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RESEARCH ARTICLE

Characterisation of hydrazides and hydrazine derivatives as novel aspartic protease inhibitors

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Abstract

Virtual screening of an in-house virtual library of synthetic compounds using FlexX, followed by enzyme inhibition, identified hydrazide and hydrazine derivatives as novel aspartic protease inhibitors. These compounds inhibited human cathepsin D and *Plasmodium falciparum* plasmepsin-II with low micromolar concentrations (IC_{50} = 1-2.5 μM). Modelling studies with plasmepsin-II predicted binding of ligands at the centre of the extended substrate-binding cleft, where hydrazide/hydrazine parts of the inhibitors acted as the transition state mimic by forming electrostatic interactions with catalytic aspartates.

Keywords: *Virtual screening; protease inhibitors; cathepsin D; plasmepsin; malaria*

Introduction

Aspartic proteases constitute one of the major protease subclasses that share a common mechanism of catalysis [1]. These proteases consist of two domains where each domain contributes one aspartic acid residue to the catalytic dyad. A beta-hairpin turn also known as the flap with the ability to interact with substrates and inhibitors covers the binding cavity [1,2]. The substrates bind to the enzyme in its extended beta-strand conformation, and substrate residues (Pn-P1/P1'-Pn') and corresponding enzyme sub-sites (Sn-S1/S1'-Sn') are denoted based on their position relative to the scissile amide bond according to the Schechter and Berger nomenclature [3]. The substrate-binding cleft of these proteases can accommodate 7/8 residues of substrates/inhibitors in S5-S3' sub-sites. The distinction in specificity and activity of various aspartic proteases are due to discrete properties of the individual amino acid residues forming sub-sites comprising the active site cleft [4,5].

Aspartic proteases play important roles in several diseases such as AIDS (HIV protease) [6], neoplastic disorders (cathepsin D and E) [7,8] Malaria (plasmepsins) [9] etc. Increased expression of human lysosomal cathepsin D is associated with a number of pathological conditions including neoplastic disorders and inflammatory diseases [7,10]. The plasmepsin family of Plasmodium aspartic proteases is involved in haemoglobin degradation during the intra-erythrocyte phase of malarial infection [9]. Plasmepsins (in particular plasmepsin-I and plasmepsin-II) have been considered as promising target for new anti-malarial drugs but Liu et al. [11] have reported that inhibition of plasmepsins does not appear to be a potential strategy, unless combined with the inhibitors of malarial parasite cysteine proteases falcipains that are also involved in haemoglobin catabolism [11]. A number of chemical functionalities and structural units have been employed as non-cleavable transition-state isosteres in plasmepsin-II inhibitors [12]. These include inhibitors encompassing statine-derived and reversed-statine-based cores [13,14], tertiary amines based on 4-aminopiperidine-tert-butylcarbamate [15], hydroxyethylamines, dihydroxyethylenes and hydroxymethylcarbonyl [16,17], diphenylurea [18] and acridinyl hydrazides [19]. Recently, new non-peptidic plasmepsin inhibitors have been reported containing achiral oligoamines and tetrahydroazepine scaffold [20,21].

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The hydrazides and their analogues are known to have antibacterial, antifungal [22,23] and antileishmanial [24] activities. They also have been reported as monoamine oxidase inhibitors [25]. We have characterised phenyl hydrazide and hydrazine derivatives as *Plasmodium falciparum* plasmepsin-II and human cathepsin D inhibitors suggested by virtual screening of an in-house library of synthetic compounds and confirmed by enzyme inhibition studies.

Material and methods

Virtual library screening

Virtual screening was carried out by FlexX ligand docking software (version 2.0) [26] using an in-house virtual library of more than 600 synthetic compounds and crystal structural coordinate sets of *Plasmodium falciparum* plasmepsin-II (PDB ids; 1M43 [27]) and human cathepsin D (PBD id; 1LYB [27]). The virtual library compounds correspond to thirty different chemical scaffolds that have been synthesised in our laboratory. 3D models of compounds in SYBYL mol2 format were utilised for binding to the active sites of both aspartic proteases. FlexX method of ligand docking involves incremental construction of ligands from smaller fragments in the cavity of a receptor. During virtual screening by FlexX, each molecule in the in-house library is docked and scored (termed as FlexX docking score) and finally the molecules are ranked according to those scores. The ranking of generated docking solutions is performed using a scoring function similar to that developed by Bohm [29] which estimates the free binding energy (ΔG) of the protein-ligand complex. Docking was carried out allowing full flexibility for the ligands, while keeping the proteins fixed. Among the two catalytic aspartates of proteases, Asp34 was considered protonated and Asp214 considered negatively charged during docking (plasmepsin-II numberings). After each ligand docking run, the top ten ranked docking solutions were saved and considered for detailed analysis.

Similar to the FlexX scoring function, the SCORE command of FlexX searches for interactions and an energy estimation for the ligand placed on a given set of coordinates (termed as FlexX binding score). The binding score of plasmepsin-II-pepstatin-A complex (PDB id; 1M43) was also calculated by SCORE command for comparison.

Synthesis of hydrazides and hydrazines

The procedure for the preparation of butanohydrazide (Mr-I-33) and 2-methoxybenzohydrazide (Mr-I-27) has been reported elsewhere [24] (Table 1).

Synthesis of 2-nitrobenzohydrazide (Mr-I-53b) and 2,2-diphenylacetohydrazide (Mr-I-179) was carried out by conventional method. In a typical reaction, hydrazine hydrate (3.5 mmol) was added to corresponding ethyl or methyl esters (3.5 mmol) in ethanol and this mixture was refluxed for 4-6 h as mentioned in Table 1. After completion of the reaction, the precipitate was obtained, filtered and washed with hexane. Hydrazides were obtained as slightly yellowish solids.

Compound Mr-I-53b (2-nitrobenzohydrazide): Yield: 92%; R_f = 0.54 (hexane/ethyl acetate, 3:7); ^{1}H-NMR (400 MHz, CD_3OD); δ 8.60 (d, 1H, $J_{3,4}$ = 8.6 Hz, H-3), 8.30 (d, 1H, $J_{6,5}$ = 8.4 Hz, H-6), 8.20 (t, 1H, $J_{4,5} = J_{4,3}$ = 7.4 Hz, H-4), 7.76 (t, 1H, $J_{5,6} = J_{5,4}$ = 8.03 Hz, H-5);IR (KBr): ν_{max} 3330, 3263, 1630, 1597, 1540, 1340, 1115, 503 cm^{-1}; UV (CH_3OH): λ_{max} 240 (log ε = 3.98) nm; EI MS: *m/z* (rel. abund. %): 181 (M^+, 17), 150 (100), 134 (15), 120.1 (60), 104 (70), 92 (50), 76 (69), 75 (28), 50 (60); Anal. calcd for $C_7H_7N_3O_3$: C, 46.41; H, 3.89; N, 23.20; Found: C, 46.40; H, 3.86; N, 23.18.

Compound MR-I-179 (2,2-diphenylacetohydrazide): Yield: 70%; R_f = 0.45 (hexane/ethyl acetate, 3:7); ^{1}H-NMR (400 MHz, CD_3OD); δ 7.23 (m, 4H, H-2, 3, 5, 6), 7.11 (m, 4H, H-2`, 3`, 5`, 6); IR (KBr): ν_{max} 3258, 2975, 1645, 1279 cm^{-1}; UV (CH_3OH): λ_{max} 270 (log ε = 4.62) nm; EI MS: *m/z* (rel. abund. %): 226 (M^+, 20), 195 (40), 167 (100), 78 (50); Anal. calcd for $C_{14}H_{14}N_2O$: C, 74.31; H, 6.24; N, 12.38. Found: C, 74.29; H, 6.24; N, 12.30.

Microwave-assisted synthesis of 2-nitrobenzohydrazide (Mr-I-53b) and 2,2-diphenylacetohydrazide (Mr-I-179)

In a typical reaction, hydrazine hydrate (3.5 mmol) was added to corresponding ethyl or methyl esters (3.9 mmol) in ethanol and these mixture were irradiated under microwave (CEM Discover system, model 908010, Matthews, North Carolina, USA) for the times mentioned in Table 1. After completion of the reaction, the precipitate was obtained, filtered and washed with hexane. Hydrazides were obtained as slightly yellowish solids.

Microwave-assisted synthesis of 1,2-diphenylhydrazine (Mr-II-10)

1,2-diphenylhydrazine was synthesised by aniline (1 mmol), copper sulphate (1 mmol) and Al_2O_3 were irradiated under microwave (CEM Discover system) for 90 s. The solid product was washed with hexane.

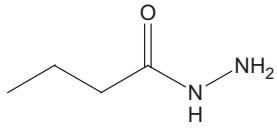
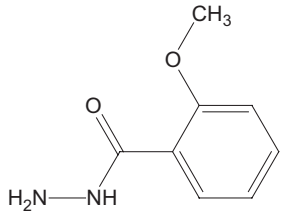
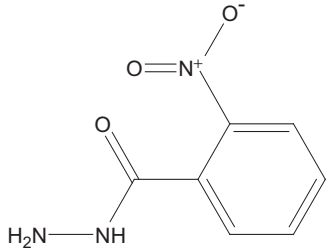
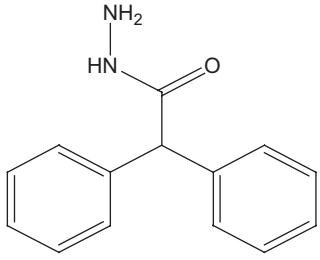
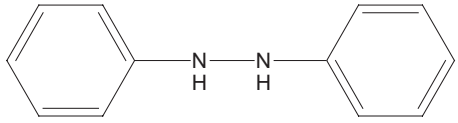
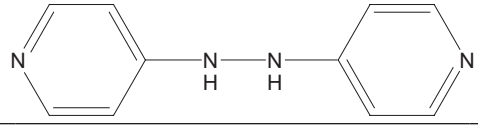
Compound Mr-II-10 (1,2-diphenylhydrazine): Yield: 92% R_f = 0.75 (hexane/ethyl acetate, 7:3); ^{1}H-NMR (400 MHz, MeOD) δ 7.89 (m, 6-H, H-10), 7.51 (m, 5-H, H-11) 7.24 (s, 7-H, H-8); IR (KBr) ν_{max} 3425, 2922, 1591, 1481;UV (CH_3OH): λ_{max} 450 (log ε = 4.01) nm; EI MS *m/z* (rel. abund. %): 184 (M^+, 1.69), 182 (11.9), 72.2 (100), 51 (50); Anal. calcd for $C_{12}H_{12}N_2$: C, 78.23; H, 6.57; N, 15.21. Found: C, 78.19; H, 6.55; N, 15.20.

Microwave-assisted synthesis of 4-[-2-(4-pyridinyl)-hydrazino]-pyridine (Mr-II-80)

For 4-[-2-(4-pyridinyl)-hydrazino]-pyridine synthesis, 4-pyridineamine (1 mmol), copper sulphate (1 mmol) and Al_2O_3 were irradiated under microwave (CEM Discover system) for 80 s. The solid product was washed with hexane.

Compound Mr-II-80 (1,2-dipyridinylhydrazine): Yield: 78%;R_f = 0.76 (hexane/ethyl acetate, 7:3); ^{1}H-NMR (400 MHz, MeOD) δ 8.26 (m, 2-H, H-13), 8.17 (s, 7-H, H-8), 7.09 (m, 5-H, H-11); IR (KBr) ν_{max} 3425, 2922, 1650, 1477;UV (CH_3OH): λ_{max} 430 (log ε = 4.02) nm; EI MS *m/z* (rel. abund. %): 186 (M^+, 2.19), 184 (15.9), 74.2 (100), 53 (20) Anal. calcd for $C_{10}H_{10}N_4$: C, 64.50; H, 5.41; N, 30.09. Found: C, 64.48; H, 5.40; N, 30.05.

Table 1. Structures and synthetic data of hydrazide and hydrazine derivatives.

No.	Compound ID	IUPAC names	Structures	Microwave-assisted synthesis		Conventional synthesis	
				Time (min:s)	Yield (%)	Time (h)	Yield (%)
1	Mr-I-33	Butanohydrazide		0:40	86	6	63
2	Mr-I-27	2-Methoxybenzohydrazide		1:30	95	5	89
3	Mr-I-53b	2-Nitrobenzohydrzide		1:50	92	4	68
4	Mr-I-179	2,2-diphenylacetohydrazide		0:50	70		62
5	Mr-II-10	1,2-diphenylhydrazine		1:30	92	-	-
6	Mr-II-80	1,2-dipyridinylhydrazine		1:20	78	-	-

Plasmepsin-II and cathepsin D inhibition assays

The enzyme activities of plasmepsin-II and cathepsin D were measured as described earlier [13,19] using a fluorescence resonance energy transfer (FRET)-based assay with the fluorogenic substrate DABCYL-Glu-Arg-Nle-Phe-Leu-Ser-Phe-Pro-EDANS (malaria FRET-1; AnaSpec, Fremont, CA, USA). Purified *Plasmodium falciparum* plasmepsin-II was provided by Daniel E. Goldberg, Howard Hughes Medical Institute, St. Louis, Missouri. Recombinant human liver cathepsin D was purchased from Biodesign International, Carmel, NA. The assay was performed with plasmepsin-II/cathepsin D (1.2 nM) and substrate (malaria FRET-1; 1.0 μM) in 0.1 M sodium acetate buffer pH 5, containing 10% glycerol and 0.01% Tween 20. The hydrazide and hydrazine compounds dissolved in DMSO were added in the reaction mixture before the addition of substrate. The assays were performed with 5% final concentration of DMSO. The enzyme inhibition experiments were performed (in triplicates) in 96-well plate format and readings were obtained on a Perkin Elmer LS55 fluorescence spectrometer, Waltham, Massachusetts, with an excitation and emission wavelengths of 336 and 490 nm respectively. IC_{50} values were calculated by nonlinear regression analysis from plots of percentage inhibition versus inhibitor concentrations. The enzyme assays using 'standard inhibitor' pepstatin-A (Sigma-Aldrich Inc., St. Louis, MO, USA) was performed in the same experimental manner as for hydrazide and hydrazine compounds.

Results and discussion

During this study, FlexX program [26] was utilised to predict the binding sites for compounds in the crystal structures of

plasmepsin-II [27] and cathepsin D [28]. FlexX scoring function provided docking scores and ranking of each compound of the in-house database. Of the library compounds subject to virtual screening with docking simulations, the following five were the highest scoring hydrazide and hydrazine derivatives and were selected as virtual hits, i.e. Mr-I-27, 2-methoxy benzohydrazide; Mr-I-53b, 2-nitro benzohydrazide; Mr-I-179, 2,2-diphenyl acetohydrazide; Mr-II-10, 1,2-diphenylhydrazine and Mr-II-80, 1,2-dipyridinylhydrazine (Tables I and 2).

The enzyme inhibition assays demonstrated that the above mentioned hit compounds inhibit both aspartic proteases in the low micromolar concentrations thereby supporting the docking predictions. The mean IC_{50} values of phenyl hydrazides for cathepsin D and plasmepsin-II are 1.38 ± 0.1 and 1.4 ± 0.3 μM, whereas mean IC_{50} values of hydrazines for these enzymes are 2.45 and 1.25 μM respectively (Table 1 and Figure 1). The inhibition data showed that these compounds are equally potent against both proteases. Recently, acridinyl hydrazides have been reported as potent aspartic protease inhibitors [19]. The compounds mentioned in the present study are alkyl (Mr-I-33) and phenyl hydrazides (M-I-27; Mr-I-53b; Mr-I-179) and hydrazine compounds (Mr-II-10; Mr-II-80).

Inhibition assays with butanohydrazide (Mr-I-33) were performed. The FlexX did not predict Mr-I-33 as 'good binder' with docking score worse than hydrazide compounds

Table 2. FlexX docking scores and human cathepsin D and *P. falciparum* Plasmepsin-II inhibition data of hydrazide and hydrazine derivatives.

	FlexX docking scores		IC_{50} values (in μM)	
Compound IDs	Docking in plasmepsin-II	Docking in cathepsin D	Plasmepsin-II inhibition	Cathepsin D inhibition
Mr-I-33	-11.2	-13.3	1.25	1.4
Mr-I-27	-22.0	-20.7	1.65	1.5
Mr-I-53b	-21.6	-23.9	1	1.3
Mr-I-179	-20.4	-19.2	1.6	1.35
Mr-II-10	-19.3	-21.3	1.05	2.4
Mr-II-80	-21.9	-20.9	1.45	2.5

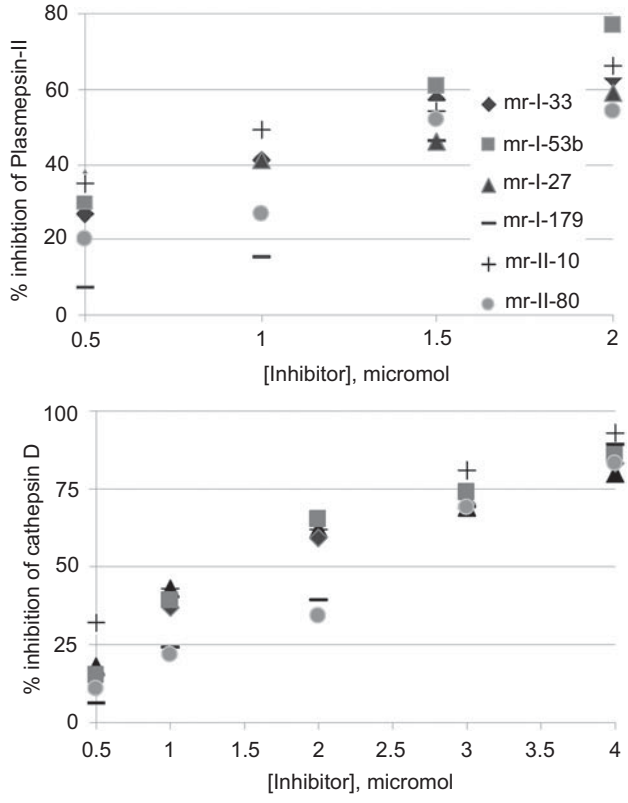


Figure 1. Enzyme inhibition plots as a function of hydrazide and hydrazine compounds concentrations.

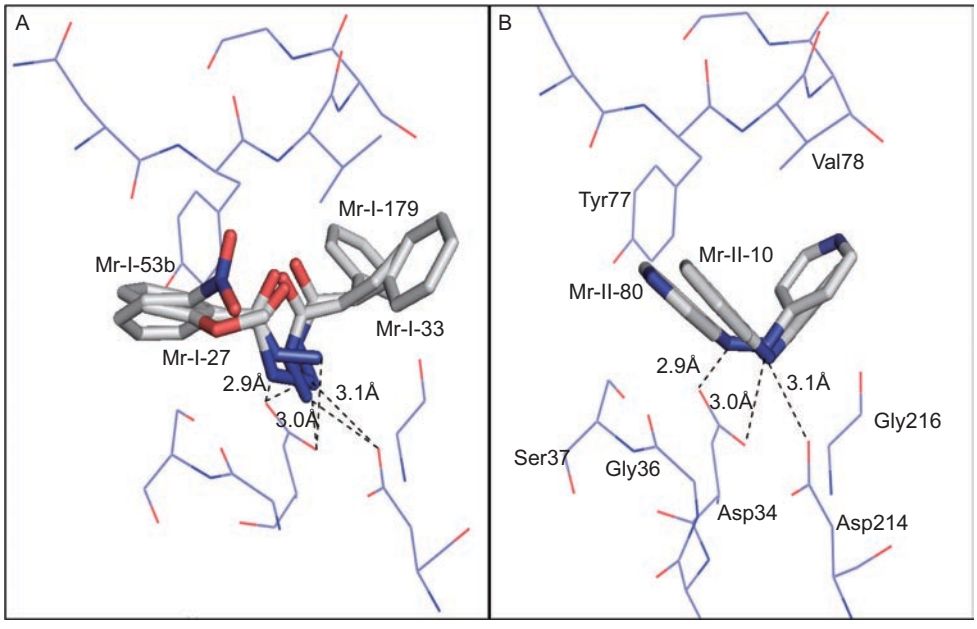


Figure 2. Interactions of (A) hydrazide and (B) hydrazine transition state isosteres with catalytic aspartates of plasmepsin-II. Electrostatic interactions of hydrazide and hydrazine moieties of inhibitors with Asp34 and Asp214 residues are shown by broken lines (distances in Angstrom).

tested in this study. However, the IC_{50} values of Mr-I-33 for plasmepsin-II and cathepsin D were estimated as 1.25 ± 0.25 and 1.4 ± 0.4 μM, respectively (Tables I and 2).

Structural analysis of the predicted binding poses of these compounds revealed important information related to the basis of inhibition. The top five binders of inhibitors were modelled into the active site of plasmepsin-II to examine interactions with protein residues. Analysis of FlexX docking solutions revealed that the enzyme-inhibitor complexes are stabilised primarily by electrostatic interactions between the side chains of catalytic aspartates, i.e. Asp34 and Asp214 and hydrazide/hydrazine moiety of ligands which operate as transition state isosteres as a central fragment [16,30] (Figure 2). Since the IC_{50} values for all tested compounds are almost the same, the inhibitory property is apparently due to the hydrazide/hydrazine isostere. Although no structure activity relationship could be found, a description of putative binding modes of these compounds would be edifying for different substitutions and heterocyclic extensions.

In the crystal structure of plasmepsin-II, two topologically similar amino-terminal and carboxy-terminal domains contact each other along the bottom of the substrate-binding cleft that contains the catalytic dyad Asp34 and Asp214. A conserved β-hairpin structure known as flap (Asn76-Thr81) lies perpendicular over the centre of the cleft and interacts with substrates/inhibitors [25]. The hydrazides are predicted to dock at the centre of the substrate-binding cleft with two binding modes. Compounds Mr-I-27 and Mr-I-53b have the same binding mode while Mr-I-179 docked differently (Figure 3A). The phenyl groups of Mr-I-27 and Mr-I-53b are placed at the same position in the cleft forming hydrophobic and aromatic interactions with S2' sub-site, structured by Met75, Leu131 and Ser37 and flap region residues Tyr77 and Val78. The ortho-nitro/methoxy side chains of these compounds are in contact with the S1' sub-site residues Tyr192 and Phe294. The ortho-nitro group of Mr-I-53b formed an H-bond with the side chain hydroxyl of Tyr192. On the other hand, the compound Mr-I-179 (with two phenyl rings termed as A and B in Figure 3A) is predicted to interact with S1-S2-S3 sub-sites residues comprising hydrophobic and aromatic side chains. Phenyl ring 'A' of this inhibitor is in contact with S2 sub-site residues Val78, Thr217, Thr221 and Ile300 whereas ring 'B' situated in the S1-S3 sub-sites structured by Ile32, Tyr77, Phe111 and Ile123. Modelling of Mr-I-33 showed superposition of its alkyl group on the biphenyl group. Hence, although the hydrazide group of these compounds has been docked at the same location as the transition state mimic; the putative binding sites of phenyl rings are in opposite directions in the active site cleft of plasmepsin-II (Figure 3A).

The compounds Mr-II-10 and Mr-II-80 docked at the centre of the substrate binding cleft where both amino nitrogens of the hydrazine moiety formed electrostatic interactions with the catalytic aspartates (Figure 2B). Phenyl and pyridinyl rings of both compounds extended towards both sides of the scissile bond forming hydrophobic and aromatic contacts with S1-S3 sub-sites residues Met15, Ile32 and Ile123; S2 sub-site residue Thr217; S1' sub-site residues Tyr192 and Ile300; and S2' sub-site and flap loop residue Tyr77, Val78 and Ser79. Moreover, both pyridinyl nitrogens of Mr-II-80 predicted to form H-bonds with Ser79 and Tyr192 side chains (Figure 3B).

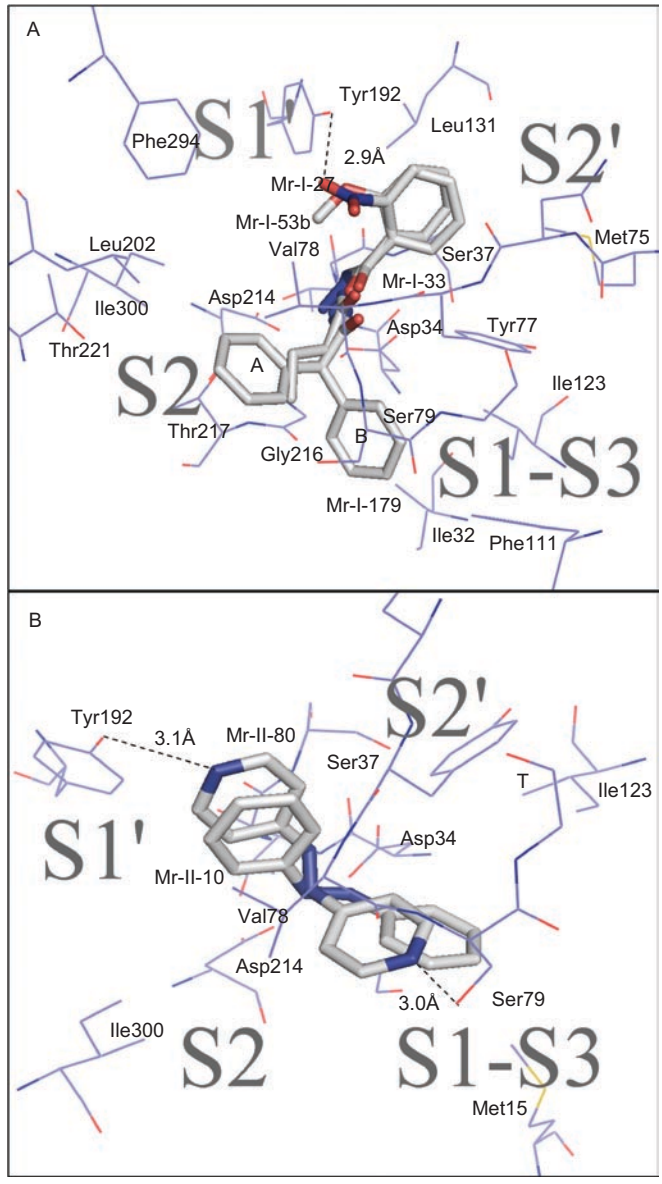


Figure 3. Docking of (A) hydrazides and (B) hydrazine compounds in the substrate-binding cleft of Plasmepsin-II. Interactions of different enzyme sub-sites with inhibitors are shown. Hydrogen bonds are indicated as broken lines (distances in Angstrom). The two phenyl rings of Mr-I-179 are denoted as 'A' and 'B'.

In conclusion, we report phenyl hydrazides/hydrazines as aspartic protease inhibitors are suggested by structure-based screen of an in-house virtual library of synthetic compounds and verified by enzyme inhibition. Different substitutions and heterocyclic extensions may lead to the discovery of novel lead compounds with better potency and selectivity towards plasmepsin-II for therapeutic intervention against malaria.

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Declaration of interest

The author reports no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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