Journal of Molecular Graphics and Modelling 64 (2016) 121–130

Contents lists available at ScienceDirect

Journal of Molecular Graphics and Modelling

journal homepage: www.elsevier.com/locate/JMGM

In silico study of subtilisin-like protease 1 (SUB1) from different *Plasmodium* species in complex with peptidyl-difluorostatones and characterization of potent pan-SUB1 inhibitors

Simone Brogi[a,b,c], Simone Giovani[a,b,c], Margherita Brindisi[a,b,c], Sandra Gemma[a,b,c,*], Ettore Novellino[a,d], Giuseppe Campiani[a,b,c,*], Michael J. Blackman[e], Stefania Butini[a,b,c]

[a] *European Research Centre for Drug Discovery and Development (NatSynDrugs), University of Siena, via Aldo Moro 2, 53100, Siena, Italy*
[b] *Dipartimento di Biotecnologie, Chimica e Farmacia, University of Siena, via Aldo Moro 2, 53100, Siena, Italy*
[c] *Centro Interuniversitario di Ricerche sulla Malaria (CIRM), University of Perugia, Perugia, Italy*
[d] *Dipartimento di Farmacia, University of Naples Federico II, Via D. Montesano 49, 80131, Naples, Italy*
[e] *Division of Parasitology, MRC National Institute for Medical Research, Mill Hill, London NW7 1AA, UK*

ARTICLE INFO

Article history:
Received 23 September 2015
Received in revised form 4 December 2015
Accepted 16 January 2016
Available online 19 January 2016

Keywords:
Homology modeling
Molecular docking
Subtilisin-like protease
Malaria
Difluorostatone-based inhibitors
Pharmacophore modeling

ABSTRACT

Plasmodium falciparum subtilisin-like protease 1 (SUB1) is a novel target for the development of innovative antimalarials. We recently described the first potent difluorostatone-based inhibitors of the enzyme ((4*S*)-(*N*-((*N*-acetyl-L-lysyl)-L-isoleucyl-L-threonyl-L-alanyl)-2,2-difluoro-3-oxo-4-aminopentanoyl)glycine (**1**) and (4*S*)-(*N*-((*N*-acetyl-L-isoleucyl)-L-threonyl-L-alanylamino)-2,2-difluoro-3-oxo-4-aminopentanoyl)glycine (**2**)). As a continuation of our efforts towards the definition of the molecular determinants of enzyme-inhibitor interaction, we herein propose the first comprehensive computational investigation of the SUB1 catalytic core from six different *Plasmodium* species, using homology modeling and molecular docking approaches. Investigation of the differences in the binding sites as well as the interactions of our inhibitors **1**,**2** with all SUB1 orthologues, allowed us to highlight the structurally relevant regions of the enzyme that could be targeted for developing pan-SUB1 inhibitors. According to our in silico predictions, compounds **1,2** have been demonstrated to be potent inhibitors of SUB1 from all three major clinically relevant *Plasmodium* species (*P. falciparum*, *P. vivax*, and *P. knowlesi*). We next derived multiple structure-based pharmacophore models that were combined in an inclusive pan-SUB1 pharmacophore (SUB1-PHA). This latter was validated by applying in silico methods, showing that it may be useful for the future development of potent antimalarial agents.

1. Introduction

Several *Plasmodium* species cause malaria in humans. Among them, *P. falciparum* is the etiological agent of the most deadly form of malaria. As a consequence, much attention has been devoted to the search for novel drugs for treating *P. falciparum* infections. *P. vivax* has historically been considered relatively avirulent compared to *P. falciparum*, so development of new chemotherapies against *P. vivax* has been relatively neglected [1]. However, morbidity due to *P. vivax* infection contributes to most of the social and economic burden of malaria outside Africa, and infections are complicated by relapses that can occur as much as 2 years following primary infection. In addition, it is now recognized that human infections by the zoonotic pathogen *P. knowlesi* are widespread in areas of South-East Asia [2]. Malaria caused by *P. knowlesi* can be severe and often fatal, so development of diagnostic tools and specific chemotherapies is urgently required. The *P. falciparum* subtilisin-like protease 1 (PfSUB1) is a serine protease which plays a key role in both egress of merozoites from infected erythrocytes and priming the developing merozoites for invasion of new erythrocytes [3–6]. This enzyme also plays an essential role in the development and egress of hepatic merozoites [7,8]. Drugs based on inhibitors of SUB1 could overcome the issue of resistance to chloroquine and several other currently available antimalarials, as well as the emerging resistance of *P. falciparum* to artemisinins [9,10]. Moreover, the same approach can be exploited for the development of new chemotherapeutics against *P. vivax* and *P. knowlesi*,

* Corresponding authors at: European Research Centre for Drug Discovery and Development (NatSynDrugs), University of Siena, via Aldo Moro 2, 53100 Siena, Italy. Fax: +39 0577 234254.
E-mail addresses: gemma@unisi.it (S. Gemma), campiani@unisi.it (G. Campiani).

http://dx.doi.org/10.1016/j.jmgm.2016.01.005
1093-3263/

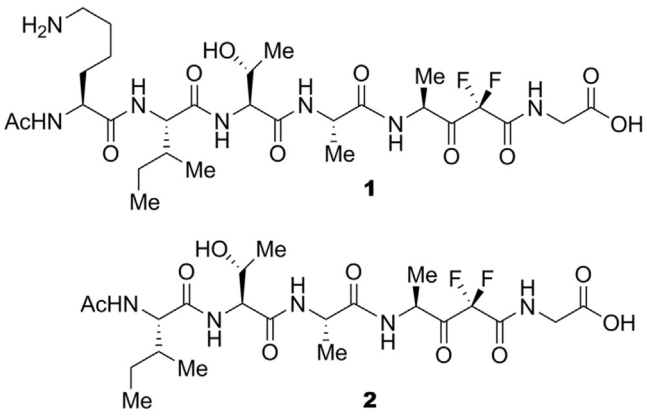

Fig. 1. Difluorostatone-based inhibitors **1** (4*S*)-(*N*-((*N*-acetyl-L-lysyl)-L-isoleucyl-L-threonyl-L-alanyl)-2,2-difluoro-3-oxo-4-aminopentanoyl)glycine) and **2** ((4*S*)-(*N*-((*N*-acetyl-L-isoleucyl)-L-threonyl-L-alanyl amino)-2,2-difluoro-3-oxo-4-aminopentanoyl)glycine).

which express orthologous SUB1 enzymes [11]. It is worth noticing that, differently from other drug targets in malaria in which rapid selection of mutants was observed (e.g., cytochrome b targeted by atovaquone [12–15]), PfSUB1 represents a particularly excellent drug target because the likelihood of simultaneous compensatory mutations in both the protease active site and the substrate cleavage sites that might result in drug resistance is low. Endogenous substrates of PfSUB1 have been investigated and some studies analyzing in silico the interaction of peptides based on endogenous sequences with PfSUB1 and PvSUB1 have been previously in depth analyzed [6,11,16–18]. Few PfSUB1 or PvSUB1 inhibitors have been described to date [11,16,19,20]. We recently developed the first potent difluorostatone-based inhibitors (**1** and **2**, Fig. 1) of PfSUB1 [21] and we later in depth analyzed the structure-activity relationships (SARs) for this series of compounds [22]. In this context, our plan is the development of pan-inhibitors that may represent an innovative approach for treating infections caused by the human malaria pathogens.

Towards this ambitious aim, we decided to dissect the similarities between the SUB1 orthologues from all three major human malaria pathogens in order to ascertain the likelihood of developing a single inhibitor for all three enzymes. We here describe the development of a homology model of the active core of PkSUB1, and the comparison of the structural features of its binding site cleft with the crystal structures of PvSUB1 [23] and PfSUB1 [24]. To expand the scope of our investigation, we also developed homology models of SUB1 from *P. berghei*, *P. chabaudi*, and *P. yoelii*, three *Plasmodium* species that specifically infect rodents and are routinely used for testing antimalarial compounds in vivo. It has been previously demonstrated that the PbSUB1 active site is significantly different from that of PfSUB1 [11], so we extended our investigation to PcSUB1 and PySUB1. Moreover we have updated the PbSUB1 model previously described [11] using the experimentally solved PfSUB1 and PvSUB1 crystal structures as templates. The overall objective of the work here described is the analysis of the binding mode of our difluorostatone-based inhibitors to the six orthologous enzymes in order to: (i) assess the feasibility of a pan-inhibitor active against all three clinically relevant parasites; (ii) derive and validate a pharmacophore model to be used as design tool for the synthesis of pan-inhibitors and/or in a virtual screening campaign to identify novel chemical entities able to inhibit SUB1s, and (iii) verify the possibility of using the rodent malarial parasites as models to assess the efficacy of inhibitors designed on the basis of the human clinically relevant parasites.

2. Materials and methods

2.1. Difluorostatone-based inhibitors

Compounds **1** and **2** were synthesized following a previously described synthetic procedure [21] and were tested against Pv- and Pk-SUB1 as described in Paragraph 2.7.

2.2. Computational details

All the calculations performed in this work were carried out on three Cooler Master Centurion 5 (Intel Core2 Quad CPU Q6600 @ 2.40 GHz; Intel Core i5–2400CPU @ 3.10 GHz Quad; Intel Core i5–2500CPU @ 3.30 GHz Quad) with Ubuntu 10.04 LTS (long-term support) operating system running Maestro 9.2 (Schrödinger, LLC, New York, NY, 2011) and GOLD software (version 5.2, Cambridge Crystallographic Data Center, UK, 2013).

2.3. Homology modeling of SUB1

The sequence of SUB1s were taken in fasta format from UniProtKB [25] (PbSUB1 UniprotKB code: Q4YVE1; PySUB1 UniprotKB code: Q7RGL7; PcSUb1 UniprotKB code: Q4XWG6; PkSUB1 UniprotKB code: B3L6J4). The SUB1 homology models were built using the recently published PfSUB1 and PvSUB1 crystal structures (PDB codes: 4LVN and 4TR2, respectively) [23,24], applying multiple template-based alignment as previously reported by us [21,26,27]. The sequence identity found by Prime during the template selection step for PbSUB1 were 4LVN 64%, 4TR2 58%; for PySUB1 were 4LVN 64%, 4TR2 58%; for PcSUB1 were 4LVN 67%, 4TR2 57%; and for PkSUB1 were 4LVN 75%, 4TR2 80%. In order to model the core catalytic domain of SUB1 orthologues Prime software [28] was used. Homology models were generated using the above-mentioned templates. These templates aligned to each query sequence were used for "Comparative Modeling" methods implemented in Prime. Since Prime offers several ways to build a model, we specified in the "build structure step" the method used for aligning multiple templates of all the SUB1 structures. Consensus model option was employed to build the model; this option allowed us to take into account all the previously selected templates since the model was built as an average of all templates. Each predicted SUB1 model for each different *Plasmodium* species was refined by means of Prime software by side-chain optimization and loop refinement. Further structure optimization was carried out using the MacroModel (MacroModel, version 9.9, Schrödinger, LLC, New York, NY, 2011) application implemented in Maestro suite 2011 using the Optimized Potentials for Liquid Simulations-all atom (OPLS-AA) force field 2005 with 10,000 maximum iterations and 0.001 as convergence threshold using PRCG method [29,30].

The quality of modelled proteins was assessed by means of Ramachandran plots generated by the RAMPAGE webserver (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php accessed date May 2015) [31]. For all the modelled SUB1, around 95% of the protein residues lie in the favoured region of the plot, around 4% lie in the additional allowed region and less than 0.6% (amino acids not involved in the binding site) of the residues were located in the disallowed regions. As previously found for the PfSUB1 homology model [21] the other generated SUB1 3D structures displayed a satisfactory and similar stereochemical quality. Accordingly, the results of the RAMPAGE webserver revealed that over 99% of the residues of our refined SUB1 models sit in the allowed regions of the Ramachandran Plot. This value is higher than the cut-off value (96.1%) defined for the most reliable models [32]. Consequently, the stereochemical quality of our SUB1 homology models was