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Synthesis of 4-aminoquinoline—pyrimidine hybrids as potent antimalarials and their mode of action studies

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Abstract

One of the most viable options to tackle the growing resistance to the antimalarial drugs such as artemisinin is to resort to synthetic drugs. The multi-target strategy involving the use of hybrid drugs has shown promise. In line with this, new hybrids of quinoline with pyrimidine have been synthesized and evaluated for their antiplasmodial activity against both CQS and CQR strains of Plasmodium falciparum. These depicted activity in nanomolar range and were found to bind to heme as well as AT rich pUC18 DNA.

1. Introduction

Malaria is one of the most widespread diseases besides tuberculosis and AIDS which affects more than 500 million people worldwide and results in around 1–3 million causalities every year [1]. In Africa alone, around 20% childhood deaths are due to malaria and a child dies every 30 s [2] and it is estimated that an African child has on an average 1.6–5.4 episodes of malaria fever each year. Of the four typically recognized Plasmodium species causing disease in humans, Plasmodium falciparum is most deadly to children below the age of five leading to mortality while Plasmodium vivax is most morbidity prone, and is responsible for latent infection that hampers current control and future elimination efforts [3]. The development of drug resistance for the common antimalarials such as 4-/8-aminoquinolines, 4-methanol quinolines, antifolate drugs, sesquiterpene lactones etc. (Fig. 1) is a rather serious issue which has stimulated considerable research efforts in the development of new drugs using different approaches [4,5] of which the molecular hybridization approach [6,7] is quite an attractive strategy which involves design of new chemical entities by covalent fusion of two drugs, both active compounds and/or pharmacophoric units derived from known bioactive molecules with complimentary activities and multiple pharmacological targets. The multiple target strategy led to the design of hybrid of 4-aminoquinoline with species such as triazine [8,9], ferrocene [10], rhodanine [11], thiazolidine-4-one [12], chalcone [13], trioxane [14], isatin [15] and recently, pyrimidines [16–18] (Fig. 2).

Quinoline containing drugs (chloroquine and primaquine, Fig. 1) are known to affect parasite metabolism and cause parasite death by blocking the polymerization of the toxic heme, into an insoluble and non-toxic pigment, hemozoin, resulting in cell lysis and parasite cell auto digestion [19–21]. On the other hand, pyrimidine-based compounds are well known for their wide range of promising antiviral [22], antitubercular [23], anti-AIDS [24], antiinflammatory [25], antifungal [26], antitumor [27] and antimalarial activities [28] apart from their role in the nucleic acid synthesis. Thus, linking of the quinoline unit with pyrimidine might furnish conjugate hybrids that are capable of showing useful antimalarial activity.

Recently, antimalarial activities of some quinoline—pyrimidine hybrids with activities in the micromolar range have been reported (Fig. 3) [16–18,29]. In yet another report on the evaluation of quinoline—pyrimidine, the activity (in micromolar range) was also reported for fixed combinations of the chloroquine and pyrimethamine. In all these reports, the pyrimidines were linked to the quinoline unit through 2-, 4- and 6-positions. We have employed...
rather conformationally flexible pyrimidine-5-carboxylate linked covalently to 4-aminoquinoline core. These novel pyrimidine carboxylate hybrids interact with the iron center of free heme within the physiological environment (pH 5.6), a key step in the accumulation of heme which is selectively toxic to the parasite. To enhance the possibility to accumulate within the digestive vacuole via weak-base trapping (the mechanism by which CQ and other quinoline antimalarials attain high concentrations inside this compartment), we developed a novel class of antimalarial hybrids by incorporating electron withdrawing substituents at C-4 phenyl of pyrimidine core, as well as by altering the basicity of diaminoalkyl spacer. We also report on their antimalarial activity against both CQ sensitive (CQ S) and CQ resistant (CQ R) strains. Finally, the mechanism of action studies with the representative compounds has also been performed.

2. Chemistry

The 4-aminoquinoline–pyrimidine-5-carboxylate hybrids were synthesized in economical way using synthetic approach outlined in Scheme 1. The key starting compound, 3,4-dihydropyrimidin-2(1H)-one 1 was prepared through NH4Cl/TFA [30,31] catalyzed three-component Biginelli condensation of an alkyl acetoacetate, urea and appropriate aldehyde or its formyl equivalent: 1,3-oxazinane derivative, in acetonitrile or under solvent-free reaction conditions, in some cases. Subsequent oxidation of 1 using 60% nitric acid readily furnished pyrimidinones 2 which upon chlorination with POCl3 yielded 3 [32]. The nucleophilic substitution reaction of 3 with appropriate 4-amino-7-chloroquinoline 4 which in turn was prepared from the commercially available 4,7-dichloroquinoline and diaminoalkanes [33], gave 5a–g in good yields (Table 1). Structures of 1–5 were unambiguously established on the basis of spectral (1H NMR, 13C NMR, MS, FT IR) as well as microanalytical analysis.

3. Results and discussion

3.1. Antimalarial activity and structure–activity relationships (SARs)

We have previously established that the 4-aminoquinoline–pyrimidine hybrids 6a–c intercepted by a diaminoalkyl spacer showed optimum potency (Table 1), when the flexible spacer consisted of three or four carbon atoms [29]. Further, the introduction of nitro substituent at ortho position of the phenyl ring at the C-4 of the pyrimidine core furnished the most potent compound 6c with antimalarial activity superior to the standard CQ and close to artesunate [29]. Keeping these observations in mind, we planned to further refine the structure of these persuasive 4-aminoquinoline–pyrimidine hybrids by incorporating electron withdrawing substituents at C-4 phenyl of pyrimidine core, as well as by altering the C-5 ester substituent and also by altering the basicity of diaminoalkyl spacer.

The in vitro antimalarial screening of the new synthesized compounds 5a–g revealed good to moderate activities in nM range against both the tested Dd2 (CQ S) and D10 strains (CQ R) of P. falciparum (Table 1). Although the tested hybrids were not as active as the standard drugs viz. CQ and ASN, interesting SARs have been drawn. Analysis of the activity of the compounds recorded in Table 1 reveals that replacing C-5 ethyl ester of the previously reported [29] compound 6a [IC50 247.5 nM (CQS); 52.2 nM (CQR)] with methyl ester 5a [IC50 659 nM (CQS); 542 nM (CQR)] led to the
decrease in antimalarial activity against both the chloroquine sensitive and chloroquine resistant strains of *P. falciparum*. However, comparison of hybrids 5b, 5c with 6b, 6c having an identical butyl spacer showed that incorporation of isopropyl ester at C-5 of pyrimidine motif (5b and 6b) increased the antimalarial activity against the CQ³ strain whereas considerable decrease in activity was observed for CQ³ strain of *P. falciparum*. Also, the most potent compound 5b of the series displayed 2-fold increase in antimalarial activity than the standard CQ against CQ³ strain of *P. falciparum*. When the diaminoalkyl linker of compound 6a was replaced with a relatively less basic alkox amino linker 5d considerable decrease in antimalarial activity was observed which in turn linked to the decreased accumulation of compound via pH trapping into the digestive vacuole. It was not unexpected since the basicity of alkyl chain linker plays crucial role in determining the antimalarial activity of this class of compounds. Furthermore, the introduction of a nitro substituent on the phenyl ring at the C-4 position of the pyrimidine core to create 5c resulted in a significant increase in antiplasmodial activity in comparison to the p-chloro/p-fluoro substituents (5e and 5f). Moreover, the hybrid 5g lacking a C-4 substituent on the pyrimidine motif led to an increase in antimalarial activity against both the CQ³ as well as CQ⁵ strains of *P. falciparum*. However, although the antimalarial activity of 5g was superior to 5c, 5e & 5f, it was less than the corresponding C-4 phenyl substituted analogs 5b as well as 6b. Thus, the SAR study suggested that both the substitution of the C-4 phenyl group with electron withdrawing groups and alterations in basicity of linker leads to better antimalarial activity. Unfortunately, these compounds suffer from high *ClogP* values which are in the range 5–8 (Table 1), which are suggestive of the fact that these possess limited aqueous solubility. However, it is not a serious limitation in view of recent advancements in formulation methods.

3.2. Cytotoxicity and antiviral activity

Compounds 5a–g were evaluated for their toxicity against various (HeLa, Vero, CRFK, HEL and MDCK) cell cultures (Table 1 & SI Table S1). Toxicity data revealed that these compounds exhibit high toxicity (low CC⁵₀) against MDCK cell cultures while CC⁵₀ values are quiet high for other cell cultures. The CC⁵₀ values for inhibition of MDCK cells summarized in Table 1 indicate that the strongest antimalarial compound 5b was mildly cytotoxic (Table 1). Further, the ratio of the cytotoxicity (CC⁵₀ in μM) and *in vitro* antimalarial activity (IC⁵₀ in nM for Dd2 strain) enabled the determination of selectivity index (SI) for these compounds. Compound 5d with alkox amino linker and compound 5g bearing a C-4 unsubstituted pyrimidine motif exhibited high CC⁵₀ values and thus led to a fairly safe selectivity index values (Table 1). Compound 5d having less basic alkyl spacer, displayed highest SI (43.6) whereas the most potent compound 5b exhibit relatively low SI value (3.92). Thus, the compounds depicted structure dependent SI values.

Chloroquine is known to elicit antiviral effects against several viruses, including human immunodeficiency virus type 1, HCoV-229E, hepatitis B virus, and herpes simplex virus type 1 [34–37]. Thus, we determined *in vitro* antiviral activities of 5a–g against (i) herpes simplex virus-1 (HSV-1; KOS), herpes simplex virus-2 (HSV-2; G); vaccinia virus, vesicular stomatitis virus, herpes simplex virus-1 (TK-KOS ACVR) in HEL cell cultures, (ii) parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxackie virus B4, Panta Toro virus in vero cell cultures, (iii) influenza A virus (H1N1 and H3N2) and influenza B virus in MDCK cell cultures, (iv) vesicular stomatitis virus, Coxackie virus B4, respiratory syncytial virus in Hela cell cultures, (v) cytomegalovirus using AD-169 and Davis strain in Hel. cells, (vi) varicella-zoster virus (VZV) in HEL cells (SI Table S1) and (vii) feline corona virus (FIPV) and feline herpes virus in CRFK cell cultures (Table 2). The anti-viral activity of most of the compounds was not impressive except compounds 5a and 5c which exhibited relatively low EC⁵₀’s only against the feline corona virus (FIPV) and feline herpes virus in CRFK cell cultures (Table 2).
Table 1
In vitro antimalarial activity of compounds 5a–g against *P. falciparum* (CQS) D10 strain and (CQR) Dd2 strain for *n* = 3 (*n* = number of replicates).

| Compound | Structure | Yield (%) | D10 IC50 (nM) | Dd2 IC50 (nM) | C log *P* | CC50 (μM) | SI |
|----------|-----------|-----------|----------------|----------------|-----------|-----------|----|
| 5a       | ![Structure of 5a](image) | 86 | 659 | 542 | 6.71 | 1.7 | 3.13 |
| 5b       | ![Structure of 5b](image) | 83 | 156 | 153 | 7.66 | 0.6 | 3.92 |
| 5c       | ![Structure of 5c](image) | 75 | 1461 | nd | 7.10 | 0.8 | – |
| 5d       | ![Structure of 5d](image) | 90 | 478 | 483 | 7.24 | 21.1 | 43.6 |
| 5e       | ![Structure of 5e](image) | 85 | 1926 | nd | 8.07 | 2.2 | – |
| 5f       | ![Structure of 5f](image) | 72 | 2759 | nd | 7.50 | 11.4 | – |
| 5g       | ![Structure of 5g](image) | 89h | 211 | 336 | 5.25 | 10.3 | 30.65 |
| 6a       | ![Structure of 6a](image) | – | 202i | 26.1h | 6.82 | 0.8 | 15.33 |

(continued on next page)
3.3. Mode of action studies

3.3.1. Heme binding studies

Quinoline antimalarials (e.g., CQ, amodiaquine and quinine) act principally by forming adducts with ferritoporphorin IX, thus blocking haemozoin formation [38]. In this study, we have evaluated the mechanistic antimalarial activity of the most potent compound 5b of the series by studying its binding with heme [Fe(III)PPIX] in solution and inhibition of β-hematin formation using UV–visible spectrophotometer. The incremental addition of 5b (0–25 μM) into monomeric heme (2.4 μM, DMSO:H2O/4:6, v/v) in 0.02 M HEPES buffer (pH 7.4) showed a substantial decrease in the intensity of the Fe(III) PPIX Soret band at 402 nm with no shift in the wavelength of the absorption maximum (Fig. 4). The titration of monomeric heme was also performed at the Plasmodial food vacuole pH 5.6 using MES buffer instead of HEPES to ensure that the compound 5b binds with heme even at acidic pH (S1). A 1:1 stoichiometry of the most stable complex of 5b with monomeric heme at pH 7.4 and 5.6 was established from the Job’s plot (SI Figure S1). The association constants (Table 3) were calculated by analyzing the titration curves obtained at pH 7.4 using HypSpec—a non-linear least square fitting programme [39]. The binding of CQ with heme under identical conditions was also determined in the similar manner and the results are presented in Table 3 for comparison. Table 3 shows that the association constants for the complexes formed between monomeric heme and 5b (log K 4.96) are comparable with those of standard antimalarial drug, CQ (log K 5.15). Furthermore, the decrease of apparent pH from 7.4 to 5.6 (Table 3) has little effect on the binding constants indicating binding is stronger even at acidic pH.

To further establish the binding of 5b with monomeric heme, 1H NMR titrations were performed and shifts in the peaks as well as peak intensity noted. The addition of 30 mol% of heme dissolved in 40% DMSO:D2O:D2SO4 (10 μl) caused a shift in the aromatic proton signals (Fig. 5), indicating binding of 5b with heme but further addition of heme led to broadening of the peaks. An equimolar (3.9 μmol) solution of hemin chloride and 5b when analyzed in mass spectrometer depicted an intense molecular ion peak at 1119.3769 Da (Fig. 6a), corresponding to the molecular formula C40H40ClFeN8O8, suggesting the formation of 1:1 complex. Thus, we propose that 5b interacts with heme by replacing chloride atom of hemin chloride and coordinating the iron atom with its endocyclic quinoline nitrogen as proposed in Fig. 6b.

Similar titration of dimers of µ-oxo type (10 μM) at pH 5.8 using standard procedure [29] with increasing concentration of

![Chemical Structures](https://example.com/structures.png)
compound 5b (0–14 μM), resulted in decrease in intensity of broad peak at 362 nm (Fig. 7a, S1). Further, Job’s plot calculations indicated a 1:1 stoichiometry for the most stable μ-oxo: 5b complex (Fig. 7b). In Table 3 the association constants of 5b (log K 5.72) are compared to that of standard CQ (log K 5.58) and also suggests that the binding of 5b is stronger with μ-oxo heme (log K 5.72) than monomeric heme (log K 4.96). Thus, the compound 5b inhibits hemooxidation by blocking the growing face of heme resulting in the observed antimalarial activity. Furthermore, the β-hematin inhibition assay (SI Table S2) shows that there is no correlation between antimalarial activity and β-hematin inhibition and also, all the compounds inhibit β-hematin formation although less than that of standard CQ.

3.3.2. DNA binding studies

The mechanism of many antimalarial drugs such as CQ, quinacrine and quinine relies upon the interaction with DNA presumably through ionic interactions between phosphate groups of DNA and protonated amine in addition to the interactions between aromatic nuclei of the drug with nucleotide bases [40,41]. Therefore, the DNA binding properties of 4-aminoquinoline–pyrimidine hybrids have been evaluated using both the UV–visible spectrophotometer and fluorescence spectrophotometry in order to probe interaction of these compounds with DNA. The addition of CT-DNA (4–200 μM) to the buffered methanolic solution of 5b (30 μM) induced hyperchromic shift of 112% in absorption band at 255 nm whereas hypochromic shift of 37% in the characteristic quinoline ring absorption at 330 nm (Fig. 8). Also, the bathochromic shift of ~3 nm was observed for both the absorptions. The observed hyperchromic as well as hypochromic shifts in absorption bands of 5b upon addition of DNA results from the intercalation of 5b with CT DNA as suggested in the literature [42]. The intercalative nature of interaction of compound 5b with CT DNA was additionally supported by thermal denaturation experiment. Intercalation of molecules into the double helix is known to stabilize the DNA against thermal strand separation and thus increases thermal melting temperature (Tm) [43,44]. The derivative melting curve presented in Fig. 9 shows an increase of 7.5 °C in thermal melting temperature of CT DNA upon addition of 5b which is less than that observed for the CQ (Table S3). Thus, both the UV–visible titrations and thermal denaturation experiment advocate partial intercalative nature of interactions between compound 5b and CT DNA.

Further, to visualize the effect of DNA base composition, the fluorescence titrations of 5b were performed with both GC-rich CT DNA and AT-rich pUC18 DNA in buffered methanol. Fig. 10 shows decrease in the intensity of the emission band of 5b at 380 nm, upon addition of increasing concentration of both the DNAs. A shift of 80 nm in emission band at 380 nm was observed upon addition of CT DNA but no such shift in emission band was observed for pUC18 DNA. Comparison of binding constant of 5b with CT DNA (log K 5.76) and pUC18 DNA (log K 5.73) calculated from titration data using HypSpec [39], suggest that 5b does not discriminate between GC rich DNA and AT rich DNA.

4. Conclusions

A series of potent 4-aminoquinoline–pyrimidine hybrids with antimalarial activity in nanomolar range were reported. The compound 5b exhibits lowest IC50 value within the series against both CQΔ and CQΔ strains of P. falciparum. These hybrids displayed mild toxicity against MDCK cell cultures. The antiviral activity profiles of these hybrids indicate that the compound 5a and 5c effectively inhibit feline corona virus and feline herpes virus. Further, the mechanism of observed antimalarial activity was established in terms of binding with heme as well as DNA.

### Table 3

| Compound | Monomeric heme, log K | μ-oxo heme, log K | CT DNA, log K | pUC18 DNA, log K |
|----------|-----------------------|-----------------|--------------|-----------------|
|          | pH 5.6                | pH 7.4          | pH 5.8       |                 |
| 5b       | 4.58 ± 0.042          | 4.96 ± 0.029    | 5.72 ± 0.025 | 5.76 ± 0.002    |
| CQ       | 4.65 ± 0.052          | 5.15 ± 0.176    | 5.58 ± 0.006 | nd              |

| Stoichiometry | 1:1 | 1:1 | nd |

* Calculated from HypSpec software.

Fig. 4. Titration of 5b with monomeric heme at (a) pH 7.4, (b) pH 5.6.

Fig. 5. The 400 MHz 1H spectra of 5b upon addition of heme (a) 0 mol%, (b) 30 mol%, (c) 50 mol% (in 40% DMF:D2O:D3SO4 (10 μl)) [Δ δ for peak: a = 0.002, b = 0.013, c = 0.023, d = 0.008, e = 0.007, f = 0.007].

Fig. 6. The 400 MHz 1H spectra of 5b with monomeric heme at (a) pH 7.4, (b) pH 5.6.
5. Experimental

5.1. General

All liquid reagents were dried/purified following recommended drying agents and/or distilled over 4Å molecular sieves. THF was dried (Na-benzophenone ketyl) under nitrogen. 1H NMR (300 MHz) and 13C (75 MHz) NMR spectra were recorded in CDCl3 on a multinuclear Jeol FT-AL-300 spectrometer with chemical shifts being reported in parts per million (δ) relative to internal tetramethylsilane (TMS, δ0.0, 1H NMR) or chloroform (CDCl3, δ77.0, 13C NMR). Mass spectra were recorded at Department of Chemistry, Guru Nanak Dev University, Amritsar on a Bruker LC-MS MICROTOF II spectrometer. Elemental analysis was performed on FLASH EA 112 (Thermo electron Corporation) analyzer at Department of Chemistry, Guru Nanak Dev University, Amritsar and the results are quoted in %. IR spectra were recorded on Perkin Elmer FTIR-C92035 Fourier transform spectrometer in the range 400–4000 cm−1 using KBr pellets. Melting points were determined in open capillaries and are uncorrected. For monitoring the progress of a reaction and for comparison purpose, thin layer chromatography (TLC) was performed on pre-coated aluminum sheets of Merck (60F254, 0.2 mm) using an appropriate solvent system. The chromatograms were visualized under UV light. For column chromatography silica gel (60–120 mesh) was employed and eluents were ethyl acetate/hexane or ethyl acetate/methanol mixtures. The steady state fluorescence experiments were carried out on Perkin Elmer LS55 fluorescence spectrometer at ambient temperature. UV–visible spectral studies were conducted on Shimadzu 1601 PC spectrophotometer with a quartz cuvette (path length, 1 cm). The absorption spectra have been recorded between 1100 and 200 nm. The cell holder of the spectrophotometer was thermostated at 25°C for consistency in the recordings.

5.2. General procedure for synthesis of 5a and 5b

To the stirred solution of 3 (2 mmol) and potassium carbonate (5 mmol) in dry THF (30 ml), a solution of appropriate 4-aminoquinoline 4 (1.0 mmol) in dry THF (50 ml) was added. The reaction mixture was stirred for 48 h at room temperature. The reaction mixture was filtered and THF was removed under vacuum. The residue was purified by column chromatography using MeOH/ethyl acetate as eluent to obtain corresponding 5, which was recrystallized from DCM/hexane. Using this procedure the following compounds were isolated.

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Fig. 6. (a) The solution phase mass spectra of 5b (3.9 μmoles) upon addition of monomeric heme (3.9 μmoles) in 40% DMSO. (b) proposed binding of heme with 5b (for optimized structure of 5b, see Figure S2).

Fig. 7. (a) Titration of 5b with μ-oxo heme at pH 5.8, (b) Job plot of μ-oxo heme complex formation at pH 5.8. \( A_0 - A_{x} \): the absorbance, when \( x = 1 \) and \( A \) is the absorbance at respective values of \( x \).

Fig. 8. Absorption spectra of 5b (30 μM) in the presence of increasing CT DNA concentration (4–200 μM); inset shows zoom between 280 and 390 nm.
5.2.1. Methyl 2-(3-(7-chloroquinolin-4-yl)amino)propylamino)-4-methyl-6-phenylpyrimidine-5-carboxylate (5a)

White solid. Rf: 0.47 (4% MeOH/ethyl acetate). Yield: 86%. m.p.105 °C. IR (KBr): νmax 770, 1267, 1709, 2928, 3427 cm⁻¹. 1H NMR (300 MHz, CDCl3, 25 °C): δ 2.00 (q, J = 6.3 Hz, 2H, CH₂), 2.49 (s, 3H, C6-CH₃), 3.46 (m, 2H, CH₂), 3.58 (s, 3H, ester-CH₃), 3.68 (q, J = 6.6 Hz, 2H, CH₂), 5.57 (br, 1H, NH), 6.41 (d, 1H, ArH), 6.44 (br, 1H, NH), 7.39−7.54 (m, 7H, ArH), 7.91 (s, 1H, ArH), 8.48 (d, 1H, ArH). 13C NMR (75 MHz, CDCl3, 25 °C): δ 14.8, 20.6, 30.0, 31.6, 43.8, 90.6, 92.3, 112.7, 116.8, 119.5, 120.2, 121.5, 126.5, 141.3, 143.6, 153.5, 159.1, 160.8. Anal. Calcld. for C₂₅H₂₄N₅O₂Cl: C, 65.00; H, 5.24; N, 15.10. Found: C, 65.14; H, 5.19; N, 14.99. MS: m/z 462 [M⁺].

5.2.2. i-Propyl 2-(4-(7-chloroquinolin-4-yl)amino)butylamino)-4-methyl-6-phenylpyrimidine-5-carboxylate (5b)

White solid. Rf: 0.41 (4% MeOH/ethyl acetate). Yield: 83%. m.p.140 °C. IR (KBr): νmax 1367, 1724, 2993, 3473 cm⁻¹. 1H NMR (400 MHz, CDCl3, 25 °C): δ 1.00 (d, J = 5.6 Hz, 6H, 2 × ester-CH₃), 1.78 (m, 4H, CH₂), 2.46 (s, 3H, C6-CH₃), 3.33 (m, 2H, CH₂), 3.50 (m, 2H, CH₂), 4.99 (m, 1H, ester-CH), 5.44 (br, 1H, NH), 5.67 (br, 1H, NH), 6.34 (d, J = 5.3 Hz, 1H, ArH), 7.27−7.63 (m, 7H, ArH), 7.93 (d, J = 1.4 Hz, 1H, ArH), 8.46 (d, J = 5.3 Hz, 1H, ArH). 13C NMR (75 MHz, CDCl3, 25 °C): δ 21.3, 22.9, 25.8, 27.4, 40.7, 42.9, 68.9, 98.9, 116.0, 117.0, 121.3, 125.3, 129.5, 135.0, 138.9, 148.4, 150.0, 151.3, 161.2, 165.8, 166.9, 168.3. Anal. Calcld. for C₂₈H₄₀N₅O₂Cl: C, 66.72; H, 6.00; N, 13.89; Found: C, 66.50; H, 5.88; N, 13.65. MS: m/z 503.2 [M⁺].

5.3. General procedure for the synthesis of compound 5c–g

To the stirred solution of appropriate 4-aminquinoline 4 in dry acetonitrile (50 ml) mixture of 3 (in a 1:2 molar ratio) and potassium carbonate in dry acetonitrile was added. The reaction mixture was refluxed for 24 h and then filtered. Acetonitrile was removed under vacuum and the residue was purified by column chromatography using MeOH/ethyl acetate as eluent to give 5 which is recrystallized from DCM/hexane.

5.3.1. Ethyl 2-(4-((7-chloroquinolin-4-yl)amino)butylamino)-4-methyl-6-(2-nitrophenyl)pyrimidine-5-carboxylate (5c)

Yellow solid. Rf: 0.28 (4% MeOH/ethyl acetate). Yield: 75%. m.p. 72 °C. IR (KBr): νmax 769, 1550, 1355, 1720, 2930, 3365 cm⁻¹. 1H NMR (400 MHz, CDCl3, 25 °C): δ 0.87 (t, J = 7.1 Hz, 3H, ester-CH₃), 1.76 (m, 4H, CH₂), 2.57 (s, 3H, C6-CH₃), 3.27 (m, 2H, CH₂), 3.47 (m, 2H, CH₂), 3.96 (q, J = 7.0 Hz, 2H, ester-CH₂), 6.07 (br, 1H, NH), 6.26 (d, J = 4.8 Hz, 1H, ArH), 6.33 (br, 1H, NH), 7.22−7.86 (m, 6H, ArH), 8.11 (s, 1H, ArH), 8.32 (d, J = 5.4 Hz, 1H, ArH). 13C NMR (100 MHz, CDCl3, 25 °C): δ 12.5, 22.1, 24.4, 28.6, 39.7, 42.2, 59.7, 97.4, 115.6, 121.3, 122.3, 124.5, 125.0, 128.6, 131.9, 134.7, 145.2, 148.3, 150.1, 159.9, 165.3, 176.3. Anal. Calcld. for C₂₅H₂₃N₅O₃Cl: C, 60.62; H, 5.09; N, 15.7; Found: C, 60.34; H, 5.01; N, 15.58. MS: m/z 534.2 [M⁺].

5.3.2. Ethyl 2-(3-(7-chloroquinolin-4-yl)amino)propoxy)-4-methyl-6-phenylpyrimidine-5-carboxylate (5d)

White solid. Rf: 0.38 (4% MeOH/ethyl acetate). Yield: 90%. m.p.110 °C. IR (KBr): νmax 1255, 1775, 2969, 3530 cm⁻¹. 1H NMR (300 MHz, CDCl3, 25 °C): δ 1.05 (t, J = 7.2 Hz, 3H, ester-CH₃), 2.43 (m, 2H, CH₂), 2.59 (s, 3H, C6-CH₃), 3.56 (m, 2H, CH₂), 4.13 (t, J = 7.2 Hz, 2H, CH₂), 4.68 (q, J = 6.0 Hz, 2H, ester-CH₂), 5.70 (br, 1H, NH), 6.41 (d, J = 5.4 Hz, 1H, ArH), 7.20−7.76 (m, 7H, ArH), 7.93 (d, J = 2.1 Hz, 1H, ArH), 8.49 (d, J = 5.4 Hz, 1H, ArH). 13C NMR (75 MHz, CDCl₃, 25 °C): δ = 13.5, 22.8, 277, 412, 61.7, 66.4, 121.3, 125.3, 130.2, 151.9. Anal. Calcld. for C₂₃H₂₈N₅O₂Cl: C, 65.47; H, 5.28; N, 11.75; Found: C, 65.12; H, 4.99; N, 11.89. MS: m/z 476.1 [M⁺].

5.3.3. Ethyl 4-(4-chlorophenyl)-2-(4-((7-chloroquinolin-4-yl)amino)butylamino)-6-methylpyrimidine-5-carboxylate (5e)

Yellow solid. Rf: 0.54 (4% MeOH/ethyl acetate). Yield: 85%. m.p. 135 °C. IR (KBr): νmax 1065, 1720, 2930, 3489 cm⁻¹. 1H NMR (300 MHz, CDCl₃, 25 °C): δ 1.03 (t, J = 6.9 Hz, 3H, ester-CH₃), 1.86 (m, 4H, CH₂), 2.46 (s, 3H, C6-CH₃), 3.40 (m, 2H, CH₂), 3.59 (m, 2H, CH₂), 4.07 (q, J = 6.0 Hz, 2H, ester-CH₂), 5.50 (br, 1H, NH), 5.78 (br, 1H, NH), 6.36 (d, J = 5.7 Hz, 1H, ArH), 7.25−7.37 (m, 5H, ArH), 7.48 (d, 60.0 Hz, 2H, ArH).
J = 8.4 Hz, 1H, ArH), 7.96 (d, J = 7.8 Hz, 1H, ArH), 8.42 (d, J = 5.7 Hz, 1H, ArH). 13C NMR (75 MHz, CDCl3, 25 °C): δ 16.5, 28.8, 30.2, 32.5, 64.1, 101.8, 128.3, 131.3, 132.2, 143.2, 152.4, 161.6, 167.7. Anal. Calcld. for C27H27N5O2Cl2: C, 61.65; H, 5.19; N, 13.35; Found: C, 61.57; H, 5.05; N, 13.12. MS: m/z 523.1 [M + ]

6.2. Cytotoxicity and antiviral activity assay

Cytotoxicity was determined by exposing different concentrations of samples to Vero, HEL, Hela and MDCK cells [29]. The antiviral assays were based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1 (HSV-1), HSV-2 (G), vaccinia virus, and vesicular stomatitis virus], Vero (parainfluenza-3, reovirus-1, Coxackie B4, and Punta Toro virus), Hela (vesicular stomatitis virus, Coxackie virus B4, and respiratory syncytial virus) and MDCK (influenza A (H1N1; H3N2) and B virus) cell cultures. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 cell culture inhibitory dose-50 (CCID50) of virus (1 CCID50 being the virus dose to infect 50% of the cell cultures) in the presence of varying concentrations of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds [29].

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.05.046.

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6. Material and methods

6.1. In vitro antimalarial activity assay

The test samples were tested in triplicate on one or two separate occasions against chloroquine sensitive (CQS) strain of P. falciparum (D10). Continuous in vitro cultures of auxsexual erythrocyte stages of P. falciparum were maintained using a modified method of Trager and Jensen [45]. Quantitative assessment of antiplasmodial activity in vitro was determined via the parasite lactate dehydrogenase assay using a modified method described by Makler et al. [46]. The test samples were prepared to a 20 mg/ml stock solution in 100% DMSO and sonicated to enhance solubility. Samples were tested as a suspension if not completely dissolved. Stock solutions were stored at −20 °C. Further dilutions were prepared on the day of the experiment. Chloroquine (CQ) was used as the reference drug in all experiments. Test samples were initially tested at three concentrations (10 μg/ml, 5 μg/ml and 2.5 μg/ml) to determine the starting concentration for the full dose–response assay. CQ was tested at three concentrations namely 30 ng/ml, 15 ng/ml and 7.5 ng/ml. A full dose–response was performed for all compounds to determine the concentration inhibiting 50% of parasite growth (IC50-value). Test samples were tested at a starting concentration of 10 μg/ml, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.02 μg/ml. The same dilution technique was used for all samples. CQ was tested at a starting concentration of 1000 ng/ml. Several compounds were tested at a starting concentration of 1000 ng/ml. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability (data not shown). The IC50-values were obtained using a non-linear dose–response curve fitting analysis via Graph Pad Prism v.4.0 software.
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