**Substrate derived peptidic ketoamides as inhibitors of the malarial protease PfSUB1**

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| ARTICLE INFO | ABSTRACT |
| Article history:  Received  Revised  Accepted  Available online | Peptidic ketoamides have been developed as inhibitors of the malarial protease PfSUB1. The design of inhibitors was based on the best known endogenous PfSUB1 substrate sequence, leading to compounds with low micromolar to submicromolar inhibitory activity. SAR studies were performed indicating the requirement of an aspartate mimicking the P1’ substituent and optimal P1-P4 length of the non-prime part. The importance of each of the P1-P4 amino acid side chains was investigated, revealing crucial interactions and size limitations. |
| Keywords:  Ketoamide  Serine proteaase  PfSUB1  Malaria  Peptides |

Malaria is a devastating disease with an estimated 207 million cases and 627 thousand lethal outcomes in 2012.1 The threat of emerging malaria strains that are resistant to all currently used drugs has motivated the search for novel drug targets to interrupt the life cycle of the parasite.2-4 As such, the malarial enzyme PfSUB1 (Plasmodium falciparum subtilisin like serine protease-1) has attracted high interest. This enzyme is released into the parasitophorous vacuole where it activates a family of protease-like proteins called SERA, leading to rupture of the vacuole and host cell membrane.5-8 Inhibition of PfSUB1 is a promising approach to block propagation of a malarial infection by preventing the egress of merozoites from the red blood cell. However, the development of small molecule inhibitors for PfSUB1 has turned out to be a difficult task. Screening of a collection of >170,000 low molecular weight compounds resulted in the identification of a structurally complex natural product **1** (MRT 12113) as a submicromolar PfSUB1 inhibitor (Figure 1).6 An independent HTS screening campaign led to the identification of a quinolylhydrazone derivative **2** as a micromolar PfSUB1 inhibitor (Figure 1).9 In addition, targeted serine protease inhibitor library screening has revealed chloroisocoumarin derivative **3** (JCP104) as a micromolar PfSUB1 inhibitor (Figure 1).7 Virtual HTS screening against homology models of SUB1 from Plasmodium vivax provided **4** and **5** as micromolar hits also active against PfSUB1 (Figure 1).10



**Figure 1.** PfSUB1 inhibitors previously identified by screening of compound libraries.

Recently, we reported the first hexapeptidic ketoamide **6** inhibitor of PfSUB1 that was designed based on the best enzyme substrate sequence - KITAQ/DDEES (Figure 2).11,12 Incorporation of ketoamide warhead instead of scissile bond ensures the binding to the protease by forming a reversible covalent bond between the keto group of the inhibitor and the serine of the catalytic triad.13-17 Additional non-covalent interactions are formed at the prime- and non-prime side of the enzyme, mimicking those of a natural substrate.



**Figure 2.** Rationally designed peptidic ketoamide **6** as PfSUB1 inhibitor based on substrate sequence KITAQ/DDEES

Here we report the synthesis and systematic SAR investigation using molecular modeling of peptidic ketoamides as PfSUB1 inhibitors to explore the crucial interactions that determine their activity.

The synthesis of peptidic ketoamides involved peptides **7a-f** and amino alcohols **8a-m** as principal building blocks (Scheme 1). Peptides **7a-f** were prepared by solution phase peptide synthesis (see SI for details). Amino alcohols **8** were prepared by using different methods depending on a substitution pattern. Azidolysis of epoxide **28** was used as a key step to prepare amino alcohols **8a-f** (Scheme 2).18 The Henry reaction of readily accessible nitro compounds **29** and **30** with ethyl glyoxylate was used to make amino alcohols **8g-j** (Scheme 3).19,20



**Scheme 1.** Synthesis of peptidic ketoamides **9-27** from peptides **7a-f** and amino alcohols **8a-m**. (a) EDC, HOBt, DIEA, DMF;(b) if TBS protected, TBAF, THF; (c) DMP, NMP, (d) TFA:DCM (1:1) or HCl in Et2O.



**Scheme 2**. Synthesis of amino alcohols **8a-f** using epoxide **29** azidolysis. (a) NaN3, Cu(NO3)\*3H2O, H2O; (b) *O-*protected amino acid or *N-*protected amide of amino acid, EDC, HOBt, DIEA, DCM or DMF; (c) anhydrous SnCl2, TEA, MeOH. Cumyl = ****-dimethylbenzyl.



**Scheme 3.** Synthesis of amino alcohols **8g-j** using Henry reaction. (a) ethyl glyoxalate, TEA toluene; (b) Ra-Ni, H2, EtOH; (c) Cbz-Cl, 1 M NaHCO3; (d) NaOH, dioxane; (e) *β*-AlaOt-Bu\*HCl, EDC, HOBt, DIEA, DCM; (f) H2, Pd/C, EtOH. For compounds **8h,i** (g) TFA:DCM (1:1); (h) cumylamine or dimethylamine, EDC, HOBt, DIEA, DCM.



**Scheme 4.** Synthesis of amino alcohols **8k-m** using homologation of aldehydes **31** with MAC reagent or isocyanate. (a) 2-(TBSO)malononitrile, *β*-AlaOt-Bu\*HCl, 4-PP, Et2O; (b) *tert*-butyl-3-isocyanopropanoate, TFA, pyridine, DCM; (c) H2, Pd/C, ethanol.

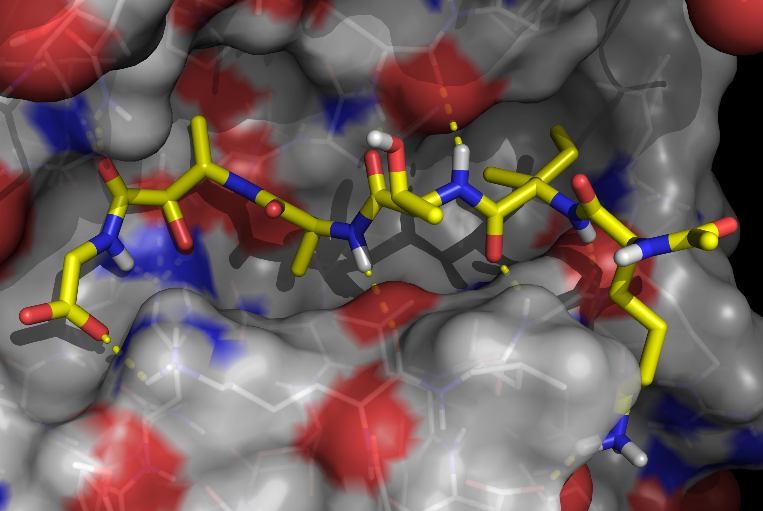
The reaction of amino aldehydes **31a**21 and **31b** with MAC reagent and amine gave O-TBS protected amino alcohols **8k,l**, after Cbz-deprotection (Scheme 4).22 Passerini reaction of aldehyde **31c** with isocyanopropionate followed by deprotection provided amino alcohol **8m** (Scheme 4).23

Studies of prime-side substituents demonstrated that the optimal linker to the terminal carboxyl group resembles an aspartic acid residue (Table 1, compounds **6**, **9-10**). Introduction of a shorter or longer chain resulted in decreased activity. The requirement for a carboxylic group at the prime side terminus was confirmed by amide analogue **11** that showed a considerable decrease in inhibitory activity. This is also in line with the tendency for the preference of acidic prime site residues of authentic PfSUB1 substrates.8

**Table 1.** Modifications of P1’ substituent



|  |  |  |
| --- | --- | --- |
| Cmpd. | R1(P1’) | IC50 (µM) |
| **6** |  | 1.0 |
| **9** |  | 3.1 |
| **10** |  | 10 |
| **11** |  | 38 |

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**Figure 3.** Binding mode of compound **6** in PfSUB1 (PDB code: 4LVO) obtained by docking calculations using the GOLD docking program. Hydrogen bonds between the compound and the PfSUB1 structure are indicated by yellow dashed lines

The SAR of prime side components in PfSUB1 inhibitors was investigated next, guided by results obtained from molecular dynamics (MD) simulations of PfSUB1 bound to different substrates24 and the binding mode of compound **6** in the active site of PfSUB1obtained by docking calculations using the X-ray structure of the enzyme (Figure 3).25 MD simulation studies indicated that the P5 substituent might not provide an important contribution to the binding free energy.24 On this basis, compound **12** (Table 2) lacking a P5 lysine residue was synthesised and found to display about two times lower enzymatic activity compared to the original inhibitor **6**. However, the structure of **12** was considerably simplified, facilitating synthesis of analogues and further SAR studies.

**Table 2.** Deletion of the P5 amino acid and importance of P2-P4 amino acids in peptidic ketoamide PfSUB1 inhibitors.



|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Cmpd. | R3, R4 (P2) | R5 (P3) | R6 (P4) | IC50 (µM) |
| **12** | Me, H |  |  | 2.5±0.1 |
| **13** | Me, Me |  |  | >50 |
| **14** | CH2CH2 |  |  | >50 |
| **15** | H, H |  |  | 0.9±0.2 |
| **16** | Me, H | Me |  | 30±1 |
| **17** | H, H |  | Me | >50 |

Based on modeling of substrate binding24 and molecular docking of **6** (Figure 3), the P2 position appears restricted to small amino acids such as alanine or glycine. Other small acids such as -Me-alanine and 1-aminocyclopropylcarboxylic acid were incorporated instead of alanine, but this resulted in inactive compounds (Table 2, compounds **13** and **14**). In contrast, incorporation of glycine instead of alanine in P2 position was beneficial leading to compound **15** with slightly increased inhibitory activity compared to analogue **12** (Table 2). The importance of the P3 and P4 residues was investigated by substitution with alanine (Table 2, compounds **16** and **17**) According to substrate binding and inhibitor **6** docking studies, the side chain of P3 points away from the binding site towards the solvent. The decreased inhibitory activity of **16** can be explained by an increased solvatation penalty resulting from replacing the polar threonine side chain with a methyl group. On the other hand, the isoleucine in P4 is well accommodated by the hydrophobic S4 pocket. Changing its side chain to a methyl group likely results in loss of favourable Van der Waals interactions, explaining the low potency of compound **17**.

To investigate the importance of the P1 substituent, we initially attempted preparation of compound **18** (Figure 4) which bears a glutamine side chain in the P1 position as in the preferred KITAQ/DDEES substrate sequence. Unfortunately we discovered that the glutamine side chain is not compatible with the electrophilic keto group. The characteristic proton signal of the ketomide was shifted up-field after protecting groups in a precursor were cleaved off, indicating the disappearance of an adjacent carbonyl group The structurally simplified model compound **33** was prepared from precursor **32** (Scheme 5).Compound **33** was investigated by 2D NMR using TOCSY and HMBC methods which revealed that the equilibrium is shifted to a cyclic tautomer of **33b** and only ~5% of **33a** was present in the solution. The formation of cyclic tautomer is expected also in the case of compound **18** which may explain its unexpectedly low enzyme inhibitory activity.



**Figure 4.** ketoamide **18** bearing a glutamine P1 side chain as an analogue of the KITAQ substrate prime side sequence.



**Scheme 5.** Synthesis of model ketoamide **33** with a deprotected glutamine side chain (a)TFA:DCM (1:1).

A limited series of pentapeptidic ketoamide analogues **19-27** was prepared to explore the importance of the P1 side chain (Table 3). These studies showed that the S1 sub pocket can accommodate only relatively small substituents which is line with MD simulations.24 Only the ethyl group in the P1 position in compound **25** produced a similar level of inhibitory activity as the methyl analogue **15**. Compound **23** bearing an *N,N’*-disubstituted glutamine side chain and compound **24** with its glutamine side chain constrained in the lactam cycle were prepared as analogues not able to form a cyclic tautomers; however these were inactive as PfSUB1 inhibitors. Given the preference for polar P1 side-chains in the substrates of PfSUB1,8,11,24 further work is needed to develop the analogues of P1 amino acids which could provide additional binding interactions and are compatible with electrophilic ketoamide warhead.

**Table 3** Investigation of P1 side chains.



|  |  |  |  |
| --- | --- | --- | --- |
| Cmpd. | R2 (P1) | R3 (P2) | IC50 (*µ*M) |
| **19** | Bn | Me | 100 |
| **20** | *n-*Pr | Me | 20±3 |
| **21** | Ph | Me | >50 |
| **22** |  | Me | 5.0±0.1 | |
| **23** |  | Me | >50 |
| **24** |  | Me | >50 |
| **25** | Et | H | 1.2±0.1 |
| **26** | -CH2OH | H | 4.0±0.1 |
| **27** | *n-*Pr | H | 4.5±0.1 |

In summary, an optimal PfSUB1 substrate sequence can be exploited to design active inhibitors by incorporating an -ketoamide moiety in place of the scissile bond. The inhibitory activity is determined by serine trap (ketoamide) and enzyme binding interactions provided by P1’-P1-P4 amino acids. These studies showed a preference for a charged -aminoalanine in the P1’ position and a preference for small size substituents limited to an ethyl group in the P1 position. In the P2 position, glycine is preferred over alanine. Threonine in P3 appears toprovide a solvent-exposed hydrophilic side chain that minimizes solvatation penalty, whereas isoleucine in P4 appears to make crucial hydrophopbic interactions important for inhibitory activity. Preliminary side chain optimization studies have resulted in pentapeptidic ketoamide **15** which is structurally less complex and less polar compared to the hexapeptidic analogue **6,** but that retains the same level of PfSUB1 inhibitory activity (IC50=0.9 uM). This makes it among the best currently available PfSUB1 inhibitors. The inhibitors are useful tool compounds to study the role of PfSUB1 as a potential antimalarial target and can be used as leads for antimalarial drug discovery.

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Supplementary Material

Synthesis description and spectroscopic characterization of compounds **9-27** and their intermediates. Description of enzymatic assay and docking procedure. Copies of 2D NMR spectra.

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