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Short communication

Synthesis, antimalarial evaluation and molecular modeling studies of hydroxyethylpiperazines, potential aspartyl protease inhibitors, Part 2

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ABSTRACT

The antimalarial acitivity of hydroxyethylamines, synthesized from the reaction of intermediated hydroxyethypiperazines with benzenesulfonyl chlorides or benzoyl chlorides, has been evaluated *in vitro* against a W2 *Plasmodium falciparum* clone. Some of the nineteen tested derivatives showed a significant activity *in vitro*, thus turning into a promising new class of antimalarials. In addition, a molecular modeling study of the most active derivative (**5l**) was performed and its most probable binding modes within plasmepsin II enzyme were identified.

1. Introduction

Malaria accounts for more than a million deaths each year, of which over 80% occur in tropical Africa, where this illness is the leading cause of mortality in children under five years of age [1]. In Brazil it is estimated that 500,000 cases occur annually, mainly in the Amazon Rain Forest. Malaria is caused by protozoal parasites of the genus *Plasmodium*. Four different *Plasmodium* species infect humans: *Plasmodium vivax*, *Plasmodium malarie*, *Plasmodium ovale* and the most dangerous specie *Plasmodium falciparum*. Despite the importance of this disease, there has been little economic incentive for the development of new drug-based antimalarial therapies. There is a growing need for effective drugs with new mechanism of action, due the high rate of mutation of the parasite, which leads to the development of resistance. One of the critical stages of the life cycle of the parasite during human infection is the degradation of hemoglobin which provides nutrients for its growth and maturation [2]. A particular family of aspartic proteases, known as the plasmepsins, appears to be involved in the initial steps of the degradation pathway and therefore converts itself in an attractive antimalarial drug target [3]. There are four aspartic proteases present in the food vacuole of *P. falciparum*, plasmepsin I, II, IV and Histo-Aspartic-Protease (HAP). The most attractive target from this group of enzymes is the plasmepsin II [4]. Recent studies have shown that HIV protease inhibitors can inhibit the plasmepsin *in vitro* [5] and *in vivo* [6] at pharmacologically relevant concentrations.

A secondary alcohol is usually the structural element of choice to inhibit aspartic protease. This element mimics the tetrahedral intermediate during peptide bond cleavage by aspartic proteases [3]. It has been successfully used to develop potent inhibitors of these enzymes, for example norstatine [7,8] and hydroxyethylamine-based [9,10] molecules (Fig. 1). In the course of our investigations on hydroxyethylamine derivatives as antimalarial agents [11,12], we describe in the present work the synthesis and *in vitro* activity of novel hydroxyethylpiperazine-based compounds against W2 clone of *P. falciparum*. The clone W2 was used for the test, as described in our previous work [13], and activity compared to that of chloroquine and lopinavir (the HIV protease inhibitor with the best antimalarial activity) [5], used in parallel as control in each test.

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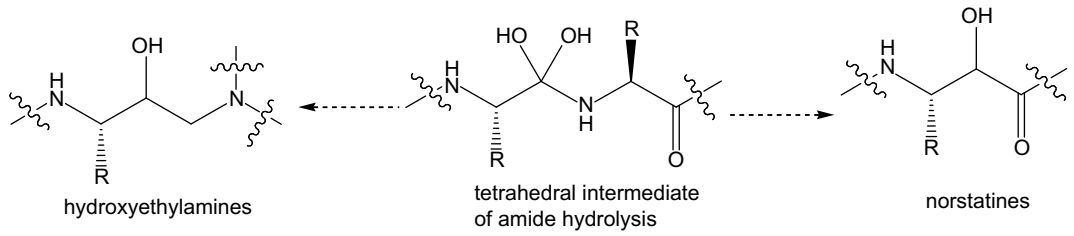


Fig. 1. Molecules developed for the synthesis of aspartyl protease inhibitors.

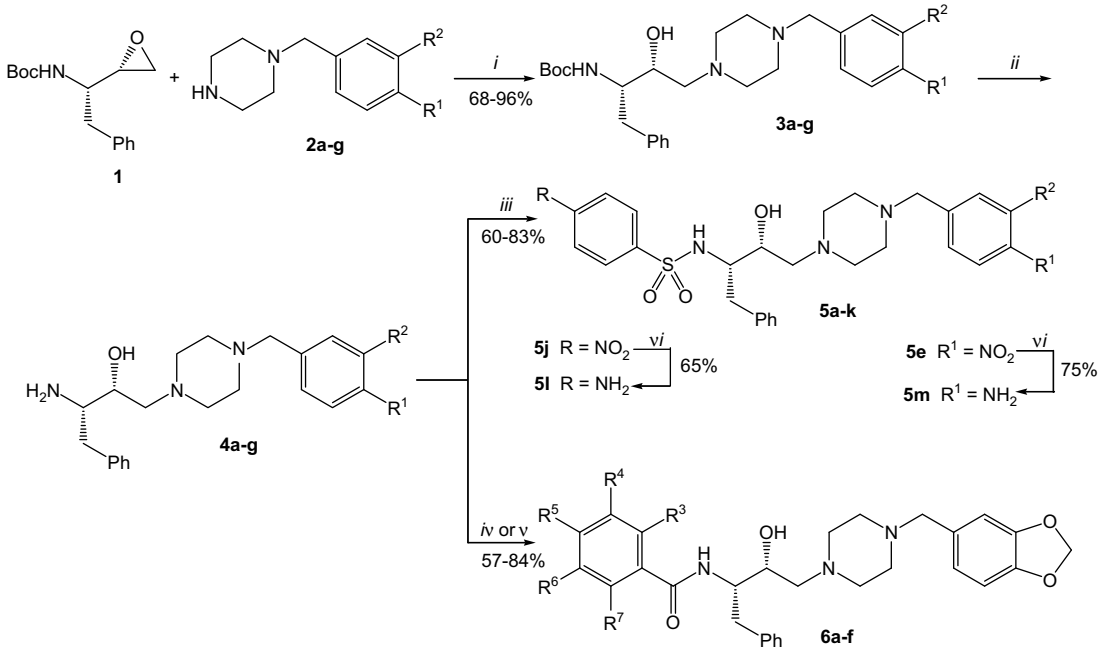
2. Pharmacological evaluation

Antimalarial assay. Parasites were cultured with human erythrocytes (blood group O+) at 5% hematocrit in RPMI 1640 supplemented with 10% human plasma as previously described [14]. Test compounds were solubilized in ethanol prior to *in vitro* tests. The antiparasitic effects of the molecules was measured by the [^{3}H]-hypoxanthine incorporation assay [15]. Briefly, trophozoite stages in sorbitol-synchronized blood, were cultured at 2% parasitaemia and 2.5% hematocrit, in the presence of the test compounds (at various concentrations), diluted with culture medium (RPMI 1640) without hypoxanthine; a chloroquine control (as a reference antimalarial drug) was used in each experiment. Inhibition of parasite growth was evaluated through the levels of [^{3}H]-hypoxanthine incorporation plotted to generate dose–response curves. The half-maximal inhibitory response (IC_{50}) compared with parasite growth in the drug-free controls was estimated by curve fitting using a software program [Microcal, Origin Software, Inc. (Northampton, MA, USA)].

Cytotoxicity assay. Cytotoxicity was determined in the murine monocyte/macrophage cell lineage J774, using the method previously described [16]. Cells were seeded in a flat bottom 96 well plate (2×10^6 cells/well) cultured for 1 h (CO_2 5% at 37 °C) in RPMI 1640 enriched with 10% bovine fetal serum, 2 mM L-glutamine, 25 μg/ml gentamicin, pH 7.4. Adherent cells were cultured in the presence of different concentrations of the compounds (0.1–100 μg/ml) for 20 h, when MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml; 22.5 μl/well) was added to the culture for 4 h. Supernatant was discharged and DMSO (150 μl/well) was added for solubilization of formazan crystals. The absorbance was read at 540 nm.

3. Chemistry

The target compounds **5a**–**m** and **6a**–**f** were prepared as outlined in Scheme 1. The hydroxyethylamine transition-state mimicking fragment was prepared by selective ring-opening of the (2*S*,3*S*)boc-phenylalanine epoxide **1** with 4-(benzyl)piperazines (**2a**–**g**) in refluxing isopropanol for 16 h to give intermediates **3a**–**g** with good yields (68–96%) [12]. Compounds **3a**–**g** were deprotected with trifluoroacetic acid in dichloromethane at room temperature for 4 h resulting the free amine **4a**–**g**, which were coupled with benzenesulfonyl chlorides by using potassium carbonate in acetonitrile at room temperature to afford the hydroxyethylpiperazines **5a**–**k** in good yields after purification (60–83%). Compounds **5l** and **5m** were prepared by a reduction reaction from compounds **5j** and **5e**, respectively. The nitro group of compounds **5e** and **5j** were reduced with H_2 and Pd/C (10%) using ethanol as the solvent, in good yields. The hydroxyethylpiperazines **6a**–**d** were synthesized from reaction of amine **4g** with



Scheme 1. Reaction conditions: *i*: IPA, reflux, 16 h; *ii*: TFA/CH_2Cl_2 (1/3), r.t., 4 h; *iii*: $ArSO_2Cl$, CH_3CN, K_2CO_3, r.t., 4 h; *iv*: ArCOCl, CH_3CN, K_2CO_3, r.t., 4 h; *v*: ArCOOH, THF, HOBT, EDC, DIPEA, 0 °C – r.t., 18 h; *vi*: H_2, Pd/C 10%, EtOH, r.t., 16 h.

Table 1
Antimalarial activity, cytotoxicity activy and lipophilicity (Log *P*) of hydroxyethylpiperazines **5a–m** and **6a–f**.

Comp.	R	R^1	R^2	R^3	R^4	R^5	R^6	R^7	IC_{50} (μM)[a]		Cytotoxicity (μg/ml)[b] % of cell viability				miLogP[c]
									W2	3D7	0.1	1	10	100	
5a	Me	H	H	–	–	–	–	–	>90	–	100	86	88	5	3.921
5b	Me	F	H	–	–	–	–	–	43.0	–	100	100	92	15	4.084
5c	Me	Cl	H	–	–	–	–	–	35.1	–	100	98	90	22	4.599
5d	Me	OMe	H	–	–	–	–	–	>90	–	100	90	84	6	3.978
5e	Me	NO_2	H	–	–	–	–	–	>90	–	91	100	93	92	3.880
5f	Me	OMe	OMe	–	–	–	–	–	>90	–	100	100	92	9	3.567
5g	Me	$-OCH_2O-$		–	–	–	–	–	16.9	–	100	100	98	49	3.811
5h	Br	$-OCH_2O-$		–	–	–	–	–	5.6	5.7	100	100	96	5	4.172
5i	OMe	$-OCH_2O-$		–	–	–	–	–	34.3	–	100	99	90	12	3.419
5j	NO_2	$-OCH_2O-$		–	–	–	–	–	44.0	–	100	100	100	40	3.321
5k	F	$-OCH_2O-$		–	–	–	–	–	42.5	–	100	94	83	4	3.526
5l	NH_2	$-OCH_2O-$		–	–	–	–	–	4.8	4.6	100	100	99	55	2.438
5m	Me	NH_2	H	–	–	–	–	–	>90	–	100	90	77	10	2.997
6a	–	$-OCH_2O-$		H	H	H	H	H	69.4	–	100	100	91	38	3.487
6b	–	$-OCH_2O-$		F	H	H	H	F	47.4	–	85	87	83	51	3.719
6c	–	$-OCH_2O-$		Cl	H	H	H	Cl	55.5	–	100	90	84	43	4.747
6d	–	$-OCH_2O-$		H	Cl	H	Cl	H	47.2	–	98	100	98	93	4.771
6e	–	$-OCH_2O-$		H	$-OCH_2O-$		H	H	25.4	–	100	100	95	61	3.378
6f	–	$-OCH_2O-$		Me	OH	H	H	H	5.9	–	100	75	8	8	3.828
Chloroquine									0.34	0.03					
Lopinavir									2.7	–					

[a] IC_{50} represents concentration inhibitory dose of the parasite growth in relation to control cultures with no drugs.
[b] Percentage of J774 cell viability 24 h after incubation with the compounds determined by the MTT method.
[c] Calculated using online www.molinspiration.com/cgi-bin/properties site (Molinspiration Property Engine v2007.04).

corresponding benzoyl chlorides in good yields (73–84%). Piperonylic acid and 3-hydroxy-2-methylbenzoic acid were used as precursors for the synthesis of compounds **6e** and **6f**, respectively. These reactions were carried out with HOBT, EDC and DIPEA using THF as the solvent in moderate yields. Both analytical and spectral data (^1H and ^{13}C NMR) of all compounds are in full agreement with the proposed structures (see Supplementary information). 2D-NMR thecniques (HMBQ, HMQC and COSY) helped us to assign the correct signals of compounds.

4. Results and discussion

Table 1 shows the antimalarial activities of all compounds expressed as 50% inhibitory concentration (IC_{50}) of *P. falciparum*. In the first series, the *p*-toluenesulfonyl group was fixed and different benzylpiperazines were attached to compounds **5a–g** and **5m**. In this preliminary study, we observed that compound **5g**, with 4-(piperonyl)piperazine moiety, presented the best antimalarial activity ($IC_{50} = 16.9$ μM). The second series was synthesized fixing the 4-(piperonyl)piperazine moiety and switched the *p*-toluenesulfonyl group for different benzenesulfonyl derivatives to give compounds **5h–l**. The hydroxyethylpiperazine **5l** ($R = NH_2$) indicated the best antimalarial activity of this work ($IC_{50} = 4.8$ μM), almost as active as standard lopinavir. The compound **5h** ($R = Br$) also showed good activity ($IC_{50} = 5.6$ μM). The compounds **5h** and **5l** were also tested against *P. falciparum* 3D7 clone (chloroquine sensitive) with $IC_{50} = 5.7$ μM and 4.6 μM, respectively. The third series was obtained from reaction of compound **4g** with benzoyl chlorides. Only compound **6f** showed good activity against *P. falciparum* ($IC_{50} = 5.9$ μM), however, this compound was cytotoxic at macrophages cells at low concentration (cell reduction of 25% at 1 μg/ml and cell reduction of 92% at 10 μg/ml concentration). The lipophilicities of the synthesized compounds **5a–m** and **6a–f**, which were expressed as log *P* values, were determined through online www.molinspiration.com/cgi-bin/properties site.

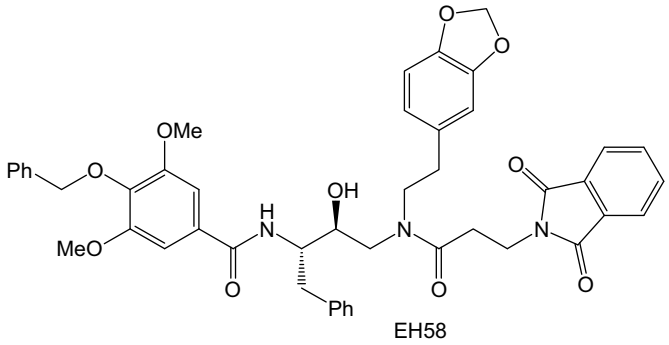


Fig. 2. Structure of EH58 ligand.

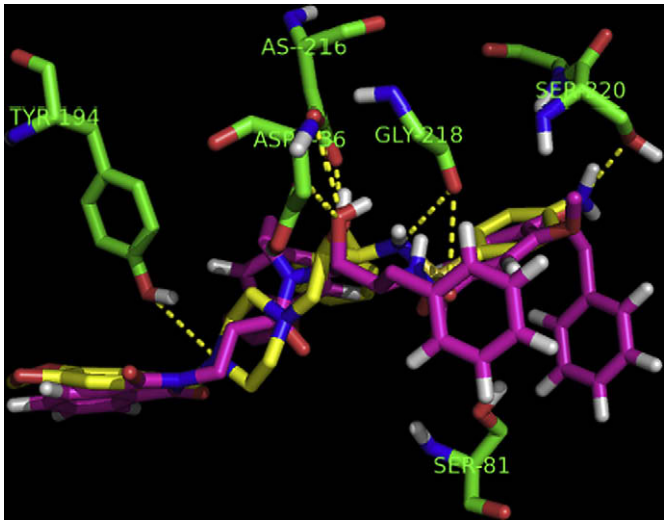


Fig. 3. Hydrogen bonds of Plm II with compound **5l** (**conf. 5** in yellow) superposed over the re-docked structure of EH58 ligand (in pink). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Results of the linear interaction energy (LIE) method.

Compound[a]	IC_{50}(μM)[b]	$\Delta G_{bind,calc}$	$\langle V_{1-s}^{vdw}\rangle_P$[c]	$\langle V_{1-s}^{el}\rangle_P$[c]	$\langle V_{1-s}^{vdw}\rangle_W$[c]	$\langle V_{1-s}^{el}\rangle_W$[c]
5l – conf. 4	4.8	0.44 (2.62)	−58.70 (1.67)	−80.00 (4.03)	−37.28 (0.88)	−91.66 (3.34)
5l – conf. 5	4.8	−1.61 (1.23)	−65.55 (1.15)	−80.78 (2.20)	−35.83 (0.86)	−91.04 (2.42)

[a] **Conf. 4** and **conf. 5** indicate the two selected docking conformations of compound **5l**.
[b] IC_{50} represents concentration inhibitory dose of the parasite growth in relation to control cultures with no drugs.
[c] The calculated average electrostatic and non-polar energies for ligand-surrounding interactions are in kcal/mol (standard deviation).

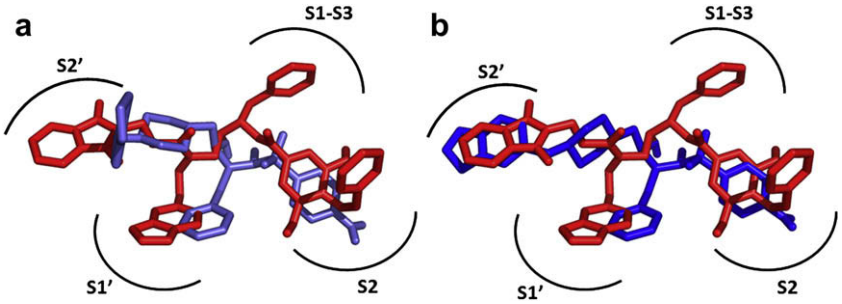


Fig. 4. Superposition of **conf. 4(a)** and **conf. 5(b)** (in blue and light blue, respectively) of compound **5l** over the crystallographic structure of the EH58 ligand (in red). The generation of the figure above was done with the software Pymol v0.99rc6, DeLano, W. L., 2002. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5. Molecular modeling

The potential binding modes and the main interactions between the most active synthesized compound **5l** and Plm II enzyme were investigated by computational analysis. Firstly, to validate the computational docking protocol, redocking analysis of the EH58 ligand into the Plm II active site was carried out with the Autodock program [17]. Since the EH58 ligand is a large and highly flexible compound (19 rotatable bonds), conformations with RMSD values up to 3.0 Å were considered correctly docked structures (Fig. 2). Taken into account this criterion, the redocking of EH58 into plasmepsin II active site presented a success rate of 60% in finding structures near the crystallographic one. The correctness of docked conformations of EH58 was also confirmed by visual inspection. Encouraged by the good agreement of docked structures with the crystallographic structure of EH58, the same docking protocol was used for automated docking of compound **5l**.

The visual inspection along with the investigation of the main interactions between the ligand and key residues of the enzyme is a helpful strategy in selecting the most favored ligand binding modes [18]. This fact prompted us to submit the 10 best conformations of compound **5l** to this kind of investigation. Visual inspection of the docked conformations interacting with key enzyme residues showed conformations **4** and **5** as the more probable binding modes of compound **5l** and their superposition upon the EH58 structure reveals equivalent interactions together with a good agreement in covering the enzyme subsites. The hydrogen bonds of the selected binding modes of compound **5l** are shown in Fig. 3.

Superposition of conformations **4** and **5** of compound **5l** suggested distinct ways in the placement of ligand groups in the Plm II subsites. In **conf. 4** the piperazine ring was situated near the S2′ pocket. The 4-aminosulfonamide group of this conformation was set near the S2 pocket and the phenyl ring is located in the vicinity of the S1′ pocket. Positions of all chemical groups of compound **5l** in **conf. 5** closely coincide with those of **conf. 4**, excepting the piperonyl group that remained parallel to the phtalimide ring of EH58, resulting in a much better free energy of binding within Plm II active site (Fig. 4).

These conformations were used as starting structures for molecular dynamics simulations and binding free energy evaluation with the LIE method [19]. The calculated free energy of binding for the two selected conformations of compound **5l** is shown in Table 2. The results revealed **conf. 5** as the most favored binding mode. When compared to **conf. 5**, **conf. 4** presented similar energies for ligand-surrounding interactions. However, the main difference was in the non-polar protein–ligand interactions. This is probably due to the absence of the ligand groups in subsites S2 and S2′ in **conf. 4**. The most favored conformation of compound **5l** (**conf. 5**) fills the Plm II subsites in a quite similar way to the experimental structure of EH58 ligand (Fig. 4). This conformation also presents a better fit of the non-polar groups of compound **5l** in the enzyme hydrophobic subsites contributing to a better overall affinity.

Molecular docking studies were also done for compound **5j**, the one with the lowest observed activity. The best automatically docked conformation of **5j** differ from the **5l** one, mainly regarding the position of the benzodioxolane and the benzylic rings at S2′ and S1′ subsites, respectively. However, the benzylic ring with the substituent group in **5j** is located at S2 pocked in a similar position to the one observed in **5l** conformation. The most active compound **5l** is hydrogen bonded to the two catalytic aspartates of Plm II enzyme and to SER218, GLY216 and TYR192. The docked conformation of compound **5j** presents two hydrogen bonds with SER218 and a hydrogen bond to only one of the catalytic aspartates. None specific interaction of the substituent groups that might explain activity was observed.

6. Conclusion

In summary, we easily synthesized nineteen hydroxyethylpiperazines derivatives in good yields. Despite the fact that the most active compounds are at least 10- to 20 times less active *in vitro* than chloroquine, their IC_{50} values are in the micromolar concentration range comparable to recently reports results [13,20]. In addition, compounds **5h**, **5l** and **6f** are as active as lopinavir, the protease inhibitor of HIV with the highest activity against *P. falciparum*. Compounds **5h** and **5l** also have low toxic activities at high concentration (100 μg/ml). The **conf. 5** was the most favored

conformation of hydroxyethylapiperazine **5l,** filling the Plm II subsites and showing a better fit of the non-polar groups in the enzyme hydrophobic subsites. The investigation of these compounds as anti-HIV agents is ongoing.

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Appendix. Supplementary information

There are supplementary informations with chemical general procedure, yields, NMR ^{1}H and ^{13}C data and LC-MS data. We also include information about molecular modeling study. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2009.03.041.

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