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Development of piperazine-based hydroxamic acid inhibitors against falcilysin, an essential malarial protease

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ABSTRACT

The human parasite *Plasmodium falciparum* kills an estimated 445,000 people a year, with the most fatalities occurring in African children. Previous studies identified falcilysin (FLN) as a malarial metalloprotease essential for parasite development in the human host. Despite its essentiality, the biological roles of this protease are not well understood. Here we describe the optimization of a piperazine-based hydroxamic acid scaffold to develop the first reported inhibitors of FLN. Inhibitors were tested against cultured parasites, and parasiticidal activity correlated with potency against FLN. This suggests these compounds kill *P. falciparum* by blocking FLN, and that FLN is a druggable target. These compounds represent an important step towards validating FLN as a therapeutic target and towards the development of chemical tools to investigate the function of this protease.

Malaria remains a serious threat to human health in many parts of the world, and it is estimated that the disease kills nearly half a million people annually.[1] The causative agent of the most serious form of human malaria is *Plasmodium falciparum*, a protozoan parasite spread by the bite of an Anopheles mosquito vector. After entering the human host at the site of the bite, the parasite transits the host liver before entering the bloodstream to begin its intraerythrocytic development. During this part of the life cycle, the parasite invades host red blood cells (RBCs) and replicates within a parasitophorous vacuole to produce 16–32 daughter merozoites. Following replication, the host cell is lysed, and the merozoite progeny reinvade new RBCs to begin another round of infection. This part of the parasite life cycle gives rise to all clinical symptoms of the disease, and despite recent management efforts malaria continues to be a severe burden on the health of subtropical regions around the world. Emerging resistance against current clinical drugs underscores the need for the identification and validation of new therapeutic targets.

We are developing small molecule inhibitors as chemical tools to investigate the function of falcilysin (FLN), a metalloprotease essential for parasite development in the RBC. It is known that FLN localizes to multiple subcellular compartments, and that FLN carries out distinct roles based on its localization. It is thought that FLN is required for protein import into the apicoplast[2] (a relict plastid involved in biosynthesis of fatty acids, heme, and isoprenoids), and for host cell hemoglobin degradation in the food vacuole (an acidic organelle involved in catabolism of host cell proteins).[3] The majority of FLN appears to be distributed throughout the cell, possibly in the ER or cytosol. Whether the protease functions in either of these compartments, or if it is simply in transit on the way to other organelles is not known. In addition, it is unclear where FLN carries out its essential function (*e.g.* does FLN carry its vital role in the apicoplast, the food vacuole, the cytosol, or some combination of these), or what the nature of this function is.

Abbreviations: RBC, Red blood cell; IPTG, Isopropyl β-D-1-thiogalactopyranoside; FLN, Falcilysin; PMSF, Phenylmethylsulfonyl fluoride; TEA, Triethylamine; ER, Endoplasmic reticulum; DMAP, 4-Dimethylaminopyridine.

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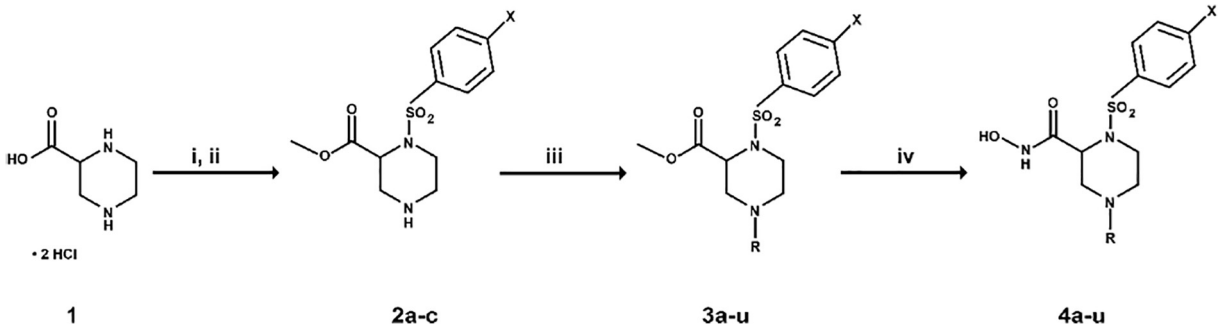
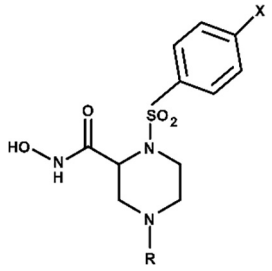


Scheme 1. Inhibitor synthesis route. Conditions: (i) di-*tert*-butyl dicarbonate, NaOH, 1,4-dioxane:H_2O (2:1), 0 °C → 20 °C, 12–24 h; aryl sulfonyl chloride, TEA, 20 °C, 12 h; (ii) Thionyl chloride, MeOH, 20 °C, 12–24 h; (iii) alkyl halides or acid halides, DMAP (cat), TEA, 1,4-dioxane, 20 °C, 4–48 h; (iv) NH_2OH, MeOH, 20 °C, 6 h.

Table 1
Activity of inhibitor series against FLN and cultured *P. falciparum*.



	X	R	IC_{50} vs FLN (μM)			IC_{50} vs. *P. falciparum* (μM)		
4a	H-	3-hydroxypropyl-		>50			>10	
4b	H-	butyl-	30	±	7		>10	
4c	H-	benzoyl-	40	±	20		>10	
4d	H-	3-F-benzoyl		>50			>10	
4e	H-	4-F-benzoyl		>50			>10	
4f	H-	2-Br-benzoyl	7	±	1		>10	
4g	H-	4-Br-benzoyl	13	±	3		>10	
4h	H-	3,4-Cl_2-benzoyl	5	±	1		>10	
4i	H-	4-phenyl-benzoyl	8	±	2		>10	
4j	MeO-	phenylsulfonyl-		>50			>10	
4k	MeO-	benzoyl-		>50			>10	
4l	MeO-	2-Br-benzoyl	8	±	2		>10	
4m	MeO-	3-Br-benzoyl	10	±	3		>10	
4n	MeO-	4-Br-benzoyl	14	±	5		>10	
4o	MeO-	3,4-Cl_2-benzoyl	6	±	1		>10	
4p	MeO-	4-phenyl-benzoyl	10	±	1	5	±	2
4q	Br-	propanoyl-		>50			>10	
4r	Br-	benzoyl-	35	±	9		>10	
4s	Br-	3-F-benzoyl	31	±	9		>10	
4t	Br-	4-F-benzoyl	16	±	3		>10	
4u	Br-	4-phenyl-benzoyl	1.5	±	0.2	1.4	±	0.1

These remaining gaps in our understanding of FLN biology are largely due to challenges in generating loss-of-function mutants (*i.e.* parasites in which FLN is inactive or absent), a result of the parasite's well-known resistance to genetic manipulation. To address this need, we are working towards the development of potent and selective chemical inhibitors of FLN. Such inhibitors could be used to block FLN activity in cultured parasites in lieu of genetic disruption and provide valuable insights into the biological roles of this essential protein. These compounds would also help to evaluate the potential of FLN as a therapeutic target. To date, no inhibitors against FLN have been reported. Here we describe the development of a panel of FLN inhibitors using a piperazine-based hydroxamic acid scaffold. This scaffold has several known advantages over peptidyl inhibitors, including good membrane permeability and an ability to target multiple binding surfaces of a protease active site.[4] These properties make this scaffold a promising platform for the development of selective, cell-permeable FLN inhibitors.

Previous studies of this scaffold against a human orthologue (MMP-3) indicate similar compounds are competitive inhibitors which utilize the hydroxamic acid moiety to coordinate the catalytic zinc atom in the protease active site, while the N1 and N4 substituents target subsites in the active site cleft involved in substrate recognition.[4] We began exploring structural requirements for FLN inhibition by synthesizing (Scheme 1) compounds with aryl sulfonyl groups at the N1 position and a variety of functional groups at the N4 position (Table 1).

We initially focused on probing SAR at N4 with various functional groups. Introduction of aryl sulfonyl or smaller alkyl or

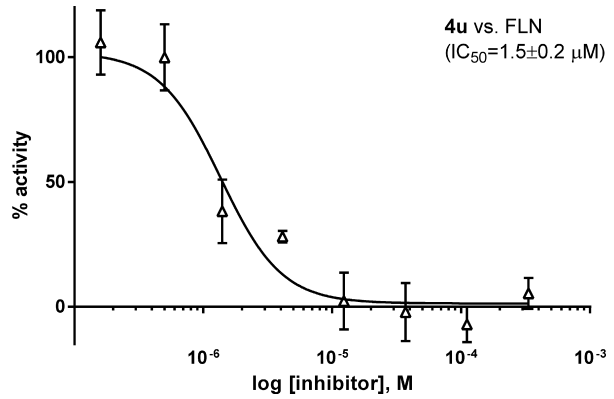


Fig. 1a. Dose response of lead compound 4u against FLN.

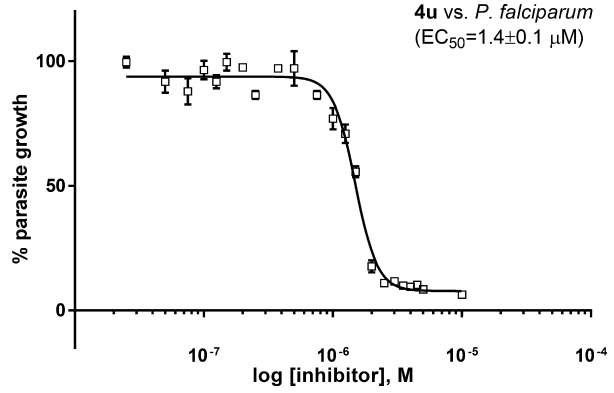


Fig. 1b. Dose response of lead compound 4u against cultured *P. falciparum*.

amide groups at N4 did not yield active inhibitors (**4a**, **4b**, **4j**, **4q**). However, installation of a phenyl ring through an amide linkage produced compounds (**4c**, **4r**) with modest activity against the metalloprotease target ($IC_{50} \sim 30–40$ μM). We expanded on this observation by testing a range of ring substitution patterns and found that introduction of smaller fluoro groups at the 3 or 4 positions of the phenyl group generally did not improve activity against FLN, while larger halogen substituents (3-Br, 4-Br, or 3,4-Cl_2) led to consistent increases in potency (3–10-fold). These data indicate that steric bulk at N4 is important driver of FLN inhibition, and we further tested this by introducing a second phenyl ring (4-Ph). This approach yielded the most potent inhibitor of the series (**4u**, IC_{50} = 1.4 μM, see Fig. 1a), indicating that the N4 substituent targets a large binding site or possibly multiple sites in the active site cleft. It is notable that the N1 position also contributes to inhibitor potency, and relatively small changes in the X substituent can result in a >5-fold change in potency (*i.e.* **4u** vs. **4p**). Future studies will focus on further optimization of the N1 and N4 aryl groups.

Inhibitors were then screened against cultured 3D7 *P. falciparum* at 1 and 10 μM. Compounds with parasiticidal activity at 1 μM were further tested in a dose response assay to determine EC_{50} values. These experiments identified 4 inhibitors with weak (<50% parasite killing) parasiticidal activity at 10 μM, as well as 2 compounds (**4p** and **4u**) with low micromolar potency against cultured parasites. It is notable that both compounds are active against FLN, and the most potent FLN inhibitor (**4u**) is also the most potent antimalarial (see Fig. 1b). Together, these data indicate FLN may be a promising point for chemotherapeutic intervention, and these inhibitors represent an important step towards the development of selective chemical tools to further probe the biological functions of this protease. As similar inhibitors have been shown to target human MMP-3,4 future studies will focus on optimizing specificity for the parasite and FLN relative to human cell lines and metalloproteases.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.04.010.

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