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2-Aminopyrimidine based 4-aminoquinolines anti-plasmodial agents. Synthesis, biological activity, structure—activity relationship and mode of action studies

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Abstract

2-Aminopyrimidine based 4-aminoquinolines were synthesized using an efficacious protocol. Some of the compounds showed in vitro anti-plasmodial activity against drug-sensitive CQ (3D7) and drug-resistant CQ (K1) strains of \textit{Plasmodium falciparum} in the nM range. In particular, 5-isopropoxycarbonyl-6-methyl-4-(2-nitrophenyl)-2-[(7-chloroquinolin-4-ylamino)butylamino] pyrimidine depicted the lowest IC	extsubscript{50} (3.6 nM) value (56-fold less than CQ) against CQR strain. Structure—activity relationship and mode of action studies

1. Introduction

Faced with the challenges of drug resistance, poor health systems, lack of affordable, safe and convenient treatment options, efficient treatment of malaria, one of the most devastating parasitic diseases, represents an unmet medical need. Malaria is a major public health concern in more than 90 countries inhabited by more than 2.4 billion people — 40% of the world’s population and is responsible for almost 1 million deaths every year [1]. The majority of malaria victims in developing countries are pregnant women or children under the age of five, possessing little or no immunological protection. The disease is estimated to result in ~250 million new annual infections worldwide. Though the majority of the cases and approximately 90% of the malaria deaths are found in sub-Saharan Africa, the disease is now increasing in Asia and Latin America. Malaria is caused by protozoan parasites of the genus \textit{Plasmodium} that infects and destroys red blood cells eventually leading to death, if untreated. The persistent threat of emergence of multidrug resistant \textit{Plasmodium falciparum}, universal chloroquine resistance [2,3], suspected resistance to artemisinins [4,5], and lack of effective, appropriate and affordable treatment options have given a new impetus to the research leading to broadening of the range of therapeutic targets. Thus, creating a new armamentarium of drugs with promising antimalarial activity coupled with understanding of their mode of action may lead to the development of a new generation of treatments both for malaria control and eradication.

The common feature of the drugs based on quinine 1 is the presence of a quinoline unit, usually a 7-chloroquinoline (chloroquine 2 and amodiaquine 3, Chart 1), and are known to 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 autodigestion [6–8]. The mode of action of 2,4-diaminopyrimidine based drugs, typified by pyrimethamine 4 [9] and the lead compound WR99210 5 [10] is through the inhibition of \textit{P. falciparum} Dihydrofolate reductase (\textit{Pf} DHFR) enzyme, required for the biosynthesis of tetrahydrofolate involved in the biotransformation of thymidylate synthase-catalysed deoxyuridylicate to deoxythymidylate (dUMP → dTMP), through a methyl group transfer reaction during DNA biosynthesis [11–16]. In addition, a number of polyamines inhibit ornithine decarboxylase activity in \textit{P. falciparum} through binding with plasmoidal DNA
Recently, investigations in hybrid antimalarial agents, combining 4-aminoquinoline with other pharmacophore having antimalarial activity have been found to be less prone to resistance to parasite and has thus offered an effective means to overcome the problem of drug resistance.

We envisaged that linking 7-chloro-4-aminoquinoline unit, critical for antimalarial activity through a diversely functionalized lateral side chain with other antimalarial moiety such as amino-pyrimidine, might furnish conjugate hybrids capable of showing useful antimalarial activity. In this communication, we report synthesis of a set of compounds that possess basic, hydrophobic as well as hydrogen bonding substituents, required for targeting either or both heme as well as DNA, thus providing new antimalarial agents active against chloroquine resistant strains of *P. falciparum*. We have evaluated their anti-plasmodial activity, cytotoxicity and cytostatic activity, binding studies with DNA and heme (monomeric as well as μ-oxo dimeric) using UV–visible, fluorescence spectrophotometry as well as NMR analysis.

2. Chemistry

Compounds 10a–s were synthesized as outlined in Scheme 1, via a common intermediate 8. 3,4-Dihydropyrimidin-2(1H)-ones 6 were prepared through HCl-catalyzed Biginelli condensation of appropriate aldehyde (R^3CHO), alkylacetoacetate (R^2CH_2COOR^1) and urea [20]. Dehydrogenation of 6 using pyridinium chlorochromate in DCM furnished pyrimidinones 7 [21]. Refluxing 7 with POCl3 yielded 8 which upon nucleophilic substitution reaction with appropriate 4-amino-7-chloroquinoline 9 gave 10a–s in a synthetically useful manner [22]. Structures of 6–10 were established on the basis of spectral (^1H NMR, ^13C NMR, MS, FT IR) as well as microanalytical analysis. The yields of the 2-aminopyrimidines 10 are reported in Table 1.

3. Results and discussion

3.1. In vitro anti-plasmodial activity and structure–activity relationships (SARS)

Antiplasmodial activity of pyrimidines linked to CQ as in 10 has not been described in literature albeit the related dihydropyrimidin-2(1H)-ones (DHPMs) have previously been reported [23]. Using the synthetic protocol shown in Scheme 1 allowed considerable diversification around the pyrimidine core for conducting SAR analysis. The in vitro anti-plasmodial activities of 10a–s were determined in primary and secondary screening against CQS and CQR strains of *P. falciparum*. The half maximal inhibitory concentration (IC₅₀) of 10a–s are summarised in Table 2 (Fig. 1). Evidently, the compounds have anti-plasmodial activity in the nM range and against the CQR strain of *P. falciparum*, in some cases activity was found to be even superior to CQ. Systematic variation of the length as well as nature of the spacer connecting the pharmacophores discerned useful trends in the anti-plasmodial activity of these analogs.

Comparing 10a–g, bearing linear alkyl spacers, revealed an increase in the anti-plasmodial activity with increase in length of the spacer up to 4 methylene groups (10a–10c, Table 2). Further lengthening of the spacer chain length resulted in significant...
reduction in activity against both the CQR as well as the CQS strains. Replacing the C-4 phenyl group in 10c by a methyl group to create 10m resulted in nearly 5 times increase in anti-plasmodial activity (IC₅₀ 42.1 nM) against the CQS strain. In fact, compound 10m was found (Table 2) to be the most active compound of the series, against the CQS strain. However, 10m exhibited a high resistance factor (~ 39) within the series but is 3.5 times less active than CQ, against the CQR strain. Replacement of the ethyl ester with iso-propyl ester (10n), in general showed a decrease in in vitro anti-plasmodial activity against the CQS strain, although a reverse trend was observed for the CQR strain, especially in case of 10s, 10p and 10r, where replacing C-4 phenyl by p-nitrophenyl (10s) or o-nitrophenyl (10p and 10r) groups, in addition to incorporating an isopropyl ester, resulted in an increase of activity in that order, rendering 10r as the most potent (IC₅₀ 3.6 nM, 56 times more potent than CQ, Table 2) of all these compounds. These results are in accordance with the trend observed in case of N, N-bis(7-chloroquinolin-4-yl)alkane diamines, wherein the alkyl spacer consisting of four carbon atoms showed optimum potency [24].

The better anti-plasmodial activity of phenyl-substituted pyrimidine compounds against the CQR strain may be attributed to optimal fitting of these compounds in the active site of Pfdhfr leading to a favorable conformation for π-π interaction with the heme functionality. Moreover, the introduction of a nitro substituent on the phenyl ring at the C-4 position of the pyrimidine core results in a significant increase in anti-plasmodial activity as well as resistance against the CQR strain, although it has little effect on activities against the CQS strain (Table 2). Also, the corresponding o-, m- or p-nitro derivatives showed considerable variation in anti-plasmodial activity (Table 2). Comparison of compounds (10n, 10q, 10s) having an identical spacer reveals that the p-NO₂ substituted 10s (IC₅₀ 175.8 nM) is more active than the o-/m-NO₂ substituted compounds 10q and 10n, respectively, but are less active than the unsubstituted ethyl ester analog 10b. Further, comparison with the butyl spacer analog suggests that o-NO₂ phenyl derivative 10r is more potent than the m-NO₂ counterpart 10o, as well as its ethyl ester analog 10c, against the CQS strain. Compound 10r was found to be the most active compound of this series against the CQR strain with anti-plasmodial activity (IC₅₀ 3.6 nM), 56 times more than CQ (IC₅₀ 201.8 nM) and comparable to artesunate (IC₅₀ 2.8 nM) (Table 2).

Comparing 10j (IC₅₀ 28096.7 nM) possessing the branched chain spacers with identical carbon linear spacer analog 10b (IC₅₀ 52.2 nM, Table 2) led to significant decrease in activity. Thus, compound 10j displayed a 540-fold decrease in activity compared to 10b, against the CQS strain. Likewise, 10k possessing a branched C-6 spacer depicted a decrease (IC₅₀ 860.4 nM, Table 2) in activity against linear alkyl (C-7) spacer analog 10d (Table 2), but higher than 10j. Comparing 10j and 10k, with branched chain spacers, not only was the anti-plasmodial activity of the latter against both CQ₅₀

Fig. 2. Titration of 10h (0–85.7 µM, DMSO:H₂O/4:6, v/v) (a) and 10i (0–8.85 µM, DMSO:H₂O/4:6, v/v) (b) with heme (2.4 µM, DMSO: H₂O/4:6, v/v).

Fig. 3. Titration of 10r (A) and 10c (B) with monomeric heme at pH 7.5.
and CQ8 strains increased, but it also showed an increased resistance factor (Table 2). The replacement of linear chain spacers with o- and p-linked aryl groups (10h and 10i, respectively), led to much higher IC50 (Table 2) values compared to former against CQ8 strain, while only moderate activity was observed in case of 10i against CQ8 strain. These findings could be attributed to the increased steric bulk (10h more than 10i) affecting the interaction of the iron center of heme with the compounds. This has been corroborated by performing the titration of monomeric heme with both 10h and 10i. While the titration of heme (2.4 μM, DMSO:H2O/4:6, v/v) with increasing concentration of 10h (0–85.7 μM, DMSO:H2O/4:6, v/v) revealed no change in the absorbance at 402 nm (Fig. 2a), the addition of 10i, in a similar way showed marked gradual decrease in absorbance at 402 nm. The decrease in absorbance continued until the concentration of 10i was 8.85 μM (Fig. 2b), representing 1:4 molar ratio of heme and 10i. Hence, the flexibility, chain length and steric constraints of the spacer linking quinoline moiety and pyrimidine unit seem to play a role in anti-plasmodial activity of these derivatives. Although the in vitro activity of 10a–c, 10p and especially 10r (IC50 3.6 nM) was superior to CQ (IC50 201.8 nM) against the CQ8 strain, these compounds suffer from high ClogP values (Table 2), which are suggestive of the fact that these possess limited aqueous solubility, which in fact is not a serious limitation in view of recent advancements in formulation methods.

3.2. Cytotoxicity and antiviral activity

Antiviral activity of the compounds 10a–c, 10i–n, 10p and 10r, which were active against the CQ8 strain was also evaluated against (i) parainfluenza-3 virus, reovirus-1, Sindbis virus, Coxsackie virus B4, Punta Toro virus in vero cell cultures, (ii) 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 ACV81), cytomegalovirus, varicella-zoster virus in HEL cell cultures, (iii) vesicular stomatitis virus, coxsackie virus B4, respiratory syncytial virus in HeLa cell cultures, (iv) influenza A virus (H1N1 and H3N2) and influenza B virus in MDCK cell cultures and (v) feline corona virus (FIPV) and feline herpes virus activity in CRFK cell cultures (SI Tables S1–S7). Unfortunately, no significant antiviral activity was noted at subtoxic concentrations. Most compounds were somewhat cytotoxic to the different cell lines (confluent non-proliferating cultures), but especially cytostatic against proliferating (Vero/MDCK/CRFK) cell cultures. Chloroquine has been shown to inhibit HIV through blockade of viral entry via inhibition of endosomal acidification [25,26]. Compounds 10a–c, 1–n, p, r were also tested against HIV-1 as well as HIV-2 (SI Table S8) in human T-lymphocyte (CEM) cell cultures. However, none of the compounds were active at subtoxic concentrations.

Generally, the compounds exhibit a relatively high cytostatic activity but displayed a fairly safe selectivity index (except 10m and 10n) (Table 2) in the range of 10.04–638 against MDCK cell cultures. The most active compound 10r with an IC50 value of 3.6 nM against CQ8 strain exhibited a highest selectivity index (SI = 638). 10i with an IC50 value of 160.8 nM against CQ8 strain exhibited a high selectivity index (361.9). However, 10m having an IC50 value of 1659.8 nM was most toxic with a selectivity index of 0.48.

3.3. Insight into the mode of action and Fe(III)PPIX-10c/10r association constants

The plausible mechanism of in vitro anti-plasmodial action against CQ8 strain has been investigated for 10c and 10r, found to be most potent of the series of the compounds reported herein (IC50 26.1 nM, 10c and IC50 3.6 nM, 10r). These compounds are also expected to bind to heme [Fe(III)PPIX] (hydroxido or aqua complex of ferrprotoporphyrin IX) in solution and inhibit aggregation to β-hematin, in much the same way as CQ itself. The higher ClogP values (Table 2) of these compounds being higher than CQ necessitated the use of aqueous DMSO as solvent. Also in 40% aqueous DMSO solution, heme is expected to be monomeric, while in purely aqueous solutions, it exists in aggregated form. Further, it is known that CQ binds to heme dimer (μ-oxo heme) in vitro and can also
inhibit the formation of β-hematin, the in vitro analog of hemozoin [27–30]. Therefore the binding assay was also extended to heme dimer also. The interaction of 10c and 10r with monomeric heme was followed by spectrophotometric titration at pH 7.4 and 5.6 (the approximate pH of food vacuole), as described previously [31]. The spectral changes (Fig. 3A, SI Figure S1), upon addition of increasing amounts (0–70 μM) of 10r to a solution of heme (2.4 μM, HEPES buffer pH 7.4) showed substantial decrease of intensity of the Fe(III) PPIX Soret band at 402 nm with no shift in the absorption maximum. The longer wavelength Q-bands (494 and 537 nm) of the metalloporphyrin also decrease in intensity (SI Figure S2) [32]. A sharp isosbestic point was observed at 416 nm (pH 7.4) and at 412 nm (pH 5.6). The association constants for the complexes formed between monomeric Fe (III) PPIX and 10r at pH 7.5 and 5.6 were calculated from titration data and are presented in Table 3.

The association constant of 10c (Fig. 2A) is found to be greater (log K 6.018) than 10r (log K 5.078). Decrease in the apparent pH from 7.5 to 5.6 caused a fairly modest decrease in log K values of both 10c and 10r, indicating a strong binding to heme even at acidic pH. The binding of 10c and 10r was also assessed from their 1H NMR titration with heme. Thus, while 1H NMR spectrum of 10r depicted considerable broadening of signals under the conditions (40% DMSO-d6 in D2O) of recording and was inconclusive, the variation in the chemical shift in signals corresponding to quinoline unit of 10c were clear (Fig. 4), upon addition of heme. The association constant of 10c and 10r with monomeric heme at pH 7.5 and 5.6 was deduced from the Job’s plot (SI Figure S3) [33,34]. The absorbance at 402 nm got to maximum when mole fraction of 10r approached 0.5. This is in good agreement with the association constant (log K) values corresponding to the most stable 1:1:heme: 10r species present in solution, obtained by fitting the titration data in Hyp Spec - a non-linear least square fitting programme. The measured log K values for both 10r and 10c (Table 3) are also in good agreement with the values reported in literature for CQ [32,35,36]. Thus, the formation of synthetic Fe(III)PPIX-10r complex, as demonstrated above is suggestive of inhibition of formation of β-hematin and presumably results in anti-plasmodial activity of these compounds in a fashion similar to that of CQ.

Titration of constant concentrations of heme in aqueous NaOH solution at the physiological pH 5.8 of Plasmodial food vacuole [35] allowed understanding of the interaction of 10r and 10c with μ-oxo heme. With addition of increasing amount of 10r to the solution of μ-oxo heme, the absorbance of Soret band (382 nm) decreased appreciably with significant red shift (362–405 nm, Fig. 5A). The association constants and stoichiometry for both 10r and 10c were determined from titration data and are presented in Table 3. The calculated association constant of 10r (log K 6.31) is greater than 10c (log K 6.09) and corresponds to 1:1 stoichiometry of the 10r:μ-oxo heme complex. The comparison of association constants of 10r and 10c
and 10c for monomeric and μ-oxo heme reveals that both compounds bind strongly with μ-oxo heme than monomeric heme and inhibit hemozoin formation by blocking the growing face of heme resulting in the observed anti-plasmodial activity. Further, all the compounds were also screened for in vitro inhibition of β-hematin formation using β-hematin inhibitory assay in order to further confirm their mechanism of action based on interference with the heme detoxification process [37,38].

As shown in Table 4, there is a general correlation between anti-plasmodial activity and inhibition of β-hematin formation, but the same generalisation does not hold for compound 10h, as, in spite of having 100% β-hematin inhibition (Table 4), it is one of the least active (IC50 27320.9 nM, CQR and IC50 14828.3 nM, CQS) compounds of the series. However, it must be noted that anti-plasmodial activity not only depends upon the β-hematin inhibition but also on other factors such as degree of accumulation of drug in food vacuole. Further, 10r showed dose dependent inhibition of β-hematin formation (SI Figure S4) with an IC50 value lower than those reported for CQ and quinine, thus demonstrating a better ability to interact with Fe(III)PPIX. Overall, these compounds showed strong β-hematin inhibition which seems to be their preferred mode of action.

3.4. DNA binding affinity

DNA binding has been considered previously as one of the possible mechanisms for anti-plasmodial activity and has been studied using spectrophotometry [39], spectrofluorimetry [40], DNA melting [41], viscometry [42] etc. Recently, some 4-aminoquinoline derivatives have been shown to interact with

![Scheme 1. Synthesis of 10.](image-url)
| S. No. | Compound |
|--------|----------|
| 1.     | 10a      | ![Structure of 10a](image) |
| 2.     | 10b      | ![Structure of 10b](image) |
| 3.     | 10c      | ![Structure of 10c](image) |
| 4.     | 10d      | ![Structure of 10d](image) |
| 5.     | 10e      | ![Structure of 10e](image) |
| 6.     | 10f      | ![Structure of 10f](image) |
| 7.     | 10g      | ![Structure of 10g](image) |
| S. No. | Compound* | Structure | Yield (%) |
|-------|-----------|-----------|-----------|
| 8.    | 10h       | ![Structure](image1) | 53        |
| 9.    | 10i       | ![Structure](image2) | 57        |
| 10.   | 10j       | ![Structure](image3) | 58        |
| 11.   | 10k       | ![Structure](image4) | 55        |
| 12.   | 10l       | ![Structure](image5) | 92        |
| 13.   | 10m       | ![Structure](image6) | 90        |
| 14.   | 10n       | ![Structure](image7) | 71        |
DNA [37] presumably through ionic interactions between phosphate groups of DNA and protonated amine sites resulting in stabilization of the helical configuration of DNA against thermal denaturation [41,43]. Additionally, interactions between aromatic nucleuses of the drug with nucleotide bases might also contribute. Binding of the plasmid DNA with CQ resulted in the elevation of the thermal melting temperature (Tm) of DNA in addition to other effects [44–47]. We therefore investigated the binding of the most active of the series, 10c and 10r toward GC rich Calf Thymus DNA (CT DNA) as well as AT rich pUC18 DNA through stepwise addition of small increments of DNA to a solution the compounds at constant concentration as well as physiological pH (Fig. 6A and B). A progressive decrease in the characteristic quinoline ring absorptions at 320 and 340 nm attributable to the intercalation of the quinoline into DNA was indicated.

The isosbestic point at 350 nm indicated that the spectra of limiting systems (i.e. the spectra of free and completely bound drug) intersect and permitted the selection of a single wavelength for study of complex formation. The binding constant (log K; 10c 4.67 and 10r 3.39) was calculated from Benesi–Hildebrand

Table 1 (continued)

| S. No. | Compound* | Structure | Yield (%) |
|-------|-----------|-----------|-----------|
| 15.   | 10o       | ![Structure](image) | 78        |
| 16.   | 10p       | ![Structure](image) | 66        |
| 17.   | 10q       | ![Structure](image) | 73        |
| 18.   | 10r       | ![Structure](image) | 85        |
| 19.   | 10s       | ![Structure](image) | 82        |

* THF, K2CO3, rt.

b MeCN, K2CO3, 80 °C.
Altering the DNA base composition to see its effect on the drug binding, we studied the interaction of 10c with GC rich CT DNA by fluorometric titration. The emission band at 380 nm of fluroxetine data is 4.67. Similar titration with AT rich DNA, the thermal behavior (SIFigure S5) of CT DNA in the presence of 10r toward AT rich pUC18 DNA unlike CQ which is known to interact strongly with GC rich DNA [46]. The strong interaction of the pyrimidine analog with AT rich pUC18 DNA further suggests that these compounds might target parasite DNA which has unusually a high AT content. Many of the anti-plasmodial agents are known to interact with Pf DHFR for their anti-plasmodial activity. Especially

### Table 2

| Compound | IC50 (nM) | Resistance factor | ClogP | Cytotoxicity (μM) | Selectivity index |
|----------|-----------|------------------|-------|------------------|------------------|
|          | CQb (3D7)^a | CQb (K1)^b |       |                  |                  |
| 10a      | 21.7      | 139.3            | 6.43  | 6.83             | 1.4              |
| 10b      | 247.5     | 52.2             | 0.210 | 6.82             | 0.8              |
| 10c      | 202.3     | 26.1             | 0.129 | 7.35             | 0.9              |
| 10d      | 1134.9    | 617.7^c          | 0.544 | 8.94             | –                |
| 10e      | 252.2     | 516.4            | 2.047 | 9.47             | –                |
| 10f      | 1776.8    | 487.7            | 0.2744| 10.53            | –                |
| 10g      | 264.8     | 853.7            | 3.22  | 11.58            | –                |
| 10h      | 14828.3   | 27320.9          | 1.842 | 20.18            | –                |
| 10i      | 664.4     | 6186.4^d         | 9.600 | 8.80             | 34.41            |
| 10j      | 15888.5   | 28096.7          | 3.00  | 8.28             | –                |
| 10k      | 286.7     | 860.4^e          | 3.00  | 8.28             | –                |
| 10l      | 63.1      | 160.8            | 2.5480 | 5.64             | 361.89           |
| 10m      | 42.1      | 1659.8^f         | 39.422| 5.75             | 0.8              |
| 10n      | 598.7     | 230.5^g          | 0.385 | 7.30             | 0.5              |
| 10o      | 697.2     | 192.1            | 0.2756| 7.30             | 0.8              |
| 10p      | 172.9     | 96.0             | 0.5555| 6.89             | 2.2              |
| 10q      | 1955.1    | 229.3            | 0.1173| 7.30             | –                |
| 10r      | 18.2      | 3.6              | 0.2   | 7.30             | –                |
| 10s      | 499.9     | 175.8            | 0.3518| 7.30             | –                |
| 10t      | 14.1      | 201.8            | 140.39| 5.1              | –                |
| 2        | 5.3       | 2.8              | 0.5418| 1.06             | –                |

**Table 3**

| Compound | Monomeric heme | μ-oxo heme | CT DNA | pH 5.6 | pH 7.5 | pH 5.8 |
|----------|----------------|------------|--------|--------|--------|--------|
| 10r^a    | 4.97 ± 0.005   | 5.075 ± 0.01 | 6.31 ± 0.01 | 3.39 ± 0.02 |
| 10c^b    | 5.284 ± 0.01   | 6.018 ± 0.008 | 6.09 ± 0.02 | 4.67 ± 0.04 |
| CQ       | 4.82           | 5.2^b       | 5.6^c   | ND     |        |
| Stoichiometry | 1:1                           | 1:1             | ND     |        |

**Table 4**

| Compound | % Inhibition^a | Compound | % Inhibition^a |
|----------|----------------|----------|----------------|
| 10a      | 96             | 10i      | 66             |
| 10b      | 100            | 10m      | 100            |
| 10c      | 100            | 10n      | 79             |
| 10d      | 89             | 10o      | 92             |
| 10e      | 56             | 10p      | –              |
| 10f      | –              | 10g      | 89             |
| 10g      | –              | 10h      | 100 (2)^b      |
| 10h      | 53             | 10i      | 94             |
| 10j      | 100            | Quinine^c| 324^b          |

**Note:**

- ^a^ Calculated from Hyp Spec.
- ^b^ Refer to text Ref. [35].
- ^c^ Refer to text Ref. [36].
- ^d^ Refer to text Ref. [37].
- ^e^ Calculated from Chem draw Ultra 11.0.
- ^f^ Determined on Madin Darby canine kidney (MDCK) cells.
- ^g^ 50% cytotoxic concentration, as determined by measuring the cell viability with the colorimetric formazan-based MTS assay (reference drugs used: Oseltamivir carboxylate CC50/MIC > 100, Ribavirin CC50/MIC > 100, Amantadine CC50/MIC > 200 and Rimantadine CC50/MIC > 200).
- ^h^ Minimum compound concentration that causes a morphologically detectable alteration of normal cell morphology.
- ^i^ Selectivity index (SI) is calculated as CC50/IC50 (K1 Strain) ratio.

**Groove binding or electrostatic binding of the phosphate backbone of DNA gives rise to small changes in thermal denaturation temperature compared to the intercalation pathway due to stabilization of Watson crick base paired duplex [43,48,49,50]. In the direction of evaluating strength of interaction of 10c and 10r with DNA, the thermal behavior (SI Figure S5) of CT DNA in the presence of drug was evaluated. The thermal denaturation temperature of CT DNA (50 °C), in the presence of 10c and 10r recorded an increase in ΔTm of 3 °C and 2.5 °C, respectively (SI Table S9), suggesting primary groove binding and/or partial intercalative nature of the interaction.**

### 3.5 Molecular docking analysis

Many of the anti-plasmodial agents are known to interact with Pf DHFR for their anti-plasmodial activity. Especially

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pyrimethamine (Chart 1) is known to produce anti-plasmodial effect by binding to Pf DHFR. The best known lead compound for this inhibitory activity is WR99210 (5). It is worth comparing the performance of the compounds designed in this work with that of 5, using molecular docking methods. The crystal structure (PDB id: 1J31, a structure of wild-type Pf DHFR-TS complexed with WR99210, NADPH, dUMP) was considered for molecular docking analysis. After appropriately preparing the protein structure of Pf DHFR, docking of 5 and 10r were carried out. Fig. 8 shows the top scoring binding pose of 10r in the active site of Pf DHFR. The docking score for 10r is −24.5, which is much better than that of the lead compound 5 (−20.1). The origin of this improvement in docking score can be traced to the increased number of stabilizing interactions between 10r and Pf DHFR, in comparison to the interactions between 5 and Pf DHFR. 10r shows hydrogen bonding interaction with Tyr170 and Asn108 residues; these two interactions are common to 10r as well as to 5. In addition, 10r shows hydrogen bonding interaction with Ser111 and Leu46 residues.

These additional stabilizing interactions ensure that 10r adopts a slightly different (and improved) pose in comparison to that of 5. These improved interactions can be considered as determinative factors for the improved anti-plasmodial activity of 10r.

4. Conclusions

An efficacious transformation for converting readily available 3, 4-dihydroxypyrimidinones (DHPMs) to 2-aminopyrimidine based 4-aminooquinolines is presented. The compounds showed anti-plasmodial activity identical to or superior to CQ. Structure–activity relationship has been drawn leading to the identification of at least one compound 10r as lead compound in this series. The tested compounds did not depict any antiviral activity and were cytotoxic.

Binding interaction of representative potent compounds with heme and μ-oxo heme using UV–visible and NMR experiments furnished log K values identical with CQ binding and pointed to 1:1 stoichiometry of the most stable complexes in solution. DNA (both GC and AT rich) binding affinity using absorption and spectrofluorometric data indicated stronger interaction of 10c (20 fold) than 10r with CT DNA, however, 10r showed higher affinity toward AT rich pUC18 DNA suggesting targeting of parasite AT rich DNA by 10r in addition to β-hematin inhibition as possible mode of action of these compounds. DNA melting experiments suggested primary groove binding and/or partial intercalative nature of interaction of 10c and 10r with CT DNA. Thus, the strong electrostatic interaction of newly designed pyrimidinyl anilines with AT rich DNA and blockage of heme polymerisation by complexation of drug with heme contribute to the observed antimalarial activity in nano molar range. Further, molecular docking analysis of 10r in the active site of Pf DHFR indicated superior binding compared to WR99210. Thus, drug 10r acts on multiple targets (Heme, Enzyme involved in biosynthesis of DNA (DHFR), parasite DNA) which accounts for its high anti-plasmodial activity.

5. Experimental

5.1 General

All liquid reagents were dried/purified following recommended drying agents and/or distilled over 4 Å 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, δ 0.0). 1H NMR) or chloroform (CDCl3, δ 77.0, 13C NMR). Mass spectra were recorded from Indian Institute of Integrative Medicine (CSIR), Jammu, under electron impact at 70 eV on a Bruker Daltonics Esquire 3000 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 recorded on FTIR Shimadzu 8400 Fourier-transform spectrophotometer in the range 400–4000 cm−1 using chloroform as medium. 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 precoated aluminum sheets 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.

5.2 General procedure for the synthesis of 2-aminopyrimidines (10a–g and 10j–s)

To the stirred solution of 8 (2 mmol) and potassium carbonate (5 mmol) in dry THF (30 ml), a solution of appropriate 4-aminooquinoline 9 (1.0 mmol) in dry THF (50 ml) was added. The reaction mixture was stirred for 16 h at room temperature. The reaction mixture was filtered and THF was removed under vacuum. The residue was purified by column chromatography using MeOH/EtOAc as eluent to obtain corresponding 10, which was recrystallized from DCM/hexane. Using this procedure the following compounds were isolated.

5.2.1 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[(7-chloroquinolin-4-ylamino)ethylamino] pyrimidine (10a)

White solid. RF: 0.4 (8% MeOH/EtOAc). Yield: 89% (IR (KBr): vmax 770, 1267, 1709, 2928, 3331 cm−1 (H 300 MHz, CDCl3, 25 C): δ 1.00 (t, J = 7.2 Hz, 3H, ester–CH3), 2.55 (s, 3H, C6–CH3), 3.47 (m, J = 4.5 Hz, 2H, CH2), 3.94 (m, 2H, CH2), 4.10 (q, J = 7.2 Hz, 2H, ester–CH2), 5.81 (br, 1H, NH), 6.34 (d, 1H, ArH), 6.44 (br, 1H, NH), 7.26–7.85 (m, 8H, ArH), 8.44 (s, 1H, ArH).13C NMR (75 MHz, CDCl3, 25 C): δ 13.5, 23.0, 61.1, 98.8, 117.2, 121.0, 125.1, 127.7, 128.4, 128.6, 138.6, 148.8, 149.9, 151.8, 162.1 and 168.3. Anal. Calc. for C25H24N5O2Cl: C, 65.00; H, 5.20; N, 15.10; Found: C, 64.92; H, 4.89; N, 15.32. MS: m/z 462 (M+1).

5.2.2 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[(7-chloroquinolin-4-ylamino)ethylamino] pyrimidine (10b)

White solid. RF: 0.6 (8% MeOH/EtOAc). Yield: 84% (IR (KBr): vmax 772, 1268, 1707, 2933, 3397 cm−1 (H 300 MHz, CDCl3, 25 C): δ 0.95 (t, J = 7.2 Hz, 3H, ester–CH3), 1.98 (q, J = 6.3 Hz, 2H, CH2), 2.50 (s, 3H, C6–CH3), 3.45 (q, J = 6.3 Hz, 2H, CH2), 3.66 (q, J = 6.6 Hz, 2H, CH2), 4.1 (q, J = 7.2 Hz, 2H, ester–CH2), 5.63 (s, 1H, NH), 6.39 (d, J = 5.4 Hz, 1H, ArH), 7.38–7.54 (m, 7H, ArH), 7.90 (d, J = 1.8 Hz, 1H, ArH), 8.48 (d, J = 5.4 Hz, 1H, ArH).13C NMR (75 MHz, CDCl3, 25 C): δ 13.5, 23.0, 38.2, 39.8, 61.1, 98.8, 117.2, 121.0, 125.1, 127.7, 128.4, 129.6, 134.7, 149.0, 149.6, 151.8, 161.6, 167.4 and 168.4. Anal. Calc. for C26H25N5O2Cl: C, 65.60; H, 5.46; N, 14.70; Found: C, 65.59; H, 5.40; N, 14.50. MS: m/z 476.1 (M+).

5.2.3 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[(7-chloroquinolin-4-ylamino)butylamino] pyrimidine (10c)

White solid. RF: 0.4 (EtOAc). Yield: 77% (IR (KBr): vmax 772, 1268, 1708, 2986, 3383 cm−1 (H 300 MHz, CDCl3, 25 C): δ 0.94 (t, J = 7.2 Hz, 3H, ester–CH3), 1.80 (q, J = 6.9 Hz, 4H, CH2), 2.47 (s, 3H, C6–CH3), 3.37 (q, J = 6.3 Hz, 2H, CH2), 3.66 (q, J = 7.2 Hz, 2H, CH2), 4.05 (q, J = 7.2 Hz, 2H, ester–CH2), 5.06 (s, 1H, NH), 5.45 (s, 1H, NH), 6.38 (d, J = 5.4 Hz, 1H, ArH), 7.3–7.58 (m, 7H, ArH), 7.94 (d, J = 2.1 Hz, 1H, ArH), 8.51 (d, J = 5.4 Hz, 1H, ArH).13C NMR (75 MHz, CDCl3, 25 C):
5.2.4. 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[7-chloroquinolin-4-ylamino]pyrimidine (10d)

White solid. RF: 0.5 (MeOH/EtOAc). Yield: 67%. IR (KBr): \( \nu_{\text{max}} \) 769, 1264, 1300, 2930, 3278 cm\(^{-1}\). 1H (300 MHz, CDC\(_3\)), 25 C): 7.95 (t, J = 7.2 Hz, 3H, ether–CH\(_3\)), 1.54 (s, 3H, CH\(_3\)), 0.95 (t, J = 7.2 Hz, 2H, ArH), 8.53 (d, J = 5.7 Hz, 1H, ArH), 13C NMR (75 MHz, CDC\(_3\)), 25 C): \( \delta \) 13.0, 22.5, 26.2, 26.6, 28.4, 28.6, 29.0, 40.7, 42.8, 98.6, 116.6, 120.3, 123.5, 124.8, 127.5, 127.7, 128.4, 128.9, 134.4, 138.8, 148.7, 149.2, 151.6, 160.9, and 168.4. Anal. Calcld. For C\(_{29}\)H\(_{32}\)N\(_5\)O\(_2\)Cl: C, 67.13; H, 6.39; N, 13.17; Found: C, 67.43; H, 6.05; N, 14.35. MS: m/z 532.1 (M\(^+\)).

5.2.5. 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[7-chloroquinolin-4-ylamino]octylamino[pyrimidine (10e)

White solid. RF: 0.6 (EtOAc). Yield: 72%. IR (KBr): \( \nu_{\text{max}} \) 770, 1263, 1712, 2928, 3269 cm\(^{-1}\). 1H (300 MHz, CDC\(_3\)), 25 C): 7.95 (t, J = 7.2 Hz, 3H, ether–CH\(_3\)), 1.54 (s, 3H, CH\(_3\)), 0.95 (t, J = 7.2 Hz, 2H, ArH), 8.53 (d, J = 5.7 Hz, 1H, ArH), 13C NMR (75 MHz, CDC\(_3\)), 25 C): \( \delta \) 13.5, 22.9, 26.7, 27.0, 28.8, 29.1, 29.2, 29.5, 41.2, 43.2, 61.0, 99.0, 115.1, 117.0, 120.8, 125.2, 127.9, 128.2, 128.8, 129.3, 134.8, 139.2, 149.0, 149.7, 152.0, 161.2 and 166.9. Anal. Calcld. For C\(_{31}\)H\(_{35}\)N\(_5\)O\(_2\)Cl: C, 67.89; H, 6.59; N, 12.8; Found: C, 67.83; H, 6.55; N, 12.02. MS: m/z 546.2 (M\(^+\)).

5.2.6. 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[7-chloroquinolin-4-ylamino]decylationo[pyrimidine (10f)

Viscous liquid. RF: 0.3 (1% MeOH/EtOAc). Yield: 64%. IR (KBr): \( \nu_{\text{max}} \) 775, 1256, 1708, 2985, 3325 cm\(^{-1}\). 1H (300 MHz, CDC\(_3\)), 25 C): 7.36 (t, J = 7.2 Hz, 3H, ether–CH\(_3\)), 1.25 (br, 14H, CH\(_2\)), 1.52 (q, J = 6.9 Hz, 2H, CH\(_2\)), 1.67 (7, J = 6.6 Hz, 2H, CH\(_2\)), 2.42 (s, 3H, CH\(_3\)), 3.22 (q, J = 6.7 Hz, 2H, CH\(_2\)), 3.41 (t, J = 6.6 Hz, 2H, CH\(_2\)), 3.97 (q, J = 6.9 Hz, 2H, ether–CH\(_2\)), 5.04 (br, 1H, NH), 5.30 (br, 1H, NH), 6.32 (d, J = 5.4 Hz, 1H, ArH), 7.19–7.61 (m, 7H, ArH), 7.88 (d, J = 2.1 Hz, 1H, ArH), 8.44 (d, J = 5.7 Hz, 1H, ArH). 13C NMR (75 MHz, CDC\(_3\)), 25 C): \( \delta \) 13.5, 14.5, 26.8, 27.1, 28.8, 29.2, 29.3, 29.4, 29.6, 41.3, 43.3, 61.0, 99.0, 115.0, 117.0, 120.8, 125.3, 127.9, 128.2, 128.8, 129.3, 134.8, 139.2, 149.0, 149.7, 152.0, 161.2 and 166.9. Anal. Calcld. For C\(_{31}\)H\(_{35}\)N\(_5\)O\(_2\)Cl: C, 68.19; H, 6.59; N, 12.20; Found: C, 68.93; H, 6.66; N, 11.82. MS: m/z 574.2 (M\(^+\)).

5.2.7. 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[7-chloroquinolin-4-ylamino]dodecylamino[pyrimidine (10g)

Viscous liquid. RF: 0.4 (1% MeOH/EtOAc). Yield: 65%. IR (KBr): \( \nu_{\text{max}} \) 772, 1270, 1700, 2998, 3300 cm\(^{-1}\). 1H (300 MHz, CDC\(_3\)), 25 C): \( \delta \) 0.93 (t, J = 7.2 Hz, 3H, ether–CH\(_3\)), 1.26 (s, 16H, CH\(_2\)), 1.57 (d, J = 6.9 Hz, 2H, CH\(_2\)), 1.71 (d, J = 6.9 Hz, 2H, CH\(_2\)), 2.48 (s, 3H, CH\(_3\)), 3.24 (s, 2H, CH\(_2\)), 3.45 (q, J = 6.6 Hz, 2H, CH\(_2\)), 4.04 (q, J = 7.2 Hz, ether–CH\(_2\)), 5.71 (br, 1H, NH), 6.18 (d, J = 5.4 Hz, 1H, ArH), 6.74 (br, 1H, NH), 7.23–7.40 (m, 7H, ArH), 7.83 (d, J = 6.9 Hz, 1H, ArH), 8.23 (s, 1H, ArH).

13C NMR (75 MHz, CDC\(_3\)), 25 C): \( \delta \) 13.5, 22.9, 26.8, 27.1, 28.9, 29.2, 29.3, 29.4, 29.5, 41.3, 43.3, 60.9, 99.0, 115.0, 117.0, 120.7, 125.2, 127.9, 128.1, 128.7, 129.3, 134.8, 149.2, 149.7, 151.9, 161.2 and 168.9. Anal. Calcld. For C\(_{31}\)H\(_{35}\)N\(_5\)O\(_2\)Cl: C, 69.82; H, 7.30; N, 11.63; Found: C, 69.53; H, 6.96; N, 11.45. MS: m/z 602 (M\(^+\)).
J:\¼C6\8.43 (d, J = 5.4 Hz, 1H, ArH), 7.27–7.30 (m, 2H, ArH), 7.81 (s, 1H, ArH), 7.92 (d, J = 8.1 Hz, 1H, ArH). 13C NMR (75 MHz, CDCl3, 25 C): δ 21.2, 25.7, 28.1, 42.8, 69.2, 98.7, 115.5, 116.7, 121.2, 123.2, 123.9, 125.2, 125.9, 128.0, 128.3, 128.4, 129.6, 130.9, 134.3, 140.5, 147.9, 150.1, 150.7, 161.0 and 167.3. Anal. Calcld. For C26H27N6O4Cl: C, 59.90; H, 4.80; N, 15.88. MS: Found: C, 60.23; H, 4.97; N, 15.45. MS: m/z 543.5 (M+)."

5.3. General procedure for the synthesis of 2-aminopyrimidines (10h & 10i)

To the stirred solution of appropriate 4-aminooquinoline 9 in dry THF (50 ml) mixture of 8 (in a 1:2 molar ratio) and potassium carbonate in dry acetonitrile was added. The reaction mixture was refluxed for 48 h and then filtered. Acetonitrile was removed under vacuum and the residue was purified by column chromatography using EtOAc/hexane as eluent to give 10h and 10i which were recrystallized from DCM/hexane.

5.3.1. 5-Ethoxycarbonyl-6-methyl-4-phenyl-2-[4-(7-chloroquinolin-4-ylamino)phenylamino] pyrimidine (10j)

Yellow solid. Yield: 57% (50% hexane/EtOAc). IR (KBr): 1653, 1629, 1614, 1524, 1508, 3282 cm⁻¹. 1H NMR (500 MHz, CDCl3, 25 C): δ 0.99 (t, J = 7.2 Hz, 3H, ester–CH3), 2.51 (s, 3H, C6–CH3), 3.81 (br, 1H, NH), 4.09 (q, J = 7.2 Hz, 2H, ester–CH2), 4.13 (d, J = 5.4 Hz, 1H, ArH), 7.52–7.57 (m, 4H, ArH), 7.87–7.90 (m, 2H, ArH), 8.02 (d, J = 1.8 Hz, 1H, ArH), 7.32 (t, J = 5.4 Hz, 1H, ArH). 13C NMR (75 MHz, CDCl3, 25 C): δ 21.1, 25.9, 29.3, 59.3, 59.4, 115.7, 117.5, 122.8, 124.1, 126.0, 126.3, 127.7 and 151.9. Anal. Calcld. For C30H29N3O3Cl: C, 74.80; H, 4.71; N, 13.70. Found: C, 74.45; H, 4.47; N, 13.57. MS: m/z 510.5 (M+).

6. Materials and methods

6.1. In vitro anti-plasmodial activity assay

Two clones of P. falciparum are used: (a) 3D7 clone of NF54 which is known to be sensitive to all anti-plasmodials, (b) K1 strain originating from Thailand that is resistant to chloroquine and pyrimethamine, but sensitive to mefloquine. The cultures were naturally asynchronous (65–75% ring stage) and were maintained in continuous log phase growth in RPMI1640 medium supplemented with 5% washed human A+ erythrocytes, 25 mM Heps, 32 mM NaHCO₃, and AlbuMAAXII (lipid-rich bovine serum albumin) (GIBCO, Grand Island, NY) (CM). All cultures and assays were conducted at 37 °C under an atmosphere of 5% CO₂ and 5% O₂, with a balance of N₂. Stock drug solutions were prepared in 100% DMSO (dimethylsulfoxide) at 20 mg/ml. The compound was further diluted to the appropriate concentration using complete medium RPMI1640 supplemented with 15 nM cold hypoxanthine and K. Singh et al. / European Journal of Medicinal Chemistry 52 (2012) 82–97.
AlbuMAXII. Assays were performed in sterile 96-well microtitre plates; each plate contained 100 μl of parasite culture (0.5% parasitemia, 2.5% hematocrit). Each compound was tested in triplicate and parasite growth compared to control and blank (uninfected erythocytes) wells. After 24 h of incubation at 37 °C, 3.7 Bq of [3H] hypoxanthine is added to each well [51]. Cultures were incubated for a further 24 h before they are harvested onto glass-fiber filter mats. The radioactivity was counted using a Wallac Microbeta 1450 scintillation counter. The results were recorded as counts per minute (CPM) per well at each drug concentration, control and blank wells. Percentage inhibition was calculated from comparison to blank and control wells, and IC50 values calculated using Graph Pad Prism 4.0. The preliminary screen uses the 3D7 strain. The compounds were tested at 6 concentrations (30, 10, 3, 1, 0.3, and 0.1 μg/ml). If the compound did not affect parasite growth at 10 μg/ml it was classified as inactive, between 10 and 1 μg/ml, the compound was designated as partially active, and if <1 μg/ml, the compound was classified as active and was further evaluated by three-fold serial dilutions in a repeat test. For secondary screening both the 3D7 clone and the K1 line were used. The compound was diluted three-fold over at 12 different concentrations with an appropriate starting concentration based on the preliminary screen. The IC50 is determined by a sigmoidal dose response analysis using Graph Pad Prism 4.0. For each assay, the IC50 and IC90 values for each parasite line were determined against the known antimalarial chloroquine and artesunate.

6.2. Cytotoxicity and antiviral assay

The antiviral assays [except anti-human immunodeficiency virus (HIV) 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 (para-influenza-3, reovirus-1, Coxsackie B4, and Punta Toro virus), HeLa (vesicular stomatitis virus, Coxsackie 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. The anti-HIV activity and anti-proliferative activity were evaluated against HIV-1 strain IIIb and HIV-2 strain ROD in human T-lymphocyte CEM cell cultures. Briefly, virus stocks were titrated in CEM cells and expressed as the 50% cell culture infective dose (CCID50). CEM cells were suspended in culture medium at ~3 × 10^5 cells/ml and infected with HIV at ~100 CCID50. Immediately after viral exposure, 100 μl of the cell suspension was placed in each well of a flat-bottomed microtiter plate containing various concentrations of the test compounds. After a 4-day incubation period at 37 °C, the giant cell formation was microscopically determined. Compounds were tested in parallel for cytostatic activity in uninfected CEM cells.

6.3. Heme binding studies

6.3.1. Binding of 10c & 10r with monomeric heme

Hemin stock solution (1.2 mM) was prepared by dissolving (7.8 mg) hemin chloride in 10 ml AR grade DMSO. Working solutions (2.4 μM) were prepared by mixing 20 μl hemin stock solution with 4 ml DMSO and 1 ml 0.2 M HEPES buffer (pH 7.4) and making it up to 10 ml with double distilled deionized water. Stock solutions of 10c and 10r (2 mM) were prepared in AR grade DMSO and were used for titration experiment. Heme (2.4 μM) was titrated with increasing concentrations (0–70 μM) of 10c and 10r. Following each addition, absorbance was recorded at 402 nm. For conducting the experiment at pH 5.6, solutions of 10c and 10r and Fe (III) PIX were prepared in exactly the same manner, except that 2-[N-morpholino]ethanesulphonate (MES) buffer (pH 5.4) was substituted for HEPES buffer [31].

6.3.2. Binding of 10c & 10r with μ-oxo dimeric heme

Stock solution (10 mM) of heme was prepared by dissolving hemin chloride in 0.1 M NaOH. Solution was sonicated for 30 min to ensure complete dissolution. Heme stock solution was diluted to 60 μM in phosphate buffer (20 mM, pH 5.8). Stock solution of 10c and 10r (2 mM) were prepared in AR grade DMSO. Titrations were performed by successive addition of aliquots of stock solution of 10c or 10r (0–15 μL) to 60 μM heme solution & changes in the absorbance at 362 nm were recorded. The association constants for both monomeric & μ-oxo dimeric heme were determined by using Hyp Spec [37].

6.3.3. Binding stoichiometry

Binding Stoichiometries of drug with monomeric & μ-oxo dimeric heme were monitored by UV–visible spectrophotometry using Job’s method of continuous variation [33,34]. The concentration of drug & heme in solution was kept constant and changes in absorbance at 402 nm (monomeric)/362 nm (dimeric) were monitored as a function of the mole fraction.

6.3.4. Ferrirhodopsin IX biominalisation inhibition test (FBIT)

96 well plate containing mixture of 50 μL of 0.5 mg/ml of hemin chloride dissolved in DMSO, 100 μL of 0.5 M sodium acetate buffer (pH 4.4) and 50 μL of different concentrations of drug solution or 50 μL of solvent (control), was incubated at 37 °C for 24 h. The plate was centrifuged at 4500 rpm for 3 min, and supernatant was discarded. The remaining pellet was re-suspended with 200 μL of DMSO to eliminate unreacted heme [38,39]. The plate was centrifuged again and supernatant similarly discarded. The precipitate was dissolved in 150 μL of 0.1 N NaOH & absorbance was read at 405 nm. The percentage of inhibition of Ferrirhodopsin IX Biominalisation was calculated using formula:

\[
\text{Inhibition} (%) = 100 \times \left( \frac{\text{Abs of control}}{- \text{(Abs of drug)}} \right) \quad \text{(Abs of control)}.
\]

IC50 values were determined by using Graph pad Prism 4.0.

6.4. DNA binding studies with 10c and 10r

6.4.1. Preparation of stock solutions

Stock solution of 10c and 10r (2 mM) were prepared in AR grade methanol. The DNA binding experiments were carried out by making dilution of the stock with 1:1 buffered methanol.

6.4.2. Preparation of CT DNA solution

Stock solution of DNA was prepared by dissolving DNA pellet in TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.4). The DNA concentration was estimated from its absorbance intensity at 260 nm with a known molar absorption coefficient value of 6600 dm^3 mol^-1 cm^-1. The purity of DNA was established from ratio of absorbance intensity at 260 nm and at 280 nm. The observed ratio of 1.8 ensured that DNA was free from protein.

6.4.3. Evaluation of binding constant

6.4.3.1. UV-spectrophotometry. The titration experiment was performed by varying the concentration of CT DNA and keeping the drug concentration constant (30 μM). All the UV–visible spectra were recorded after equilibration of solution for 5 min. The binding
constants were calculated from absorption at 331 nm by using Benesi–Hildebrand equation [48].

6.4.3.2. Spectrofluorometric titrations. The steady state fluoro-
cence experiments were carried out on Varian Cary Eclipse Spec-
 trometer at ambient temperature. A slit width of 5 nm was used with \( \lambda_{ex} = 320 \) nm and \( \lambda_{em} = 380 \) nm. The titration experi-
 ment was accomplished by varying the concentration of DNA in
cuvette (0.3–70 \( \mu \)M) and keeping the compound concentration
constant (30 \( \mu \)M). In fluorescence titration experiment working
solutions were prepared by making dilution of DNA stock and drug
stock with methanol.

6.4.3.3. DNA thermal denaturation. DNA melting experiment were carried out by monitoring the absorbance of CT DNA (151 \( \mu \)M NP) at
260 nm at various temperature in the presence and absence of drug
in a 5:1 ratio of the DNA and drug with a ramp rate of 0.5 \( ^\circ \) C/min in
a 40% DMSO/TE buffer (pH 7.4) with 0.5 mM NaCl on a Shimadzu
1601 PC spectrophotometer equipped with a Feltier thermo regu-
lator. The thermal melting temperature was calculated by plotting
\( \Delta A/\Delta T \) vs temperature using Microsoft Excel.

6.4.4. Molecular docking method

The molecular docking analysis was carried out using FlexX
molecular docking module [52,53] (FlexX 11.35) available in the
SYBYL 7.1 software package from Tripos [54]. This docking
approach adopts an incremental construction algorithm for iden-
tifying appropriate pose of the substrate in the active site of the
enzyme. It generates about 30 possible poses of the substrate in the
active site. Most of the 30 poses obtained in this analysis
adopting appropriate pose of the substrate in the active site of the
enzyme. It generates about 30 possible poses of the substrate in the
active site. Most of the 30 poses obtained in this analysis
were selected after verification by applying FlexX module
(52,53). The 30 poses were selected after verification by applying
FlexX module (52,53).

Appendix A. Supplementary data

Supplementary data related to this article can be found online at
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References

[1] T.N.C. Wells, P.L. Alonso, W.E. Gutteridge, New medicines to improve
control and contribute to the eradication of malaria, Nature 8 (2009) 879–891.
[2] W.E. Martin, R.V. Marchetti, A.J. Cowan, S.M. Howart, S. Broer, K. Kirk, Chloro-
quine transport via the malaria parasite's chloroquine resistance transporter,
Science 325 (2009) 1680–1682.
[3] T.E. Wellemes, Plasmodium chloroquine resistance and the search for
a replacement antimalarial drug, Science 298 (2002) 124–126.
[4] A.M. Dondorp, F. Nosten, W. Hanphitakpong, S.J. Lee, P. Ringwald, K. Silamut,
M. Imwong, K. Chotivanich, P. Lim, T.A.S. Herdman, S. Yeung, P. Singhavisavan,
N.P. Day, N. Lindegardh, D. Socheat, A. White, Artemisinin resistance in
Plasmodium falciparum malaria, N. Engl. J. Med. 361 (2009) 455–467.
[5] H. Noedl, D. Socheat, W. Satimal, Artemisinin-resistant malaria in Asia, N. Engl.
J. Med. 361 (2009) 540–541.
[6] R. Ettari, F. Bova, M. Zappala, S. Grasso, M. Nicale, Falcipain-2 inhibitors, Med.
Res. Rev. 30 (2010) 136–167.
[7] M. Foley, L. Tilley, Quinoline antimalarial: mechanisms of action and resis-
tance, Int. J. Parasitol. 27 (1997) 231–240.
[8] M.J. Kelly, M.J. Smilkstein, R. Brun, W. Sergio, A.R. Cooper, K.D. Lane,
A. Janowsky, R.A. Johnson, R.A. Dodean, R. Winter, D.J. Hinrichs, M.K. Risoe.
Discovery of dual function acridones as a new antimalarial chemotype, Nature
459 (2009) 270–273.
[9] D. Mazier, L. Rébia, G. Sounou, A pre-emptive strike against malaria's stealthy
hepatitis forms, Nat. Rev. Drug Discov. 8 (2009) 855–864.
[10] L. Adane, P.V. Bharatam, Modelling and informatics in the analysis of
P. falciparum DHFR enzyme inhibitors,Curr. Med. Chem. 15 (2008)
1522–1569.
[11] R.D. Powell, G.J. Brewer, Effects of pyrimethamine, chloroquine and prima-
quine against exoerythrocytic forms of a strain of chloroquine-resistant
Plasmodium falciparum from Thailand, Am. J. Trop. Med. Hyg. 16 (1967)
693–698.
[12] S. Vangapandu, S. Sachdeva, M. Jain, S. Singh, P. Singh, C.L. Kaul, R. Jain,
8-Quinolylaminines and their pro-drug conjugates as potent blood-
schizontocidal antimalarial agents, Bioorg. Med. Chem. 11 (2003) 4557–4568.
[13] A. Garawal, K. Srivastava, S.K. Puri, M.S. Chauhan, Synthesis of 4-pyrido-6-
dihydropyrimidin-2(1H)-ones using pyridinium chlorochromate as catalyst.
J. Med. Chem. 325 (2009) 270.
[14] L. Adane, P.V. Bharatam, Modelling and informatics in the analysis of
P. falciparum DHFR enzyme inhibitors, Curr. Med. Chem. 15 (2008)
1522–1569.
[15] A.M. Dondrop, F. Nosten, W. Satimal, Artemisinin-resistant malaria in Asia, N. Engl.
J. Med. 361 (2009) 540–541.
[16] R. Ettari, F. Bova, M. Zappala, S. Grasso, M. Nicale, Falcipain-2 inhibitors, Med.
Res. Rev. 30 (2010) 136–167.
[17] M. Foley, L. Tilley, Quinoline antimalarial: mechanisms of action and resis-
tance, Int. J. Parasitol. 27 (1997) 231–240.
[18] M.J. Kelly, M.J. Smilkstein, R. Brun, W. Sergio, A.R. Cooper, K.D. Lane,
A. Janowsky, R.A. Johnson, R.A. Dodean, R. Winter, D.J. Hinrichs, M.K. Risoe.
Discovery of dual function acridones as a new antimalarial chemotype, Nature
459 (2009) 270–273.
[19] D. Mazier, L. Rébia, G. Sounou, A pre-emptive strike against malaria's stealthy
hepatitis forms, Nat. Rev. Drug Discov. 8 (2009) 855–864.
[20] L. Adane, P.V. Bharatam, Modelling and informatics in the analysis of
P. falciparum DHFR enzyme inhibitors, Curr. Med. Chem. 15 (2008)
1522–1569.
[21] R.D. Powell, G.J. Brewer, Effects of pyrimethamine, chloroquine and prima-
quine against exoerythrocytic forms of a strain of chloroquine-resistant
Plasmodium falciparum from Thailand, Am. J. Trop. Med. Hyg. 16 (1967)
693–698.
[22] S. Vangapandu, S. Sachdeva, M. Jain, S. Singh, P. Singh, C.L. Kaul, R. Jain,
8-Quinolylaminines and their pro-drug conjugates as potent blood-
schizontocidal antimalarial agents, Bioorg. Med. Chem. 11 (2003) 4557–4568.
[23] A. Garawal, K. Srivastava, S.K. Puri, M.S. Chauhan, Synthesis of 4-pyrido-6-
dihydropyrimidin-2(1H)-ones using pyridinium chlorochromate as catalyst.
J. Med. Chem. 325 (2009) 270.
[34] C.Y. Huang, Determination of binding stoichiometry by the continuous variation method—the job plot, Methods Enzymol. 87 (1982) 509–525.

[35] J.K. Natarajan, J.N. Alumasa, K. Yearick, K.A. Ekoue-Kovi, I.R. Casabianca, A.C.D. Dios, C. Wolf, P.D. Roepe, 4-N-, 4-S-, and 4-O-Chloroquine analogues: Influence of side chain length and quinolyl nitrogen pK, on activity vs chloroquine resistant malaria, J. Med. Chem. 51 (2008) 3466–3479.

[36] J.X. Kelly, R. Wüster, M. Riscoe, D.H. Peryton, A spectroscopic investigation of the binding interaction between 4, 5-dihydroxyxanthone and heme, J. Inorg. Biochem. 86 (2001) 617–625.

[37] F. Rodríguez, I. Rozas, M. Kaser, R. Brun, B. Nguyen, W.D. Wilson, R.N. García, A.C.D. Dios, C. Wolf, P.D. Roepe, 4-N-, 4-S-, and 4-O-Chloroquine analogues: Influence of side chain length and quinolyl nitrogen pKa on activity vs chloroquine resistant malaria, J. Med. Chem. 51 (2008) 3466–3479.

[38] J.L. Allison, R.L. O’Brien, F.E. Hahn, DNA: reaction with chloroquine, Science 149 (1965) 1111–1113.

[39] N.B. Kurnick, I.E. Radcliffe, Reaction between DNA and quinacridine and other antimalarials, J. Lab. Clin. Med. 60 (1962) 669–688.

[40] E.T. Mudaris, D.H. Wahyuni, N. Tjahjono, H. Yoshihiko, Inoue, Spectroscopic studies on the thermodynamic and thermal denaturation of the CT-DNA binding of methylene blue, Spectrochim. Acta Part A 77 (2010) 528–534.

[41] J. Cheng, R. Zeidan, S. Mishra, A. Liu, S.H. Pun, R.P. Kulkarni, G.S. Jensen, N.C. Belloq, M.E. Davis, Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery, J. Med. Chem. 49 (2006) 6522–6531.

[42] Y. Pollack, A.L. Katzen, D.T. Spira, J. Golenser, The genome of Plasmodium falciparum I: DNA base composition, Nucleic Acids Res. 10 (1982) 539–546.

[43] J.M. Woynarowsk, M. Krugliak, H. Ginsburg, Pharmacogenomic analyses of targeting the AT-rich malaria parasite genome with AT-specific alkylating drugs, Mol. Biochem. Parasitol. 154 (2007) 70–81.

[44] N.I. Wenzel, N. Chavain, Y. Wang, W. Friebolin, L. Maes, B. Pradines, M. Lanzer, V. Yardley, R. Brun, C.H. Mende, C. Biot, K.T. Oth, E.D. Charvet, Antimalarial versus cytotoxic properties of dual drugs derived from 4-aminoquinolines and Mannich bases: interaction with DNA, J. Med. Chem. 53 (2010) 3214–3226.

[45] J.L. Allison, R.L. O’Brien, F.E. Hahn, DNA: reaction with chloroquine, Science 149 (1965) 1111–1113.

[46] N.B. Kurnick, I.E. Radcliffe, Reaction between DNA and quinacridine and other antimalarials, J. Lab. Clin. Med. 60 (1962) 669–688.

[47] E.T. Mudaris, D.H. Wahyuni, N. Tjahjono, H. Yoshihiko, Inoue, Spectroscopic studies on the thermodynamic and thermal denaturation of the CT-DNA binding of methylene blue, Spectrochim. Acta Part A 77 (2010) 528–534.

[48] J. Cheng, R. Zeidan, S. Mishra, A. Liu, S.H. Pun, R.P. Kulkarni, G.S. Jensen, N.C. Belloq, M.E. Davis, Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery, J. Med. Chem. 49 (2006) 6522–6531.

[49] Y. Pollack, A.L. Katzen, D.T. Spira, J. Golenser, The genome of Plasmodium falciparum I: DNA base composition, Nucleic Acids Res. 10 (1982) 539–546.

[50] J.M. Woynarowsk, M. Krugliak, H. Ginsburg, Pharmacogenomic analyses of targeting the AT-rich malaria parasite genome with AT-specific alkylating drugs, Mol. Biochem. Parasitol. 154 (2007) 70–81.

[51] N.I. Wenzel, N. Chavain, Y. Wang, W. Friebolin, L. Maes, B. Pradines, M. Lanzer, V. Yardley, R. Brun, C.H. Mende, C. Biot, K.T. Oth, E.D. Charvet, Antimalarial versus cytotoxic properties of dual drugs derived from 4-aminoquinolines and Mannich bases: interaction with DNA, J. Med. Chem. 53 (2010) 3214–3226.

[52] J.L. Allison, R.L. O’Brien, F.E. Hahn, DNA: reaction with chloroquine, Science 149 (1965) 1111–1113.

[53] N.B. Kurnick, I.E. Radcliffe, Reaction between DNA and quinacridine and other antimalarials, J. Lab. Clin. Med. 60 (1962) 669–688.

[54] E.T. Mudaris, D.H. Wahyuni, N. Tjahjono, H. Yoshihiko, Inoue, Spectroscopic studies on the thermodynamic and thermal denaturation of the CT-DNA binding of methylene blue, Spectrochim. Acta Part A 77 (2010) 528–534.

[55] J. Cheng, R. Zeidan, S. Mishra, A. Liu, S.H. Pun, R.P. Kulkarni, G.S. Jensen, N.C. Belloq, M.E. Davis, Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery, J. Med. Chem. 49 (2006) 6522–6531.

[56] Y. Pollack, A.L. Katzen, D.T. Spira, J. Golenser, The genome of Plasmodium falciparum I: DNA base composition, Nucleic Acids Res. 10 (1982) 539–546.