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New inhibitors of proteolytic enzymes Cathepsin D and Plasmepsin II

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Abstract: Aspartic proteases plays very important role in posttranslational processing of proteins and many of them are important for organism viability. Here we report five different inhibitor compounds against two Aspartic proteases which are Catephsin D and Plasmepsin II. Cathepsin D and Plasmepsin II are drug targets for treatment of breast cancer and malaria respectively. It is also noted in the present study that the docking results of these compounds were good which ranges from -23.87 to -43.39 in case of Cathepsin D while from -22.31 to -31.68 in case of Plasmepsin II. Aspartic proteases Plasmepsin II catalyzes the initial step in the breakdown of hemoglobin by the Plasmodium falciparum, which causes a big percentage of malaria deaths. In this study, two compounds showed inhibition for Plasmepsin II and four compounds showed inhibition for Cathepsin D among five compounds 7223, 11994, 13161, 28427, 32656 (Compounds received from National Cancer Institute, New York, USA for this research). The compounds were further experimentally confirmed by enzyme inhibition studies after docking analysis. These compounds can be use in combination therapy to increase the resistance as antimalarial drugs and for breast cancer treatment.

Keywords: Plasmepsin II, cathepsin D, malaria, enzyme inhibition, aspartic proteases.
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INTRODUCTION

Malaria is still a devastating global problem, each year Malaria affects 350-500 million people and causes 1 million deaths[1]. Malaria is a complex disease caused by protozoan parasites belonging to the genus *Plasmodium*, Four species account for almost all human infections *(P. falciparum, P. vivax, P. malariae and P. ovale)*, of which *P. falciparum* is responsible for most severe disease[2]. The parasite degrades most of the host cell hemoglobin during the morphologically separate phases inside the erythrocyte[3]. Since the parasite has a limited capacity to synthesize amino acids de novo or to take them up exogenously, the hemoglobin is thought to be broken down to provide amino acids for its growth and maturation[4,5]. Plasmepsins and cathepsin D comprise a family of aspartic proteases. Plasmepsins are known to play a key role in the breakdown of hemoglobin during intraerythrocytic stage of plasmodial infection and hence are considered as potential anti-malarial drug target[6,7,8]. Plasmepsin I and Plasmepsin II both are capable of cleaving indentured hemoglobin between phenylalanine and leucine residues are located in a conserved domain, which is involved in stabilizing the overall structure of hemoglobin[9,10]. In addition, a dipeptidyl aminopeptidase (DPAP) activity has been identified within the food vacuole[11,12]. Proposed pathways have been reported for hemoglobin degradation in P. *falciparum* by Plasmepsin into smaller peptides and hemozoin (malarial pigment)[13].

Most of the aspartic proteases, including the Plasmepsins and cathepsin D are the member of the pepsin family found only in the eukaryotes, which together with the viral retropepsin, including the HIV-I protease, constitute a clan[14]. Aspartic proteases play important role in several diseases such as AIDS (HIV protease)[15], neoplastic disorders (Cathepsin D and E)[16,17], Malaria (Plasmepsins) etc. Virtual screening of Acridinyl hydrazide compounds [18] hydrazine and hydrazide derivatives[19] Piperidine derivatives[20] and Sulfomyl benzamide derivatives[21] followed by enzyme inhibition assays showed that these compounds inhibited human catepsin D and plasmodium flciparanuum plasmepsin II in different low ranges. In the present study, five inhibitors compounds were selected as proposed inhibitors for plasmepsin II and cathepsin D. The compounds as inhibitor were further confirmed by enzyme inhibition studies and Docking analysis. Results infer the inhibition against Plasmepsin II and Cathepsin D which have shown that these compounds requires further research for antimalarial and anticancer drugs.

MATERIALS AND METHODS

Enzyme inhibition assay

The activity of Plasmepsin II and cathepsin D were measured as described earlier[22] using a total reaction volume of 100µl with buffer. Purified Plasmodium falciparum Plasmepsin-II (Provided by Daniel Goldberg, USA) was used. The concentration of Plasmepsin II and Cathepsin D (Purchased from Biodesign Intrnational, USA) were (1.2nm), incubated separately in the reaction buffer at room temperature for 40 minutes. The activity was measured in fluorescence resonance energy transfer (FRET) based assay with flurogenic Substrate Malaria Fret-I used was DABCYL-Glu-Arg-Nle-Phe-Leu-Ser-Phe-Pro-EDANS (Purchased from Anaspec, USA). The reaction was initiated by using 3µM substrate and hydrolysis was recorded as increase in fluorescence intensity over a 10 minutes interval during which rate increased in a linear

fashion. The standard inhibitor Pepstatin A (Purchased from sigma inc. USA) with a concentration of 1µM was used for the experiment. The assay was performed with plasmepsin-II (1.2 nm) and substrate (malaria FRET-1;1.0 uM) in 0.1 M Sodium acetate buffer pH 5.0 containing 10% Glycerol and 0.01% Tween 20. The compounds (proposed inhibitor) dissolved in DMSO stock solution was added in the reaction mixture before the addition of substrate. Assays were performed in 5.0% concentration of DMSO to ensure dissolution of inhibitors and initially the inhibitors concentration is 1µM. The assays were performed with 5.0 % final concentration of DMSO. The enzyme inhibition experiment were performed (in triplicate) in 96 well plate format and readings were obtained on a Perkin Elmer LS55 Fluorescenece spectrometer with an excitation and emission wavelengths of 336 and 490 nm respectively with 430 emission cut off filter. Percentage of inhibition was calculated. The enzyme assays using 'standard inhibition' Pepstatin-A (Sigma Inc, USA) was performed in the same experimental manner as for compounds (proposed inhibitor).

Molecular docking

FlexX ligand docking software (version 2.0)[23] was utilized for docking using crystal structural coordinates of Plasmepsin-II[8] in complexed with Pepstatin A and Catepsin D (PDB id; 1LYB and 1M43 respectively). FlexX method of ligand docking involves incremental construction of ligands from smaller fragments in the cavity of a receptor (Potein). The ranking of the generated docking solutions is performed using a scoring function similar to that developed by Bohm[24], which estimates the free binding energy (ΔG) of protein-ligand complex. After each ligand docking run, 10 top ranking docking solutions were saved and considered for detailed analysis.

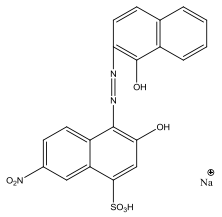
DISCUSSION

In this study we identified five compounds as for inhibition studies of Plasmepsin II and Cathepsin D (Fig 1). As a result two compounds apparently act out of these five compounds, as active inhibitors compounds (11994 and 13161) 1,1'-(1,2-Propanediyl)diurea and ([3-(Carbamoylamino)-4-hydroxy-5-iodophenyl]) arsonic acid have shown 17% and 14% inhibition with Plasmepsin II (Table 1) whereas other compound showed very weak binding, against aspartic protease Plasmepsin-II from *P. falciparum* at 1.0 µM concentration, Whereas standard inhibitor Pepstatin A at the same concentration showed 100% inhibition. The structure-activity relationship of 11994 (1,1'-(1,2-Propanediyl)diurea) proposed inhibitors compound exhibited good percentage of inhibition (i.e. 17%) (Table 1 and Fig2), has shown that the amino group and methyl plays a key role in Plasmepsin-II inhibition. On the other hand compound 13161 ([3-(Carbamoylamino)-4-hydroxy-5-iodophenyl])arsonic acid has shown 14% inhibition due to the presence of Carbamoylamino at position 3. These results indicated that compounds to be 'good binders' with scores comparable to Pepstatin A, the well-known aspartic protease inhibitor. The analysis of FlexX docking solutions revealed that the enzyme-inhibitor complexes were stabilised by different interactions. It was also determined experimentally the good docking score -23.75 and -22.31 of compound 11994 and 13161 respectively (Table 1). We identified four compounds showed inhibition with Cathepsin D in 35%, 42%, 59% and 14%. Compound 13161 showed 59% inhibition for Cathepsin D due to presence of carbamoylamino group at position 3 while compound 11994 showed 42% inhibition which is also good inhibition due to presence of amino and methyl group.

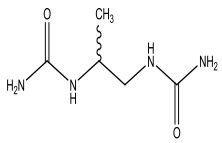
Table 1: FlexX docking scores and human Cathepsin D and P. *falciparum* Plasmepsin II inhibition data of compounds.

No.	Docking scores In Plasmepsin II	Docking scores in Cathepsin D	Inhibition Plasmepsin II (%)	Inhibition Cathepsin D (%)
			100	100
1	-31.62	-23.87	---	35
2	-23.75	-25.9	17	42
3	-22.31	-28.01	14	59
4	-24.39	-24.63	---	---
5	-36.5	-43.39	---	14

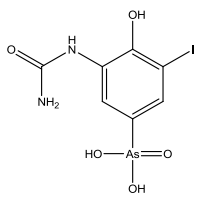
We have been determined previously that benzimidazole compounds were active inhibitors of P. *falciparum* plasmepsin II and human cathepsin D by virtual screening of an internal library of synthetic compounds. This was confirmed by enzyme inhibition studies that gave IC (50) values in the low micro molar range (2-48µM)[25]. We have also identified experimentally that hydrazide and hydrazine derivatives as novel aspartic protease inhibitors by virtual screening of an in-house virtual library of synthetic compounds using FlexX, followed by enzyme inhibition. These compounds inhibited human Cathepsin D and P. *falciparum* Plasmepsin II with low micro molar concentrations (IC50=1-2.5µM).



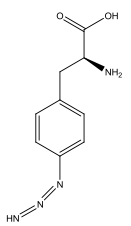
Compound 1(7223) 1-Naphthalene sulfonic acid,3-hydroxy-4-[2-(1-hydroxy-2-naphthalenyl) diazenyl]-



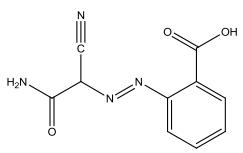
Compound 2 (11994) 1,1/ -Propane-1,2-diyldiurea



Compound 3 (13161) 3-((Aminocarbonyl) amino)-4-hydroxy 5-iodophenyl arsonic acid



Compound 5 (32656) 4-Azido-L-Phenylalanine



Compound 4 (28427) 2-{ (E)-[(1R)-2-Amino-1-cyano-2-oxoethyl] diazenyl}benzoic acid

Figure 1: Compounds used for docking and enzyme inhibition assay.

Modelling studies with Plasmepsin II predicted binding of ligands, where hydrazide/hydrazine parts of the inhibitors acted as the transition state imitate by forming electrostatic interactions with catalytic aspartates[19]. Furthermore we have identified acridinyl derivatives as proposed 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 P. *falciparum* Plasmepsin II in nano molar ranges[18]. We have also identified Piperidine derivatives which are reported to exhibit a variety of pharmacological activities. In this article, synthesis and aspartic protease inhibitory activity of three nitrophenacyl derivatives of N-methyl-4-hydroxy piperidine are reported and enzyme assays showed that the attachment of a nitro group in the benzene ring plays an important role in the inhibition of Plasmepsin II of *P. falciparum*[20]. We have also identified that some sulfomyl benzamide derivatives showed good inhibition in low ranges[21].

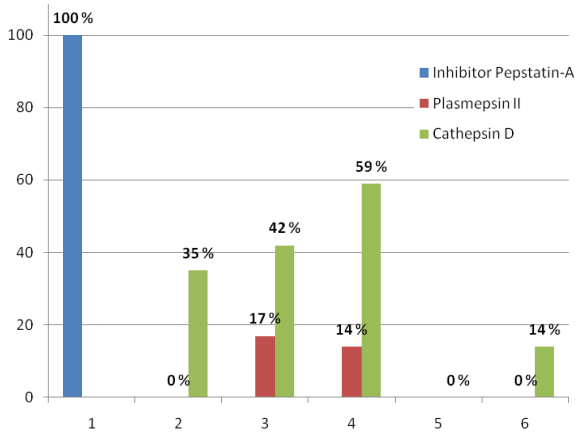


Figure 2: Comparative inhibition of enzymes Plasmepsin II and Cathepsin D with Pepstatin A as standard inhibitor.

CONCLUSION

In conclusion, we have recognized active inhibitory compounds that produce rapid and irreversible *P. falciparum* killing of developing parasites. Our results revealed that selected compounds would target almost all life-cycle phase of malaria parasites. The study of these five compounds, two proposed inhibitors compounds (11994 and 13161) 1,1'-(1,2-Propanediyl)diurea and ([3-(Carbamoylamino)-4-hydroxy-5-iodophenyl]) arsonic acid have identified as an active inhibitory compounds. Proposed inhibitor compound revealed that the position of amino group in 1,1'-(1,2-Propanediyl)diurea (11994) and iodophenyl or Carbamoylamino ([3(Carbamoylamino)-4-hydroxy-5-iodophenyl]) arsonic acid (13161) played significant role in the inhibition of Plasmepsin II exhibited 17% and 14% inhibition while in Cathepsin D have shown high percentage 42% and 59% of inhibition due to presence of amino, methyl and carbamoyl amino group, respectively, at a concentration of 1.0µM in comparison to the 100%

inhibition by the standard. Compound 7223 have shown 35% of inhibition against Cathepsin D while no inhibition was observed in case of Plasmepsin II whereas others compounds were very weak binder in case of Plasmepsin II. It is proposed that related derivatives of substituted piperidine may exhibit interesting anti-malarial, anti-breast cancer and analgesic activity.

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