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Identification of Potent and Selective Non-covalent Inhibitors of the *Plasmodium falciparum* Proteasome

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Supporting Information

ABSTRACT: We have identified short N,C-capped peptides that selectively inhibit the proteasome of the malaria-causing pathogen *Plasmodium falciparum*. These compounds are highly potent in culture with no toxicity in host cells. One cyclic biphenyl ether compound inhibited intraerythrocytic growth of *P. falciparum* with an IC_{50} of 35 nM, and we show that even a pulse treatment with this cyclic peptide induced parasite death due to proteasome inhibition. These compounds represent promising new antimalarial agents that target the essential proteasomal machinery of the parasite without toxicity toward the host.

Plasmodium falciparum is a pathogenic parasite that causes malaria, a disease prevalent in the developing world.[1] This parasite belongs to the phylum of Apicomplexa, which is a highly divergent family of eukaryotic protozoans.[2] All eukaryotic organisms express the proteasome, a multisubunit enzyme complex that is the key regulator of cellular catabolism.[3] The proteasome is well-characterized in yeast and mammalian species, facilitating the development of inhibitors both as biochemical tools and as clinical anticancer agents.[4] *P. falciparum* expresses a proteasome complex that is typical of eukyarotic organisms.[5] The catalytic core comprises two rings of seven distinct α-subunits that flank two rings of seven distinct β-subunits. Sequence comparison of *P. falciparum* with other eukaryotic organisms indicates that *P. falciparum* has three active β-subunits, β1, β2, and β5, though the specificity of these subunits has yet to be defined.[6]

As *P. falciparum* has a high rate of replication and is subjected to oxidative and temperature stress during host infection, it is likely that the parasite proteasome is a key regulator of growth. Early studies using a panel of highly potent covalent inhibitors designed for the mammalian proteasome confirmed the essential nature of this enzyme complex in the asexual, sexual, and liver stages of the *P. falciparum* life cycle.[7,8] Therefore, the proteasome is a significant potential antimalarial target whose inhibition could block parasite growth and prevent transmission. However, all of the inhibitors described to date also inhibit the host proteasome, which would limit their tolerated dose and thereby limit their use as antimalarial agents.[5] Herein we report a class of non-covalent small-molecule inhibitors that selectively inhibit the *P. falciparum* proteasome over the host enzyme. These compounds attenuate parasite growth at concentrations that have no significant effect on the activity of the host proteasome.

As a starting point for new inhibitor discovery, we screened a recently reported library of 1600 non-covalent proteasome inhibitors. This library of peptidic inhibitors was recently used to identify selective and potent inhibitors of the human[9,10] and *Mycobacterium tuberculosis*[11] proteasomes. We reasoned that non-covalent inhibitors could have advantages over the existing covalent inhibitors as they will not be permanently sequestered by irreversible reaction with the red blood cell proteasome and can thus be more efficiently distributed into parasites. Furthermore, this library of compounds was designed to be selective for the β5 (chymotrypsin-like) active site, which we have recently shown to be an essential subunit in *P. falciparum*.[12]

We screened the library against purified *P. falciparum* proteasome (Pf20S) activated with PA28α, using Suc-LLVY-AMC as the reporter substrate for chymotryptic-like activity. From this initial in vitro screen (Figure S1 in the Supporting Information), we identified 69 compounds that had >50% inhibition at 3.3 μM. We further triaged the hits by selecting the most potent compounds that were also weak inhibitors of the human proteasome (h20S) (IC_{50} > 1 μM). We screened the resulting 42 compounds in dose−response assays using purified h20S and Pf20S activated with PA28α. From this secondary screen, we identified nine compounds that have selectivity for *P. falciparum* β5 activity over the human enzyme, with selectivities ranging from 2- to 62-fold (Table 1 and Figure 1).

Structural and biochemical studies have identified the S1 and S3 sites of the β5 proteasome subunits as well-defined pockets for the respective binding of the P1 and P3 side chains of peptide-based substrates and inhibitors.[13] This initial set of nine lead compounds gave us insight into residues that are preferred in these positions, thereby conferring selective inhibition of Pf20S. All nine of the selective compounds have 4-methylbenzyl at their C-termini, suggesting favorable interactions with hydrophobic side chains in the S1 pocket of the β5 subunit (Figure 1). Also, eight of the nine compounds have a P3 homo-Phe residue. To further investigate the preference for homo-Phe at P3, we used the available crystal structure of the yeast proteasome[14] bound to a member of this library[9] to

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Table 1. IC_{50} and EC_{50} Values for *P. falciparum* 20S-Selective Compounds

ID	h20S IC_{50} (μM)	Pf20S IC_{50} (μM)	HFF[a] EC_{50} (μM)	Pf EC_{50} (μM)	selectivity[b]
1	9.22	1.25	>50[c]	0.0345	>1450
2	1.97	0.0789	61	0.104	587
3	6.54	0.105	35	0.127	276
4	2.09	0.229	>50[c]	0.221	>226
5	0.171	0.00924	68	0.357	189
6	0.807	0.0671	14	0.277	51
7	0.159	0.0472	2.9	0.0644	45
8	>100	2.09	>100[c]	4.22	>24
9	0.489	0.311	>50[c]	7.76	>6

[a]Human foreskin fibroblasts (HFF) were non-confluent. [b]Determined as the ratio of HFF EC_{50} to Pf EC_{50} for a 72 h treatment period. [c]Compounds were tested up to solubility limit.

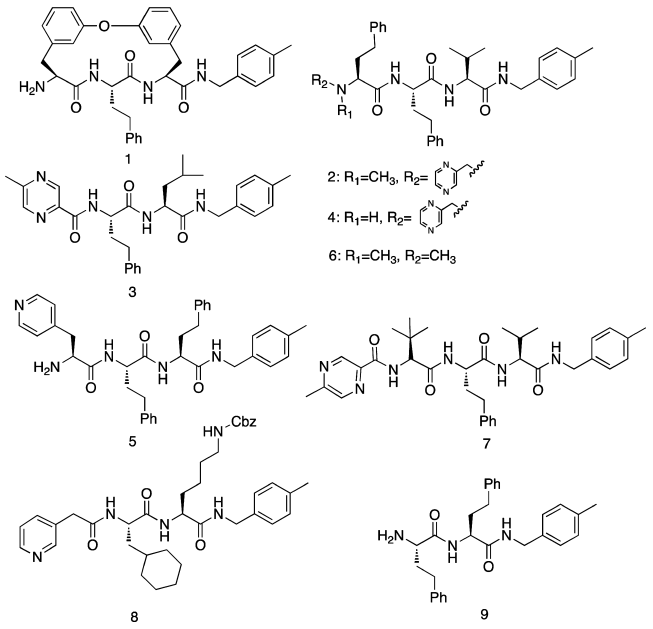


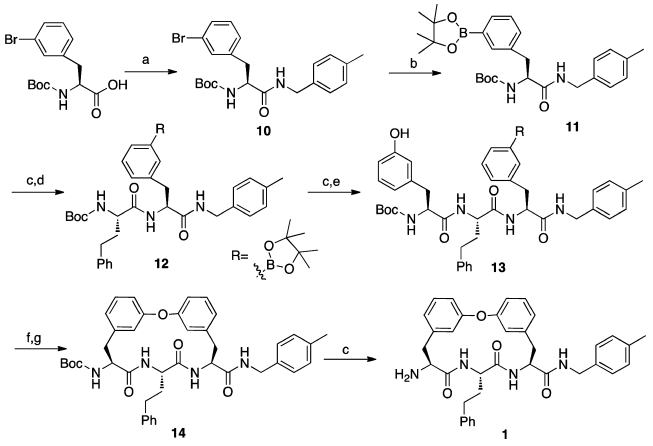
Figure 1. Structures of *P. falciparum* proteasome-selective compounds.

perform active-site docking studies with compound 1. We then created a homology model for the *P. falciparum* β5 active site and performed similar docking studies. We found that homo-Phe is a highly optimal fit for the S3 pocket in the *Plasmodium* β5 active site (Figure S2 in the Supporting Information). On the contrary, the S3 pocket in the yeast and mammalian β5 active sites is more accommodating for residues with longer, less bulky alkyl chains. Therefore, we postulate that the S1 and S3 binding pockets in the β5 active site in *Plasmodium* have a major role in conferring selective binding of inhibitors. Interestingly, the larger S3 site of the *M. tubercuolosis* proteasome also allows for its selective inhibition by related N,C-capped dipeptides.[11]

To test whether the selective compounds identified in our screen have activity in parasite culture, we treated replicating *P. falciparum* at the ring stage (early blood stage) for 72 h and assessed parasite viability. We also performed the same treatment on non-confluent, replicating human foreskin fibroblast (HFF) cells, which predominantly have constitutive proteasome, to assess the overall host cell toxicity. To our surprise, all nine of the lead compounds had an enhanced therapeutic window in culture compared with our in vitro data using purified enzymes (Table 1). In particular, five compounds showed greater than 100-fold selectivity for the parasite over the host cell, killing parasites in the concentration range of 35–360 nM. This high selectivity resulted in overall low or complete absence of toxicity to HFFs.

We are especially intrigued with compound 1, which is highly potent toward *P. falciparum* but is virtually nontoxic toward mammalian cells (Table 1 and Figure S3 in the Supporting Information). Compound 1 is also a poor inhibitor of the immunoproteasome (Table S1 in the Supporting Information). This compound was designed in part on the basis of biphenyl ether clamp analogues of TMC95A,[15,16] which is a natural product that inhibits the proteasome in a non-covalent manner. TMC95A shows high-affinity binding, partly due to its constrained structure that likely drives favorable entropic binding to the active site. To confirm the activity of compound 1, we resynthesized it starting from Boc-protected 3-bromophenylalanine, which allows convenient installation of the pinacol-protected boronate (Scheme 1). We then used

Scheme 1. Synthesis of Cyclic Peptide 1[a]



[a]Reagents and conditions: (a) 4-methylbenzylamine, HBTU, DIEA in DCM, RT; (b) bis(pinacolato)diboron, $PdCl_2$(dppf), 1,4-dioxane, 80 °C, 17 h; (c) 4 M HCl in dioxane, 1 h, RT; (d) Boc-L-homoPhe, EDC, HOBt, DIEA in DCM, RT; (e) Boc-*m*-tyrosine, EDC, HOBt, DIEA in DCM, RT; (f) $NaIO_4$, NH_4OAc in acetone/water, RT, 17 h; (g) $Cu(OAc)_2$, MeOH, triethylamine, 4 Å MS in DCM, RT, 6 h.

standard peptide coupling to extend the peptide backbone with L-homophenylalanine and L-*m*-tyrosine. The biphenyl ether clamp was formed using Cu(II)-mediated cyclization of the *m*-tyrosine and the boronate,[17] and subsequent Boc-group deprotection yielded the cyclic peptide.

To assess the subunit selectivity of 1, we performed inhibitor competition assays with Suc-LLVY-AMC (β5 substrate), Boc-LRR-AMC (β2 substrate), and Z-LLE-AMC (β1 substrate) using human and *P. falciparum* 20S proteasomes (Figure 2A,B). Compound 1 showed nearly absolute selectivity for the chymotrypsin-like site in h20S within the solubility limit of the compound but lost this exquisite selectivity for the β5 subunit of *Plasmodium*. We have previously shown that at least partial coinhibition of β5 with β2/β1 is required for killing at all stages of the blood cycle in *P. falciparum* when an inhibitor is administered for only a short time period.[12] Therefore, the general reactivity of compound 1 for all three catalytic subunits suggests that it should be effective at all stages of the blood cycle even after a short pulse treatment.

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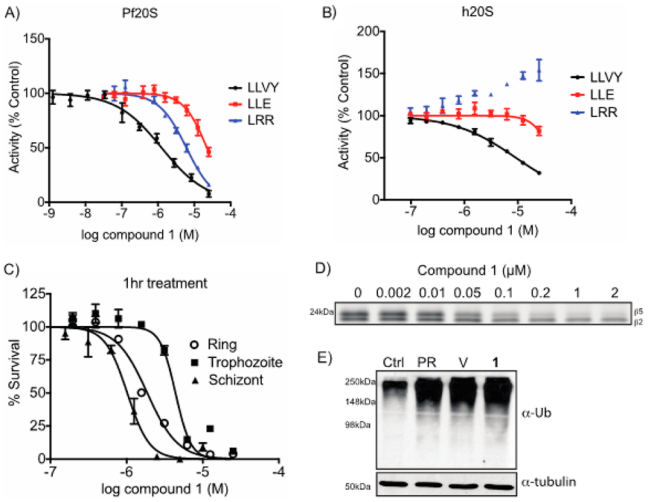

Figure 2. Compound 1 inhibits *P. falciparum* proteasome. (A, B) Substrate assays to assess chymotryptic (LLVY), tryptic (LRR), and caspase-like (LLE) activities in the presence of compound 1 in (A) *P. falciparum* and (B) human proteasome. (C) Survival curves of 1 h pulse treatment of compound 1 at three different stages in the parasite life cycle ($EC_{50,ring}$ = 1.87 μM, $EC_{50,trophozoite}$ = 4.38 μM, $EC_{50,schizont}$ = 1.02 μM). (D) Inhibition of *P. falciparum* proteasome after 1 h treatment with compound 1 in live culture. The residual proteasome activity after compound treatment was labeled using the activity-based probe MV151. (E) Accumulation of ubiquitinated species in *P. falciparum* schizonts after 2 h treatment with 10 μM carfilzomib (PR), 10 μM Velcade (V), or 2 μM compound 1.

To test this hypothesis, we treated synchronized cultures of rings, trophozoites, and schizonts (three distinct life stages in the blood cycle of *Plasmodium*) for 1 h with compound 1 (Figure 2C). We found that *P. falciparum* was sensitive to the drug at all stages of the blood cycle, and the trend of the stage-specific sensitivity to the inhibitor followed that of our previously validated covalent proteasome inibitors.[12] To further confirm direct inhibition of the *Plasmodium* proteasome by compound 1, we performed the same pulse treatment as described above in *P. falciparum* culture and labeled the residual proteasome activity in live parasites using the cell-permeable activity-based probe MV151 (Figure 2D). Previously, we demonstrated that MV151 labels the β2 and β5 subunits of the *Plasmodium* proteasome.[18] Using this assay, we observed that compound 1 has enhanced potency in live parasite treatment, and this inhibition of the catalytic subunits corresponds well to parasite killing (Figure S4 in the Supporting Information). Interestingly, we observed that compound 1 has increased potency in live parasite treatment compared with compound 2, which is a stronger inhibitor in vitro (Figure S4). As a further confirmation of proteasome inhibition by compound 1, we assessed the effects of the compound on global ubiquitinylation of *P. falciparum*. We found that inhibition of the *Plasmodium* proteasome by compound 1 leads to rapid accumulation of ubiquitinylated species to the same extent as treatment with other known covalent proteasome inhibitors (Figure 2E).

In summary, we have identified potent and selective inhibitors of the *Plasmodium* proteasome that have significant antiparasitic activity in culture. We have found that similar to the *M. tubercuolosis* proteasome, the P1 and P3 amino acid residues on the inhibitor confer selective binding to the target proteasome. Our screen identified compound 1, a non-natural cyclic peptide that has the strongest parasite killing activity and can coinhibit all of the catalytic subunits in the *Plasmodium* proteasome while showing weak proteasome inhibition toward the host. We have demonstrated that the effect on parasite viability is associated with inhibition of the parasite proteasome and furthermore that the compound has selective parasite cell toxicity when used in infected red blood cell cultures. This enhanced activity of compound 1 in live parasites is most likely due to increased uptake and accumulation of the inhibitor. Since there is high homology for the proteasome subunits among the different *Plasmodium* species,[5] we are currently investigating the in vivo effects of compound 1 in a rodent model of malaria. We believe that further development of this compound might lead to novel and highly effective antimalarial therapy.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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