Journal of Enzyme Inhibition and Medicinal Chemistry, 2012; 27(1): 69–77

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.3109/14756366.2011.575071

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RESEARCH ARTICLE

Synthesis and biological screening of some pyridine derivatives as anti-malarial agents

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Abstract

Two series of pyridine derivatives were synthesised and evaluated for their *in vivo* anti-malarial activity against *Plasmodium berghei*. The anti-malarial activity was determined *in vivo* by applying 4-day standard suppressive test using chloroquine (CQ)-sensitive *P. berghei* ANKA strain–infected mice. Compounds **2a**, **2g** and **2h** showed inhibition of the parasite multiplication by 90, 91 and 80%, respectively, at a dose level of 50 µmol/kg. Moreover, The most active compounds (**2a**, **2g** and **2h**) were tested *in vitro* against CQ-resistant *Plasmodium falciparum* RKL9 strains where compound **2g** showed promising activity with IC_{50} = 0.0402 µM. The compounds were non-toxic at 300 and 100 mg/kg through the oral and parenteral routes, respectively. The docking pose of the most active compounds (**2a**, **2g** and **2h**) in the active site of dihydrofolate reductase enzyme revealed several hydrogen and hydrophobic interactions that contribute to the observed anti-malarial activities.

Keywords: Pyridine derivatives, *in vivo*, anti-malarial, docking, acute toxicity, dihydrofolate reductase

Introduction

Malaria has infected humans and may have been a human pathogen for the entire history of the mankind[1]. There are a limited number of drugs that can be used to treat or prevent malaria, but increased resistance to these drugs is of concern to health practitioners. This resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. Newer anti-malarials were discovered in an effort to tackle this problem, but all these drugs are either expensive or have undesirable side effects.

Even the new generation of anti-malaria drugs appears to be less effective after a variable length of time due to the development of resistance in the parasites, especially the *Plasmodium falciparum* species. Recently, scientists reported the first evidence of resistance to the world's most effective drug coartem in western Cambodian subjects[2]. With the continuous resistance to commonly used anti-malarial drugs is spreading, the search for new effective anti-malarial drugs is an urgent and a necessity in large parts of the world to contain and control the disease.

A number of scaffolds, including the pyridine nucleus, showed anti-malarial activities. Thus, in an effort to discover novel anti-malarial agents, new compounds were designed in the present work by incorporating some pharmacophoric assemblies to the pyridine nucleus.

The pyridine derivative 4-isonicotinic hydrazide, which is a leading drug in the treatment of tuberculosis, is an inhibitor of enoyl-ACP reductase, an important enzyme in the fatty acid biosynthesis. Thus, pyridine analogues may inhibit the biosynthesis of fatty acids that are fundamental for the survival of *P. falciparum* in the host[3]. Compounds that act on more than one target site are more liable to be active.

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(Received 23 June 2010; revised 07 March 2011; accepted 22 March 2011)

Several lead compounds containing a pyridine moiety attached to various cyclic and/or acyclic moieties have been reported (Figure 1). Pyridine derivatives **I**, **II**[4,5], adamantylthiopyridine **III**[6], benzoylpyridinethiosemicarbazones **IV**[7] and dichloropyridine derivative **V**[8] showed pronounced anti-malarial activity (Figure 1).

Many of the clinically relevant anti-malarial agents that have a known mechanism of action directly or indirectly affect pyrimidine metabolism. Drugs targeting dihydrofolate reductase (DHFR) or dihydropteroate synthetase (e.g., pyrimethamine, cycloguanil, sulphonamides and sulphones) disrupt folate metabolism, which is essential for the formation of thymidine[9]. Also the lactate dehydrogenase enzyme of *P. falciparum* has the ability to rapidly use 3-acetyl pyridine NAD as a coenzyme in the reaction leading to the formation of pyruvate from lactate[10].

In the present study, various substituents at different positions on the pyridine nucleus were introduced to study the effect of such molecular variation on the anti-malarial activity. In addition to the targeted anti-malarial activity, docking pose of the most active compounds (**2a**, **2g** and **2h**) in the active site of DHFR enzyme and their acute toxicity profile were obtained.

Material and methods

Melting points were determined in open glass capillaries using an electro thermal BUCHI (B-540) melting point apparatus and are uncorrected. Infra red (IR) spectra were recorded on Perkin-Elmer (Postfach, Switzerland) 1430 IR spectrophotometer (Waltham, MA) using the KBr disc technique. ^{1}H NMR spectra was recorded on a Bruker Avance DMX400 FT-NMR spectrometer (Rheinstetten, Germany) and the chemical shifts are given in δ (ppm) downfield from tetramethylsilane, which served as an internal standard.

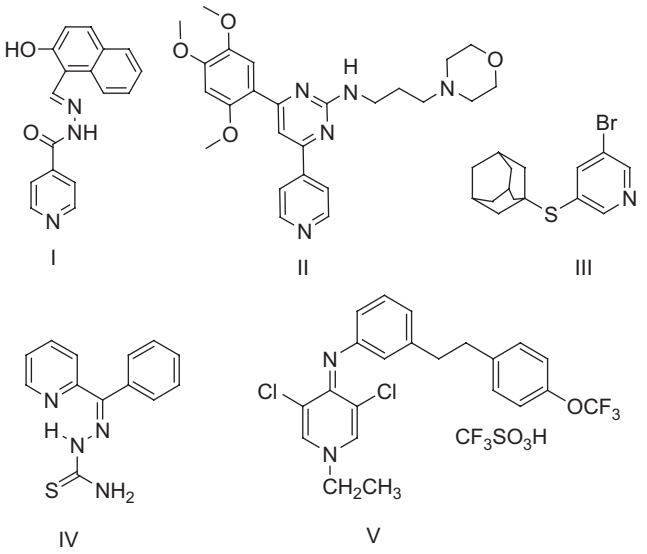


Figure 1. Chemical structures of pyridine derivatives having anti-malarial activity.

Splitting patterns were designated as follows: s: singlet; d: doublet; m: multiplet. Elemental analyses were performed on Perkin-Elmer 2400 elemental analyzer and were found within ±0.4% of the theoretical values. Follow up of the reactions and checking the purity of the compounds was made by thin layer chromatography on silica gel–pre-coated aluminium sheets (Type 60 GF254; Merck, Darmstadt, Germany) and the spots were detected by exposure to UV-lamp at $\lambda = 254$ nm and/or iodine chamber. Parasites were counted using BIO-PLUS microscope. All the chemicals were from Sigma Chemical Co. (St. Louis, MO) and the solvents were from AnalaR BDH (UK) unless otherwise stated.

Synthesis

N′-arylideneisonicotinohydrazide (2a–h)

A mixture of selected aldehyde (10 mM), isonicotinic hydrazide **1** (1.37 g, 10 mM) and one drop HCl in ethanol (50 mL) was heated under reflux for 6–8 h. The reaction mixture was allowed to cool and the precipitate formed was filtered, washed with ethanol, dried and crystallized from ethanol–water mixture (7:3) (Scheme 1, Tables 1 and 2).

4-Aryl-1,2-dihydro-2-oxo-6-phenylpyridine-3-carbonitrile (4a–e)

A mixture of acetophenone **3** (1.20 g, 10 mM), ethyl cyanoacetate (1.13 g, 10 mM), the appropriate aldehyde (10 mM) and ammonium acetate (6.16 g, 80 mM) in ethanol (50 mL) was heated under reflux for 6 h. The reaction mixture was cooled and the formed precipitate was filtered, washed with ethanol, then washed successively with water, dried and crystallized from ethanol–water mixture (7:4) (Tables 1 and 2).

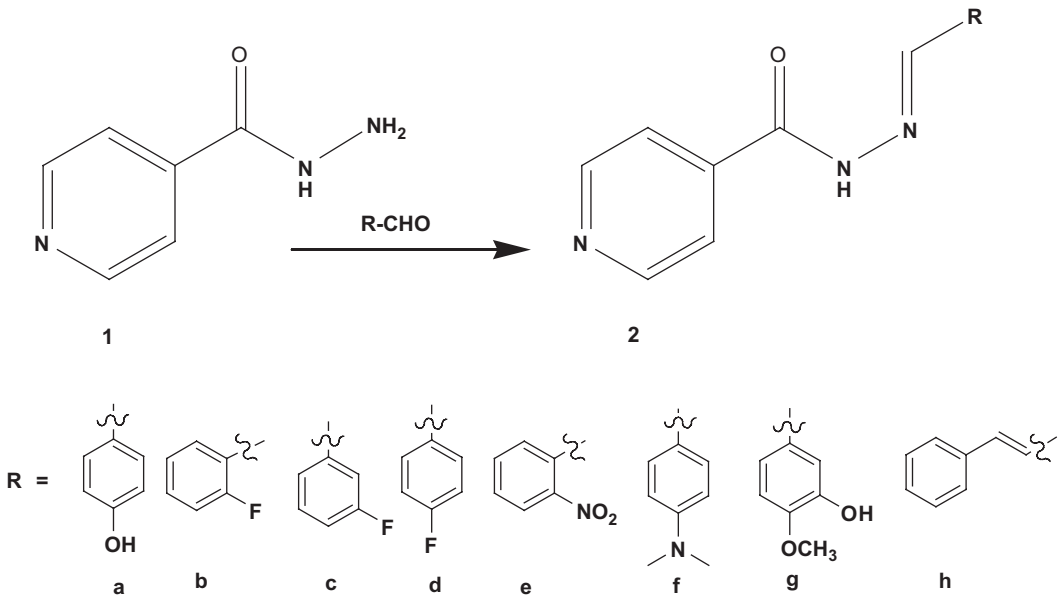
Ethyl 2-cyano-3-(4-(dimethylamino)phenyl)acrylate (5)

This compound was obtained as an unexpected product applying method used to prepare compounds **4a–e** using 4-(dimethylamino)benzaldehyde **A**. To ensure the structure, this compound was synthesized by heating under reflux a mixture of 4-(dimethylamino)benzaldehyde **A** (1.49 g, 10 mM), ethyl cyanoacetate (1.13 g, 10 mM) and ammonium acetate (1.54 g, 20 mL) in ethanol (50 mL) for 5 h. The reaction mixture was concentrated, cooled and the formed precipitate was filtered, washed with ethanol, then washed successively with water, dried and crystallized from ethanol–water mixture (4:1) (Tables 1 and 2).

In vivo anti-malarial activity

In vivo anti-malarial activity test of the synthesized compounds was performed using a 4-day standard suppressive test[11] with some modifications. This is the most widely used preliminary test, in which the efficacy of a compound is assessed by the comparison of blood parasitaemia and mouse survival times in treated and untreated mice[12].

On day 0, the test mice were injected with 0.2 ml of 2×10^7 parasitised erythrocytes, (*P. berghei* ANKA strain) intra venously. After 2 h, the infected mice were weighed and randomly divided into 15 groups of six mice each per cage. Groups 1–13 received the synthesized compounds



Scheme 1. Synthesis of hydrazone derivatives.

at 50 μmol/kg dose levels and served as treatment group[13]. Group 14 received the vehicle (7% Tween, 3% ethanol in water) and served as a negative control. Group 15 received the standard drug Choloroquine diphosphate (CQ, Sigma-Aldrich, Deisenhofen, Germany) at the same dose level (50 μmol/kg) and served as a positive control[14].

On days 1–3, animals in the experimental groups were treated again (with the same dose of the synthesized compounds and same route daily) as in day 0. On day 4 (i.e. 24 hr after the last dose or 96 hr post-infection), blood smear from all test animals was prepared using Giemsa stain. Level of parasitaemia was determined microscopically by counting 4 fields of approximately 100 erythrocytes per field. The difference between the mean value for the control group (taken as 100%) and those of the experimental groups was calculated and expressed as percent reduction or activity.

Untreated control mice typically die approximately 1 week after infection[15]. For treated mice the survival time (in days) was recorded and the mean survival time was calculated in comparison with that of the negative control group[16].

Percentage parasitaemia and suppression were calculated using the following formulae:

$$\%\text{Parasitaemia} = \frac{\text{No. of infected RBC}}{\text{No. of total RBC}} \times 100$$

$$\%\text{Suppression} = \frac{\text{Parasitaemia in negative control} - \text{Parasitaemia in study group}}{\text{Parasitaemia in negative control}} \times 100$$

In vitro anti-malarial assay

P. falciparum strains RKL9 (CQ resistant) was maintained in a continuous culture using the standard method described by Trager and Jensen[12]. Parasites were cultured in human AB (+) erythrocytes in RPMI-1640 media (GIBCOBRL, Paisely, Scotland) supplemented with 25 mM HEPES buffer, 10% human AB (+) serum and 0.2% sodium bicarbonate (Sigma, St. Louis, MO) and maintained at 5% CO_2. Cultures containing predominantly early ring stages were synchronized by addition of 5% D-sorbitol (Sigma) lysis[17] and used for testing. Initial culture was maintained in small vials with 10% haematocrit, i.e., 10 mL erythrocytes containing 1.0% ring stage parasite in 100 μl complete media. The culture volume per well for the assay was 100 mL. Number of parasites for the assay was adjusted at 1–1.5% by diluting with fresh human AB (+) RBC. Assay was done in 96-well microtitre flat-bottomed tissue culture plates incubated at 37°C for 24 h in presence of three-fold serial dilutions of compounds

Table 1. Physical and analytical data of compounds **2a–h**, **4a–e** and **5**.

Compound	Yield %	Melting point (°C)	Molecular formula (MW)
2a	81	245–250	$C_{13}H_{11}N_3O_2$ (241.08)
2b	84	258–259	$C_{13}H_{10}FN_3O$ (243.23)
2c	85	251–252	$C_{13}H_{10}FN_3O$ (243.23)
2d	82	260–261	$C_{13}H_{10}FN_3O$ (243.23)
2e	86	225–227	$C_{13}H_{10}N_4O_3$ (270.08)
2f	78	237–239	$C_{15}H_{16}N_4O$ (268.13)
2g	87	228–230	$C_{14}H_{13}N_3O_3$ (271.09)
2h	58	208–210	$C_{15}H_{13}N_3O$ (251.10)
4a	84	272–273	$C_{18}H_{11}FN_2O$ (290.29)
4b	89	281–282	$C_{18}H_{11}FN_2O$ (290.29)
4c	87	267–268	$C_{18}H_{11}FN_2O$ (290.29)
4d	89	280–281	$C_{18}H_{11}N_3O_3$ (317.08)
4e	61	271–272	$C_{20}H_{14}N_2O$ (298.11)
5	76	127–128	$C_{14}H_{16}N_2O_2$ (244.28)

Table 2. Spectral data of compounds **2a**–**h**, **4a**–**e** and **5**.

Compound	IR (KBr, cm^{-1})	^1H NMR (DMSO-d_6)
2a	3522 (OH), 3207 (NH), 1668 (C=O), 1652 (C=N)	6.91 (d, 2H, J = 8.80 Hz, phenyl-$C_{2,6}$H), 7.75 (d, 2H, J = 8.80 Hz, phenyl-$C_{3,5}$H), 7.95 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.37 (s, 1H, CH=N), 8.82 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H).
2b	3218 (NH), 1669 (C=O), 1654 (C=N)	7.21–7.62 (m, 4H, phenyl-H), 7.93 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.32 (s, 1H, CH=N), 8.81 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H).
2c	3220 (NH), 1666 (C=O), 1655 (C=N)	7.01–7.72 (m, 4H, phenyl-H), 7.89 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.40 (s, 1H, CH=N), 8.84 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H).
2d	3215 (NH), 1667 (C=O), 1653 (C=N)	7.18 (d, 2H, J = 8.80 Hz, phenyl-$C_{3,5}$H), 7.72(d, 2H, J = 8.80 Hz, phenyl-$C_{2,6}$H), 7.91 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.39 (s, 1H, CH=N), 8.81 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H).
2e	3209 (NH), 1670 (C=O), 1656 (C=N), 1522, 1305 (NO_2)	7.72–8.11 (m, 3H, phenyl-$C_{4,5,6}$H), 8.22 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.39 (s, 1H, CH=N), 8.85 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H), 8.90 (s, 1H, phenyl-C_3H).
2f	3219 (NH), 1664 (C=O), 1656 (C=N)	2.16 (s, 6H, N-$CH_3)_2$), 6.52(d, 2H, J = 8.80 Hz, phenyl-$C_{3,5}$H), 6.73(d, 2H, J = 8.80 Hz, phenyl-$C_{2,6}$H), 7.93 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.21 (s, 1H, CH=N), 8.82 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H).
2g	3542 (OH), 3227 (NH), 1665 (C=O), 1657 (C=N)	3.81 (s, 3H,–OCH_3), 6.90 (d, 1H, J = 8.42 Hz, phenyl-C_5H), 7.15 (d, 1H, J = 8.42 Hz, phenyl-C_6H), 7.41(s, 1H, phenyl-C_2H), 8.15 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.42 (s, 1H, CH=N), 8.91 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H).
2h	3124 (NH), 1662 (C=O), 1651 (C=N)	6.95 (m, 2H, phenyl-*CH*=*CH*-), 7.32–7.61 (m, 5H, phenyl-H), 8.23 (d, 1H, CH=N), 8.38 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\beta}$H), 8.89 (d, 2H, J = 5.78 Hz, pyridine-$C_{-\alpha}$H).
4a	3284 (NH), 2220 (CN), 1654 (C=O)	7.09 (s, 1H, pyridine-C_5H), 7.28–7.65 (m, 9H, phenyl-H), 12.87 (br s, 1H, NH, D_2O exchangeable).
4b	3278 (NH), 2215 (CN), 1652 (C=O)	7.13 (s, 1H, pyridine-C_5H), 7.19–7.86 (m, 9H, phenyl-H), 12.88 (br s, 1H, NH, D_2O exchangeable).
4c	3274 (NH), 2217 (CN), 1649 (C=O)	6.92 (d, 2H, J = 8.80 Hz, flourophenyl-$C_{2,6}$H), 7.11 (s, 1H, pyridine-C_5H), 7.36–7.78 (m, flourophenyl-$C_{3,5}$ H & phenyl-H), 12.91 (br s, 1H, NH, D_2O exchangeable).
4d	3280 (NH), 2220 (CN), 1656 (C=O)	7.10 (s, 1H, pyridine-C_5H), 7.22–7.94 (m, 8H, nitrophenyl-$C_{4,5,6}$H & phenyl-H), 8.65 (d, 1H, J = 8.64 Hz, nitrophenyl-C_3 H), 12.88 (br s, 1H, NH, D_2O exchangeable).
4e	3279 (NH), 2219 (CN), 1655 (C=O)	6.67 (d, 1H, J = 5.22 Hz, Phenyl-*CH*=CH-),7.12 (s, 1H, pyridine-C_5 H), 1.18 (d, 1H, J = 5.22 Hz, Phenyl-*CH*=*CH*-), 7.20–8.02 (m, 10H, phenyl-H), 12.89 (br s, 1H, NH, D_2O exchangeable).
5	2214 (CN); 1704 (C=O); 1256, 1123 (C–O–C)	1.24 (t, 3H, CH_3), 3.15 (s, 6H,–$N(CH_3)_2$), 4.35 (m, 2H, CH_2), 6.71 (d, 2H, J = 8.75 Hz, phenyl $C_{2,6}$H), 7.92 (d, 2H, J = 8.75 Hz, phenyl $C_{3,5}$H), 8.12 (s, 1H,–C=*CH*).

and CQ diphosphate for their effect on schizont maturation. Test compounds were dissolved in ethanol and further diluted with RPMI-1640 medium (the final ethanol concentration did not exceed 0.5%, which did not affect parasite growth). CQ diphosphate was dissolved in aqueous medium. Test was done in duplicate wells for each dose of the drugs. Solvent control culture containing the same concentrations of the solvent as present in the test wells was done with RPMI-1640 containing 10% AB (+) serum.

Parasite growth was found to be unaffected by the solvent concentrations used in the test. Growth of the parasites from duplicate wells of each concentration was monitored in Giemsa-stained blood smears by counting number of schizont per 100 asexual parasites. Percent schizont maturation inhibition was calculated by the formula: $(1 - N_t/N_c) \times 100$ where, N_t and N_c represent the number of schizont in the test and control wells, respectively.

Acute toxicity

The oral acute toxicity of the most active compounds **2a**, **2g** and **2h** was investigated using male mice (20 g each, Medical Research Institute, Alexandria University) according to previously reported methods. The animals were divided into groups of six mice each. The compounds were given orally, suspended in 1% gum acacia, in doses of 1, 10, 100, 200, 250, 300 mg/kg. The mortality percentage in each group was recorded after 24 h[18]. Additionally the test compounds were investigated for their parenteral acute toxicity in groups of mice of six animals each. The compounds or their vehicle, propylene glycol (control), were given by intra-peritoneal injection in doses of 10, 25, 50, 75, 100 mg/kg. The percentage survival was followed up to 7 days[19].

Docking

The co-ordinate from the X-ray crystal structure of dehydrofolate reductase (DHFR) enzyme used in this simulation was obtained from the Protein Data Bank (PDB ID: 1J3I), where the selective DHFR inhibitor **WR99210** is bound to the active site. The ligand molecules were constructed using the builder module and were energy minimized. The active site of DHFR was generated using the MOE-Alpha Site Finder, Molecular Operating Environment's (MOE-Dock 2005) module to

rationalize the observed biological results in the *in vivo* study[20]. Then ligands were docked within this active site using the MOE-Dock. The lowest energy conformation was selected and the ligand interactions (hydrogen bonding and hydrophobic interaction) with DHFR were determined.

Results and discussion

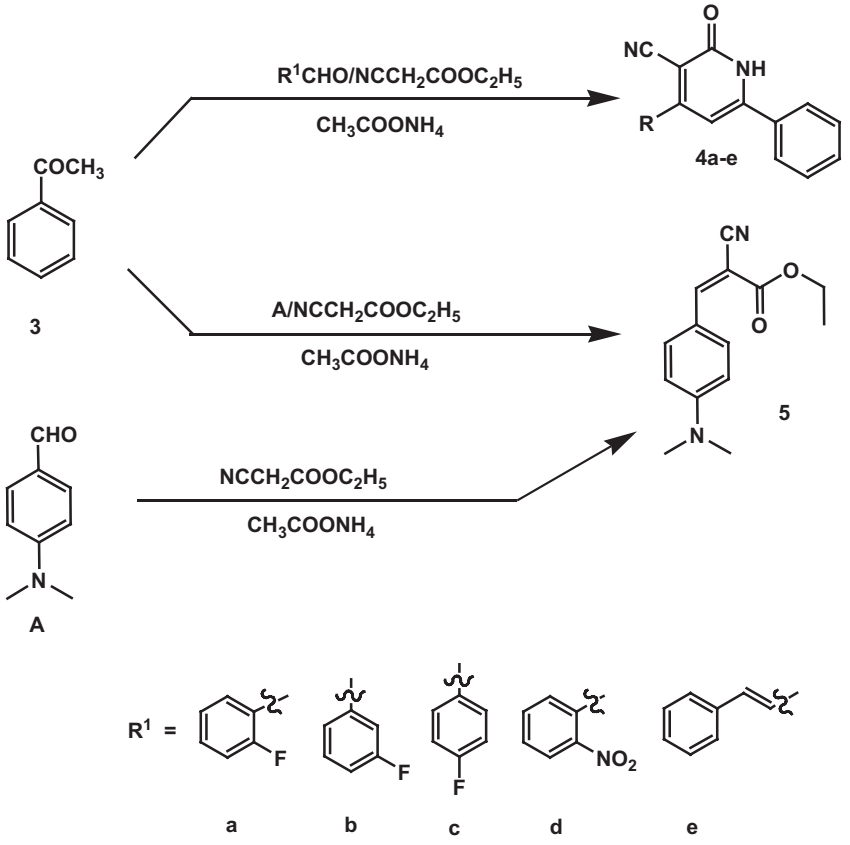
Chemistry

The target compounds were synthesized according to the steps outlined in Schemes 1 and 2. Condensation of the appropriate aldehyde derivatives **a**–**h** with isonicotinic hydrazide **1** in ethanol afforded the corresponding hydrazones **2a**–**h**. The structures of these compounds were confirmed by IR spectra that showed lack of the absorption bands characteristic for NH_2 group. The absence of this primary amine in the IR spectrum and aldehydic proton in the ^{1}H NMR spectrum confirmed that the target compounds were formed.

One of the synthetic procedures applied in the present work (Scheme 2) is regarded to be a multicomponent reaction[21,22]. Applying one pot reaction, the selected aldehyde, ethyl cyanoacetate, acetophenone **3** and ammonium acetate in ethanol were heated under reflux to obtain compound **4a**–**e**. The presence of amidic carbonyl group in the IR spectrum and the absence of aldehydic proton in the ^{1}H NMR spectrum showed that the product obtained is in agreement with the proposed structure. In contrast, one pot reaction with *p*-dimethylaminobenzaldehyde, ethyl cyanoacetate, ammonium acetate and acetophenone did not produce the target compound. This may be attributed to the weak electrophilic property of *p*-dimethylbenzaldehyde, due to the electron donating effect of the *p*-dimethylamino group. Trials to obtain the target compound 1,2-dihydro-4-(4-dimethylaminophenyl)-2-oxo-6-phenylpyridine-3-carbonitrile by preparing the α,β-unsaturated ketone *via* condensation of acetophenone and *p*-dimethylbenzaldehyde in the presence of KOH, then cyclisation by ethyl cyanoacetate and ammonium acetate went in vain. The results obtained from elemental and spectral analyses clearly demonstrated that the compound obtained was not the targeted one, instead ethyl 2-cyano-3-(4-(dimethylamino)phenyl)acrylate **5** was obtained. To ensure the structure of compound **5**, this compound was further synthesized by the reaction of 4-(dimethylamino)benzaldehyde **A**, ethyl cyanoacetate and ammonium acetate in ethanol. It is worth mentioning that the presence of ester functional group in the IR spectra and the number of protons from the ^{1}H NMR spectral integration were exactly the same in the structure of the above unexpected compound and the targeted compound.

In vivo anti-malarial activity

Table 3 shows the effect of the synthesised compounds against *P. berghei* infected mice. Compounds **2a**, **2b**, **2c**,



Scheme 2. Synthesis of pyridone derivatives.

2d, **2f**, **2g**, **2h** and **4c** showed more than 50% suppression of parasitaemia compared to the control and CQ treated groups. Compounds **2a**, **2g** and **2h** are the most active compounds in this study with 90, 91 and 80% suppression, respectively. The only cyanopyridine derivative that showed promising activity was **4c**. In general, isonicotinohydrazide derivatives were more active than cyanopyridine derivatives.

The *in vivo* biological activities of some of the synthesised isonicotinohydrazide derivatives showed encouraging results against *P. berghei*. Substitution of phenyl ring at the *para*-position with hydroxyl, flouro or methoxy group (**2a**, **2d** and **2g**) showed remarkable activity, which might be due the formation of hydrogen bonding with backbone of the receptor. Compound **2d** showed promising activity. Moreover, compound having styryl moiety **2h** showed good activity but still less than **2a** and **2g**. The presence of dimethylamino group at the *para*-position of the phenyl ring might decrease the activity of compound **2f**. When the phenyl ring was substituted at *para*-position with hydroxyl group **2a**, the activity was higher than that of compound **2e**, which has nitro group at the *para*-position of the phenyl ring. This could be speculated to be due to the large nitro group that may not be able to interact with the backbone of the receptor.

In vitro anti-plasmodial activity

On testing the *in vitro* anti-plasmodial activity (Table 4), compounds **2a**, **2g** and **2h** showed better activity than CQ diphosphate (IC_{50} = 0.188 μM) against CQ-resistant (RKL9) strain of parasite. Compound **2g** was found to be the most potent against RKL9 (IC_{50} = 0.0402 μM). This may be attributed to the OH group at the *para*-position, which is capable of forming hydrogen bonding with the backbone of the receptor.

In vivo acute toxicity test

Compounds **2a**, **2g** and **2h** were further evaluated for their oral acute toxicity in male mice using a previously reported method[18,19]. The results indicated that the test compounds proved to be non-toxic and well tolerated by experimental animals up to 300 mg/kg. Moreover, these compounds were tested for their toxicity through the parenteral route. The results revealed that all test compounds were non-toxic up to 100 mg/kg.

Docking

Molecular docking studies further helps in understanding the various interactions between the ligands and enzyme active sites in detail. The determination of the 3D co-crystal structure of DHFR (PDB ID: 1J3I), complexed with a selective inhibitor, **WR99210** (Figure 2), has led to the development of a model for the topography of the anti-malarial drugs binding site in DHFR enzyme. This compound displayed hydrogen bond interactions with Ile 14, Asp 54 and Ile 164 in addition to hydrophobic interactions with Ile 14, Cys 15, Ala 16, Asp 54, Met 55, Phe 58 and Pro 113.

Figures 3–5 show the binding interactions of compounds **2a**, **2g** and **2h** to the active site of DHFR, respectively, where they exhibited some similar interactions to **WR99210**. Compound **2a** displayed hydrogen bond interactions with Phe 116, in addition to hydrophobic interactions with Ala 16, Leu 46, Asp 54, Met 55, Phe 58, Phe 116 and Arg 122. On the other hand, compound **2g** showed hydrogen bond interactions with Phe 116 and Ser 120, in addition to hydrophobic interactions with Ser 111, Ile 112, Phe 116, Leu 119, Ser 120 and Arg 122. However, compound **2h** displayed hydrogen bond interactions with Ser 111 in addition to hydrophobic interactions with Gly 44, Val 45, Leu 46, Met 55, Ser 108, Ser 111, Phe 116 and Leu 119. The higher activity of compounds **2a** and **2g** over compound **2h** may be attributed to the arene–cation interaction between hydroxyphenyl group and Arg 122. The hydrogen bonds profile and their scores of the active compounds **2a**, **2g** and **2h** and standard compound **WR99210** are listed in Table 5. Although all compounds showed hydrogen bond interactions with the backbone of the enzyme, the residue of interaction may be different. Both compounds **2a**, **2g** interact with Phe 116 with hydrogen donor bond, but compound **2g** had additional hydrogen acceptor bond with Ser 120 of excellent score. The standard compound **WR99210** showed three hydrogen donor bonds with backbone of the enzyme, those with Ile 14 and Ile 164 are of weak score while that with Asp 54 of medium score.

Table 3. *In vivo* anti-malarial activity of compounds **2a**–**h** and **4a**–**e**.

Compound	% Parasitaemia	% Suppression	Mean survival time (days)
2a	8 ± 0.8	90	11.5 ± 0.6
2b	34 ± 1.2	58	8.2 ± 1.6
2c	32 ± 1.6	61	8.6 ± 1.9
2d	22 ± 0.4	74	9.1 ± 1.2
2e	81 ± 2.4	1	2.4 ± 0.8
2f	31 ± 1.0	63	9.22 ± 1.4
2g	15 ± 0.6	91	9.8 ± 0.5
2h	17 ± 1.3	80	9.4 ± 1.0
4a	46 ± 3.4	44	7.4 ± 1.1
4b	52 ± 2.8	37	6.8 ± 1.8
4c	31 ± 3.8	63	8.8 ± 1.4
4d	64 ± 1.8	22	4.2 ± 2.4
4e	47 ± 2.8	43	7.1 ± 1.5
Control	82 ± 1.6	0.0	6.3 ± 0.5
Chloroquine	0.0	100	ND

Table 4. *In vitro* anti-plasmodial activity against chloroquine-resistant (RKL9) strain of *Plasmodium falciparum*.

Compound	IC_{50}, μM ± SD*
2a	0.0422 ± 0.004
2g	0.0402 ± 0.005
2h	0.0660 ± 0.012
Chloroquine	0.188 ± 0.003

*Results of two separate determinations.

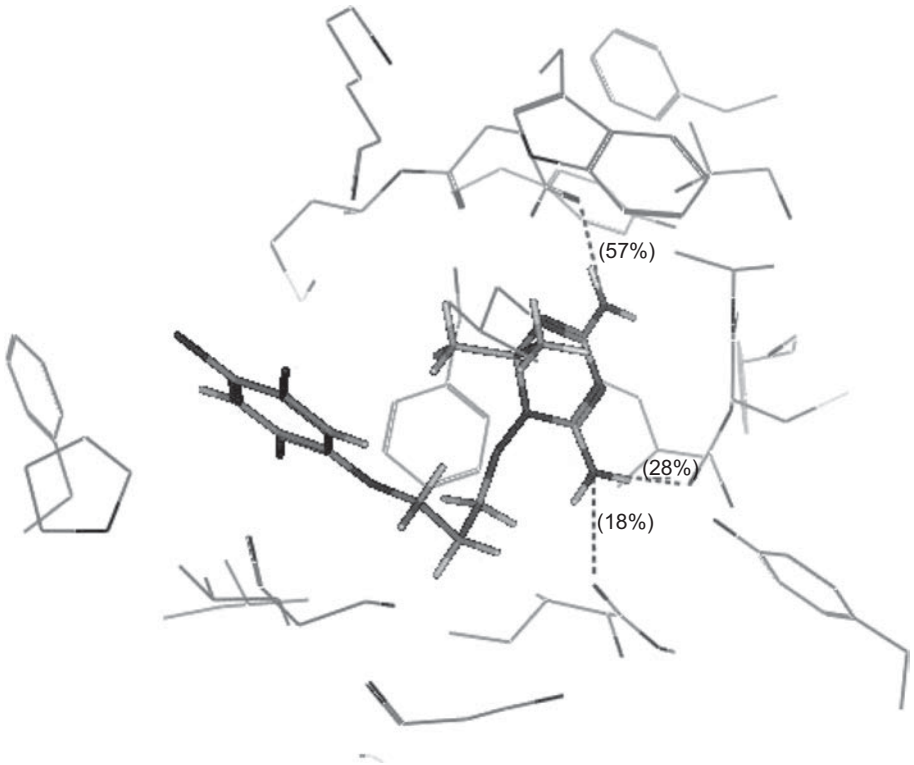


Figure 2. 3D view from a molecular modelling study, of the minimum-energy structure of the complex of **WR99210** docked in DHFRE (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

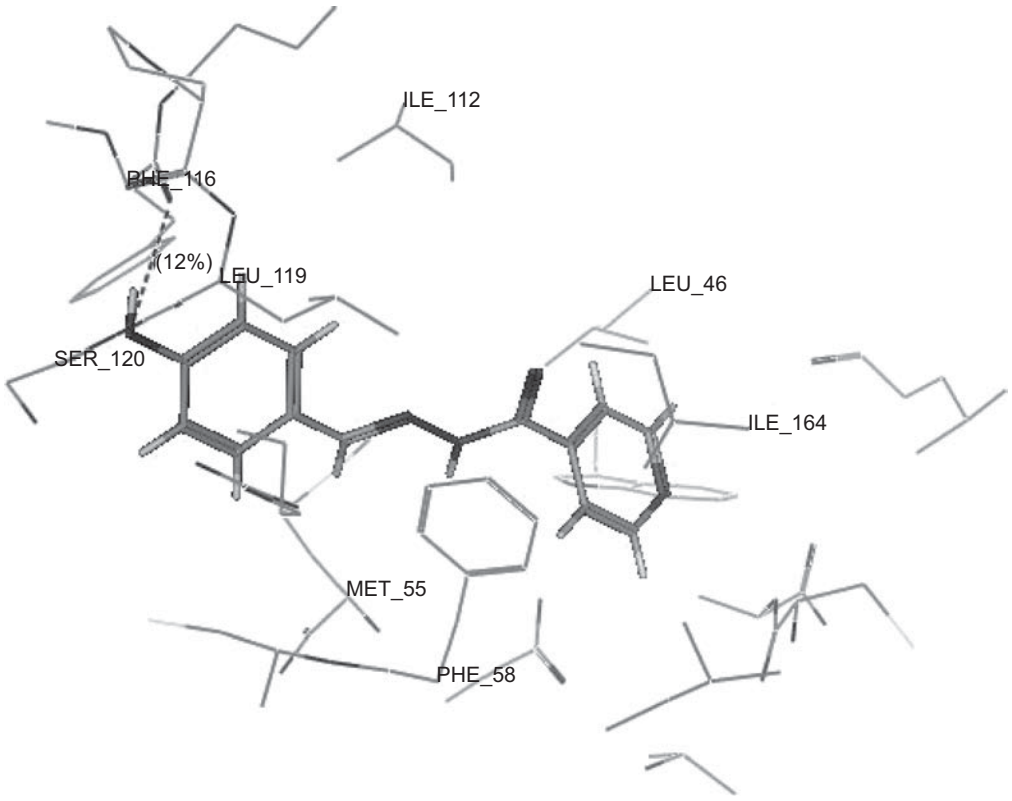


Figure 3. 3D view from a molecular modelling study, of the minimum-energy structure of the complex of **2a** docked in DHFRE (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

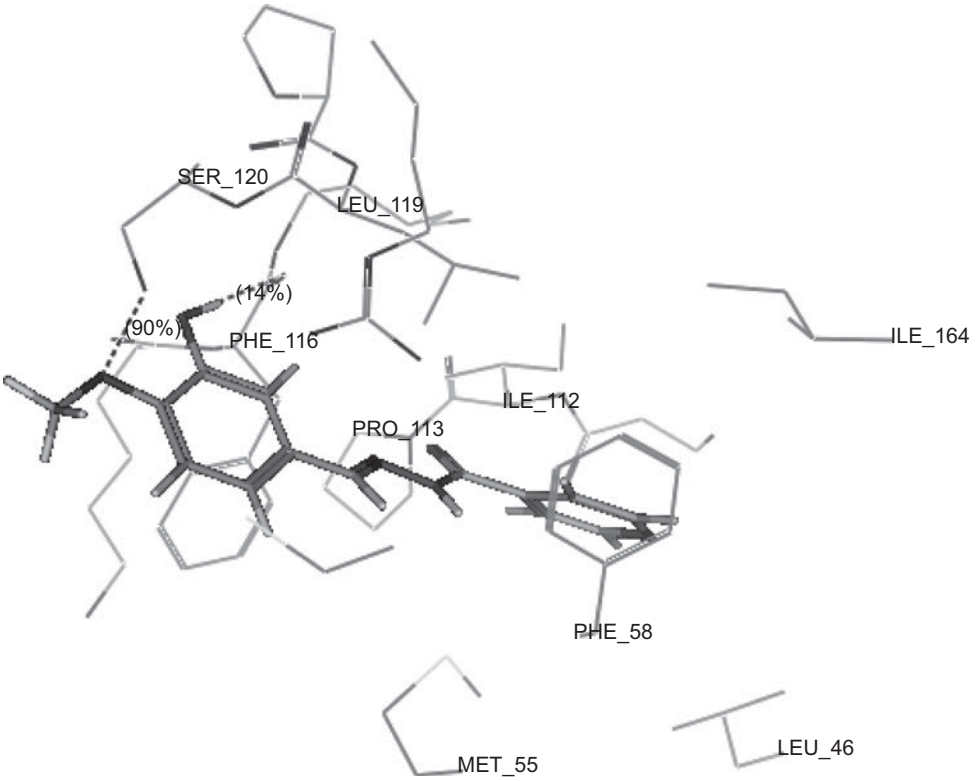


Figure 4. 3D view from a molecular modelling study, of the minimum-energy structure of the complex of **2g** docked in DHFRE (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

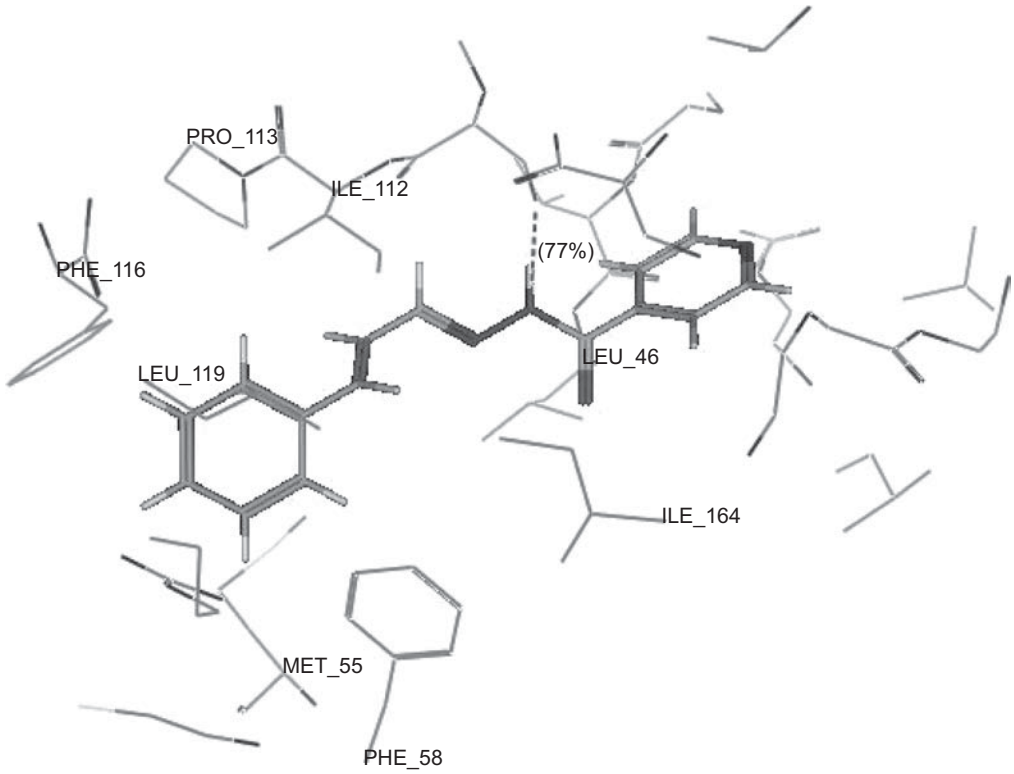


Figure 5. 3D view from a molecular modelling study, of the minimum-energy structure of the complex of **2h** docked in DHFRE (PDB ID: 1J3I). Viewed using Molecular Operating Environment (MOE) module.

Table 5. Calculated docking hydrogen bonding results for compounds **WR99210**, **2a**, **2g** and **2h**.

Compound	Residue	Type	Score (%)	Distance
WR99210	Ile 14	H-don	28.2	1.98
	Asp 54	H-don	57.1	1.87
	Ile 164	H-don	17.8	2.20
2a	Phe 116	H-don	72.3	2.17
2g	Phe 116	H-don	13.6	2.02
	Ser 120	H-acc	90.2	2.44
2h	Ser 111	H-don	76.7	1.67

Conclusions

In this study, several pyridine derivatives were synthesised and tested for their anti-malarial activity both *in vivo* and *in vitro* against CQ-resistant and CQ-sensitive plasmodia. Compounds **2a**, **2g** and **2h** exhibited promising anti-malarial activities against both malaria strains. Compound **2g** was the most active in the present work. Acute toxicity studies showed that compounds **2a**, **2g** and **2h** have good safety margin. Docking studies for **2a**, **2g** and **2h** with the active site of DHFR (PDB ID: 1J3I) showed good binding profile. Therefore, compounds **2a**, **2g** and **2h** would represent a fruitful matrix for the development of a new class of anti-malarial agents that would deserve further investigation and derivatisation.

It is worth to mention that the newly synthesised pyridine derivatives **2a**, **2g** and **2h** (IC_{50} = 0.0402–0.0660 μM) exhibited higher anti-malarial activity against *P. falciparum* than the thiopyridine II (IC_{50} = 1–10 μM)[5]. The current compounds also displayed pronounced activity compared to benzoylpyridinethiosemicarbazones **IV** (IC_{50} = 0.1 μM)[7]. Furthermore, **2a**, **2g** and **2h** showed superior activity over the dichloropyridine derivative **VII** (IC_{50} = 0.94 μM)[8], although the postulated anti-malarial mechanism in both groups of compounds is different.

Acknowledgements

The authors wish to thank the Egyptian Fund for Technical Cooperation with Africa, Ministry of Foreign Affairs, Egypt, for the financial support that enabled Prof. Adnan Bekhit and Prof. Abdel Maaboud Mohamed to be available in School of Pharmacy, Addis Ababa University.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Joy DA, Feng X, Mu J, Furuya T, Chotivanich K, Krettli AU, Ho M, Wang A, White NJ, Suh E, Beerli P, Su XZ. Early origin and recent expansion of *Plasmodium falciparum*. Science 2003;300:318–321.
2. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 2009;361:455–467.
3. Surolia A, Ramya TN, Ramya V, Surolia N. 'FAS't inhibition of malaria. Biochem J 2004;383:401–412.
4. Agarwal A, Kumkum Srivastava, Puri SK, Prem MSC. Synthesis of 4-pyrido-6-aryl-2-substituted amino pyrimidines as a new class of antimalarial agents. Bioorg Med Chem 2005;13:6226–6232.
5. Rollas S, Küçükgüzel SG. Biological activities of hydrazone derivatives. Molecules 2007; 12:1910–1939.
6. Prachayasittikul S, Treeratanapiboon L, Ruchirawat S, Prachayasittikul V. Novel activities of 1-adamantylthiopyridines as antibacterials, antimalarials and anticancers. EXCLI J 2009;8:121–129.
7. Pingaew R, Prachayasittikul S, Ruchirawat S. Synthesis, cytotoxic and antimalarial activities of benzoyl thiosemicarbazone analogs of isoquinoline and related compounds. Molecules 2010; 15:988–996.
8. Rodrigues T, Guedes RC, dos Santos DJ, Carrasco M, Gut J, Rosenthal PJ, Moreira R, Lopes F. Design, synthesis and structure-activity relationships of (1H-pyridin-4-ylidene)amines as potential antimalarials. Bioorg Med Chem Lett 2009; 19:3476–3480.
9. Phillips MA, Rathod PK. *Plasmodium* dihydroorotate dehydrogenase: a promising target for novel anti-malarial chemotherapy. Infect Disord Drug Targets 2010; 10:226–239.
10. Makler MT, Hinrichs DJ. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. Am J Trop Med Hyg 1993; 48:205–210.
11. Peters W, Robinson BL. (1999) Parasitic infection models: Handbook of animal models of infection. London: Academic Press, 757–773.
12. Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976;193:673–675.
13. Loiseaul PM, Xuong ND. *Plasmodium berghei* mouse model: Antimalarial activity of new alkaloid salts and of thiosemicarbazone and acridine derivatives. Trop Med and Inter Health 1996;1:379–384.
14. Kiseko K, Hiroyuki M, Syun-ichi F, Ryuiichi F, Tomotaka K, Seiji M. Anti-malarial activity of leaf-extract of *Hydrangea macrophyla*, a common Japanese plant. Acta Med Okayama 2000;54:227–232.
15. Kalra BS, Chawla S, Gupta P, Valecha N. Screening of antimalarial drug. Ind J Pharmacol 2006;38:1–12.
16. Cravo P, Culleton R, Hunt P, Walliker D, Mackinnon MJ. Antimalarial drugs clear resistant parasites from partially immune hosts. Antimicrob Agents Chemother 2001; 45:2897–2901.
17. Lambros C, Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 1979; 65:418–420.
18. Verma M, Tripathi M, Saxena AK, Shanker K. Antiinflammatory activity of novel indole dervatives. Eur J Med Chem 1994;29:941–946.
19. Bekhit AA, Fahmy HTY. Design and synthesis of some substituted 1H-pyrazolyl-oxazolidines or 1H-pyrazolyl-thiazolidines as anti-inflammatory-antimicrobial agents. Arch Pharm Pharm Med Chem 2003; 336:111–118.
20. Molecular Operating Environment (MOE). Version 2005–06. Chemical Computing Group, Inc: Montreal, Canada. Available at: http://www.chemcomp.com
21. Bekhit AA, Baraka AM. Novel milrinone analogs of pyridine-3-carbonitrile derivatives as promising cardiotonic agents. Eur J Med Chem 2005;40:1405–1413.
22. Domling A. Isocyanide based multi component reactions in combinatorial chemistry. High Throughput Screen 1998;1:1–22.