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PII: S1001-8417(17)30510-7
DOI: https://doi.org/10.1016/j.cclet.2017.11.041
Reference: CCLET 4356

To appear in: *Chinese Chemical Letters*

Received date: 27-9-2017
Revised date: 25-11-2017
Accepted date: 30-11-2017

Please cite this article as: Wenhua Chen, Xue Yao, Zhenghui Huang, Fei Mao, Longfei Guan, Yun Tang, Hualiang Jiang, Jian Li, Jin Huang, Lubin Jiang, Jin Zhu, Novel dual inhibitors against FP-2 and PfDHFR as potential antimalarial agents: Design, synthesis and biological evaluation, Chinese Chemical Letters https://doi.org/10.1016/j.cclet.2017.11.041

Communication

Novel dual inhibitors against FP-2 and PfDHFR as potential antimalarial agents: Design, synthesis and biological evaluation

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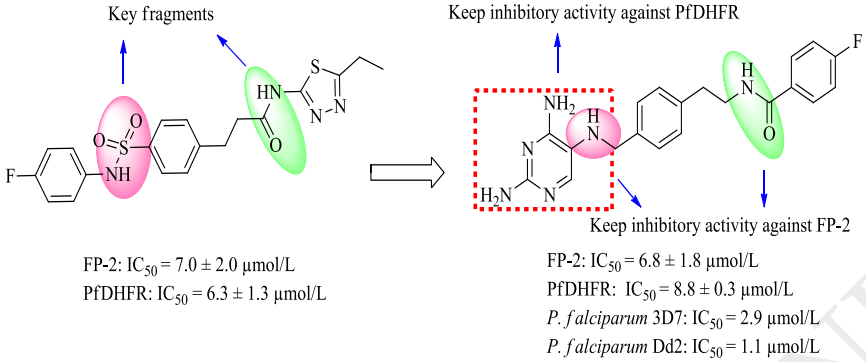
Graphical Abstract



A series of novel 2, 4-diaminopyrimidine-modified compounds were designed and synthesized. Compound **14** showed micromolar dual inhibitory effect on both FP-2 and PfDHFR, and potential inhibition to the proliferation of *P. falciparum* 3D7 strain and chloroquine-resistant *P. falciparum* Dd2 strain.

ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Antimalarial drug
Plasmodium falciparum
FP-2
PfDHFR
Dual inhibitor

ABSTRACT

Resistance to malaria parasites has quickly developed to almost all used antimalarial drugs. Cysteine protease falcipain-2 (FP-2) and *Plasmodium falciparum* dihydrofolate reductase (PfDHFR) have crucial roles, which are absolutely necessary, in the parasite life cycle. In this study, based on the uniform pharmacophores of reported PfDHFR inhibitors and the first-generation dual inhibitors against FP-2 and PfDHFR, we identified a novel series of dual inhibitors through fragments assembly. Lead optimization led to the identification of **14**, which showed potent inhibition against FP-2 and PfDHFR enzyme (IC_{50} = 6.8 ± 1.8 μmol/L and IC_{50} = 8.8 ± 0.3 μmol/L) and *P. falciparum* 3D7 strain (IC_{50} = 2.9 μmol/L). Additionally, **14** exhibited more potent inhibition to the proliferation of chloroquine-resistant *P. falciparum* Dd2 strain (IC_{50} = 1.1 μmol/L) than pyrimethamine (IC_{50} > 10 μmol/L), and **14** displayed micromolar inhibitory activities against two clinical isolated strains Fab9 (IC_{50} = 2.6 μmol/L) and GB4 (IC_{50} = 1.0 μmol/L). Collectively, these data demonstrated that **14** might be a good lead compound for the treatment of malaria.

Malaria, a mosquito-borne disease caused by infection with *Plasmodium* parasites, is the world's most deadly parasitic infection [1,2]. Almost half the world's population live in malaria endemic areas, and an estimated 1.2 billion people are at high risk of contracting the disease [3-5]. According to WHO 2016, malaria caused 429,000 deaths and there were approximately 212 million clinical cases of infection globally in 2015. Since the control of malaria has been severely compromised in recent years by the widespread resistance to nearly all frontline therapeutics which were used for both prophylaxes and treatments [3, 6-8], hence, there is an urgent need for the development and discovery of new antimalarial drugs, which are structurally distinct from existing drugs and endowed with novel mechanisms of action [9].

Cysteine protease falcipain-2 (FP-2) of *P. falciparum* is an essential hemoglobinase of erythrocytic *P. falciparum* trophozoites and provides the amino acids for the growth and proliferation of *Plasmodium*. Many *in vitro* and *in vivo* studies have confirmed that inhibitors of FP-2 could block parasite hemoglobin hydrolysis, halt the development of culture parasites, and are effective against

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murine malaria [10,11]. *P. falciparum* dihydrofolate reductase (PfDHFR) has received considerable attention for the prophylaxes and treatments of *P. falciparum* infection. PfDHFR is one of the key enzymes in the process of DNA replication, and could catalyze 7,8-dihydrofolate to transform into tetrahydrofolate [12,13]. Tetrahydrofolate serves as a necessary cofactor in the important one-carbon transfer reactions in the biosynthetic pathways of pyrimidines, purines, and amino acids [14]. Thus, a more powerful pesticidal effect could be achieved by inhibiting FP-2 and PfDHFR simultaneously. Such dual inhibitors might show a good synergetic effect, and overcome the drug-resistance and be capable of providing "a combination therapy" in a single agent [15].

Previously, we reported the first-generation dual inhibitors against FP-2 and PfDHFR based on the compound **27**, which was randomly identified by screening FP-2 inhibitors in our laboratory, and gained compound **2o** (**28**) which exhibited a high enzymatic inhibition and a moderate *in vivo* antimalarial efficacy [15] (Fig. 1). Based on the SAR of the first-generation dual inhibitors [15], to gain new scaffold dual inhibitors with more potencies, a novel series of dual inhibitors were designed, synthesized by displacing 4-fluorophenyl with the uniform pharmacophores of reported PfDHFR inhibitors, 2,4-diamino heterocyclic fragments (Table S1 in Supportion information). Considering the feasibility of the synthesis and novelty of scaffold, the sulfonamide was displaced with the secondary amine (green, Fig. 1), and the amide position was interchanged (gray, Fig. 1). Therefore, 2,4-diaminopyrimidine analogues with substituent (R, Fig. 1) at the terminal amide (**7**−**26**) were synthesized to obtain better dual target inhibitors and explore the SAR.

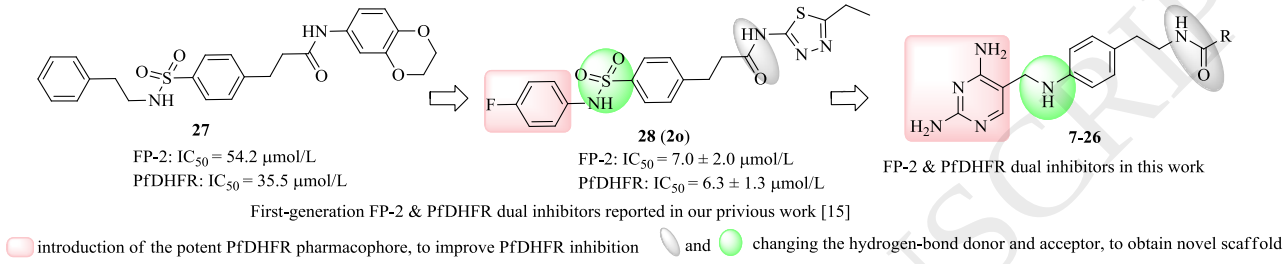
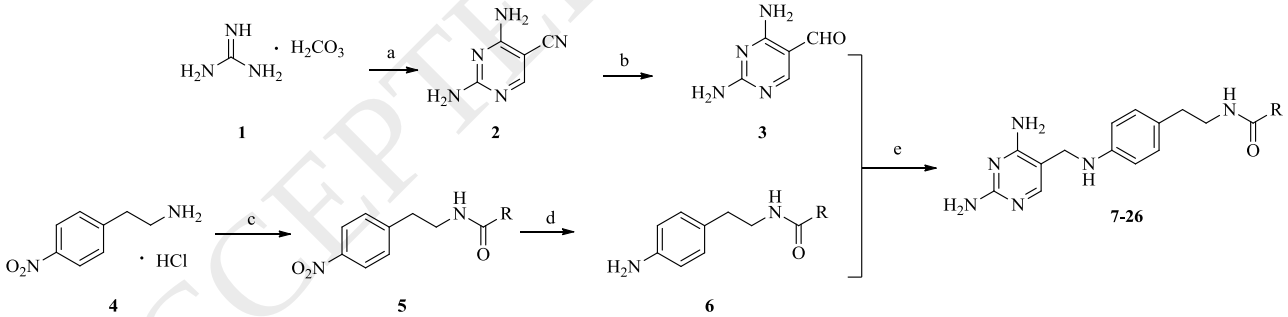


Fig. 1. Chemical modification strategies for the second-generation dual inhibitors.

First of all, we synthesized all the 2,4-diaminopyrimidine analogues (**7**−**26**) as described in Scheme 1. 2,4-Diaminopyrimidine-5-carbonitrile (**2**) was prepared by reaction of guanidine carbonate (**1**) with ethoxymethylenemalononitrile in EtONa and EtOH in a 71% yield. Further treatment of **2** with Nickel in MeOH under H_2 for 24 h gave rise to 2,4-diaminopyrimidine-5-carbaldehyde (**3**) in an 80% yield. Subsequent condensation of commercially available 4-nitrophenethylamine hydrochloride (**4**) with appropriate acids RCOOH in the presence of HOBt, EDCI and DIPEA in *N*,*N*-dimethylacetamide afforded *N*-(4-nitrophenethyl)amide **5** in good yields. Reduction of **5** in the presence of 10% Pd/C and H_2 in MeOH overnight in good yields provided *N*-(4-aminophenethyl)amide (**6**). Analogues **7**−**26** were performed by the reaction of **3** and **6**, *via* the condition using $NaCNBH_3$ in MeOH under reflux overnight in good yields (30%-70%) and were characterized by ^{1}H NMR and HRMS. The purity was over 95%, as determined by HPLC analysis (Table S2 in Supportion information). Supplementary data associated with experiment section, reported PfDHFR inhibitors and HPLC analysis data of compounds **7**−**26** can be found in Supporting information.



Scheme 1. Reagents and conditions: (a) Ethoxymethylenemalononitrile, EtONa/EtOH, 5 °C, 8 h; (b) Ni/H_2, MeOH, 25 °C, 24 h; (c) HOBt, EDCI, DIPEA, RCOOH, DMA, 25 °C, overnight; (d) 10% Pd/C, H_2, MeOH, reflux; (e) $NaCNBH_3$, MeOH, reflux, overnight.

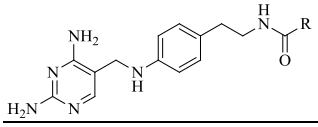
Then the twenty analogues were evaluated for FP-2 enzymatic inhibitory activity and for PfDHFR enzymatic inhibitory activity using the cysteine protease inhibitor (E-64) and pyrimethamine as the reference standard in the assay, respectively. And we assessed the inhibition rate (IR) of all the analogues against FP-2 and PfDHFR at 10 μmol/L firstly. A set of analogues with various substituents (R), including electron-donating substituted phenyl ring (**9**−**13**), electron-withdrawing substituted phenyl ring (**14**−**17**), hetero-aryl rings (**18**−**22**) and alkyl groups (**23**−**26**), were evaluated for IR against FP-2 and PfDHFR, respectively. From the results in Table 1, 3 analogues, *i.e.*, **11**, **14** and **15**, were identified as inhibitors against FP-2 with IR > 40% at 10 μmol/L and 19 analogues, *i.e.*, **7**−**24**, **26**, were identified as potent inhibitors against PfDHFR with IR > 70% at 10 μmol/L. Therefore, the IC_{50} value of them for the enzymatic inhibitory activity were further evaluated.

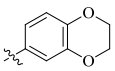
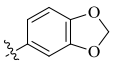
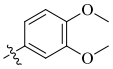
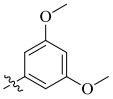
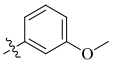
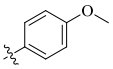
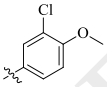
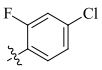
Analysis of the data shown in Table 1 revealed some noteworthy observations from the SAR study of analogues **7**−**26**: (1) The inhibitory activities against FP-2 showed the R substituents which were phenyl rings with electron-donating groups (EDGs) preferred 3-position (**9** *vs.* **10** and **11** *vs.* **12**) and the R substituents which were phenyl rings with electron-withdrawing groups (EWGs) favored

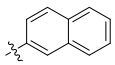
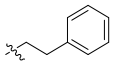
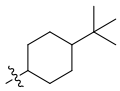
4-position (**14** *vs.* **15** *vs.* **16**); (2) In the studied sets of the R substituents, the potency against FP-2 substantially increased in the order of EWG-aryl > EDG-aryl > (aryl)alkyl; (3) In the test of inhibitory activities against PfDHFR, the R substituents could be well tolerated and 19 analogues displayed high potencies (IR at 10 μmol/L > 70%); the potency against PfDHFR substantially increased in the order of EDG-aryl > (aryl)alkyl > EWG-aryl.

From the results described above, analogue **14** was identified as a potent inhibitor against both FP-2 and PfDHFR (IC_{50} = 6.8 μmol/L against FP-2, IC_{50} = 8.8 μmol/L against PfDHFR). Therefore, analogue **14** and the lead compound **28** were next evaluated in the inhibitory activity against the blood stage of the multi-drug-sensitive *P. falciparum* 3D7 strain and *P. falciparum* Dd2 strain, which carry a phenotype of resistance to chloroquine [16,17]. Compound **28** displayed poor potencies against 3D7 strain (IR = 7.9% @ 20 μmol/L) and Dd2 strain (IR = 6.2% @ 20 μmol/L). As shown in Fig. S1 (Supporting information), analogue **14** together with artemisinin (Art) were tested in 3D7 cells. Analogue **14** performed micromolar potencies against 3D7 parasites (IC_{50} = 2.9 μmol/L). Moreover, as shown in Fig. S2 (Supporting information), **14** displayed micromolar potency against Dd2 (IC_{50} = 1.1 μmol/L) while pyrimethamine presented less effecitive inhibition against Dd2 (IC_{50} > 10 μmol/L), which indicated analogue **14** could also inhibit the growth of resistant *P. falciparum*. Furthermore, we evaluated the inhibitory activities of compound **14** against two clinical isolated strains, Fab9 and GB4. To our delight, as shown in Fig. S3 (Supporting information), **14** also displayed micromolar potencies against both strains (IC_{50} = 2.6 μmol/L against Fab9 and IC_{50} = 1.0 μmol/L against GB4).

Table 1
In vitro inhibitory activities against FP-2 and PfDHFR of analogues **7**-**26**.



Compound	R	FP-2		PfDHFR	
		Inhibition @ 10 μmol/L (%)	IC_{50} ± SD [a] (μmol/L)	Inhibition @ 10 μmol/L (%)	IC_{50} ± SD [a] (μmol/L)
28 (**2o**)		64.7	7.0 ± 2.0	59.2	6.3 ± 1.3
E-64		n.t.[b]	57.2 ± 8.2 nmol/L		
Pyrimethamine					25.5 ± 6.7 nmol/L
7		23.3		100.0	1.6 ± 0.1
8		30.0		89.7	6.3 ± 0.9
9		35.8		100.0	1.9±0.3
10		39.7		100.0	0.7±0.1
11		42.0	55.5 ± 11.0	87.1	3.1 ± 0.2
12		29.6		81.7	4.2 ± 0.3
13		38.4		91.8	1.3 ± 0.1
14		74.0	6.8 ± 1.8	74.5	8.8 ± 0.3
15		49.7	36.8 ± 2.5	87.0	3.0 ± 0.3
16		38.5		100.0	1.4 ± 0.2
17		37.1		100.0	1.5 ± 0.2
18		10.9		81.5	1.7±0.2
19		28.9		100.0	0.5 ± 0.2
20		10.2		82.7	1.5 ± 0.2
21		11.7		78.8	6.1 ± 0.4

22		34.5	94.6	0.7 ± 0.2
23		5.6	100.0	1.5 ± 0.3
24		12.2	91.5	1.9 ± 0.2
25		25.6	42.8	
26		24.7	97.9	3.2 ± 0.2

[a] Average of more than two experiments.
[b] Indicates no test.

Moreover, to understand the structural basis for the inhibitory activities of the inhibitors against FP-2 and PfDHFR, the 3D binding models of analogue **14** with FP-2 and PfDHFR were studied by molecular docking (Fig. 2). Fig. 2A shows the predicted binding poses of **14** in the catalytic site of FP-2. The amide moiety of **14** directly interacted with Asn156 *via* H-bonds, meanwhile, the terminal 2,4-diaminopyrimidine group and secondary amine interacted with Asp18 and Lys20 *via* H-bonds. For PfDHFR (Fig. 2B), the catalytic subpocket formed around Asp54 were occupied by the 2,4-diaminopyrimidine group in **14**. The 2,4-diaminopyrimidine ring formed a complicated hydrogen-bond network with Asp54, Ile14, and Ile164, respectively, which contributed significantly to the higher affinity of **14**. Moreover, the amide group formed an H-bond with the key residue Arg122.

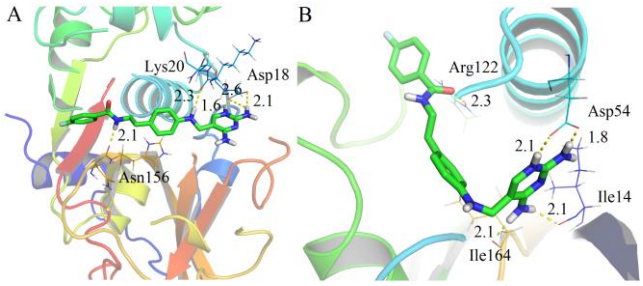


Fig. 2. Molecular docking studies on **14**. Docked poses of **14** (green sticks) in the active sites of FP-2 (A) and PfDHFR (B), respectively. Key residues of the binding pockets are shown as lines. Hydrogen bonds are shown with yellow dash lines.

In summary, we have identified a novel series of 2,4-diaminopyrimidine analogues (**7**−**26**) derived from **28** as FP-2 and PfDHFR dual inhibitors. On the basis of the structure of the lead compound **28**, 20 novel 2,4-diaminopyrimidine analogues have been synthesized and tested in FP-2 and PfDHFR enzymatic inhibitory activity assays. Molecular docking studies showed that the amide and secondary amine groups of new analogues could form hydrogen bonds with the surrounding amino acid residues, which were the same as the first generation dual inhibitors [15], combining with the preliminary SARs, we speculated the amide and secondary amine groups inherited FP-2 inhibitory activity from **28** and 2,4-diaminopyrimidine groups contributed to the inhibitory activity against PfDHFR. The *in vitro* inhibitory activity against *P. falciparum* assays with multi-drug-sensitive strain 3D7 further confirmed that analogue **14** was a good parasites inhibitor (IC_{50} = 2.9 μmol/L). To our delight, the inhibition of **14** against chloroquine-resistant *Plasmodium falciparum* strain Dd2 (IC_{50} = 1.1 μmol/L) was more superior than pyrimethamine (IC_{50} > 10 μmol/L) and analogue **14** displayed micromolar inhibitory activities against two clinical isolated strains Fab9 (IC_{50} = 2.6 μmol/L) and GB4 (IC_{50} = 1.0 μmol/L). Overall, **14** has the potential to be developed as a lead compound of antimalarial drugs.

Acknowledgement

Financial support for this research provided by the National Natural Science Foundation of China (Nos. 21372001 and 21672064), the “Shu Guang” project supported by the Shanghai Municipal Education Commission and Shanghai Education Development Foundation (No. 14SG28), and the Fundamental Research Funds for the Central Universities are gratefully acknowledged.

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