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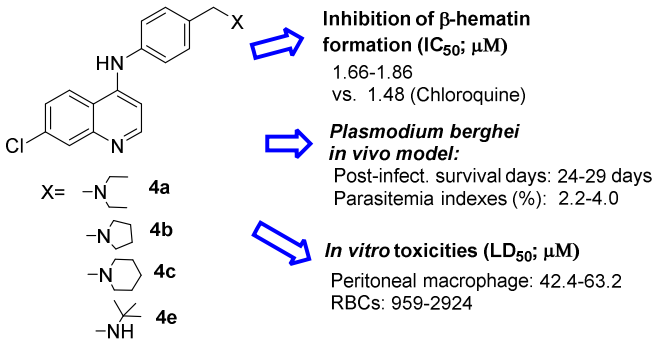


Synthesis, *β*-hematin inhibition studies and antimalarial evaluation of new dehydroxy isoquine derivatives against *Plasmodium berghei*: A promising antimalarial agent

Edgar A. Valverde,[a] Angel H. Romero[a*], María E. Acosta,[b] Neira Gamboa,[b] Genesis Henriques,[b] Juan R. Rodrigues,[b] Carlos Ciangherotti,[b] Simón E. López[a*]

[a]*Laboratorio de Química Medicinal y Heterociclos, Departamento de Química, Universidad Simón Bolívar, Valle de Sartenejas, Baruta, Caracas 1080-A, Apartado 89000, Venezuela.*

[b]*Unidad de Bioquímica, Facultad de Farmacia, Universidad Central de Venezuela, Aptdo. 47206, Los Chaguaramos, 1041-A Caracas, Venezuela.*

[a]***Corresponding authors:** E-mail: angel.ucv.usb@gmail.com (AH Romero); slopez@usb.ve (SE López)

ABSTRACT: Many people are affected by Malaria around the world, and the parasite is developing resistance against available drugs. Currently, isoquine and *N-tert*-butyl isoquine are some of the most promising antimalarial candidates that have already reached Phase I and II clinical trials, respectively. Nevertheless, pharmacodynamic studies have demonstrated that isoquine is highly sensitive to form *O*-glucuronide metabolite, which may affect its accumulation in tissues. To avoid the *O*-glucuronide formation and its negative influence in the accumulation process, a series of novel five dehydroxy isoquine derivatives were designed and prepared herein as potential antimalarial agents. By a simple three-step procedure, five dehydroxy isoquines were prepared and subsequently examined on the inhibition of haemozoin formation, the main target of the 4-aminoquinolines. Four derivatives displayed significant inhibitory activities at low IC_{50} values from 1.66 to 1.86 μM comparable to CQ. On the basis of the results, these four compounds were subsequently tested against *Plasmodium berghei* ANKA model in mice, showing to be as active as CQ with significant curative responses and parasitemia suppresion in mice infected. On the other hand, these four compounds showed an acceptable non specific cytotoxicity on murine peritoneal macrophague and human erythrocyte cells. Thus, the presented data indicate that the dehydroxy isoquines **4b, 4c** and **4e** constitute promising cost-effective leads for the development of new antiplasmodial targeted at blood-stage malaria parasites.

Keywords: Malaria, Isoquine, 4-aminoquinoline, antimalarial activity, *Plasmodium berghei*, Lipinski rule.

1. Introduction

Malaria is one of the largest tropical diseases caused by parasites of the genus *Plasmodium* spp., which are transmitted to humans via the bites of an infected female *Anopheles* mosquito. *Plasmodium falciparum* is the most virulent species in human malaria parasite, responsible for about 1 million deaths every year [1-2]. According to the World Health Organization (WHO) 2015 report, about 3.2 billion people are at risk of malaria, particularly in poor or developing countries, registering 250 million clinical

cases of malaria and more than 400,000 deaths annually, especially among children and pregnant women [3].

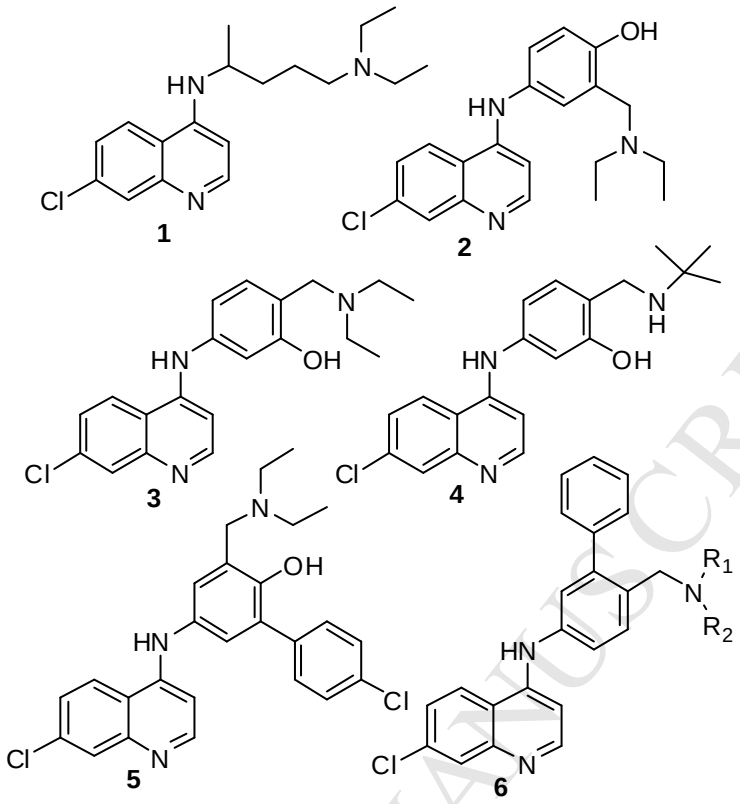


Figure 1. Structure of most important 4-aminoquinolines antimalarial derivatives **1-6.**

Classically, chloroquine (CQ) **1** has been used widely as a standard antimalarial drug for more than 60 years due to many advantage such as excellent availability, low toxicity, effectiveness and facile preparation [1-4]. However, its efficacy has been compromised by the increasing resistant *P. falciparum* in worldwide [5]. As a consequence of the emergence of chloroquine resistance, alternative 4-aminoquinoline such as amodiaquine (AQ) **2**, isoquine (IQ) **3** and tebuquine (TBQ) **5** were subsequently designed and prepared (see structures in Figure 1**)** [5a,6]. In particular, AQ is effective against many chloroquine resistant strain of *P. falciparum*, although its clinical use has been restricted by the hepatotoxic effect and agranulocytosis found in the treated patients [7]. The AQ toxicities are mainly associated to the formation "*in vivo*" of a quinoneimine reactive intermediate from oxidation of the phenolic ring. The oxidation of AQ is facilitated by the *para* position of hydroxyl group on the aniline ring. Although structure activity relationship (SAR) studies have demonstrated that the phenolic group plays an important role in the antimalarial activity of AQ [8-14]. To prevent oxidation to toxic metabolites in *in vivo* models, isoquine (IQ), that posses the hydroxyl group placed at *meta*-position of aniline ring, was designed and evaluated as an alternative antimalarial agent [6a,15]. IQ and derivatives have exhibited excellent *in vitro* and *in*

vivo antimalarial results, with good bioavailabilities. In contrast to AQ, IQ can be excreted primarily by the formation of its *O*-glucuronide derivative (metabolism Phase II), which has been found in bile and urine samples of treated host. This last may affect the accumulation of drug in tissues. Currently, isoquine and *N-tert*-butyl isoquine **4** are in phase I and II clinical trials [16a-c].

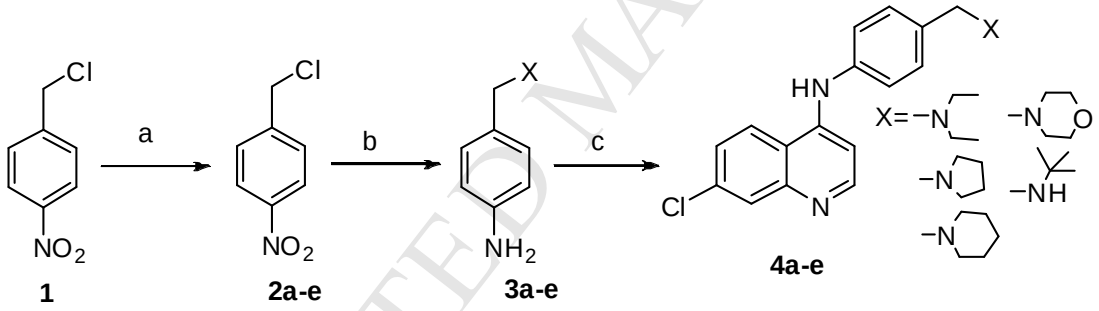
Other group of novel 4-aminoquinolines have been demonstrated excellent antimalarial activity against resistant *P. falciparum* strains [16d-i]. Recently, as part of a preliminary investigation to the present report, we designed and synthesized a series of new dehydroxy isotebuquine derivatives **6** to prevent Phase II-glucuronidation and clarify the relevance of the hydroxy group on the antimalarial response of isotebuquine derivatives. These dehydroxy isotebuquines showed excellent *in vitro* and *in vivo* antimalarial results comparable to standard CQ [17]. Then, in order to complement the mentioned investigation [17], we propused herein the design, synthesis and antimalarial evaluation of new low-cost dehydroxy isoquines **4a-e** (see Scheme 1) as potential antimalarial targeted at blood-stage malarie parasite. Unable to produce the *O*-glucuronide, these new dehydroxy isoquines may exhibit similar or better antimalarial activity than isoquine derivatives.

2. Results and discussion

A series of novel dehydroxy isoquines were designed herein as an alternative isoquine analogue incapable of producing *O*-glucuronide metabolites. This type of metabolite derived of hydroxylated compounds can affect the accumulation of the drug inside host. It is important to mention that the absence of hydroxyl substitution in isoquine derivatives may have profound effect on physicochemical properties such as lipid solubility, water solubility, pKa and hydrogen bondings. The hydroxyl substitution has demonstrated to play an important role on the antimalarial efficacy of AQ, where the replacement of hydroxyl group by either fluorine or chlorine in AQ led a decrease in the antimalarial response against *Plasmodium falciparum* strains [8,15]. The relevance of the hydroxyl substitution in the antimalarial activity of isoquine derivatives has not been investigated previously, which motivated the design, synthesis and antimalarial evaluation of the new dehydroxy isoquine derivatives **4a-e**.

2.1. Chemistry

New dehydroxy isoquine derivatives **4a-e** were synthesized employing a straightforward and efficient protocol reported recently by our group [17], which is depicted in Scheme 1. The synthetic sequence involves a facile three-step procedure from low cost starting materials. Initially, 4-nitrobenzyl chloride **1** was reacted with different secondary amines by a nucleophilic sustitution (SN_2) to lead the corresponding benzylamines **2a-e**. These resulting derivatives **2a-e** were subjected to a subsequent reduction reaction using tin in hydrochloric acid, affording the desired anilines **3a-e** in good yields. Stage 3 of the sequence involves the heteroaromatic nucleophilic substitution (SNAr) reaction of 4,7-dichloroquinolines at position 4 with the synthesized anilines **3a-e** under refluxing ethanol. The reaction requires a catalytic amount of hydrochloric acid (HCl) to give the corresponding substituted 4-aminoquinolines **4a-e** in good 70-80% yields (see Table 1). Particularly, the synthesis of *tert*-butyl quinoline **4** required at least 0.4 eq. of HCl to complete the reaction. In this synthetic sequence, only the anilines derivatives **3a-e** and dehydroxy isoquines **4a-e** were well purified and characterized.



Scheme 1. Synthesis of novel dehydroxy isoquine derivatives **4a-e**. Conditions: (a) dialkylamine- or alkylamine (5 eq.), toluene, 90 ºC, 5 h; (b) Sn, HCl, 70ºC, 2 h. (c) 4,7-dichloroquinoline (1.2 eq.), ethanol, HCl (cat.), reflux, 6 h.

Table 1. Yields for the last step of the synthesis of dehydroxy isoquine derivatives **4a-e**.

Compounds	X[a]	Yield (%)
3a		79
3b		80
3c		81
3d		83
3e		79[b]

[a]The molecular structure of **4a-e** are shown in Scheme 1 and Supplementary material.

[b]The *tert*-butyl substituted quinoline **4e** was obtained as the monohydrochloride salt.

2.2. Biological evaluations

In vitro studies on the haem polymerization. Initially, the ability of the five synthesized dehydroxy isoquine **4a-e** to inhibit *β*-hematin formation was assessed by previously reported methods [17-18]. The percentage of inhibition of haem polymerization (% IHP) and IC_{50} values for each compound are listed in Table 2. The *β*-hematin was formed *in vitro* from hemin under acidic and low oxygen conditions, simulating the natural conditions found in the food vacuole of the parasite. It is well known that the inhibition of the *β*-hematin formation is the main mode of action of diverse 4-amino-7-chloroquinolines such as CQ, AQ, IQ and TBQ [18-23]. The formation of the *β*-hematin is a *Plasmodium*-specific defense mechanism in which the toxic ferriprotoporphyrin IX (FPIX) is converted into an insoluble non-toxic crystalline polymer [24-25]. It is not completely clear how 4-amino-7-chloroquinoline agents inhibit the *β*-hematin formation *in vivo*; however, several evidences have demonstrated that CQ and other 4-aminoquinolines interfere with the haem polymerization through a *π*-*π* stacking interaction between the porphyrin ring of the hematin and the quinoline ring, which facilitate an effective accumulation of the heme/drug complex within the erythrocyte leading the parasite death by oxidative stress [26-30]. On these basis, the inhibition of the *β*-hematin represents the main mode of action of our designed dehydroxy isoquine **4a-e** as antimalarial compounds.

In general, four of the five synthesized quinolines showed an excellent capability to block the haem polymerization (IHP), exhibiting relatively low IC_{50} values from 1.66 to 1.86 μM (see Table 2). It should be noted that these four derivatives presented IC_{50} values against the IHP comparable to that obtained for standard chloroquine (1.48 μM). Only derivative **4d** bearing a morpholine group showed a marginal inhibition of the haem polymerization with a minimum percentage of 5.17% at 50 μM. In addition, it is important to mention that the capacity of the tested dehydroxy isoquine derivatives to inhibit the haem polymerization is comparable to those obtained for diverse hydroxylated 4-amino-7-chloroquinolines such as AQ, IQ or TBQ [18-20], which reflects that this inhibition process is not affected by the absence of the hydroxyl substitution on the aniline ring of the 4-amino7-chloroquinolines. On the other hand, the inhibitory activity found for the dehydroxy isoquines are completely expected because these derivatives keep some important pharmacophore such as 7-chloroquinoline

scaffold, the terminal amine moiety and nonpolar linker between these two mentioned fragments.

Table 2. Results of inhibition of haem polymerization (IHP) and toxicity evaluations for compounds **4a-e**.

Entries	% IHP[a,b] (± SD)	IC_{50}[c] (μM) ± SD	LD_{50}[d] (μM) ± SD	$LyticC_{50}$[e] (μM) ± SD
4a	98.21 ± 1.52	1.78 ± 0.13	42.4 ± 2.3	2924.1 ± 13.3
4b	97.98 ± 1.79	1.86 ± 0.22	52.3 ± 2.8	1405.7 ± 23.4
4c	98.11 ± 2.14	1.81 ± 0.23	63.2 ± 3.4	959.2 ± 24.8
4d	5.17 ± 1.05	>50.0	36.2 ± 3.1	591.3 ± 13.7
4e[f]	98.56 ± 2.13	1.66 ± 0.11	47.1 ± 2.5	1485.4 ± 9.8
CQ[g]	98.57 ± 2.01	1.48 ± 0.14	134.5 ± 1.7	> 1500.0

[a]**SD**: Standard desviation; [b] **%IHP**: Percentage of inhibition of haem polymerization at 50 μM; [c] Inhibitory doses for the inhibition of haem polymerization.[d]Lethal dose of the tested compounds against murine peritoneal macrophague cells.[e] $LyticC_{50}$, half-lytic concentration on red blood cells of the tested compounds. [f] Monohydrochloride quinoline; [g]Positive control: CQ (chloroquine).

In vitro toxicity on murine peritoneal macrophage and human red blood cells (RBCs). Initially, toxicity of the synthesized derivatives was determined using peritoneal macrophages derived from murine model. Macrophages are resident cells found in almost all tissues of the mammal bodies, where they assume specific phenotypes and develop distinct functions. Tissue macrophages are considered as immune sentinels because of their strategic localization and their ability to initiate and modulate immune responses during pathogenic infection or tissue injury and to contribute to the maintenance of tissue homeostasis [31]. LD_{50} was used as parameter to evaluate the toxicity against murine peritoneal macrophages. In general, four of the five tested dehydroxy isoquine derivatives showed an acceptable toxicity toward murine peritoneal macrophages with LD_{50} values ranging from 40 to 63 μM [32]. In particular, the derivatives **4b** (52.3 μM) and **4c** (63.2 μM) exhibited the lowest toxicity ranges against peritoneal macrophagues, while the compound **4d** (36.2 μM) was relatively the most toxic among the tested quinolines. All synthesized compounds were relatively more toxic than CQ against murine peritoneal macrophagues.

Secondly, cytotoxicity effect of these five derivatives was evaluated on human red blood cells (RBCs). RBCs are crucially important because of these compounds act on the blood-stage malaria parasites. To evaluate the *in vitro* toxicological effect of the most active dehydroxyl isoquine derivative, we used a model based on the lysis of RBCs, measuring the haemoglobin released in the supernatant fraction [33]. The release of haemoglobin by an equal number of RBCs by hypotonic lysis in 0.05 volumes of

water was used as a 100% positive control, while RBCs treated with saline solution served as negative controls. Their toxicity results were expressed as the concentration at which half of the RBCs lysed (LyticCC_{50}). Generally speaking, all tested derivatives **4a-e** showed a significant cytotoxicity against human RBCs with high LyticC_{50} values from 591 to 2924 μM, indicating that the action of these compounds has a negligible effect on the viability of the RBCs in *in vivo* models. This result is very promising because our dehydroxy isoquines act on the blood stage of parasite. Similarly to toxicity against macrophages, the compound **4d** exhibited the highest toxicity among the tested quinolines on RBCs. On the basis of inhibition of haem polymerization and toxicity results presented in table 2, the compounds **4a, 4b, 4c** and **4e** were selected for further studies in the mouse model of malaria.

In vivo antimalarial effect of the dehydroxy isoquines. In order to know the therapeutic efficacy of the synthesized dehydroxy isoquines the best compounds **4a, 4b, 4c** and **4e** were evaluated in a standard "4-day test" in mice infected intravenously with murine pathogen *Plasmodium berghei* ANKA, a chloroquine-susceptible strain. Mice were treated with the compound (CQ or **4a-e**, ip once daily) for four consecutive days (days 0-4 post-infection). Then, parasitemias in peripheral blood were measured at day fourth post-infection and the survival days were monitored for the treated mice. These results on treated mice were compared with control mice receiving saline (untreated mice) (see Table 3). In general, dehydroxy isoquines **4a, 4b, 4d** and **4e** have the ability to increase significantly the survival time in more than 20 days post-infection, results comparable to those obtained for CQ drug. It is important to emphasize that the untreated mice died between days 6 and 7 post-infection, demonstrating the significant *in vivo* efficacy of the four tested compounds. On other hand, the treated mice presented a remarkable suppression of parasitemia with percentages from 2 to 4 % post-infection, which are lower than those found for untreated mice of 63% post-infection. Thus, the significant survival time of infected mice is associated to a curative response of the compounds on the parasitemia (see Table 3). Finally, the satisfactory correlation between the antimalarial *in vivo* and inhibition of *β*-hematin formation results indicates that the significant antimalarial efficacy of our designed dihydroxy isoquines may be associated mainly to the inhibition of haem polymerization within the erythrocytes.

Table 3. The effect of dehydroxy isoquine derivatives on *P.berghei* infected mice.

Entries	Post-inf. days of survival (± SD)[a]	%P (± SD)[b,c]
4a	24.90 (2.95)	3.80(1.20)
4b	27.70 (1.80)	2.50 (0.75)
4c	28.20 (1.50)	2.20 (0.86)
4e	25.80 (2.05)	4.00 (0.25)
CQ[d,e]	29.40 (0.40)	0.60 (0.08)
Untreated control	6.80 (0.70)	65.20 (2.58)

[a]**Results are expressed as the media±standard desviation (SD)**. n=6 (number of treated mice); Standard desviation; [b]**%P**: Percentage of parasitemia; [c]P<0,05 comparing to control treated group; [d]Positive control treated with cholorquine (25 mg kg^{-1}); [e]P<'0,01 comparing to control treated group.

In general, the nature of the alkylamine moiety seems to have an important influence on the effectiveness of these classes of derivatives. Particularly, the pyrrolidine and piperidine derivatives exhibited the best values of inhibition of haem polymerization, parasitemia and survival days for infected mice with *P. berghei* among the compounds tested, while the morpholine substituted quinoline was discarded as potential antimalarial agent due to its poor inhibitory activity on the haem polymerization. The effect of the alkylamine group on the antimalarial activity of the dehydroxy isoquines is in good agreement with those reported for amodiaquine and isoquine derivatives [6]. The particular negative effect of morpholine moiety was also found for the dehydroxy isotebuquine reported recently by our group [17]. Curiously, the less toxic compounds **4b**, **4c** and **CQ** against macrophague cells exhibited the best *in vivo* antimalarial effect (compared Tables 2 and 3). Therefore, the new dehydroxy isoquines **4b** and **4c** represent promising antimalarial candidates for further studies with a significant curative antimalarial response and low toxicological effect.

The significant antimalarial activity found for the dehydroxy isoquine herein and those previously reported for the dehydroxy isotebuquine derivatives [17], make us to conclude that the hydroxyl group is not essential to lead a significant antimalarial activity for the studied isoquine and isotebuquine compounds. In addition, the inclusion of hydroxyl group in isoquine derivatives contributes to its fast elimination in an *in vivo* model by excretion as glucuronide, which affect its effective concentration in animal tissues. This fast elimination mechanism is not possible in our designed structures; although additional pharmacodynamics, pharmacokinetic and metabolism assays are necessary for our derivative to know the real positive effect of the absence of the hydroxyl group. On the other hand, no appreciable differences in antimalarial activities between the dehydroxy isoquine **4a-e** and reported dehydroxy isotebuquine[17] were

observed, by which the inclusion of additional phenyl ring on the aniline ring of dehydroxy isoquine seem to have a negligible effect in the biological response of these interesting promising 4-aminoquinolines.

2.3. Molecular Docking studies

In order to know if the action of dehydroxy isoquines may affect other stages of the erythrocytic cycle of the parasite, a molecular docking study was performed on a protease involved in hemoglobin degradation such as the plasmepsins of *Plasmodium falciparum*. In general, there are several proteases involved in the hemoglobin degradation pathway such as plasmepsins (Plm) I, II and IV and HAP (a histo-aspartic protease). These proteases are located in the acidic food vacuole of the parasite and are essential for the survival and reproduction of the most virulent malaria parasite, *Plasmodium falciparum* [34a]. In particular, the plasmepsins are aspartic proteases with the catalytic aspartic residues situated in an active site cleft formed by two domains. The catalytic mechanism of peptide bond cleavage in aspartic proteases is generally considered to involve the activation of a water molecule by one of the aspartates accompanied by nucleophilic attack of the water/hydroxide on the carbonyl carbon of the substrate [34b]. Thus, taken into account the well known good acumulation of 4-aminoquinolines in the acidic vacuole of the parasite, these type of proteases emerge as highly promising drug targets for theraphies against malaria [35]. In this sense, molecular docking of our five derivatives were carried out on Plm I and Plm II enzymes, deposited in the Protein Data Bank website under codes 1LEE and 1LF2, respectively. Molecular docking calculations were performed using the AMBER force field, applied to the complete structure of both enzymes. The molecular docking results of the tested compounds **4a-e**, CQ and references drugs (R36 and R37) **1-5** are shown in Table 4 and Figure 2.

The molecular docking results showed clearly that our dehydroxy isoquines exhibited weaker affinity towards both Plm I and Plm II enzymes than the reference drugs, by which seem, at least theoretically, that our dehydroxy isoquines are should not be good inhibitors of these enzymes. On the other hand, a detailed analysis of ligand-enzyme complex showed that reference drugs R36 and R37 are more extended along active site than our dehydroxy isoquines (see Figure 2), which permits to facilitate the stabilization of the ligand-enzyme complex and explain the remarkable binding affinity found for R36 and R37 compared to our derivatives. Despite the exclusive theoretical nature of this study, it is possible to conclude that a good inhibitor of the Plm I and Plm II

enzymes is represented by a molecule containing long, flexible and coordinative chains (see structure of R36 and R37). Then, based on molecular features, this mode of action is practically discared for our synthesized compounds, and the significant antimalarial response of our synthesized compounds is associated mainly to the inhibition of haem polymerization. Ligand-enzyme interactions into active site of the Plm I and PlmII enzymes are shown clearly in Supplementary material.

Table 4 shows the hydrogen bonding interactions exhibited by the five derivatives with essential residues of active sites. In addition, all derivatives exhibited two π-π interactions between quinoline ring of **4a-e** with the phenyl ring of Tyr-77 and Phe-111 residues in the Plm I enzyme.

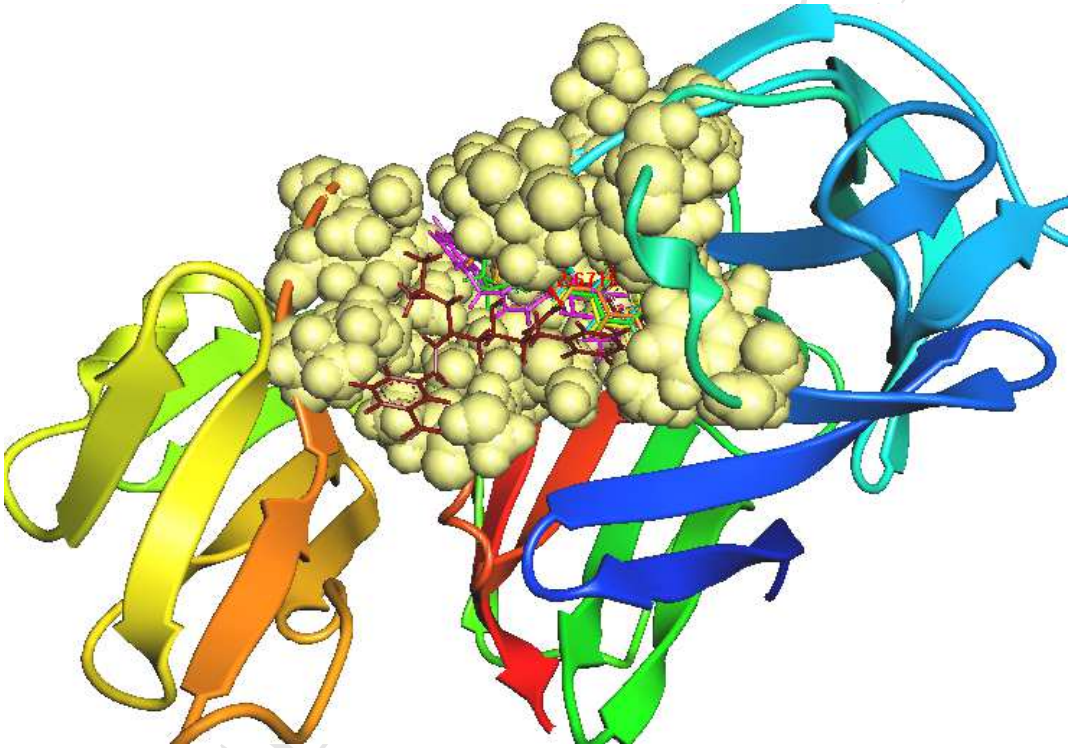


Figure 2. Representation of ligand-enzyme complex for the dehydroxy isoquines **4a-e** in the active site of the Plm I enzyme.

Table 4. Docking statistics on the Plm I and Plm II enzymes for the derivatives **4a-e**.

Molecules	Plm I		Plm II	
	E (kcal/mol)	*H*-interactions[c]	*E(kcal/mol)*	*H*-interactions[d]
4a	-10.751	No interactions	-10.342	Asp-214 (3.00Aº) and Thr-217 (2.75Aº)
4b	**-11.855**	Ser-79 (2.94 Aº)	**-11.485**	Asp-34 (2.88Aº), Gly-36 (2.78Aº), Tyr-192 (3.00 Aº)
4c	**-11.287**	Ser-79 (2.81 Aº)	-10.674	Ser-79 (2.53Aº)
4d	-11.034	Try-192 (2.53 Aº)	-10.163	Ser-218 (2.34 Aº), Thr-217 (2.62 Aº)
4e	-10.888	Asp-214 (3.00 Aº)	-10.996	Ser-79 (2.98 Aº), Gly-36 (3.00 Aº)
CQ	-10.125	Ser-79 (2.98 Aº)	-9.781	Ser-79 (2.52 Aº)
R36[a]	-14.137	Ser-79 (2.67 Aº), Ser-218 (2.64 Aº), Ala-219 (2.88 Aº).	----------	----------
R37[b]	---------	----------	-13.452	Ser-79 (2.23 A º), Gly-216 (2.31 Aº), Thr-217 (2.03 Aº)

[a]Reference drug for the Plm I enzyme (PDB code: 1LEE). Name: 4-amino-*N*-{4-[2-(2,6-dimethyl-phenoxy)-acetylamino]- 3-hydroxy-1-isobutyl-5-phenyl-pentyl}-benzamide.

[b]Reference drug for the Plm II enzyme (PDB code: 1LF2). Name: 3-amino-*N*-{4-[2-(2,6-dimethyl-phenoxy)-acetylamino]- 3-hydroxy-1-isobutyl-5-phenyl-pentyl}-benzamide.

[c]*H*-interactions (in Aº) in Plm I is through the NH of aniline ring. **4d** interact through oxygen of morpholine ring.

[d]*H*-interactions (in Aº) in Plm II is through the NH of aniline ring and the nitrogen of the dialkylamine moiety.

As last topic, in order to identify quinolines orally active, the five Lipinski´s parameters such as partition coefficient (LogP), molacular weight (MW), number of *H*-donors (*H*-D) and *H*-acceptors (*H*-A), were calculated for the tested dehydroxy isoquines **4a-e** (see Table 5). The rule of five is an important criteria for the design of new orally active drug candidate. By applying the rule of five (RO5) and its variants,[36] all our derivatives showed good drug-like properties, except to their Log *P* whose values are near to the upper limit of lipophilicity. This fact is very important due to that the absence of hydroxy moiety could compromise the bioavailability of these new derivatives; however still it is required to make additional studies of bioavailability on the most promising compounds.

Table 5. Calculation of Lipinski´s parameters for the dehydroxy isoquines **4a-e** and CQ.[a]

	Compounds	Log $P_{4.5}$	Log $P_{7.4}$	MW	nON	nOHNH	nViol
		<5	<5	<500	<10	<5	
1	**4a**	4.77	4.76	339.86	3	1	0
2	**4b**	4.82	4.87	351.87	3	1	0
3	**4c**	4.40	4.42	337.85	3	1	0
4	**4d**	3.68	3.42	353.85	4	1	0
5	**4e**[b]	4.77	4.66	375.13	3	2	0
6	**CQ**	3.73	3.68	319.18	3	1	0

[a]nViol, number of violations; MW, molecular weight; nON, number of hydrogen bond acceptors; nOHNH, number of hydrogen bond donors. [b] Monohydrochloride quinoline.

3. Conclusion

In summary, novel dehydroxyl-isoquines **4b**, **4c** and **4e** emerged as excellent candidates for further studies with significant activities on the inhibition of the haem polymerization, satisfactory *in vivo* results and acceptable toxicity ranges. The dehydroxy isoquines **4a-e** were prepared in good yields using a simple three step synthetic sequence from low-cost starting materials. The good correlation between the antimalarial data and inhibition of the haem polymerization suggests that the inhibition of the *β*-hematin is the main mechanism of action of these novel dehydroxyl isoquines. A structure-activity relationship indicates that the 3-hydroxyl substitution on the isoquine compound is not necessary to lead a potent antimalarial effect such as was observed in our dehydroxyl isotebuquines. However, further *in vitro* and *in vivo* assays on resistant strain of *Plasmodium falciparum* are required to evaluate the real potential of the most active derivatives **4b**, **4c** and **4e**.

4. Experimental section

4.1. Chemistry

All reagents and starting materials were purchased from commercial sources and used without further purification; solvents were anhydrous HPLC grade. Melting points are recorded with a micro melting point apparatus und uncorrected. ^{1}H NMR and ^{13}C NMR spectra are recorded at JEOL Eclipse Plus 400 or JEOL Eclipse Plus 270. Multiplicity is indicated as follows: s (singlet), d (doublet), t (triple), m (multiplet), dd (doublet of doublets), br s (broad singlet); chemical shifts were measured in parts per million (δ) and coupling constant (*J*) are given in Hz. Proton chemical shifts were given in relative to tetramethylsilane (δ 0.00 ppm) in $CDCl_3$ or CD_3OD solvents. Carbon chemical shifts are internally referenced to the deuterated solvent signals in $CDCl_3$ (δ 77.00 ppm) or CD_3OD (δ 48.67 ppm). IR spectra were recorded on NICOLET Magna IR 760 using KBr tablets. Elemental analyses of the synthesized compounds were performed using a Perkin Elmer 2400 CHN analyser: results fell in the range of 0.4% of the required theoretical values.

4.1.1. General procedure for the preparation of 4-(*N*,*N*-dialkylamine-1-methyl)aniline 3a-e: These derivatives were prepared following a similar procedure reported in literature [17]. To a solution of 1-(chloromethyl)-4-nitrobenzene **1** (2 mmol, 1 equiv.) in toluene was added slowly the corresponding dialkylamine or *tert*-butylamine

(10 mmol, 5 equiv.). The mixture was stirred under reflux for 4-5 hours and monitored by TLC using solvent mixture Hex/AcOEt (7:3). The reaction mixture was then cooled to room temperature, the solvent and the unreacted alkyl(dialkyl)amine was evaporated under reduced pressure to give a yellow-white solid of 1-(alkyl(dialkyl)amine-methyl)-4-nitrobenzenes **2a-e** (see ^{1}H-NMR and ^{13}C-NMR data in supplementary data from page 8 to page 23), which was pure enough to proceed with the oxidation step. These 1-(dialkylaminemethyl)-4-nitrobenzene **2a-e** (2 mmol, 1 equiv.) were treated with a solution of hydrochloric acid (20 mmol) and tin powder (6 mmol, 3 equiv.) under reflux for 2 hours. The reaction mixture was then cooled to room temperature and neutralized carefully with sodium hydroxide solution (aq. 10%) and extracted with dichloromethane (3x20 mL). The organic lawyer was washed with distilled water, dried using anh. $MgSO_4$, filtered and the solvent evaporated under reduced pressure to give the aniline product, which was purified by flash chromatography column using as eluent dichloromethane: methanol (9/1) to give a yellow-orange compound **3a-e**.

4.1.1.1. 4-(diethylamino)methyl-aniline 3a. Orange oil, yield 72%. FT-IR (KBr, ν cm^{-1}): 3354 (st. N-H), 3158 (st. N-H), 2934-2890 (st. C-H), 1608-1522 (st. C=C, Ar). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.09 (dd, 2H, *J*=8.40; *J*=2.52); 6.62 (dd, 2H, *J*=8.20; *J*=1.80); 3.61 (s, 2H, NH_2); 3.46 (s, 2H, CH_2-N); 2.49 (q, 4H, CH_2-N); 1.03 (t, 6H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 145.2; 130.3 (2C); 129.2; 115.0 (2C); 56.8 (CH_2-N); 46.4 (2C, CH_2-N); 11.6 (2C, CH_3). DEPT-135 (100 MHz, $CDCl_3$): 130.3 (2C); 115.0 (2C); 56.8 (1C, CH_2-N); 46.4 (2C, CH_2-N); 11.6 (2C, CH_3). Anal. Calc. for $C_{11}H_{18}N_2$: C, 74.11; H, 10.18; N, 15.71. Found: C, 74.03; H, 10.12; N, 15.58.

4.1.1.2. 4-(piperidino-1-ylmethyl)-aniline 3b. Orange oil, yield 78%. FT-IR (KBr, ν cm^{-1}): 3332 (st. N-H), 3142 (st. N-H), 2934-2890 (st. C-H), 1601-1526 (st. C=C, Ar). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.07 (dd, 2H, *J*=8.44; *J*=2.56); 6.62 (dd, 2H, *J*=8.30; *J*=1.84); 3.60 (s, 2H, NH_2); 3.39 (s, 2H, CH_2-N); 2.37 (s, 4H, CH_2-N); 1.56 (m, 4H, CH_2); 1.40 (m, 2H, CH_2). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 145.8; 130.8 (2C); 128.2; 114.9 (2C); 63.2 (CH_2-N); 54.1 (2C, N-CH_2); 25.7 (2C, CH_2); 24.3 (1C, CH_2). DEPT-135 (100 MHz, $CDCl_3$): 130.8 (2C); 114.9 (2C); 63.2 (CH_2-N); 54.1 (2C, N-CH_2); 25.7 (2C, CH_2); 24.3 (2C, CH_2). Anal. Calc. for $C_{12}H_{18}N_2$: C, 75.74; H, 9.53; N, 14.72. Found: C, 75.68; H, 9.48; N. 14.63.

4.1.1.3. 4-(pyrrolidino-1-ylmethyl)-aniline 3c. Orange oil, yield 81%. FT-IR (KBr, ν cm^{-1}): 3351 (st. N-H), 3167 (st. N-H), 2934-2890 (st. C-H), 1599-1529 (st. C=C, Ar).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.10 (d, 2H, *J*=8.08); 6.63 (dd, 2H, *J*=8.20; *J*=2.60); 3.57 (s, 2H, NH_2); 3.51 (s, 2H, CH_2-N); 2.50 (s, 4H, N-CH_2); 1.75 (m, 4H, CH_2). DEPT-135 (100 MHz, $CDCl_3$): 129.8 (2C); 115.4 (2C); 68.2 (CH_2-N); 46.6 (2C, N-CH_2); 20.5 (2C, CH_2). Anal. Calc. for $C_{11}H_{16}N_2$: C, 74.96; H, 9.15; N, 15.89. Found: C, 74.83; H, 9.06; N, 15.80.

4.1.1.4. 4-(morpholinomethyl)-aniline 3d. Yellow solid, 84%, m.p.: 82-84°C. FT-IR (KBr, ν cm^{-1}): 3359 (st. N-H), 3175 (st. N-H), 2934-2890 (st. C-H), 1603-1524 (st. C=C, Ar).). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.07 (dd, 2H, *J*=8.44; *J*=2.56); 6.61 (dd, 2H, *J*=8.44; *J*=2.54); 3.70 (t, 4H, CH_2-O); 3.62 (s, 2H, NH_2); 3.37 (s, 2H, CH_2-N); 2.40 (s, 4H, CH_2-N). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 145.6; 130.5 (2C); 127.5; 115.0 (2C); 67.1 (CH_2-N); 63.1 (2C, N-CH_2); 53.6 (2C, CH_2). Anal. Calc. for $C_{11}H_{16}N_2O$: C, 68.72; H, 8.39; N, 14.57. Found: C, 68.61; H, 8.28; N, 14.45.

4.1.1.5. 4-(*tert*-butylamino)methyl-aniline 3e. Yellow oil, yield, 87%. FT-IR (KBr, ν cm^{-1}): 3354 (st. N-H), 3150 (st. N-H), 2934-2890 (st. C-H), 1602-1524 (st. C=C, Ar). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.10 (d, 2H, *J*=8.04); 6.63 (dd, 2H, *J*=8.20; *J*=1.84); 3.60 (s, 2H, CH_2); 1.11 (s, 9H, CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$): 145.2; 131.4 (2C); 129.5; 115.3 (2C); 50.7 (CH_2-N); 46.8; 29.2 (3C, CH_3). Anal. Calc. for $C_{11}H_{18}N_2$: C, 74.11; H, 10.18; N, 15.71. Found: C, 74.07; H, 10.02; N, 15.59.

4.1.2. General procedure for the preparation of 4-amino-quinolines 4a-e: These derivatives were prepared following a similar procedure reported by our group [17]. To a solution of 4,7-dichloroquinoline (1 mmol, 1.2 equiv.) in ethanol (20 mL) was added concentrated hydrochloric acid (catalytic amount from one to two drops) and stirred by 4 minutes. To this mixture was added the corresponding aniline **3a-e** (1 equiv.) and stirred under reflux for 4 hours. During the reaction a yellow precipitate was observed, the mixture was then cooled to room temperature and the precipitated solid filtered, washed with ethanol and dried under reduced pressure to yield the desired quinolines **4a-e**.

4.1.2.1. 7-chloro-*N*-(4-((diethylamino)methyl)phenyl)quinolin-4-amine 4a. Yellow solid, yield 79%, m.p.: 198-200°C. FT-IR (KBr, ν cm^{-1}): 3165-3090 (st. N-H), 2967-2933 (st. C-H), 1614-1590-1534-1511 (st. C=C, Ar). ^{1}H-NMR (400 MHz, CD_3OD): δ 8.28 (d, 1H, *J*=5.48); 8.24 (d, 1H, *J*=9.0); 7.82 (d, 1H, *J*=1.64); 7.46 (d, 1H, *J*=8.96, *J*=1.72); 7.37 (d, 2H, *J*=8.2); 7.30 (d, 2H, *J*=8.2); 6.88 (d, 1H, *J*=5.48); 4.89 (s, 1H, NH); 3.30 (s, 2H); 2.54 (q, 4H, CH_2); 1.08 (t, 6H, CH_3). ^{13}C-NMR (100 MHz, CD_3OD):

δ 152.4; 151.1; 150.2; 140.2; 136.7; 136.1; 131.8 (2C); 127.8; 126.7; 124.6; 124.4 (2C); 119.5; 102.6; 58.8 (CH_2-N); 47.8 (2C,N-CH_2); 11.4 (2C, CH_3). Anal. Calc. for $C_{20}H_{22}ClN_3$: C,70.68; H, 6.52; N, 12.36. Found: C, 70.71; H, 6.37; N, 12.14.

4.1.2.2. 7-chloro-*N*-(4-(piperidino-1-ylmethyl)phenyl)quinolin-4-amine 4b. Yellow solid, yield 80%, m.p.:229-231°C. FT-IR (KBr, ν cm^{-1}): 3156-3093 (st. N-H), 2967-2925 (st. C-H), 1611-1590-1530 (st. C=C, Ar). ^{1}H-NMR (400 MHz, CD_3OD): δ 8.36 (d, 1H, *J*=4.56); 8.27 (d, 1H, *J*=8.8); 7.85 (s, 1H); 7.48 (d, 1H, *J*=8.8); 7.41 (d, 2H, *J*=7.0); 7.33 (d, 2H, *J*=7.3); 6.93 (d, 1H, *J*=4.6); 4.61 (s, 1H, NH); 3.58 (s, 2H, CH_2-N); 2.52 (q, 4H, N-CH_2); 1.64 (s, 4H, CH_2); 1.49 (m, 2H, CH_2). ^{13}C-NMR (100 MHz, CD_3OD): δ 152.4; 151.1; 150.2; 140.7; 136.8; 135.1; 132.4 (2C); 127.8; 126.7; 124.7; 124.3 (2C); 119.6; 102.8; 63.8 (CH_2-N); 55.0 (2C, N-CH_2); 26.2 (2C, CH_2); 24.9 (1C, CH_2). Anal. Calc. for $C_{20}H_{22}ClN_3$: C,70.68; H, 6.52; N, 12.36. Found: C, 70.71; H, 6.37; N, 12.14. Anal. Calc. for $C_{21}H_{22}ClN_3$: C, 71.68; H, 6.30; N, 11.94. Found: C, 71.59; H, 6.09; N, 11.42.

4.1.2.3. 7-chloro-*N*-(4-(pyrrolidino-1-ylmethyl)phenyl)quinolin-4-amine 4c. Yellow solid, yield 81%, m.p.: 196-198°C. FT-IR (KBr, ν cm^{-1}): 3160-3096 (st. N-H), 2964-2930 (st. C-H), 1612-1575-1532-1512 (st. C=C, Ar). ^{1}H-NMR (400 MHz, CD_3OD): δ 8.33 (d, 1H, *J*=4.56); 8.25 (d, 1H, *J*=8.8); 7.83 (s, 1H); 7.45 (d, 1H, *J*=8.8); 7.39 (d, 2H, *J*=7.0); 7.31 (d, 2H, *J*=7.3); 6.89 (d, 1H, *J*=4.6); 4.61 (s, 1H, NH); 3.63 (s, 2H, CH_2-N); 2.56 (q, 4H, N-CH_2); 1.81 (m, 2H, CH_2). ^{13}C-NMR (100 MHz, CD_3OD): δ 152.4; 151.1; 150.2; 140.4; 136.8; 136.0; 131.7 (2C); 127.9; 126.7; 124.7; 124.4 (2C); 119.6; 102.7; 60.9 (CH_2-N); 54.9 (2C, N-CH_2); 24.1 (2C, CH_2). Anal. Calc. for $C_{20}H_{20}ClN_3$: C, 71.10; H, 5.97; N, 12.44. Found: C, 71.25; H, 6.02; N, 11.77.

4.1.2.4. 7-chloro-*N*-(4-(morpholinomethyl)phenyl)quinolin-4-amine 4d. Yellow solid, yield 83%, m.p.: 219-221°C. FT-IR (KBr, ν cm^{-1}): 3160-3080 (st. N-H), 2953-2926 (st. C-H), 1613-1593-1535-1511 (st. C=C, Ar). ^{1}H-NMR (400 MHz, CD_3OD): δ 8.36 (d, 1H, *J*=5.6); 8.29 (d, 1H, *J*=9.1); 7.87 (d, 1H, *J*=2.1); 7.52 (dd, 1H, *J*=9.0; *J*=2.1); 7.42 (d, 2H, *J*=8.5); 7.33 (d, 2H, *J*=8.5); 6.91 (d, 1H, *J*=5.6); 4.6 (s, 1H); 3.71 (t, 4H, CH_2-O); 3.57 (s, 2H, CH_2-N); 2.52 (t, 4H, N-CH_2). ^{13}C-NMR (100 MHz, CD_3OD): δ 151.9; 151.6; 149.6; 140.3; 137.1; 134.9; 132.1 (2C); 127.3; 126.9; 124.8; 124.6 (2C); 119.4; 102.6; 67.6 (2C, CH_2-O); 63.7 (1C, N-CH_2); 52.4 (2C, CH_2-N). Anal. Calc. for $C_{20}H_{20}ClN_3O$.(0,06.CH_3OH): C, 66.06; H, 6.11; N, 11.17. Found: C, 66.05; H, 5.39; N, 10.81.

4.1.2.5. *N*-(4-((*tert*-butylamino)methyl)phenyl)-7-chloroquinolin-4-amine hydrochloride 4e. In this case, the resulting compound was not treated with alcaline solution, and the product was recrystalizated directly to give the monohidrochloride salt. Yellow solid, yield 79%, m.p.: 220-222 °C. FT-IR (KBr, ν cm^{-1}): 3284 (st. N-H), 2960 (st. C-H), 1613-1575-1528-1514 (st. C=C, Ar). ^{1}H-NMR (400 MHz, CD_3OD): δ 9.06 (s, 1H, N-H); 8.43 (s, 1H); 8.42 (d, 1H, *J*=2.92); 7.88 (d, 1H, *J*=2.20); 7.55 (d, 1H, *J*=8.80; *J*=2.20); 7.39 (d, 2H, *J*=8.44); 7.27 (d, 2H, *J*=8.44); 6.83 (d, 1H, *J*=5.52); 3.66 (s, 2H, CH_2-N); 1.11 (s, 9H, CH_3). ^{13}C-NMR (100 MHz, CD_3OD): δ 152.4; 151.3; 150.2; 140.1; 138.0; 136.7; 131.0 (2C); 127.8; 126.7; 124.8; 124.7 (2C); 119.6; 102.5; 52.5 (CH_2-N); 48.2; 28.6 (3C, CH_3). Anal. Calc. for $C_{20}H_{23}Cl_2N_3$: C, 63.83; H, 6.16; N, 11.17. Found: C, 64.35; H, 6.28; N, 10.73.

4.2. Biological assays

4.2.1. Inhibition of haemozoin formation

The haemozoin formation assay was performed according to the literature [31]. Briefly, a solution of hemin chloride (50 μL, 4 mM), dissolved in DMSO (5.2 $mg.mL^{-1}$), was distributed in 96-well micro plates. Different concentrations (50-5 μM) of the quinoline compounds **4a-e** dissolved in DMSO, were added in triplicate in test wells (50 μL). Controls contained either water (50 μL) or DMSO (50 μL). *β*-Hematin formation was initiated by the addition Acetate buffer (100 μL 0.2 M, pH 4.4). Plates were incubated at 37 °C for 48 h to allow completion of the reaction and centrifuged (4000 rpm × 15 min, IEC-CENTRA, MP4R). After discarding the supernatant, the pellet was washed twice with DMSO (200 μL) and finally, dissolved in NaOH (200 μL, 0.2 N). The solubilized aggregates were further diluted 1:2 with NaOH (0.1 N) and absorbances recorded at 405 nm (Microplate Reader, BIORAD-550). The results were expressed as a percentage of inhibition of haemozin formation (% IHF). CQ was used as positive control.

4.2.2. Parasite, experimental host and strain maintenance

Male Balb-C mice, weighing 18-22 g were maintained on a commercial pellet diet and housed under conditions approved by Ethics Committee. *P. berghei* (ANKA strain), a rodent malaria parasite, was used for infection. Mice were infected by i.p. injection with 1×10^6 infected erythrocytes diluted in phosphate buffered saline solution (PBS, 10 mM, pH 7.4, 0.1 mL). Parasitemia was monitored by microscopic examination of Giemsa stained smears [37].

4.2.3. Four-days suppressive test

Balb-C mice (18-22 g) were infected i.v. (using caudal vein) with 10^6 infected RBC with *P. berghei* (n=6). Two hours after infection, treatment began with the best compounds tested in the *in vitro* assays. These quinolines **4a, 4b, 4c** and **4e** were dissolved in DMSO (0.1 M), diluted with Saline-Tween 20 solution (2%). Each compound (20 $mg.kg^{-1}$) was administered once by i.p. for 4 days. At day 4, the parasitemia was counted by examination of Giemsa stained smears. Chloroquine (25 $mg.kg^{-1}$) was used as a positive control. The survival time beyond the control group (saline treated) was recorded. The results were expressed as percentage of parasitemia (% of parasitemia) and survival days of each compound treated-group over the control (saline treated group) [38].

4.2.4. Murine peritonial macrophages growth inhibition assay.

Murine peritoneal macrophages were were plated in 96-well plates at $5x10^4$ cell per well in 150 mL culture medium composed of DMEM supplemented with 5 mM L-glutamine, 10% (v/v) fetal bovine serum and antibiotics (penicillin/streptopmycin/ amphotericin B). After an overnight incubation in a 5% CO_2 humidified incubator, different concentrations of the tested dehydroxy isoquines **4a, 4b, 4c** and **4e** were added to the cultures (duplicate wells; 200 mL final culture volume) and incubation continued for an additional 48 h. Cell viability in individual wells was assessed by using the colorimetric MTT reduction assay and expressed as percentage of MTT reduction relative to control wells containing untreated cell and used to derive LD_{50} values from dose response plots of % cell viability vs. log (compound concentration) using the non-linear regression function of GraphPad Prism v.5.02 [39].

4.2.5. In vitro toxicity on human red blood cells (RBCs)

To evaluate the *in vitro* toxicological effect of the dehydroxyl isoquine derivative **4a-e**, we used a model based on the lysis of red blood cells (RBCs), measuring the haemoglobin released in the supernatant fraction [33]. The haemoglobin released is measured using spectrophotometer at 550 nm. RBCs in 50% Alsever's solution were centrifuged at 800*g* for 10 min and then washed three times with saline solution to obtain RBCs in 100%. The synthesized compound (5-5000 μM) was incubated with a 2% final suspension of RBCs at 37°C for 45 min. The release of haemoglobin by an equal number of RBCs by hypotonic lysis in 0.05 volumes of water was used as a 100%

positive control, while RBCs treated with saline solution served as negative controls. Results were expressed as the concentration at which half of the RBCs lysed ($LyticC_{50}$).

4.2.6. Molecular docking studies

Five dehydroxylisoquines **4a-e** were taken from our previous investi-gations as potential inhibitors of the Plasmepsin I and II enzymes. Each quinoline structures were built using ArgusLab v4.0.1 [40] and their respectives geometry was optimized using semiempiricalmethods: PM3 [41a] and AM1 [41b]. In the optimization geometry calculations of **4a-e**, the requested convergence on density matrix was 10^{-12} and the maximum gradient component was 0.000084. Each optimized quinoline was exported to Protein document for the corresponding molecular docking. The X-ray crystallographic structure of the *P. falciparum* enzymes: Plm I (PDB Code: 1LEE) and Plm II (PDB Code: 1LF2) (see Tables 4 and Figure 2). Addition of hydrogens atoms to the tested proteins was performed. Energy minimization of the proteins was performed with the AMBER force field by using conjugate gradient method with a RMA gradient of 0.01 kcal/A◦mol on Swiss-PdbViewer v4.0.1 [42] software. Binding site definition was determined by comparison with reported interactions for the respective co-crystallized ligand. The prepared protein was exported to ArgusLab v.4.0.1 program package and save as Agl document.4.2. Molecular docking of the 102 diastereomers over the three selected proteins was performed using ArgusLab (v4.0.1) package program under Windows 7.0 enviroment, using AMBER force field. The protein and ligand molecules were prepared as described above. The docking experiment on the tested enzymes was carried out between the optimized ligand into the binding site through their respective grid map dimensions and with a grid point spacing of 0.375 A◦. Flexible ligand model was used in the docking and subsequent optimization scheme. As a test of docking accuracy and for docking energy comparison, co-crystallized ligands were re-docked into the protein structures (see Tables 4 and Figure 2). Different orientations of the ligands were scanned and ranked from their energy scores. Reproducibility of the calculated affinity energy and the minimum energy pose were evaluated through 10 replicates for each ligand [43]. Affinity energy is reported as mean of the 10 replicates. The lowest energy (strongest docking) poses for each ligand in each protein target are summarized in Tables 4.

4.2.6. Data analysis

Data were statistically analyzed using one-way ANOVA and t-tests for specific group comparisons; assuming 95% of confidence according to GraphPad Prism 3.02 [44].

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Supporting Information

Information on compound synthesis and characterization data, and protocols and results for various antimalarial analyses is listed in this file. This material is available at http://sciencedirect.com.

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Highlights

- Novel dehydroxy isoquines were synthesized and evaluated as antimalarial agents.
- Derivatives **4b** and **4c** exhibited high potent activity against *Plasmodium berghei*.
- **4a, 4b, 4c** and **4e** inhibited the *β*-hematin formation at low sub-micromolar ranges.
- The prepared dehydroxy isoquines exhibited acceptable *in vitro* cytotoxicities.