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Identification of novel class of falcipain-2 inhibitors as potential antimalarial agents

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ABSTRACT

Falcipain-2 is a papain family cysteine protease and an emerging antimalarial drug target. A pseudo-tripeptide scaffold I was designed using in silico screening tools and the three dimensional structures of falcipain-2, falcipain-3, and papain. This scaffold was investigated at four positions, T_1, T_2, T_3, and T'_3, with various targeted substitutions to understand the structure–activity relationships. Inhibitor synthesis was accomplished by first obtaining the appropriate dipeptide precursors with common structural components. The pyrrolidine moiety introduced interesting rotamers in a number of synthesized molecules, which was confirmed using high-temperature ^{1}H NMR spectroscopy. Among the synthesized compounds, **61**, **62**, and **66** inhibited falcipain-2 activity with inhibition constants (K_i) of 1.8 ± 1.1, 0.2 ± 0.1 and 7.0 ± 2.3 μM, respectively. A group of molecules with a pyrrolidine moiety at the T_2 position (**68**, **70**, **71**, **72**, and **73**) also potently inhibited falcipain-2 activity (K_i = 0.4 ± 0.1, 2.5 ± 0.5, 3.3 ± 1.1, 7.5 ± 1.9, and 4.6 ± 0.7 μM, respectively). Overall, compound **74** exhibited potent anti-parasitic activity (IC_{50} = 0.9 ± 0.1 μM), corresponding with its inhibitory activity against falcipain-2, with a K_i of 1.1 ± 0.1 μM. Compounds **62** and **67** inhibited the growth of the drug resistant parasite Dd2 with better efficacy, and compound **74** exhibited a 7- to 12-fold higher potency against Dd2 and MCamp isolates, than the laboratory strain (3D7). These data suggest that this novel series of compounds should be further investigated as potential antimalarial agents.

1. Introduction

Malaria is a parasitic disease caused by *Plasmodia*, and continues to be a major global health problem. According to World Malaria Report 2012, 154–289 million malaria cases were reported worldwide in 2010, causing up to 836,000 deaths.[1] Malaria is potentially a preventable and curable infection, if appropriate treatment strategies, including novel drugs, are available.[2] Malaria prevention is further complicated by the resistance of mosquitos to insecticides, which has been reported in 64 countries, raising concerns about vector control measures.[1] To date, drug resistance to artemisinins, the key compounds in artemisinin combination therapies (ACTs), has been detected in at least 4 countries in Southeast Asia. Overall, drug resistance to nearly all existing antimalarial agents is of great concern;[3–5] development of novel antimalarial agents is of wide interest. There is also a need to identify novel molecular targets for the development of clinical agents yet. Recently, we identified several potent inhibitors of orotidine-5′-monophosphate decarboxylase (ODCase).[6–8] Among these compounds, 6-iodo-uridine exhibited potential for further preclinical development as an antimalarial agent.[9,10] ODCase catalyzes the decarboxylation of orotidine-5′-monophosphate into uridine monophosphate (UMP), and is an essential enzyme for the synthesis of pyrimidine nucleotides in *Plasmodia* species.[11,12]

Plasmodia have developed a specialized lysosome-like organelle, the food vacuole, which has an acidic environment. This organelle contains proteases with hemoglobinase activity, including plasmepsins and falcipains.[13–17] Among these proteases, falcipains have been investigated as potential drug targets. Falcipain-2, which shares 95% amino acid sequence homology with

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falcipain-2′, is involved in the degradation of hemoglobin and erythrocyte-membrane skeletal proteins, leading to host cell rupture and the release of the mature merozoites.[18,19] Falcipain-2 is a cysteine protease, and its biochemical mechanism of proteolysis is similar to that of other cysteine proteases.[12,20] Inhibitors that target falcipain-2 will block hemoglobin hydrolysis in the parasite food vacuole, thereby inhibiting parasite development.[21–23] Thus, we explored falcipain-2 as a potential drug target for the design of antimalarials.

It has been suggested that members of several classes of plasmodia proteases have redundancy in function. For example, Goldberg's group has shown that plasmepsins I, II, and IV and histo-aspartic protease have overlapping function as hemoglobin-degradation in food vacuole.[24] These studies may suggest that proteases of similar classes and with redundant functions are to be inhibited together using more than one inhibitor to completely block a specific pathway and kill the parasites. Similarly, *Plasmodium falciparum* has two copies of falcipain-2 (viz. falcipain-2 and falcipain-2′);[16,17] Falcipain 2/2′ are shown to be major hemoglobinase in the parasite; however falcipain-2 and -2′ proteins are nearly identical in sequence (97.5% amino acid identity between the mature proteases), therefore the inhibitors of falcipain-2 are expected to inhibit falciapin-2″ as well.

Falcipain-2 inhibitors have previously been investigated using peptidomimetic approaches.[25–27] In vitro screening of chemical libraries and medicinal chemistry approaches have also been undertaken to identify potent inhibitors of falcipain-2 activity.[23,28,29] There have been a number of efforts towards development of inhibitors against falcipain-2, most of which are peptide-based inhibitors.[30] However, peptidyl-based inhibitors, in spite of their ability to inhibit enzymatic activity of falcipains at very low nanomolar range, have low utility as therapeutic agents because of their susceptibility to proteolytic degradation and their poor absorption through cell membranes. O'Neill and co-workers designed and synthesized peptidomimetic falcipain-2/3 inhibitors with antimalarial activity in the micromolar range.[31] Other peptidyl reversible inhibitors of falcipains include peptidyl aldehyde and β-ketoamide derivatives developed by Rosenthal and co-workers.[32] Several groups have designed non-peptidic small inhibitors of falcipains using computer based designing and virtual screening studies; however, most of these inhibited falcipain-2 in micromolar range concentrations.[33,34]

Recently, vinyl esters were designed using molecular modeling, and their binding to falcipain-2 was evaluated.[35] These inhibitors of falcipain-2 exhibited antiplasmodial activities (IC_{50}) in the low micromolar range, due to the ester core, which was capable of trapping the active site Cys through a covalent bond and functioning as a Michael acceptor.[36,37] Rizzi et al. designed peptidomimetics based on the interaction of cystatin with the active site of falcipain-2.[38] These compounds specifically inhibited falcipain-2 activity and parasite growth at low micromolar range.[39] In addition, natural products and their derivatives are potential inhibitors of falcipain-2.[39,40]

As a part of our antimalarial drug discovery program, we envisioned utilizing structure-based tools to design novel scaffolds for non-covalent inhibitors of falcipain-2 as potential antimalarial drugs. Here, we report the design, identification, and evaluation of a chemically diverse set of compounds as potential falcipain-2 inhibitors, paving the way for non-covalent antimalarial therapeutic agents.

2. Results

Our overall approach was to identify initial hits, re-design the core structural elements, and build a novel library of compounds as potential inhibitors of falcipain-2. Thus, the process was initiated with the three-dimensional structures of falcipain-2.

2.1. In silico screening and identification of initial hits

The three dimensional structures of falcipain-2, falcipain-3 and papain (RCSB codes: 1YVB, 3BPF, 3BPM, and 3E1Z) were initially investigated to understand the similarities and differences between these three proteases.[14,41,42] Overall, the catalytic site of these cysteine proteases is composed of hydrophobic pockets in close proximity to the catalytic residues (Cys25 and His159) (Fig. 1). Using the three-dimensional structure of falcipain-2 from *P. falciparum* (RCSB code: 1YVB)[14] as a template, the pharmacophore features were selected for in silico screening of commercial chemical compounds libraries (Fig. 1). The in silico analysis used the UNITY database search program and Surflex-Dock suite.[43–46] A small molecule library containing more than 250,000 commercially available compounds was screened against the active site of falcipain-2 employing UNITY software. Initial hits were selected based on features that were complimentary to the pharmacophore features. Criteria for a hit included a match with two of the three hydrophobic features in the S1′, S1, and S2 pockets (yellow spheres in Fig. 1), and at least one additional feature, such as a hydrogen bond donor, hydrogen bond acceptor, cationic feature, or other hydrophobic features. During the in silico analysis, surface constraint and flex search were employed to allow the torsions in the inhibitor molecules to be flexible and to remove any sterically prohibited poses in the active site of falcipain-2. Further, the filter for Lipinski's rule of five was turned off; at this early stage of screen, Lipinski's rule of five would not be a major determining factor for the compounds since they would be redesigned anyway (vide infra). Following the search criteria, 2084 initial hits were obtained and subjected to Surflex-Dock™-based docking in the active site of falcipain-2. These poses were scored using the empirical scoring function in Surflex-Dock, and the top 200 compounds, with their corresponding pose based on the best-fit score,

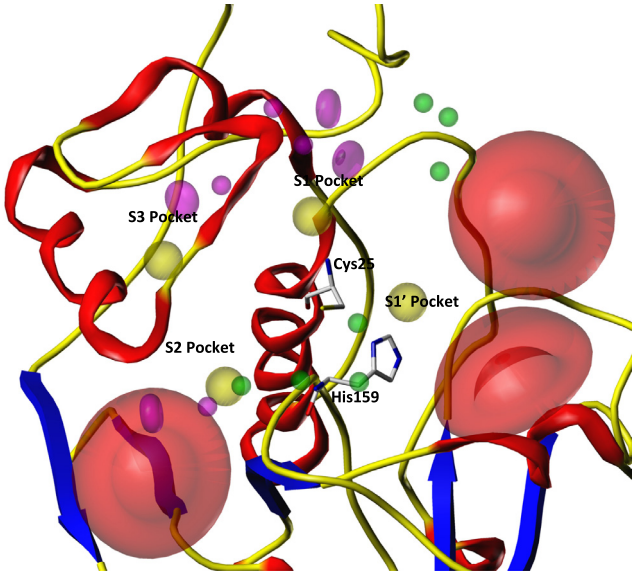

Figure 1. Molecular features of the falcipain-2 binding site used for the in silico screen. Falcipain-2 is rendered according to the secondary structural features (β-sheets: blue, α-helices: red, loops and turns: yellow) using its three-dimensional structure (PDB ID: 1YVB). Catalytic residues Cys25 and His159 are shown in capped-stick representation. Yellow spheres indicate hydrophobic features, red caps indicate the cationic regions on the enzyme, green spheres represent hydrogen bond donor sites on the enzyme, and magenta spheres show the hydrogen bond acceptor regions on the enzyme.