Bioorganic & Medicinal Chemistry Letters 22 (2012) 5317–5321

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Quinolylhydrazones as novel inhibitors of *Plasmodium falciparum* serine protease PfSUB1

Sandra Gemma [a,b,c], Simone Giovani [a,b,c], Margherita Brindisi [a,b,c], Pierangela Tripaldi [a,b,c], Simone Brogi [a,b,c], Luisa Savini [a,b,c], Isabella Fiorini [a,b,c], Ettore Novellino [a,d], Stefania Butini [a,b,c], Giuseppe Campiani [a,b,c,*], Maria Penzo [e], Michael J. Blackman [e]

[a] *European Research Centre for Drug Discovery and Development (NatSynDrugs), University of Siena, Via A. Moro, 53100 Siena, Italy*
[b] *Dipartimento Farmaco Chimico Tecnologico, University of Siena, Via A. Moro, 53100 Siena, Italy*
[c] *Centro Interuniversitario di Ricerche sulla Malaria (CIRM), University of Torino, Torino, Italy*
[d] *Dipartimento di Chimica Farmaceutica e Tossicologica, 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, UK*

ARTICLE INFO

Article history:
Received 21 May 2012
Revised 7 June 2012
Accepted 10 June 2012
Available online 23 June 2012

Keywords:
Malaria
Egress
Serine protease inhibitors
PfSUB1
Hydrazones

ABSTRACT

Plasmodium falciparum subtilisin-like protease 1 (PfSUB1) is a serine protease that plays key roles in the egress of the parasite from red blood cells and in preparing the released merozoites for the subsequent invasion of new erythrocytes. The development of potent and selective PfSUB1 inhibitors could pave the way to the discovery of potential antimalarial drugs endowed with an innovative mode of action and consequently able to overcome the current problems of resistance to established chemotherapies. Through the screening of a proprietary library of compounds against PfSUB1, we identified hydrazone **2** as a hit compound. Here we report a preliminary investigation of the structure–activity relationships for a class of PfSUB1 inhibitors related to our identified hit.

Malaria is one of the major causes of morbidity and mortality in the world.[1] Malaria is endemic in many low-income countries and despite considerable scientific advances and the intensification of elimination campaigns, the goal of eradicating malaria is still elusive.[2–4] According to WHO data, 225 million malaria cases and approximately 781,000 deaths globally were reported in 2009.[1] The continued emergence of drug-resistant parasites compromises efforts to control the disease.[5] This dramatic scenario is further worsened by the fact that currently only a single class of drugs (artemisinins) is active against drug-resistant parasites.[6,7] Indeed, besides the recently approval of arterolane in India,[8,9] no new class of antimalarials has been introduced into clinical practise for decades, and very few prospective clinical candidates with novel mechanisms of action are under development.[10,11]

Human malaria is caused by one of five species of *Plasmodium* parasites, of which *Plasmodium falciparum* is the most deadly. The life cycle of *P. falciparum* is shared between an *Anopheline* mosquito vector and the human host and involves several phases of development. During the erythrocytic phase, occurring inside the human body, the parasite lives inside red blood cells (RBCs), encircled by a membrane forming the parasitophorous vacuole (PV). The parasite matures within the PV through several developmental stages until the resulting schizont eventually divides to form 16 to 32 mature merozoites. Merozoites are the invasive forms of the parasite and are released upon rupture of the RBC, in a process known as egress.[12] Egress and subsequent invasion of RBCs by the released merozoites are highly regulated events, and over the last decade it has been demonstrated that several proteases are mediators of both egress and invasion.[12–16]

The activity of a subtilisin-like serine protease (PfSUB1) has been demonstrated to be essential in the egress of invasive parasites from the host RBC.[17–20] In the context of a drug discovery process both invasion and egress appear excellent processes to be targeted not only because they are critical for parasite survival but also because many components of the invasion and/or egress machinery are conserved across the phylum. Anti-invasion and/or anti-egress drugs developed for malarial species may therefore prove to be applicable to others. As a result, PfSUB1 represents a highly promising potential drug target for the development of inhibitors useful in the fight against malaria. In previous work,[18] the PfSUB1 inhibitor MRT12113 (**1**, Fig. 1) was found to block egress at high concentrations, whilst allowing egress of invasion-defective merozoites at lower concentrations. Other than **1**, the

* Corresponding author.
E-mail address: campiani@unisi.it (G. Campiani).

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http://dx.doi.org/10.1016/j.bmcl.2012.06.023

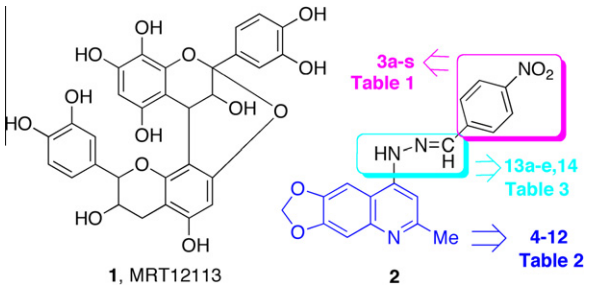


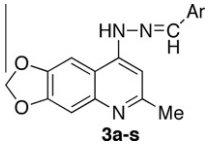
Figure 1. Reference compound **1**, PfSUB1 inhibitor identified during the screening (**2**) and site of structural modifications at the hydrazone scaffold.

only inhibitors of PfSUB1 identified to date are a biotinylated chloroisocoumarin[19] or very recently developed peptidyl α-ketoamides[21] based on authentic substrates of the protease.

In the search for new small molecule PfSUB1 inhibitors, we performed a high-throughput screening (HTS) campaign of a proprietary library of compounds (~450 peptidic and non peptidic compounds) against PfSUB1, leading to the identification of the quinolylhydrazone **2** (Fig. 1) as a hit compound. The library was formed by several chemotypes belonging to different classes of biologically active compounds. Our hit compound is characterised by good predicted log*P* and log*D* values, molecular weight lower than 350, and most importantly low cost of the synthetic pathway for its preparation allowing fast exploration of the structure-activity relationships (SARs). However, we had concerns due to the presence of the nitro group. Here we present a preliminary exploration of the SARs for this hydrazone class of compounds as PfSUB1 inhibitors through the synthesis of several structural analogues bearing modifications at the aromatic aryl substituent (compounds **3a–s**, Table 1), at the quinoline moiety (compounds **4–12**, Table 2) and at the hydrazone linker (compounds **13a–e**, **14**, Table 3).

In order to explore the effect of the substituent at the arylhydrazone moiety on the potency of PfSUB1 inhibition, we replaced the nitro group of **2** with several electron withdrawing and/or H-bond donor/acceptor groups (as in **3a–s**, Table 1). Compounds **2** and **3a–s** were synthesized as described in Scheme 1. Accordingly, hydrazine **15** was reacted with equimolar amounts of the suitable carboxaldehydes **16** and **17a–p** in refluxing EtOH to furnish final compounds **2** and **3a–p**, respectively (Scheme 1 and Table 1).[22] Aldehydes **16**, **17a–c,f,h–p** were commercially available while aldehydes **17d,e,g** were prepared following described procedures.[23–25] Compound **3a**, containing a methyl ester in the *p*-position of the aromatic ring, was hydrolyzed with 20% aqueous NaOH in a 1:2 MeOH/THF mixture to give **3q** in high yield. Aldehyde derivative **3r** was obtained from **3b** through acidic hydrolysis and finally, amidoxime **3s** was obtained after exposure of the cyano-derivative **3p** to hydroxylamine hydrochloride in the presence of potassium *tert*-butoxide.[26] Hydrazones **4–12** and **13a,b** (Tables 2 and 3) were synthesized as described in Scheme 2. 4-Quinolinhydrazines **18a–f**, 4-pyridylhydrazine **19**, and 2-benzimidazolylhydrazine **20**, prepared following described procedures,[27,28] were coupled with *p*-nitrobenzaldehyde **16**, *p*-cyanobenzaldehyde **17p** or with *p*-nitrophenylmethyl ketone to afford the final hydrazones **4–12** and **13a** in reasonable yields. For the synthesis of derivative **13b**, hydrazine **15** was firstly reduced to the corresponding aniline derivative **21** using a mixture of nickel chloride and sodium borohydride in methanol,[29] the resulting compound **21** was then coupled with *p*-nitrobenzaldehyde **16** in refluxing ethanol to afford **13b**. Finally, hydrazides **13c–e** and **14** were synthesized as described in Scheme 3, by coupling the appropriate carboxylic acids **22a–c** with hydrazines **15** and **20**, in the presence of DCC and HOBt.

Table 1
PfSUB1 inhibitory activity of compounds **2** and **3a–s**[a]



3a-s

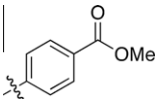
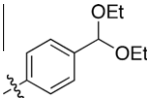
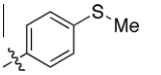
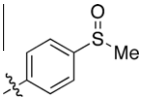
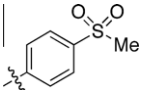
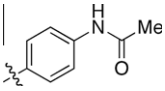
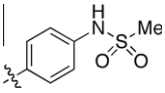
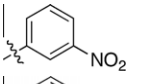
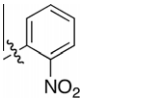
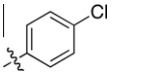
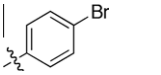
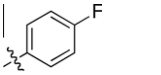
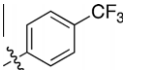
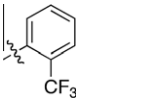
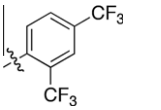
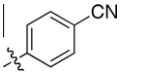
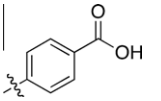
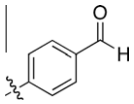
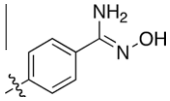
Compd	Ar	% Residual hydrolytic activity at 50 μM ± S.D.	IC_{50} (μM)
3a		85.0 ± 7.7	–
3b		75.6 ± 6.5	–
3c		77.5 ± 7.3	–
3d		92.4 ± 3.1	–
3e		76.4 ± 2.1	–
3f		70.9 ± 4.2	–
3g		72.2 ± 8.5	–
3h		97.2 ± 4.2	–
3i		38.6 ± 21.3	$40 < IC_{50} < 50$
3j[30]		77.9 ± 2.7	–
3k[30]		78.2 ± 1.2	–
3l[30,31]		97.0 ± 4.4	–
3m		86.1 ± 4.8	–
3n		100 ± 6	–
3o		62.1 ± 15	–
3p		29.2 ± 3.3	40
3q		75.5 ± 0.6	–

Table 1 (*continued*)

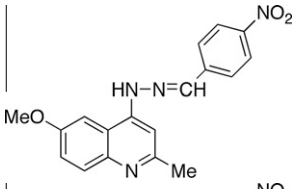
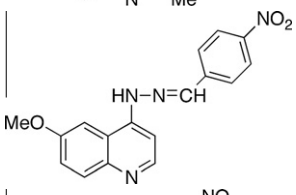
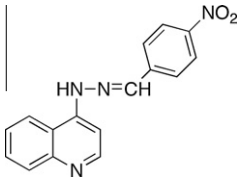
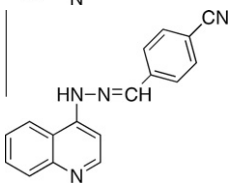
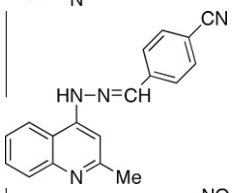
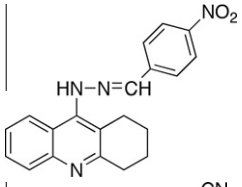
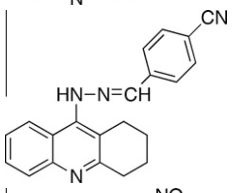
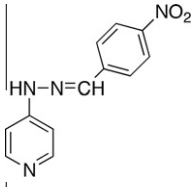
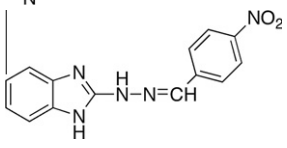
Compd	Ar	% Residual hydrolytic activity at 50 μM ± S.D.	IC_{50} (μM)
3r		86.5 ± 3.4	–
3s		73.9 ± 1.6	–
2	–	27.2 ± 2.5	20

[a] Physical and spectroscopic data for compounds **2** and **3j–l** are consistent with those reported in Ref. [30,31].

The inhibitory activity of the synthesized hydrazones and hydrazides against PfSUB1 was assessed using a previously described fluorimetric assay.[21] PfSUB1 inhibitory potencies are expressed as percent residual hydrolytic activity after incubation of PfSUB1 with the tested compounds. Compounds showing levels of inhibition greater than 50% at the starting concentration of 50 μM were further examined at a range of lower concentrations. Results are reported in Tables 1–3.

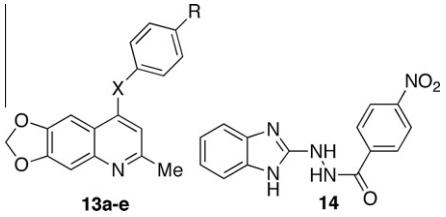
The inhibition activity data given in Table 1 clearly highlight that H-bond acceptor/donor groups such as **3a–c**, **3e–g**, and **3q–s** are not beneficial substituents at the para position. This points out that the importance of the nitro group for the activity of **2** probably relies on its high electron withdrawing properties. This observation is further supported by the fact that the *o*-nitro derivative **3i** has a slightly lower inhibitory activity against the enzyme compared with the *p*-nitro analogue **2**, while the *m*-nitro derivative **3h** is inactive at the tested concentration. With the aim of replacing the potentially toxic nitro group with safer substituents, we synthesized a series of derivatives bearing various electron withdrawing groups such as halogens (**3j–l**), trifluoromethyl (**3m–o**) and the cyano group **3p**. Interestingly, introduction of the cyano group resulted in a compound with only slightly reduced potency with respect to **2**. The observed potencies are in agreement with the electron withdrawing potencies of the groups (NO_2 > CN > -SO_2Me > hal). Next we examined the role of the quinoline substituents on the inhibitory potency and we discovered that the substitution pattern of the quinoline ring can sensibly modulate the inhibitory potency (hydrazones **4–11**, Table 2). From our screening, compound **4**, in which the fused dioxolane ring was replaced by a 6-methoxy group, was slightly less potent than **2**, and removal of the 2-methyl group (**5**) further reduced potency. The low activity of compounds **6–8** is due to the lack of ether function at the quinoline ring (**5** *vs* **6**). The pyridyl-hydrazone **11** was also not active at the tested concentration while the 1,2,3,4-tetrahydroacridinyl-derivative **9** showed a minimal level of inhibition. A visual inspection of the Novartis in vitro antimalarial screening dataset[34,35] revealed that compound **12**, containing a *p*-nitrophenylhydrazone moiety had antimalarial activity that might be due to a capacity to inhibit PfSUB1. We therefore synthesized and tested compound **12**, which showed some degree of inhibition activity against PfSUB1, although this latter activity might not be related to its antiplasmodial potency (EC_{50} = 0.81 and 0.74 μM against 3D7 and W2,[34,35] respectively). Finally, in this preliminary evaluation of the SARs we investigated the role of the hydrazone linker (**13a–e** and **14**, Table 3) on PfSUB1 inhibitory potency. Methylation of the benzylidene moiety (as in **13a**), or conversion of the hydrazone moiety to an imine (as in **13b**) or to a hydrazide (as in **13c–e**) always resulted in a drastic decrease in inhibitory potency. Only in the case of the benzimidazole derivatives, both hydrazone **12** and hydrazide **14** showed similar inhibition potency. The strict

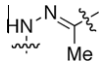
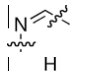
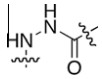
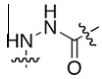
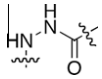
Table 2
PfSUB1 inhibitory activity of compounds **4–12**[a]

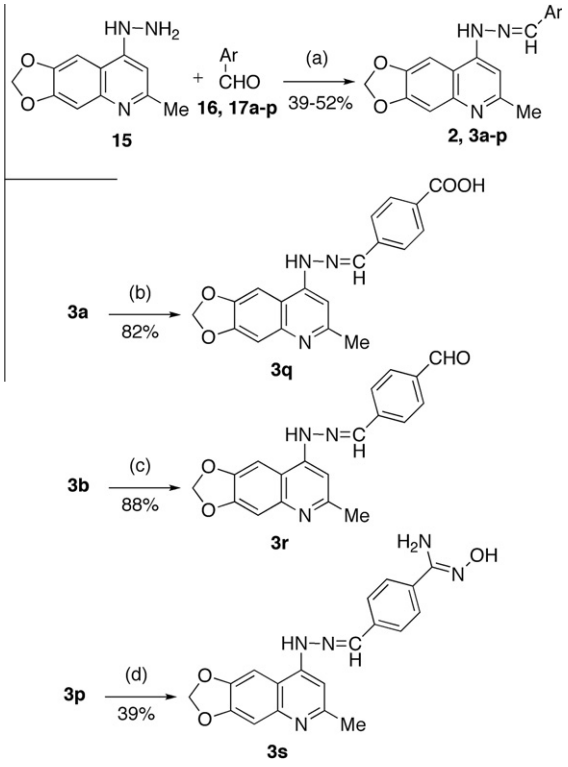
Compd	Structure	% Residual hydrolytic activity at 50 μM ± S.D	IC_{50} (μM)
4[30]		12.3 ± 1.6	20 < IC_{50} < 30
5		32.4 ± 4.3	50
6		80.6 ± 4.0	–
7		74.3 ± 12.1	–
8		85.0 ± 4.5	–
9		55.8 ± 10.0	>50
10		81.5 ± 20.9	–
11[32]		84.6 ± 12.3	–
12[33]		43.1 ± 11.6	>50
2	–	27.2 ± 2.5	20

[a] Physical and spectroscopic data for compounds **4**, **11**, and **12** are consistent with those reported in Ref. [30,32,33].

Table 3
PfSUB1 inhibitory activity of compounds **13a–e** and **14**

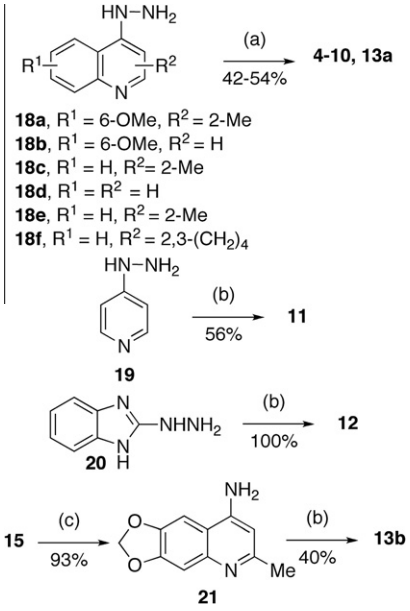


Compd	X	R^1	% Residual hydrolytic activity at 50 μM ± S.D
13a		NO_2	60.3 ± 0.5
13b		NO_2	72.2 ± 9.8
13c		NO_2	64.4 ± 4.0
13d		CN	85.5 ± 5.8
13e		SO_2Me	68.7 ± 5.1
14	—	—	46.7 ± 11.4
2	—	—	27.2 ± 2.5

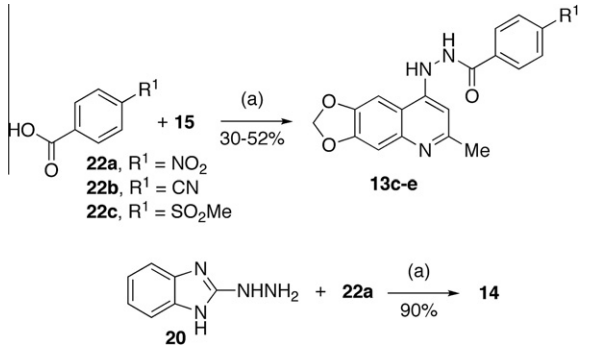


Scheme 1. Synthesis of hydrazones **2**, **3a–s** reported in Table 1. Reagents and conditions: (a) EtOH, reflux, 14 h; (b) 20% NaOH, 1:2 MeOH/THF, 25 °C, 30 min; (c) 0.5 N HCl, acetone, 25 °C, 14 h; (d) hydroxylamine hydrochloride, potassium *tert*-butoxide, dry DMSO, 25 °C, 16 h.

dependence of the inhibitory activity of this set of quinolylhydrazones on an intact hydrazone moiety linked to an aromatic ring



Scheme 2. Synthetic route to the hydrazones **4–12** reported in Table 2 and **13a**,**b** reported in Table 3. Reagents and conditions: (a) **16**, or **17p**, or *p*-nitrophenylmethyl ketone, EtOH, reflux, 14 h; (b) **16**, EtOH, reflux, 14 h; (c) $NiCl_2$, $NaBH_4$, MeOH, 25 °C, 1 h.



Scheme 3. Synthetic route to the hydrazones **13c–e** and **14** reported in Table 3. Reagents and conditions: (a) DCC, HOBt, dry DMF, 25 °C, 12 h.

bearing a strong electron withdrawing group led us to speculate whether a covalent mechanism of inhibition, involving a direct attack of the active site serine at the benzylidene carbon, could be at the basis of the observed SARs. Experiments designed to explore this issue revealed that the enzyme recovered its catalytic activity after removal of inhibitor **2**, highlighting a mechanism of inhibition that could be either competitive or covalent reversible.

In conclusion, here we presented the initial exploration of the SARs for a class of quinoline hydrazones as novel PfSUB1 inhibitors. The key role played by this enzyme in the life cycle of the parasite, and the current lack of antimalarial agents endowed with innovative mechanisms of action make compound **2** and its cyano-derivative **3p** important lead structures for further development. Other than **1** and a ketoamide-based inhibitor,[18,21] quinolylhydrazones here described represent the first series of PfSUB1 inhibitors with clear-cut SARs.

Acknowledgments

The authors thank MIUR (PRIN) for financial support. M.P. was funded by the European Union Framework Programme 7-funded

Marie Curie Initial Training Network STARS (contract number PITN-GA-2009-238490).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.06.023.

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