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Synthesis and Antimalarial Screening of Some New Isoquine Analogues

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ABSTRACT : Amodiaquine is a 4-aminoquinoline antimalarial that can cause adverse side effects including hepatic and haematological toxicity. The drug toxicity involves the formation of a reactive metabolite, amodiaquine quinoneimine (AQQI), which binds to cellular macromolecules, leading to hepatotoxicity and agranulocytosis. Interchange of the 3´ hydroxyl and the 4´ Mannich side-chain function of amodiaquine provide an amodiaquine regioisomer (isoquine) that can not form toxic quinoneimine metabolites. By a simple two-step procedure, four isoquine analogues were synthesized and subsequently evaluated against the chloroquine sensitive RKL-2 strain of *Plasmodium falciparum in vitro*. All synthesized analogues demonstrated differential level of antimalarial activity against the test strain. However, no compound was found to exhibit better antimalarial property as compared to chloroquine.

Key-words: Amodiaquine, isoquine, antimalarial, RKL-2.

INTRODUCTION

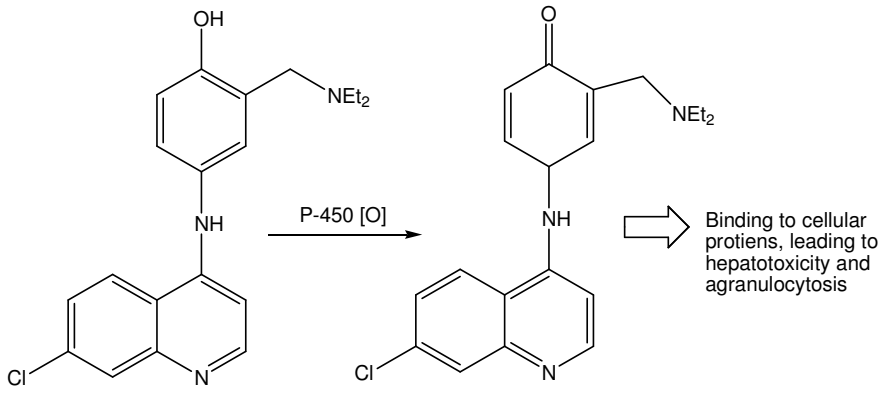
Malaria is the most serious, complex and refractory health problems facing humanity. Almost one-half of the world's population is exposed to the threat of malaria and the disease is responsible for two million deaths each year, either directly or in association with acute respiratory infections and anaemia and upto 1 million of those deaths are children. Malaria is a leading cause of morbidity and mortality in developing world[1]. Chloroquine was a mainstream drug in the fight against *Plasmodium falciparum*, but its efficacy is being eroded by the emergence of resistant parasites. The spread of chloroquine resistance has prompted the re-investigation of the chemistry and pharmacology of alternative antimalarials such as amodiaquine, an other 4-aminoquinoline which proved to be effective against chloroquine-resistant strains.[2,3]

Amodiaquine is a 4-aminoquinoline antimalarial which is effective against many chloroquine resistant strains of *P. falciparum*. However, clinical use of amodiaquine has been severely restricted because of associations with hepatotoxicity and agranulocytosis [4,5]. It has been suggested that the toxicity of amodiaquine is related to the reactive electrophillic metabolites formed by oxidation of its phenolic side chian, especially to the formation of a quinineimine by cytochrome P-450-catalyzed biological oxidation (Scheme 1). It has been found that amodiaquine is excreted in bile exclusively as the 5´ thioether conjugates (glutathione and cysteinyl) in rats[6]. This observation indicates that the parent drug undergoes extensive bioactivation *in vivo* to form amodiaquine quinoneimine (AQQI) or semiquinoneimine (AQSQI) with subsequent conjugation of glutathione[7].

Scheme 1: Bioactivation of amodiaquine to toxic quinoneimine metabolite by P-450.

Structure activity relationship (SAR) studies on amodiaquine had previously shown that wide variations in the side chain can be accommodated with retention of antimalarial activity. Blocking of bioactivation pathways by removal of the phenolic group or introduction of non reactive substituents has been the main strategy. Reducing bioactivation also seems to result in compounds with slower elimination (enhanced biological half life), and increased tissue accumulation[8].

From SAR studies it has been noted that in the amodiaquine and tebuquine series of 4-aminoquinoline analogues, the presence of the 4´ hydroxyl group within the aromatic ring imparts greater inherent antimalarial



Amodiaquine Amodiaquine quinone imine (AQQI)

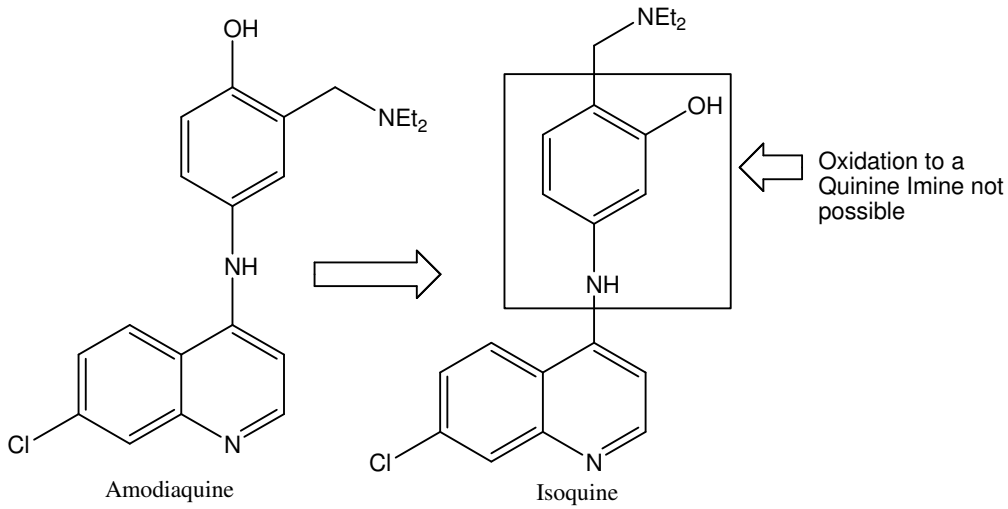
activity against chloroquine resistant parasites than the corresponding deoxo analogues.[9,10] Interchange of the hydroxyl group and the Mannich side chain provides a means of preventing oxidation to toxic metabolites while retaining possible important bonding interactions with the aromatic hydroxyl function. This amodiaquine regioisomer (isoquine) cannot form toxic metabolites by simple oxidation and is potent against chloroquine resistant parasites *in vitro* (Scheme 2). Isoquine itself has been reported to possess potent *in vitro* and oral *in vivo* antimalarial activity in experimental animal models and it does not undergo *in vivo* biotransformation to quinineimine metabolites[11]. Apart from an excellent antiparasitic profile, isoquine and its different side-chain analogues are rather inexpensive antimalarials to synthesize and may represent new leads for development of a safe, cheap, affordable, and effective antimalarial for both prophylaxis and treatment of malaria. Considering the above said facts we have designed and synthesized a few new isoquine analogues (Table 1). The present paper reports the synthesis and *in vitro* antimalarial screening of those analogues.

CHEMISTRY

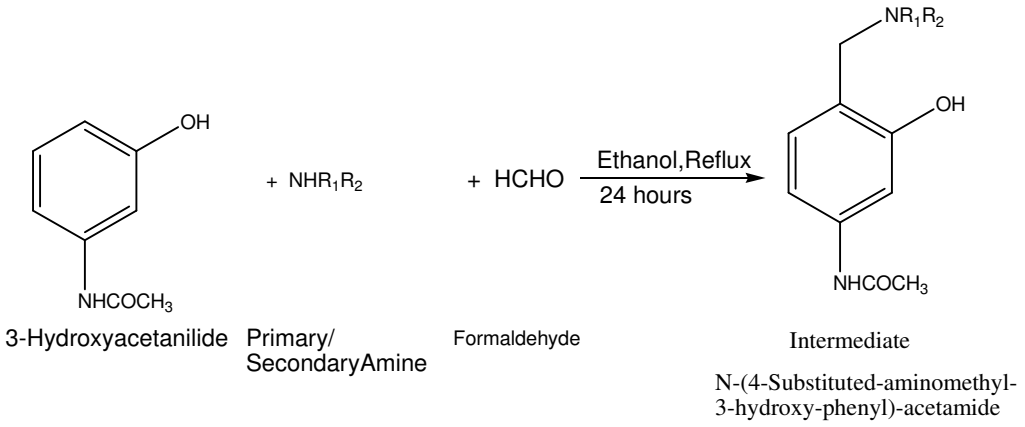
The synthesis of designed isoquine analogues involves a two-step procedure from commercially available starting materials according to a method originally utilized by Burkhalter and co-workers[12].

Step-I: This step involves a Mannich reaction of the commercially available 3-hydroxyacetanilide to provide the Mannich product in yields ranging from 50% to 90% (Scheme 3).

Scheme 2: Redesign of Amodiaquine.



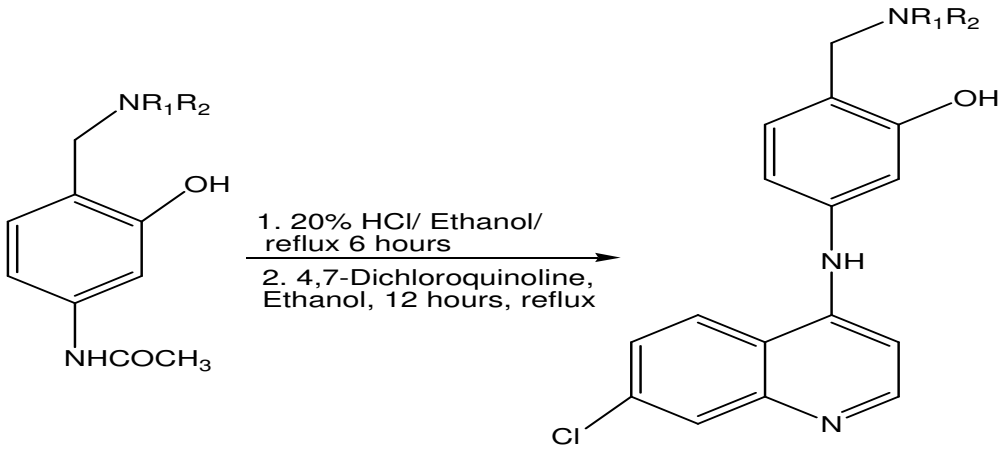
Amodiaquine Isoquine



3-Hydroxyacetanilide | Primary/ SecondaryAmine | Formaldehyde | Intermediate

N-(4-Substituted-aminomethyl-3-hydroxy-phenyl)-acetamide

Scheme 3



Intermediate | Final Product

Scheme 4

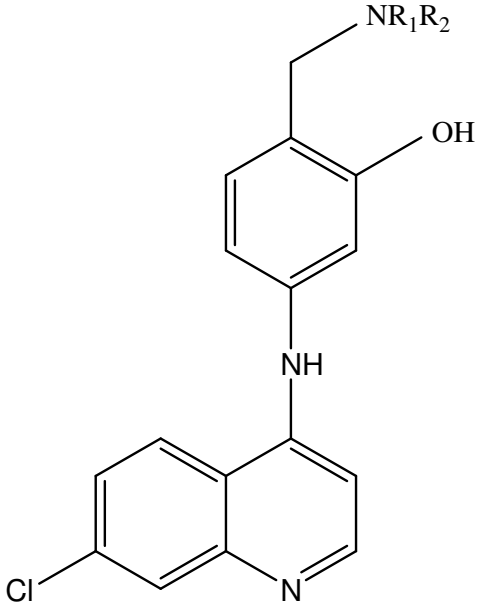
Step-II: This step involves the hydrolysis of the amide function to provide the corresponding Mannich-substituted 3-aminophenol which is subsequently coupled with 4, 7-dichloroquinoline (Scheme 4) to provide the target compounds shown in Table 1.

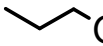
EXPERIMENTAL

4,7-dichloroquinoline was obtained from Mangalam Drug and Organics Ltd., Mumbai as gift sample. All the other chemicals used were of synthetic grade chemicals of Aldrich and Rankem, without further purification and obtained from commercial suppliers.

The completion of reactions was tested by analytical thin layer chromatography on aluminum sheets pre-coated with silica gel obtained from Merck. Visualization was attempted by iodine vapour and UV light. Melting point of the synthesized compounds was determined on *Veego, Model No. MPI,* by open capillary method. The UV absorption maximum (λ_{max}) of the synthesized compounds was recorded on Shimadzu *UV-1700* UV-Visible spectrophotometer. The FTIR spectra of the synthesized compounds were recorded on *Hitachi 270-50* spectrophotometers using potassium bromide pellets. The ^{1}H-NMR and ^{13}C-NMR spectra of the synthesized compounds in DMSO were recorded at 400 MHz and 100 MHz respectively by *Bruker* 400 NMR spectrometer. Chemical shift values are given in δ (ppm) scale using TMS as an internal standard. Significant ^{1}H-NMR data are written in order: number of protons, multiplicity (b, broad; s, singlet; d, doublet; t, triplet; m, multiple), coupling constants in hertz, assignment. The mass spectra of the synthesized compounds were recorded on *Waters Micromass Q-Tof Micro* Mass spectrometer. The m/z values of the more intense peaks are mentioned.

Table 1. List of the designed compounds.



Compounds	R_1	R_2
CS-1	Phenyl	H
CS-2	H	$CSNH_2$
CS-3		
CS-4	Isopropyl	Isopropyl

SYNTHESIS OF THE DESIGNED COMPOUNDS

All the designed compounds (CS-1 to CS-4) were synthesized as per the scheme described in step-I & step-II of synthesis by using the following method.

Step I: Ethanol was added to 3-Hydroxyacetanilide in a 100 ml round-bottom flask followed by one equivalent of primary or secondary amine and aqueous formaldehyde was added and the solution was allowed to heat under reflux for 24 hours. After this reflux period, the solvent was removed under reduced pressure and the crude material (intermediate amide) was purified by flash column chromatography using 20-80% MeOH/dichloromethane as eluent.

Step II: Aqueous hydrochloric acid (20%) (25 ml) was added to a round-bottom flask containing the amide (intermediate) and the solution was heated under reflux for 6 hours. The solvent was then removed *in vacuo* and the resulting residue co-evaporated with ethanol to give the corresponding hydrochloride salt. 4,7-Dichloroquinoline and ethanol (30 ml) were added, and the reaction mixture was heated under reflux for around 12 hours until completion of the reaction (checked by analytical TLC). A crude product was obtained upon removing the solvent under reduced pressure; this was subsequently purified by flash column chromatography using 20-80% MeOH/dichloromethane as eluent to yield the quinoline hydrochloride salt. To liberate the free base compound, this solid was dissolved in distilled water (18 ml) and the solution was basified by careful drop wise addition of saturated sodium bicarbonate (added until no more precipitate was formed). Dichloromethane (100 ml) was added, and the free base was extracted into the organic layer. Subsequent drying and removal of solvent *in vacuo* afforded the desired product.

CS-1: 5-(7-Chloroquinolin-4-ylamino)-2-[(phenylamino)-methyl]-phenol. CS-1 was obtained as brownish yellow solid (75.87 % yield). mp = 88-90^{o}C; **UV λ_{max}:** 368 nm (DMSO); **^{1}H NMR (400 MHz, DMSO):** δ 8.56 (d, 1H, *J* = 5.24 Hz, quinoline-H), δ 8.02 (d, 1H, *J* = 2.08 Hz, quinoline-H), δ 7.84 (d, 1H, *J* = 8.91 Hz, phenyl-H),δ 7.45 (dd, 1H, *J* = 8.91, 2.06 Hz, quinoline-H), δ 6.98 (d, 1H, *J* = 7.92 Hz, Ar-H), δ 6.73 (d, 1H, *J* = 2.20 Hz, Ar-H), δ 6.58 (bs, 1H, OH), δ 5.69 (s, 1H, Ar-H), δ 4.48 (s, 1H, methylene), δ 4.18 (d, 1H, Ar-H); **^{13}C NMR (100 MHz, DMSO):** δ 152.50, 151.23, 149.66, 149.40, 142.65, 135.65, 129.60, 129.25, 128.61, 125.56, 124.97, 124.45, 123.52, 121.42, 120.45, 119.86, 117.81, 117.16, 108.23, 100.97, 37.65; **IR (in KBr disc):** 3200, 1604, 1541, 1450, 1354, 1211, 1095, 866, 815 cm^{-1}; **MS (m/z):** 375.01 (m+).

CS-2: 1-[4-(7-Chloroquinolin-4-ylamino)-2-hydroxyphenyl] methyl thiourea. CS-2 was obtained as dark brown solid (61.09 % yield); mp = 155-158^{o}C; **UV λ_{max}:** 365.2 nm (DMSO); **^{1}H NMR (400 MHz, DMSO):** δ 8.60 (d, 1H, *J* = 5.32 Hz, quinoline-H), δ 8.08 (d, 1H, quinoline-H), δ 7.85 (d, 1H, *J* = 8.90 Hz, quinoline-H), δ 7.43 (dd, 1H, *J* = 8.59, 2.14 Hz, quinoline-H), δ 7.09 (d, 1H, *J* = 5.32 Hz, quinoline-H), δ 6.76 (d, 1H, *J* = 2.20 Hz, Ar-H), δ 6.55 (s, 1H, OH), δ 5.68 (s, 1H, Ar-H), δ 4.14 (s, 2H, CH_2), δ 3.56 (s, 2H, CH_2), δ 2.02 (s, 2H, amine); **^{13}C NMR (100 MHz, DMSO):** δ 178.66, 157.00, 151.84, 149.59, 149.46, 135.13, 129.86, 128.68, 125.64, 125.25, 125.09, 123.60, 121.41, 117.51, 117.07, 101.30; **IR (in KBr disc):** 3200, 2831, 1690, 1609, 1500,

1445, 1363, 1200, 1082, 827, 775 cm^{-1}; **MS (m/z):** 357.97 (m+).

CS-3: 2-[bis (2-hydroxyethyl) amino methyl]-5-(6-chloronapthalen-1-ylamino) phenol. CS-3 was obtained a pale brown solid (71.56 % yield); mp = 77-79^{o}C; **UV λ_{max}:** 363.6 nm (DMSO); **^{1}H NMR (400 MHz, DMSO):** δ δ 8.56 (d, 1H, *J* = 5.24 Hz, quinoline-H), δ 8.02 (d, 1H, *J* = 2.08 Hz, quinoline-H), δ 7.86 (d, 1H, *J* = 8.92 Hz, quinoline-H), δ 7.47 (dd, 1H, *J* = 8.91, 2.06 Hz, quinoline-H), δ 7.03 (d, 1H, *J* = 5.30, quinoline-H), δ 6.95 (d, 1H, *J* = 7.92 Hz, Ar-H), δ 6.58 (bs, 1H, OH), δ 5.28 (s, 1H, Ar-OH), δ 4.20 (s, 1H, Ar-H), δ 3.76 (d, 1H, methylene), δ 1.99 (s, 1H, acetylene); **^{13}C NMR (100 MHz, DMSO):** δ 159.15, 153.64, 153.40, 149.31, 141.49, 140.29, 134.94, 127.29, 121.08, 119.75, 117.0, 115.93, 111.74, 102.97, 65.11, 55.74, 40.48. **IR (in KBr disc):** 3424, 3205, 2883, 2777, 1636, 1518, 1454, 1367, 1203, 1050, 906, 816, 740 cm^{-1}; **MS (m/z):** 385.30 (m+)

CS-4: 4-(7-Chloroquinolin-4-ylamino)-2-[(diisopropylamino) methyl] phenol. CS-1 was obtained as yellowish brown solid (73.95% yield). mp = 180-183ºC; **UV λ_{max}:** 364.0 nm (DMSO); **^{1}H NMR (400 MHz, DMSO):** δ 8.37 (d, 1H, *J* = 5.20 Hz, quinoline-H), δ 8.07 (d, 1H, *J* = 8.88 Hz, quinoline-H), δ 7.89 (d, 1H, *J* = 1.12 Hz, quinoline-H), δ 7.34 (dd, 1H, *J* = 1.92, 1.92 Hz, quinoline-H), δ 7.29 (d, 1H, *J* = 8.16 Hz, quinoline-H), δ 6.97 (d, 1H, *J* = 7.68 Hz, Ar-H), δ 6.72 (d, 1H, *J* = 8.40 Hz, Ar-H), δ 6.85 (s, 1H, OH), δ 6.58 (dd, 1H, *J* = 5.28, 5.20 Hz, Ar-H), δ 3.76 (s, 2H, CH_2), δ 3.10 (m, 2H, diisopropyl-H), δ 1.05 (d, 12H, diisopropyl-H); **^{13}C NMR (100 MHz, DMSO):** δ 157.00, 151.84, 149.59, 149.46, 135.13, 129.86, 128.68, 125.64, 125.25, 125.09, 123.60, 121.41, 117.51, 117.07, 101.30, 49.56, 48.14, 19.77; **IR (in KBr disc):** 3425, 3061, 2970, 2897, 2876, 1612, 1568, 1454, 1367, 1261, 1123, 874, 812 cm^{-1}; **MS (m/z):** 384.0 (m+).

IN VITRO ANTIMALARIAL SCREENING

All the synthesized compounds were screened for antimalarial activity in the Regional Medical Research Centre (Indian Council of Medical Research), N.E. Region, Dibrugarh, India.

The *in vitro* antimalarial assay was carried out in 96 well microtitre plates according to the microassay protocol of Rieckmann and co-workers with minor modifications[13,14]. The cultures of *P. falciparum* RKL-2 strain were maintained in medium RPMI 1640 supplemented with 25 mM HEPES, 1% D-glucose, 0.23% sodium bicarbonate and 10% heat inactivated human serum[15]. The asynchronous parasites of *P. falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage parasitized cells[16]. For carrying out the assay, an initial ring stage parasitaemia of 0.8 to 1.5% at 3% haematocrit in a total volume of 200 µl of medium RPMI-1640 was determined by Jaswant Singh Bhattacharya (JSB) staining to assess the percent parasitaemia (rings) and uniformally maintained with 50% RBCs (O^{+}) [17]. A stock solution of 5 mg/ml of each of the test samples was prepared in DMSO and subsequent dilutions were prepared with culture medium. The diluted samples in 20 µl volume were added to the test wells so as to obtain final concentrations (at five fold dilutions) ranging between 0.4 µg/ml to 100 µg/ml in duplicate well containing parasitized cell preparation. The culture plates were incubated at 37^{o}C in a candle jar. After 36 to 40 h incubation, thin blood smears from each well were prepared and stained with JSB stain[17,18]. The slides were microscopically observed to record maturation of ring stage parasites into trophozoites and schizonts in presence of different concentrations of the test agents. The test concentration which inhibited the complete maturation into schizonts was recorded as the minimum inhibitory concentrations (MIC). Chloroquine was used as the reference drug.

OBSERVATIONS OF THE *IN VITRO* ANTIMALARIAL SCREENING

The mean number of rings, trophozoites and schizonts recorded per 100 parasites from duplicate wells after incubation for 38 hours, and percent maturation inhibition with respect to control group are shown in the Table 2. The minimum inhibitory concentration (MIC) values (in the Table 2) are indicated in italic bold form.

DISCUSSION AND CONCLUSION

A series of new 7-chloro-4-aminoquinoline Mannich base derivatives were synthesized from commercially available starting materials. In this series the 4´-diethylamino function of isoquine is replaced by a 4´-primary or secondary amino function. The synthesis involved the preparation of Mannich base by Mannich reaction of the 3-hydroxyacetanilide followed by hydrolysis of the amide function of the Mannich base. The hydrolysis product (Mannich substituted 3-aminophenol) was subsequently coupled with 4, 7-dichloroquinoline to provide the four designed compounds. The compounds were characterized by various spectrometric analysis and the results of which are characteristic of the anticipated structure of the synthesized compounds.

All the synthesized compounds constitute a series with having modification at the lateral amino group of the side chain (CS-1 to CS-4). The compounds were evaluated for their *in vitro* antimalarial activity against the chloroquine sensitive RKL-2 strain of *P. falciparum*. The *in vitro* antimalarial assay was carried out by JSB stained slide method. All the tested compounds showed negligible to average percentage of killing the parasites. Two of the synthesized compounds (CS-3 and CS-4) showed comparatively better antimalarial activity under the given test conditions with MIC values of 50 and 10 µg/ml respectively. But none of the compounds demonstrated any appreciable activity better than the reference drug, chloroquine.

The antimalarial screening result reflects that the compound (CS-4) with alkyl substituted amino group side chain (diisopropylamine) showed comparatively

higher activity than the compound (CS-3) containing amino group with alcohol side chian (diethanolamine). The compound (CS-1) with aromatic ring (aniline) and the compound with thiourea side chain (CS-2) exhibited negligible activity. Though none of the synthesized compound (CS-1 to CS-4) demonstrated promising level of antimalarial activity as compared to chloroquine but compounds with aliphatic side chain to the amino side chain (CS-3, CS-4) showed significant level of activity at a concentration dependent manner. There is another provision to check the significant level of activity against the other strains and species of *Plasmodium*. The novel 7-chloro-4-aminoquinoline derivatives synthesized and screened in the present work may be of help for further modification of the isoquine structure in the antimalarial research for the development of a new generation of 4-aminoquinoline antimalarials in due course.

Table 2: *In vitro* antimalarial activity of synthesized compounds against chloroquine sensitive RKL-2 strain of *Plasmodium falciperum*

Code No. (Compound)	Concentrations Employed (µg/ml)	Number of parasites/ 100 infected RBCs			Percentage Inhibition of Schizont Maturation
		Rings	Trophozoites	Schizonts	
CS – 4	100	0	0	0	100
	50	0	0	0	100
	10	***0***	***100***	***0***	***100***
	2.0	55	23	16	82.22
	1.0	40	30	26	71.11
	0.5	25	16	59	34.44
	0.25	0	15	85	5.55
	0.125	51	74	100	0
CS – 3	100	100	0	0	100
	50	***100***	***0***	***0***	***100***
	10	100	14	19	78.89
	2.0	100	0	52	42.22
	1.0	100	0	87	3.33
	0.5	68	58	100	0
CS – 2	100	0	45	56	37.78
	50	0	29	77	14.44
	25	0	22	88	2.22
CS-1	100	0	57	75	16.67
	50	0	36	100	0
Chloroquine	0.50	100	0	0	100
	0.25	***100***	***0***	***0***	***100***
	0.125	95	3	2	97.1
	0.0625	70	32	8	88.4
Control	-	0	10	90	-

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REFERENCES

1. World Health Organization, Malaria Fact sheet No. 94 (2006).http://www.who.int/mediacentre/factsheets/fs94/en/.
2. World Health Organisation (WHO). Conquering, Suffering, and Enriching Humanity: The World Health Report; World Health Organisation Publishers: Geneva, 1997.
3. (a)Watkins, W. M.; Sixsmith, D. G.; Spencer, H. G.; Boriga, D. A.; Karjuki, D. M.; Kipingor, T.; Koech, D. K. Effectiveness of Amodiaquine as a Treatment for Chloroquine Resistant *Plasmodium falciparum*. Lancet I, 1984, 357-359. (b) White, N. J. Can Amodiaquine be resurrected*?* Lancet, 1996, 348, 1184- 1185. (c) Olliaro, P.; Nevill, C.; Lebras, J.; Ringwald, P.; Mussano, P.; Garner, P.; Brasseur, P. Systematic Review of Amodiaquine Treatment in Uncomplicated Malaria. Lancet 1996, 348, 1196-1201.
4. Neftel, K. A.; Woodtly, W.; Schmid, M. Amodiaquine Induced Agranulocytosis and Liver Damage. Br. Med. J. 1986, 292, 721- 723.
5. Lind, D. E.; Levi, J. A.; Vincent, P. C. Amodiaquine Induced Agranulocytosis; Toxic Effects of Amodiaquine in Bone Marrow Culture *In vitro*. *Br. Med. J.* 1973, *1*, 458-460.
6. Harrison, A. C.; Kitteringham, N. R.; Clarke, J. B.; Park, B. K. The Mechanism of Bioactivation and Antigen Formation of Amodiaquine in the Rat. Biochem. Pharmacol. 1992, 43, 1421- 1430.
7. Maggs, J. L.; Kitteringham, N. R.; Park, B. K. Drug Protein Conjugates-XIV. Mechanism of Formation of Protein Arylating Intermediates from Amodiaquine a Myelotoxin and Hepatotoxin in Man. Biochem. Pharmacol. 1988, 37, 303-311.
8. Casteel D. A. Antimalarial agents. In: Burger's Medicinal Chemistry and Drug Discovery, 6th Ed., Vol. 5: Chemotherapeutic agents, Abraham, D. J., Editor; John Wiley & Sons Inc., Hoboken and New Jersey; 2003, 920-1031.
9. (a) O'Neill, P. M.; Hawley, S. R.; Bray, P. G.; Ward, S. A.; Park, B. K. The 4-Aminoquinolines-Past, Present and Future-A Chemical Perspective. Pharmacol. Ther. 1998, 77, 29-58.
10. O'Neill, P. M.; Willock, D. J.; Hawley, S. R.; Bray, P. G.; Storr, R. C.; Ward, S. A.; B. K. Park. Synthesis, Antimalarial Activity, and Molecular Modeling of Tebuquine Analogues. J. Med. Chem. 1997, 40, 437-448.
11. O'Neil, P. M.; Mukhtar, A.; Stocks, P. A.; Randle, L. E.; Hindley, S.; Ward, S. A.; Storr, R. C.; Bickley, J. F.; O'Neil, I. A.; Maggs, J. L.; Hughes, R. H.; Winstanley, P. A.; Bray, P. G. and Park, B. K.; Isoquine and related amodiaquine analogues: A new generation of improved 4-aminoquinoline antimalarials. J. Med Chem. 2003, 46, 4933-4945.
12. Burckhalter, J. H.; Tendwick, J. H.; Jones, F. H.; Jones, P. A.; Holcombe, W. F.; Rawlins, A. L. Aminoalkylphenols as Antimalarials II (Heterocyclic-amino)-R-amino-*o*-cresols; The Synthesis of Camoquine. J. Am. Chem. Soc. 1948, 70, 1363-1373.
13. Rieckmann K.H, Campbell G.H., Sax L.J., Mrema J. E. Drug sensitivity of *Plasmodium falciperum*, an in vitro microtechnique. Lancet 1978, 1, 221-223.
14. Desjardins R.E. *In vitro* techniques for antimalarial development and evaluation. In: Peters, W. and Richards, W.H.G. editors. Handbook of Experimental Pharmacology. Springer-Verlag, Germany; 1984, 179-200.
15. Trager, W. and Jensen, J.B.; Human Malaria Parasites in Continuous Culture. Science 1976, 193, 673-675.
16. Lambros C, Vanderberg J. P. Synchronization of *Plasmodium falciparum* intraerythrocytic stages in culture. J Parasitol. 1979, 65, 418-420.
17. Singh, J. J.S.B. stain: A review. Indian Journal of Malariology 1956, 10, 117-129.
18. Panjarathinam, R. Text Book of Medical Parasitology, 2nd Ed., Orient Longman Pvt. Ltd.,Chennai; 2007, 329-331.

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