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Design, Synthesis and Antimalarial Activity of Novel, Quinoline-Based, Zinc Metallo-Aminopeptidase Inhibitors

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Abstract—PfA-M1, a neutral zinc aminopeptidase of *Plasmodium falciparum*, is a new potential target for the discovery of antimalarials. The design and synthesis of a library of 45 quinoline-based inhibitors of PfA-M1 is reported. The best inhibitor displays an IC₅₀ of 854 nM. The antimalarial activity on a CQ-resistant strain and the specificity towards mammalian aminopeptidase N are also discussed.

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Introduction

Malaria, caused by the parasite *Plasmodium falciparum*, is still prevalent and lethal in many countries.¹ Chloroquine (CQ), exerting its antimalarial activity by inhibiting haemozoin formation in the food vacuole of the parasite, has been the standard drug for many decades. Unfortunately, the spread of resistant *P. falciparum* to this molecule has created an urgent need to develop new antimalarial treatments.² In a project to find new quinoline-based antimalarials that would not induce resistance, unlike CQ, we have designed several compounds displaying a piperazine linker. Several of these compounds were active on the CQ-resistant strain FcB1.³

The recent publication of the genome of the parasite opens the opportunities of a better understanding of the biology of the parasite.⁴ This will hopefully result in the discovery of new specific targets to tackle with drugs. Proteases expressed in the erythrocytic stage of *P. falciparum*, for example, can be considered as interesting targets for the design of antimalarials.^{5,6}

In particular, PfA-M1,⁷ a neutral zinc-aminopeptidase (M1 family), inhibited by the classical aminopeptidase inhibitor bestatin, has been proposed:

(1) to hydrolyze the small peptides generated in the food vacuole, during hemoglobin digestion by acidic endopeptidases (aspartyl, cysteine and metallo-endopeptidases), into amino-acids at the level of the cytoplasm, and: (2) to play a role in the erythrocyte re-invasion by the parasite.⁸ PfA-M1 shares a maximal homology in the active site region (44%) with two human proteases: the human aminopeptidase-N and leukotriene A4 hydrolase.⁹

It is the only aminopeptidase of *P. falciparum* that has been purified and biochemically characterized. This enables the design and testing of potential inhibitors. Such compounds would help understanding the role of this enzyme and validate it as a therapeutic target.

In a project aiming at decreasing the potential for resistance occurrence of our quinoline-based antimalarials, we have designed dual inhibitors that would inhibit both the haemozoin formation and one of the proteases potentially involved in globin digestion. Such a strategy has been developed by Avery for the design of malarial cysteine proteases inhibitors based on mefloquine and chloroquine.¹⁰ The screening of several of our in-house quinoline-based compounds allowed us to identify compound **1** as an interesting inhibitor of PfA-M1, that served as a starting point for analoguing (Fig. 1).

We report here the design and parallel synthesis of a library of 45 non-peptidic analogues of **1**, and its biological

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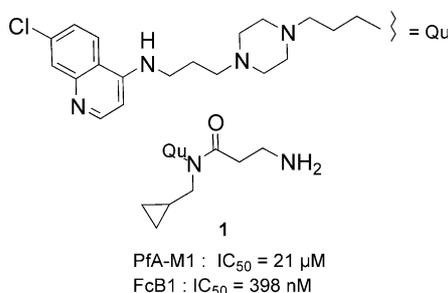


Figure 1.

evaluation on PfA-M1. Preliminary results on the anti-malarial activity on FcB1 strain of the most active enzyme inhibitors, and preliminary specificity data on mammalian aminopeptidase N, are presented and discussed.

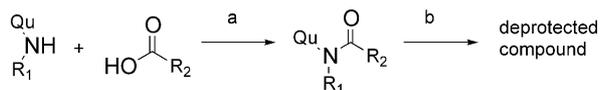
Chemistry

PfA-M1 is a zinc-metalloprotease, of the M1 family, preferably cleaving basic, hydrophobic, as well as aromatic amino-acids.⁸ Zn-chelating groups are critical for the inhibition of such enzymes.¹¹ To fulfill this function, we incorporated at least a carboxylic acid or an hydroxamate group in our molecules. In order to mimic the side chain of a Leucine residue, an isobutyl group (Ibu), analogue of the methyl-cyclopropyl moiety in **1**, was also added in our potential inhibitors.

The 45 analogues (5 amines × 9 carboxylic acids) have been synthesized at a 5-μmol scale, in parallel, according to the two-step procedure described in Scheme 1. The first step consists in the formation of the amide bond using PyBrop as the activator of the carboxylic acid followed by deprotection of the Boc and tBu groups of amines, hydroxamates and carboxylic acids.

The synthesis of this full combinatorial library required specific protected amines and carboxylic acids precursors to be synthesized prior to the coupling step.

Amines **3–6** (Fig. 2) all derived from compound **2** previously obtained from 4,7-dichloro-quinoline and 1,4-bis-(3-aminopropyl)-piperazine by nucleophilic aromatic substitution.¹² They were synthesized either by



Scheme 1. (a) 1.1 equiv PyBrop, 2 equiv DIEA in DCM, overnight, rt; (b) TFA/DCM (20/80), 72 h, rt.

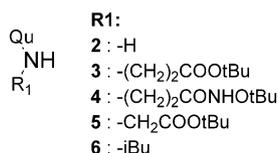
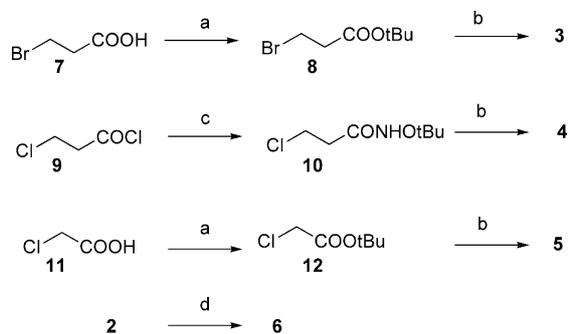


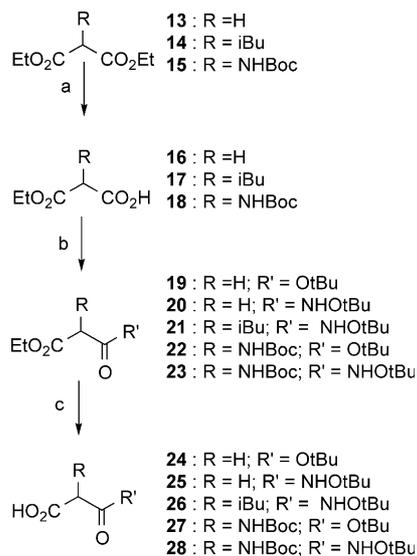
Figure 2. Amine precursors selected and synthesized for the library.



Scheme 2. (a) 1 equiv carboxylic acid, 0.7 equiv BF₃·Et₂O in *t*-Bu-COOCH₃, 4 h, rt; (b) 1.1 equiv amine **2**, 1.1 equiv DIEA, EtOH, 15 h, reflux; (c) 1.2 equiv H₂N-OtBu·HCl, 2.4 equiv pyridine, THF, 1 h, rt; (d) 1.1 HCOCH(CH₃)₂, molecular sieves, 0.4 equiv DIEA, 6 equiv NaBH₄, MeOH, overnight, rt.

reductive amination of an aldehyde or *N*-alkylation with the appropriate halide (Scheme 2).

As for the carboxylic acid precursors, we synthesized malonic derivatives (**24–28**) according to the procedure described in Scheme 3. These moieties have been previously described in inhibitors of matrix-metalloproteases (MMPs).¹³ We also added to these in-house precursors, some commercially available *N*-protected amino-acids Lys, Leu, Phe, Asp (**29–32**, Fig. 3).



Scheme 3. (a) 1 equiv KOH, EtOH, 2 h, rt; (b) 1.2 equiv *t*-Bu-O-NH₂·HCl, 1.2 equiv DIEA, 1.1 equiv EDCI and 1.1 equiv HOBT, THF, overnight, rt or 5 equiv *t*-Bu-OH, 4 equiv DIEA, 2 equiv 2-chloro-1-methylpyridinium iodide, acetonitrile, 2 h, rt; (c) 3 equiv KOH, EtOH, overnight, rt.

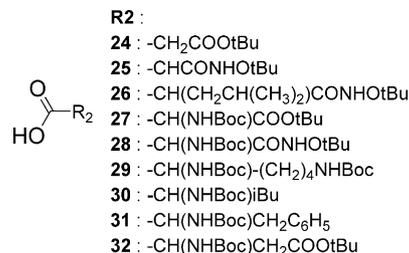


Figure 3. Carboxylic acids selected for the library.

Analytical control

Each crude product was tested for purity using HPLC and identity using MALDI-TOF. In all cases, the purity exceeded 80% and the mass spectrum was consistent with the anticipated product structure.

Biological Assays

The analogues and the deprotected precursors were screened for their ability to inhibit PfA-M1 at 10 μ M.¹⁴ Crude products displaying an inhibition percentage above 50% were selected for re-synthesis and IC₅₀ determination on fully purified and controlled samples. The selected compounds were then tested for their ability to inhibit FcB1 parasite growth.¹⁵ The specificity against other aminopeptidases was evaluated in a model of mammalian aminopeptidase-N from porcine kidney.¹⁶

Structure–activity relationships on PfA-M1

Out of the 45 compounds tested at 10 μ M, three compounds (**33–35**, Fig. 4),¹⁷ displayed a percentage of inhibition above threshold. Interestingly, they all derived from the same amine **6**. After resynthesis and testing, they displayed IC₅₀s from 0.85 to 1.54 μ M on PfA-M1, that is, a 10–25 times increase in potency comparing to compound **1** (Table 1).

The presence of a Zn-chelating group is essential for the activity. Compound **33**, the closest analogue of **1**, bearing an hydroxamate is 10 times more active on the enzyme. In contrast, amine **6** is inactive. The hydroxamate group is more efficient than the carboxylic acid group. Carboxylic acids analogues of **33** and **34** were inactive. The isobutyl group is essential for activity, since

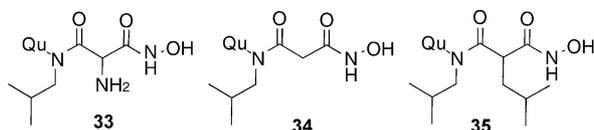


Figure 4. Best hits from the library on PfA-M1.

Table 1. Inhibition of PfA-M1 and of parasite growth, and specificity towards mammalian aminopeptidase-N of compounds **33–35**

Compd	PfA-M1 inhibition IC ₅₀ , μ M	Parasite growth inhibition IC ₅₀ , μ M ^a	Aminopeptidase N (APN) inhibition IC ₅₀ , μ M	Ratio IC ₅₀ PfA-M1/IC ₅₀ APN
CQ	ND ^b	0.126 (0.026)	ND ^b	—
Bestatin	0.284	29	2.700	0.1
1	21	0.398 (0.056)	28	0.7
33	2.503	0.178 (0.037)	1.345	1.9
34	0.854	0.317 (0.063)	0.028	30.5
35	1.540	0.151 (0.013)	1.628	0.9
38	> 10 ^c	1.380 (0.050)	ND ^b	—

^aValues are means of three experiments, standard deviation is given in parentheses. Strain is FcB1.

^bND, not determined.

^c22% inhib (10 μ M).

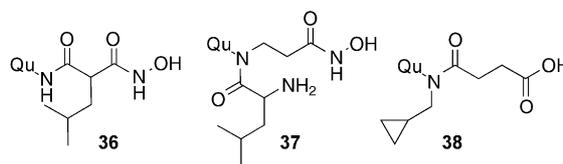


Figure 5. Inactive analogues **36**, **37** from the screening and reference compound **38**.

the analogues of **33–35** without this moiety (derivatives of **2**) were inactive.

The relative positions of the Zn-chelating group and the isobutyl group are critical for the activity. For example, compounds **36–37** (Fig. 5) are inactive while displaying the same groups in a different 3D arrangement as respectively in **34** and **33**. None of the analogues derived from the amino-acids (**37** for instance) supposed to be recognized by the enzyme, was active, suggesting that the Zn-chelating group should be supported by the carboxylic acid precursor.

The central position in malonic derivatives seems tolerant since it can accommodate with basic (NH₂) or hydrophobic (isobutyl) groups. Nevertheless, the best analogue, compound **34**, is not substituted in that position. In particular, this suggests that an amino-group is not essential for the recognition by the enzyme although it is an aminopeptidase.

Antimalarial activity and specificity

The three PfA-M1 inhibitors are sub-micromolar inhibitors of the parasite growth. Their antimalarial activity is due to the inhibition of both PfA-M1 and haem detoxification.¹⁸ Interestingly, reference compound **38**, displaying a COOH moiety at the same position as the hydroxamate OH in compound **34** (and a cyclopropylmethyl group instead of an isobutyl group), is weakly active on PfA-M1, and less active than **34** on parasite growth inhibition. Specificity for compounds **33–35**, as expressed with the ratio of the IC₅₀s on the two targets, varies from 0.9 to 30.5. The size of the substituent on the malonic methylene appears to be critical.

Conclusion

We have successfully designed non-peptidic inhibitors of PfA-M1, an aminopeptidase of *P. falciparum*. The quinoline moiety allows these compounds also to be inhibitors of haem detoxification, making these compounds potentially dual inhibitors of hemoglobin digestion. Yet the relative part of each mode of action needs to be determined. The three selected inhibitors **33–35** are active against CQ-resistant strain, thus validating their ability to cross membranes. The activity on PfA-M1, and the specificity against other aminopeptidases such as mammalian aminopeptidase-N need to be improved. Such issues are under investigation and results will be reported in due course. Antimalarial activities and rough SARs emerging from this study,

have encouraged us to pursue the optimization of PfA-M1 inhibitors. Especially, we are now focusing on inhibitors devoided of the quinoline moiety.

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14. Native PfA-M1 was purified according to the procedure described in ref 9, and diluted 10 times in Tris-HCl buffer (25 mM; pH 7.4) before use. The assays were set up in 96-well plates. The compounds were tested at a final concentration of 10 μ M. 33 μ L of purified PfA-M1 were pre-incubated 10 min at rt with 33 μ L of the inhibitor (30 μ M in Tris-HCl buffer, 0.3% DMSO). 33 μ L of the substrate Leu-pNA (K_m =0.1 mM) 0.3 mM in Tris-HCl buffer were then added. The reaction kinetics was followed on a UV-microplate reader MultiskanRC (Labsystems, Finland) at 405 nm. The control activity was determined by incubating the enzyme in the same conditions without inhibitor. Bestatin was used as the reference inhibitor. The statistical Z' factor for the test (Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. *J. Biomol. Screen.* **1999**, *4*, 67) was 0.82, allowing activities to be determined with a single point with a 95% confidence. Initial velocities are expressed in μ mol. min⁻¹. Data were normalized to the controls that represent V_{max} . For the IC₅₀ values determination, initial velocities were plotted as a function of inhibitor concentration.
15. *P. falciparum* strains were maintained continuously in culture on human erythrocytes as described by Trager and Jensen (*Science* **1976**, *193*, 673). In vitro antiplasmodial activity was determined using a modification of the semi-automated micro-dilution technique of Desjardins et al. (*Antimicrob. Agents Chemother.* **1979**, *16*, 710). *P. falciparum* CQ-resistant FcB1 (Colombia) strain was used. Stock solutions of CQ-diphosphate and test compounds were prepared in sterile distilled water and DMSO, respectively. Drug solutions were serially diluted with culture medium and introduced to asynchronous parasite cultures (0.5% parasitemia and 1% final hematocrite) on 96-well plates for 24 h at 37°C prior to the addition of 0.5 μ Ci of [³H]hypoxanthine (1–5 Ci/mmol; Amersham, France) per well, for 24 h. The growth inhibition of each drug concentration was determined by comparison of the radioactivity incorporated into the treated culture with that in the control culture (without drug) maintained on the same plate. The IC₅₀, as obtained from the drug concentration–response curve were expressed as mean \pm standard deviations, determined from three independent experiments. The DMSO concentration never exceeded 0.1% and did not inhibit the parasite growth.
16. Microsomal aminopeptidase N from porcine kidney (EC 3.4.11.2) was purchased from Sigma-Aldrich France, as a suspension in 3.5 M (NH₄)₂SO₄ solution. Before use, it was diluted 600 times in Tris-HCl buffer (25 mM; pH 7.4). The assays were set up in 96-well plates and proceeded like for PfA-M1 with the same substrate Leu-pNA (K_m =0.13 mM). The Z' factor of the test was 0.80 allowing activities to be determined with a single point with a 95% confidence. The reference inhibitor was bestatin.
17. **33**. (3TFA): NMR (DMSO-*d*₆) ¹H δ ppm 0.80–0.84 (m; 14H); 1.10–1.26 (m; 5H); 1.27–1.90 (m; 6H); 2.75–3.58 (m; 14H); 6.89 (d; J =7.1 Hz; 1H); 7.76 (dd; J =9.0 Hz; J =1.1 Hz; 1H); 7.95 (s; 1H); 8.50–8.56 (m; 2H); 9.30 (br s; 1H; 2NH⁺); 9.73 (br s; 0.5H; NHO); MALDI-TOF: 534.2 (M+H⁺). **34**. (2TFA): NMR (CD₃OD) ¹H δ ppm 0.80 (d; J =6.6 Hz; 2H); 0.86 (d; J =6.6 Hz; 4H); 1.86–1.97 (m; 5H); 2.72 (br t; J =6.5 Hz; 2H); 2.76–2.95 (m; 4H); 2.96–3.21 (m; 10H); 3.43 (br t; J =6.6 Hz; 2H); 3.62 (br t; J =6.7 Hz; 2H); 6.83 (d; J =6.8 Hz; 1H); 7.60 (dd; J =1.7 Hz; J =9.1 Hz; 1H); 7.79 (br s; 1H); 8.25–8.33 (m; 2H); MALDI-TOF: 519.2 (M+H⁺). **35**. (2TFA): NMR (DMSO-*d*₆) ¹H δ ppm 0.80–0.84 (m; 14H); 1.10–1.26 (m; 5H); 1.27–1.90 (m; 6H); 2.75–3.58 (m; 14H); 6.90 (d; J =7.1 Hz; 1H); 7.78 (dd; J =9.0 Hz; J =1.9 Hz; 1H); 7.95 (d; J =1.8 Hz; 1H); 8.51 (d; J =9.0 Hz; 1H); 8.56 (d; J =7.0 Hz; 1H); 8.91 (br s; 0.6H; NHO); 9.34 (br s; 1.3H; 2NH⁺); 10.75 (br s; 0.6H; OH); MALDI-TOF: 575.3 (M+H⁺).
18. In-vitro inhibition of haem polymerization induced by lipids, unpublished data (see ref 3 for protocol).