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Bioorganic & Medicinal Chemistry Letters 18 (2008) 3011–3015
Bioorganic & Medicinal Chemistry Letters

# Identification of acridinyl hydrazides as potent aspartic protease inhibitors☆

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Received 10 September 2007; revised 5 February 2008; accepted 25 February 2008
Available online 29 February 2008

**Abstract**—We have identified acridinyl derivatives as potent aspartic protease inhibitors by virtual screening of in-house library of synthetic compounds. Enzyme inhibition experiments showed that both compounds inhibit human cathepsin D and *Plasmodium falciparum* plasmepsin-II in nanomolar ranges. The $IC_{50}$ values against cathepsin D and plasmepsin-II of compound-Nar103 were found to be 9.0 ± 2.0 and 4.0 ± 1.0 nM and of compound-Nar110 were 0.5 ± 0.05 and 0.13 ± 0.03 nM, respectively. Ligand docking predicted the binding of acridinyl derivatives at the substrate-binding cleft, where hydrazide part of the inhibitors interact with the S1–S1′ subsite residues including catalytic aspartates. The phenyl ring and acridinyl moiety of the inhibitors were predicted to interact with S2/S3 and S2′/S3′ subsite residues.


The family of aspartic proteases is characterized by an optimum pH in the acidic range and by their susceptibility to inhibition by Pepstatin, an acylated pentapeptide isolated from *Streptomyces*.[1] Aspartic proteases play important roles in several diseases such as AIDS (HIV protease),[2] neoplastic disorders (cathepsin D and E),[3,4] and malaria (plasmepsins).[5] As an intracellular aspartic protease, human cathepsin D functions primarily in protein degradation. However, over-expression of cathepsin D has been correlated with breast cancer as well as neurodegenerative conditions.[3,6] The malarial parasites (*Plasmodium* species; *P. falciparum* and *P. vivax*) produce a family of hemoglobin degrading aspartic proteases termed as plasmepsins. These enzymes have been identified as potential anti-malarial drug targets[5] and the blockage of these enzymes has been demonstrated to result in parasites death.[7a,b]

The bilobal structure of aspartic proteases is made up of nearly equivalent N- and C-terminal domains containing predominantly β-sheets with small α-helical segments. The active site located at the interface between the two domains encloses two invariant aspartates.[8] Despite the overall primary and tertiary structural homology among the members of aspartic proteases (cathepsin D exhibits ~35% sequence homology to plasmepsins); there are subtle distinctions particularly at the extended substrate-binding cleft which can accommodate at least seven residues of substrate/inhibitor.[9,10]

Here, we report acridinyl derivatives as potent aspartic protease inhibitors with the help of (a) virtual screening for the binding of >600 synthetic compounds to the substrate-binding sites of human cathepsin D and *P. falciparum* plasmepsin-II and (b) enzyme inhibition studies. The acridines and their derivatives are well known for their DNA-intercalating property.[11] They have activities against a number of solid tumors.[12–14]

*Keywords*: Virtual screening; Protease inhibitors; Cathepsin D; Plasmepsin; Acridines.

☆ Nomenclature for amino acid residues of substrate used in proteolytic enzymology is P*n*, ...P2, P1, P1′, P2′, ...P*n*′, where P1–P1′ denotes the scissile bond. Substrate/inhibitor amino acids extending away from the scissile bond toward the amino terminus are denoted P1, P2, ...P*n*, whereas those extending toward the carboxyl terminus are denoted P1′, P2′, ...P*n*′. The corresponding binding sites in the proteases are denoted as S1, S2, ...S*n* and S1′, S2′, ...S*n*′, respectively.[10]

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doi:10.1016/j.bmcl.2008.02.060

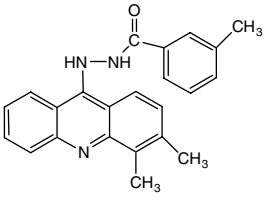
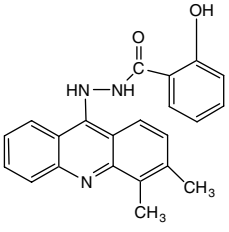
In this study, virtual screening was carried out by FlexX ligand docking software (version 2.0)[15] using crystal structural coordinates of cathepsin D and plasmepsin-II in complexed with Pepstatin-A (PDB id; 1LYB[16] and 1M43,[17] respectively). FlexX method of ligand docking involves incremental construction of ligands from smaller fragments in the cavity of a receptor. 3D models of >600 compounds in SYBYL mol2 format (from the in-house combinatorial library) were utilized for binding to the active sites of both aspartic proteases. The compounds in the virtual library represent thirty chemical scaffolds that have been synthesized in our laboratory. The ranking of the generated docking solutions is performed using a scoring function similar to that developed by Bohm[18] which estimates the free binding energy ($\Delta G$) of the protein–ligand complex. After each ligand docking run, 10 top ranking docking solutions were saved and considered for detailed analysis.

The enzyme activities of cathepsin D (BioDesign Inter., USA) and plasmepsin-II were measured as described earlier[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 Inc., USA) with an excitation and emission wavelengths of 336 and 490 nm, respectively. The assay was performed in 0.1 M Sodium acetate buffer pH 5.0, containing 10% Glycerol and 0.01% Tween 20. The acridinyl derivatives (1.0 μM) dissolved in DMSO was added in the reaction mixture before the addition of a substrate. The assays were performed with 5.0% final concentration of DMSO. The inhibition assays using Pepstatin-A (Sigma) and $IC_{50}$ values estimation in the same experimental manner as for Nar-compounds were also performed. The concentration–response curves were plotted for $IC_{50}$ determination by plotting the data as percentage inhibition as a function of inhibitor concentration. The $IC_{50}$ defined by the midpoint (50% inhibition) of the inhibitor titration. $IC_{50}$ values of Pepstatin-A against both enzymes determined by us and reported in the literature[21] are given in Table 1.

During this study, FlexX program was utilized to predict the binding sites for the compounds in the crystal structures of cathepsin D[16] and plasmepsin-II.[17] To determine potential aspartic protease inhibitors present in our in-house database, we established a criterion based on the FlexX binding scores of enzyme–Pepstatin-A complexes which is mentioned below.

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 calculated binding score for cathepsin D–Pepstatin-A (PDB id; 1LYB) and plasmepsin-II–Pepstatin-A (PDB id; 1M43) complexes are −10.0 and −18.0, respectively. Therefore, we considered those docked compounds as 'potentially good' inhibitors that were having FlexX docking scores better than −20.0. Based on this 'filtering criterion', we identified two acridinyl derivatives in the virtual library screened; that is, Nar103 [*N*′-(3,4-dimethyl-9-acridinyl)-3-methylbenzohydrazide] and Nar110

**Table 1.** FlexX docking scores and enzyme inhibition data of acridinyl derivatives and Pepstatin-A

| Acridinyl derivatives and Pepstatin-A | FlexX docking scores | | $IC_{50}$ (nM) | |
|---|---|---|---|---|
| | Docking in CD | Docking in P-II | Inhibition of CD | Inhibition of P-II |
| **Nar103**  | −27.7 ± 0.82 | −28.0 ± 1.3 | 9.0 ± 2.0 | 4.0 ± 1.0 |
| **Nar110**  | −30.3 ± 0.93 | −26.5 ± 1.5 | 0.5 ± 0.05 | 0.13 ± 0.03 |
| Pepstatin-A | −10.0 | −18.0 | 0.55 ± 0.05<br>0.64[20] | 0.68 ± 0.07<br>0.44[20] |

Docking scores of Nar-compounds are average values of 10 top ranking scores whereas values for Pepstatin-A are binding scores.

[*N*′-(3,4-dimethyl-9-acridinyl)-2-hydroxybenzohydrazide] (Table 1). Synthesis of these acridinyl derivatives has been reported elsewhere.[20] The FlexX docking scores of Nar103 and Nar110 for cathepsin D were $-27.7 \pm 0.82$ and $-30.3 \pm 0.93$ and for plasmepsin-II were $-28.0 \pm 1.3$ and $-26.5 \pm 1.5$, respectively. The consistent docking scores indicate that these compounds would inhibit both aspartic proteases with similar affinities.

Results of enzyme inhibition assays are inline with docking predictions which demonstrated that both aspartic proteases are inhibited by acridinyl compounds in the nanomolar ranges. The $IC_{50}$ values of Nar103 for cathepsin D and plasmepsin-II are $9.0 \pm 2.0$ and $4.0 \pm 1.0$ nM; whereas, $IC_{50}$ values of Nar110 for these enzymes are $0.5 \pm 0.05$ and $0.13 \pm 0.03$ nM, respectively (Table 1 and Fig. 1). Inhibition data pointed out (a) that Nar110 is several times more potent as inhibitor compared to Nar103 and (b) potencies of both inhibitors are comparable with Pepstatin-A (Table 1 and Fig. 1).

Modeling of enzyme–inhibitor interactions provided the basis of inhibition from a structural point of view. The acridinyl compounds (Nar103 and Nar110) are composed of three-ring acridinyl moiety attached to the phenyl-hydrazide side chain. The only difference in compounds Nar103 and Nar110 is the substitution in the phenyl ring; Nar103 contains a meta-methyl and Nar110 has *ortho*-hydroxyl groups. Analysis of docking solutions of acridinyl compounds revealed that the conformations of both compounds at substrate-binding clefts of cathepsin D and plasmepsin-II are strikingly different. In fact, the orientations of inhibitors docked in the substrate-binding clefts of both enzymes are approximately 'anti-parallel' to each other (Fig. 2).

Docking solutions showed that enzyme–inhibitor complexes are stabilized primarily by hydrogen bonds between hydrazide part of the inhibitors and both main chain and side chain atoms of the enzymes. Regardless of different orientations, Nar103 and Nar110 have docked at the center of the extended substrate-binding cleft with charged-hydrogen bonds between hydrazine part of the inhibitors and the residues forming S1–S1′ subsites of enzymes which includes catalytic aspartates (Asps 33 and 231 in cathepsin D; Asps 34 and 214 in plasmepsin-II) and the 'flap region' (the β-hairpin structure composed of residues 72–87; cathepsin D numberings; Figs. 3 and 4). In cathepsin D–inhibitor complexes, hydrazide group formed hydrogen bonds with main chain atoms of Gly79 and Gly233 as well as with Thr234 side chain. These amino acid residues interact with P1/P1′ and P2 side chains, respectively, of substrates/peptide inhibitors (Fig. 3b and c). Likewise, in the plasmepsin-II–inhibitor complexes, hydrazide group has been predicted to form hydrogen bonds with P1/P1′ side chain binding residues Asp34 (or Asp214), Gly36, and

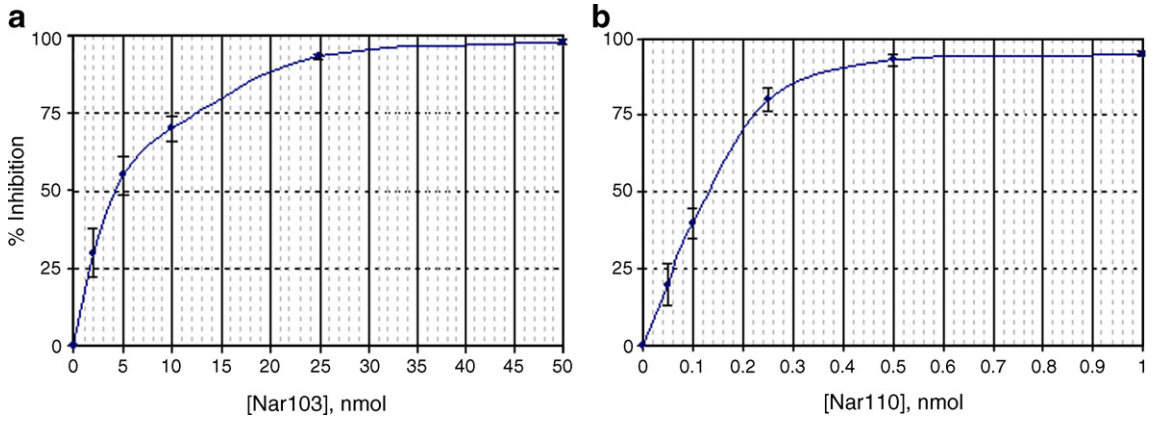



**Figure 1.** Plasmepsin-II inhibition plots as a function of (a) Nar103 and (b) Nar110 concentrations.

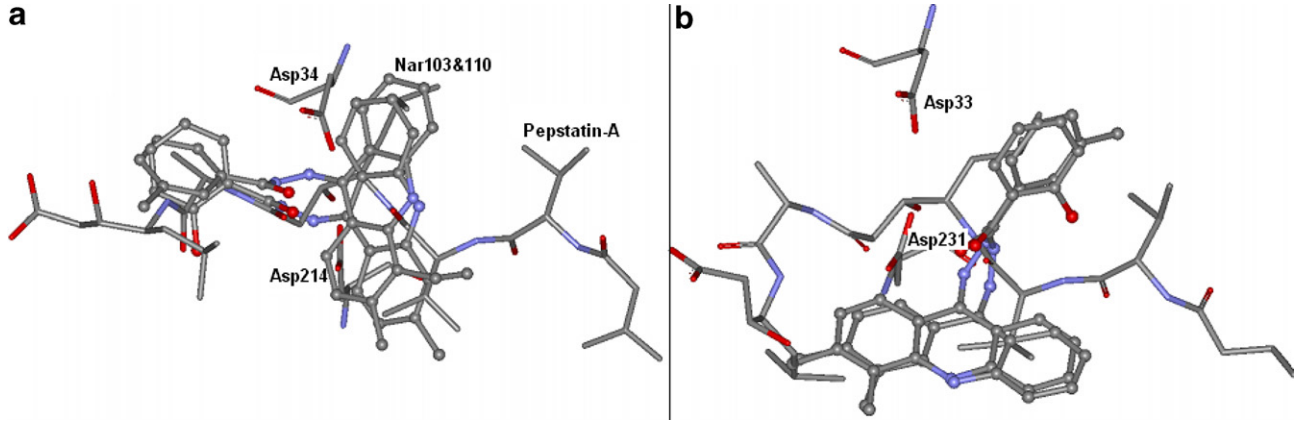



**Figure 2.** (a) Comparison of Pepstatin-A, Nar103 and Nar110 binding at the active site cleft of (a) Plasmepsin-II and (b) cathepsin D. Catalytic aspartates of both enzymes and Pepstatin-A are shown in stick and Nar-compounds in ball-n-stick representation.

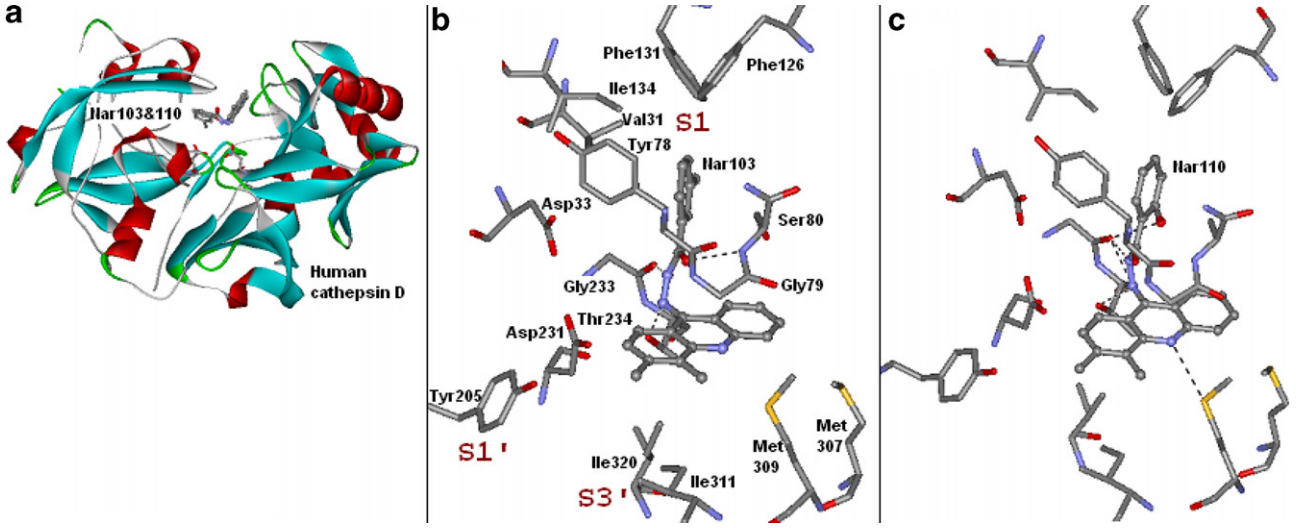



**Figure 3.** (a) Docking of acridinyl compounds in the substrate-binding cleft of cathepsin D. (a) Tertiary structure of cathepsin D is depicted in ribbon and inhibitors in ball-n-stick representations. Interaction of enzyme residues with Nar103 (b) and Nar110 (b) are shown. Enzyme residues and inhibitors are shown in stick and ball-n-stick representations, respectively. Hydrogen bonds are indicated as dashed lines. Positions of different subsites are indicated.

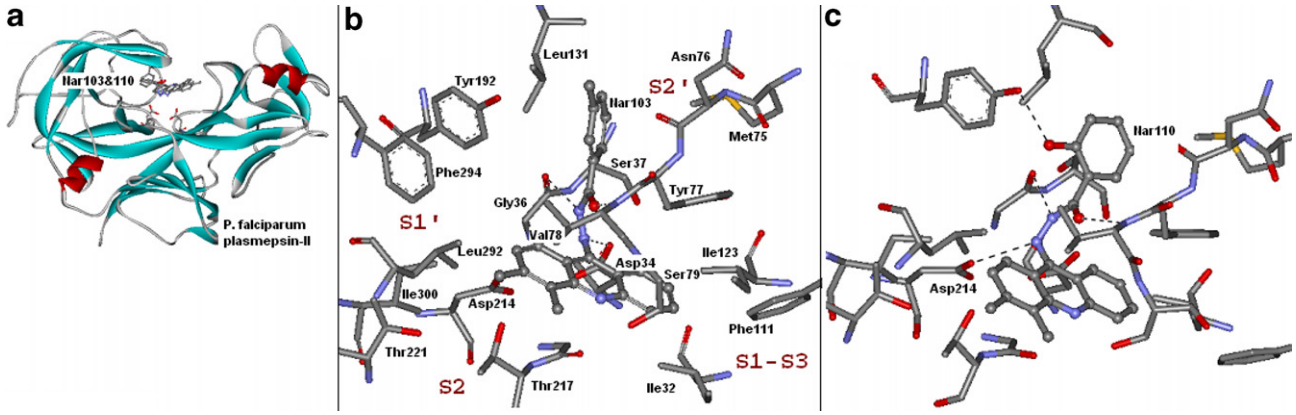



**Figure 4.** Docking of acridinyl compounds in the substrate-binding cleft of plasmepsin-II. (a) Tertiary structure of plasmepsin-II is depicted in ribbon and inhibitors in ball-n-stick representations. Interaction of enzyme residues with Nar103 (b) and Nar110 (b) are shown. Enzyme residues and inhibitors are shown in stick and ball-n-stick representations, respectively. Hydrogen bonds are indicated as dashed lines. Positions of different subsites are indicated.

Val78 (Fig. 4b and c). In cathepsin D–inhibitor complexes, acridinyl compounds are not predicted to form Hbond with any of the catalytic aspartates, although the distance between the side chain carboxylate of Asp231 and one of the two amino groups of hydrazide is 4.0 Å.

Docking of hydrazine part at S1–S1′ subsites placed the inhibitors in cathepsin D binding site such that the phenyl ring has formed hydrophobic and aromatic interactions with Val31, Ile134, Phe131, and Phe126 residues which form S1 subsite; whereas acridinyl moiety is in contact with S1′–S3′ subsite residues, that is, Tyr205, Ile320, and Ile311 and nearby residues including Met307 and Met309 (Fig. 3b and c). On the contrary, in plasmepsin-II–inhibitor complexes, phenyl ring has been predicted to be accommodated in the S2′ subsite structured by Met75-Asn76-Tyr77, and Leu131 and acridinyl moiety are predicted to interact with S1–S2–S3 subsites (Fig. 4b and c). In plasmepsin-II, S1–S3 subsites are made up of residues Ile32, Tyr77, Ile111, and Ile123, whereas Threonines at positions 217 and 221 form S2 subsite. Therefore, the acridinyl moiety was modeled to bind at opposite sites of the scissile bond in the substrate-binding cleft of both proteases; a phenomenon termed as anti-parallel orientation of inhibitors (i.e., in cathepsin D complex acridinyl moiety is in contact with S1′–S3′ subsites whereas in plasmepsin-II it is situated in S1–S3 subsites).

The *ortho*-hydroxyl group of Nar110 was predicted to form additional Hbond in both enzymes. In the cathepsin D complex, the hydroxyl group interacts with the main chain of Gly233 (Fig. 3c), whereas in the plasmepsin-II complex it forms Hbond with Tyr192 side chain (Fig. 4c). Moreover, in cathepsin D complex, Nar110 can form another polar interaction with Met309 (Fig. 3c). These additional interactions provide structural basis for enhanced inhibiting property of Nar110 (Table 1).

In summary, we applied virtual screening by using an in-house library of synthetic compounds to identify novel aspartic protease inhibitors effective in nanomolar range. Different substitutions and heterocyclic ring extensions for the acridinyl ring system and phenyl group may (a) improve the potency of these compounds and (b) provide selective aspartic protease inhibitors with therapeutic potential.

## Acknowledgments

We are thankful to (i) Dr. Daniel E. Goldberg (Howard Hughes Medical Institute, Department of Medicine and Molecular Biology, Washington University School of Medicine, St. Louis, Missouri, USA) for providing purified *P. falciparum* plasmepsin-II and (ii) Asif Iqbal for help during docking studies.

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