylated isatins **12** were then utilized in Cu(*I*)Cl catalyzed Mannich reaction with **9** in the presence of formaldehyde in dry 1,4-dioxane (Scheme 3) (50). Mechanistically, the reaction is thought to proceed *via* an initial formation of Cu-acetylide, which undergoes a nucleophilic addition to the iminium ion formed by the condensation reaction between **9** and formaldehyde. The progress of reactions was monitored by TLC. On completion and after workup with ethyl acetate and water, the organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure resulting into the crude product that was purified by column chromatography using a chloroform/methanol (95:5) mixture. The structure of the synthesized Mannich adducts has been assigned on the basis of spectral data and analytical data, the details of which are provided in the experimental section.



Scheme 2: Synthesis of 7-chloroquinoline–isatin conjugates **5a**–**5p**.



Scheme 3: Synthesis of 7-chloroquinoline–isatin Mannich adducts **6a**–**6d**.

In vitro *antimalarial, antitubercular, and cytotoxic evaluation*

The test compounds were evaluated for their antimalarial profiles against the CQ-resistant W2 strain of *P. falciparum*. Analysis of Table 1 revealed that these were not as active as standard drug *viz.* chloroquine (CQ), although compounds **5c**, **5f**, **5h**, and **5j** have shown good antimalarial activity among the test compounds with IC_{50} values ranging from 0.22 to 0.27 μM. The observed activity profiles were found to be independent of the length of alkyl chain especially among butyl, pentyl, and hexyl spacer, while a fifteen times decrease (compare **5c**

Table 1: Antiplasmodial activity against a chloroquine-resistant strain, anti-TB activity against *Mycobacterium tuberculosis* mc^{2}6230, and cytotoxicity of compounds **5a**–**5p** and **6a**–**6d**

			Antiplasmodial activity			
Code	R	*n*	W2 (CQ-R) IC_{50} (μM)	SI[a]	Anti-TB activity MIC (μm)	Cytotoxicity IC_{50} (μM) ± Standard error
5a	H	1	1.12	1	55.80	1.81 ± 0.01
5b	F	1	1.17	5	107.29	6.77 ± 0.09
5c	Cl	1	0.27	28	10.35	7.57 ± 0.01
5d	CH_3	1	0.81	7	54.11	5.82 ± 0.06
5e	H	2	0.46	4	43.29	2.01 ± 0.02
5f	F	2	0.27	23	<10.41	6.35 ± 0.10
5g	Cl	2	0.42	36	20.12	15.40 ± 0.10
5h	CH_3	2	0.25	27	52.52	6.79 ± 0.02
5i	H	3	0.54	13	52.52	7.55 ± 0.03
5j	F	3	0.22	143	<10.12	31.62 ± 0.13
5k	Cl	3	0.92	16	19.56	14.72 ± 0.08
5l	CH_3	3	0.62	13	ND	8.35 ± 0.01
5m	H	5	1.39	0.8	39.60	1.22 ± 0.07
5n	F	5	0.90	ND	38.24	ND
5o	Cl	5	3.79	ND	166.97	ND
5p	CH_3	5	7.52	ND	>192.67	ND
6a	H	–	1.01	4	>225	4.81 ± 0.01
6b	F	–	0.58	26	>216	15.56 ± 0.03
6c	Cl	–	0.42	17	135.69	7.32 ± 0.07
6d	CH_3	–	1.12	9	54.58	11.04 ± 0.12
Chloroquine			0.036			
Cephalexin					68.41	
Isoniazid (INH)					0.18	

ND, Not determined.
[a]SI: Selective index is ratio of IC_{50} of 3T6 cell line to that of chloroquine-resistant (W2) strain.

and **5o**) in antimalarial efficacy has been observed in octyl spacers. The activity profiles at shorter alkyl chain length *viz.* $n = 1$, 2, and 3 seemed to be independent over the nature of substituent at C-5 position of the isatin ring, while a preference for flouro substituent for good activity has been observed at $n = 5$. Similar preference for the halogen substituent for good activity has been observed in case of 7-chloroquinoline–isatin Mannich adducts with compounds **6c** having chloro substituent showed an IC_{50} of 0.42 μM. The test compounds were further evaluated for their antitubercular profiles, and the results are listed in Table 1. As evident, compounds are not as active as that of isoniazid. Analysis of Table 1 explicated that the presence of substituent at C-5 position of the isatin ring tends to influence the activity profile, while longer alkyl chain lengths adversely affect the anti-TB efficacy of the test compounds. A clear preference for fluoro substituent is evident for good anti-TB efficacy as observed in case of **5f** and **5j**, being six times more potent than cephalexin. The Mannich adducts on the other hand proved to be inefficient in inhibiting the growth of *M. tuberculosis*.

The synthesized scaffolds have also been evaluated for their cytotoxic profiles against 3T6 cell line, a permanent mouse embryonic fibroblast cell line. As evident, the compounds *viz*. **5c**, **5f**, and **5j** with good antimalarial efficacy also possess higher cytotoxicity against the embryonic fibroblast cell line. However, the compound **5j** with an IC_{50} of 0.22 μM against W2 strain of *P. falciparum* and 31.62 μM against the embryonic fibroblast cell line (cytotoxicity) also possess a high selective index of 143.73.

Conclusion

In conclusion, the present manuscript describes the synthesis of piperazine-tethered 7-chloroquinoline–isatin conjugates prepared *via* nucleophilic substitution and Cu-catalyzed Mannich addition protocols along with their antimalarial, antitubercular, and cytotoxic evaluation. SAR studies showed a preference for halogen substituent at C-5 position of isatin ring for good activity, while longer alkyl chain lengths adversely affected the activity profiles.

Acknowledgment

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Conflict of Interest

The authors declare no competing financial interest.

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Notes

[a]WHO, World Malaria Report (2012), http://www.who.int/malaria/publications/world malaria report 2012/en/index.html.
[b]World Health Organization, Global Tuberculosis Report (2012), http://www.who.int/tb/publications/global_report/en/.

Supporting Information

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

Data S1. The details of characterisation of compounds **5a**–**5p** and **6a**–**6d**, along with scanned ^{1}H and ^{13}C spectra of **5a**, **5b**, **5l**, and **6a** are given in supplementary file.