Assessing Subunit Dependency of the *Plasmodium* Proteasome Using Small Molecule Inhibitors and Active Site Probes

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**Supporting Information**

**ABSTRACT:** The ubiquitin-proteasome system (UPS) is a potential pathway for therapeutic intervention for pathogens such as *Plasmodium*, the causative agent of malaria. However, due to the essential nature of this proteolytic pathway, proteasome inhibitors must avoid inhibition of the host enzyme complex to prevent toxic side effects. The *Plasmodium* proteasome is poorly characterized, making rational design of inhibitors that induce selective parasite killing difficult. In this study, we developed a chemical probe that labels all catalytic sites of the *Plasmodium* proteasome. Using this probe, we identified several subunit selective small molecule inhibitors of the parasite enzyme complex. Treatment with an inhibitor that is specific for the β5 subunit during blood stage schizogony led to a dramatic decrease in parasite replication while short-term inhibition of the β2 subunit did not affect viability. Interestingly, coinhibition of both the β2 and β5 catalytic subunits resulted in enhanced parasite killing at all stages of the blood stage life cycle and reduced parasite levels *in vivo* to barely detectable levels. Parasite killing was achieved with overall low host toxicity, something that has not been possible with existing proteasome inhibitors. Our results highlight differences in the subunit dependency of the parasite and human proteasome, thus providing a strategy for development of potent antimalarial drugs with overall low host toxicity.

Malaria is a disease caused by the Apicomplexan parasite *Plasmodium*. Of the five *Plasmodium* species that infect human, *Plasmodium falciparum* is responsible for the most severe form of malaria. *P. falciparum* has an estimated disease burden of 219 million people in 2010 and is mostly prevalent in sub-Saharan Africa.† The disease is especially lethal to young children, with the majority of death by malaria occurring in children less than five years old.‡ *P. falciparum* can rapidly evolve resistance to drugs, and this emergence of drug resistance to many current drug targets highlights the need to develop new antimalarial therapeutics.† The *Plasmodium* proteasome has been proposed as a potential drug target for the treatment of malaria. Proteasome inhibitors attenuate parasite growth during the asexual intraerythrocytic stages, the sexual stage as well as the liver stage of *Plasmodium*.§ This makes the *Plasmodium* proteasome an ideal target for drug development, as inhibitors could not only reduce or eliminate the acute stage of the disease but also potentially block transmission.

The proteasome is a multisubunit enzyme complex that is conserved across the eukaryota.§ It is made up of a 20S catalytic core that is capped by regulatory subunits that control the entry of substrates. The 20S core has a barrel-shaped structure made up of two heptameric rings of β subunits sandwiched between two heptameric rings of α subunits.¶ Only the β1, β2, and β5 subunits have catalytic activity. The β1 subunit has caspase-like activity, favoring cleavage after acidic residues, while the β2 subunit has trypsin-like activity (cleaves after basic residues), and the β5 subunit has chymotrypsin-like activity (cleaves after nonpolar residues).¶ Studies using yeast mutants of each catalytic subunit showed preliminary evidence that the β5 subunit is the most essential catalytic subunit as its inactivation caused significant growth defects.¶,¶ The β2 catalytic mutant, on the other hand, only showed slightly reduced growth, and the β1 and β2 double mutants show a stronger growth defect, though not as severe as the β5 mutant.† From these studies, it was concluded that selective inhibition of the catalytic site in
the β5 subunit alone is able to reduce cell viability. However, recent studies using subunit selective inhibitors on a panel of mammalian cancer cells found that most cells are not sensitive to inhibition of the β5 subunit alone but instead require coinhibition of β5 with β2 and/or β1 to induce efficient cell killing. Normal human cells are resistant to selective inhibition of the chymotrypsin-like site, and only a few cancer cell lines are sensitive to inhibition of β5 alone.

The *Plasmodium* proteasome remains poorly studied by biochemical methods, and it is unclear if the *Plasmodium* parasite has similar sensitivity to subunit selective inhibition as its mammalian host cells. Our previous efforts identified an epoxycetone proteasome inhibitor that can effectively attenuate parasite growth both in culture and in vivo. As this inhibitor targets multiple subunits of the *P. falciparum* proteasome, it was not useful for assessment of the effects of specific proteasome subunit inhibition. In this study, we designed and synthesized an activity-based probe for the *P. falciparum* proteasome that covalently labels all three catalytic sites of the *P. falciparum* proteasome. This allowed us to identify compounds that could be used to chemically knockdown activity of individual catalytic subunits and correlate loss of activity with parasite schizont growth. Furthermore, coinhibition of the *Plasmodium* β5 and β2 proteasome subunits results in effective parasite killing at all stages of the asexual form of *P. falciparum* with minimal host cell toxicity. These results provide us guidelines to design parasite-killing proteasome inhibitors as a new class of antimalarial drugs.

### RESULTS AND DISCUSSION

#### Design and Synthesis of *Plasmodium* Proteasome Activity-Based Probe.

Analysis of the sequence homology of the *P. falciparum* proteasome suggest that it contains caspase-like, trypsin-like, and chymotrypsin-like active sites. We have previously used the activity-based proteasome probe MV15 to contain a vinyl sulfone to label the β2 and β5 subunits of the parasite proteasome. However, we were unable to label the β1 subunit using this reagent. In an effort to identify a more broad-spectrum probe that would allow analysis of all three active sites, we synthesized an activity-based probe BMV037 (Figure 1 A) that contains an epoxycetone peptidic scaffold based on the recently FDA-approved proteasome inhibitor Kyprolis (carfilzomib/PR171; Supporting Information Figure 1). This probe contains epoxycetone as the electrophile which covalently reacts with the active site threonine of all three catalytic β-subunits. As this covalent interaction only occurs in the presence of active enzyme, BMV037 can be used to directly assess activity of the proteasome in both the purified *Plasmodium* 20S proteasome and native proteasome populations within parasite lysates (Figure 1B). We find that BMV037 labeling of the proteasome corresponds well to inhibition of model substrate cleavage (Supporting Information Figure 2A). Furthermore, we were able to confirm the identity of each labeled subunit by mass spectrometry (Figure 1C, D). Pretreatment with inhibitors followed by incubation with the probe allows us to simultaneously monitor activities of the three catalytic subunits (Supporting Information Figure 2B and C).

#### Subunit Selective Inhibitors Highlight Differences between Host and Parasite Proteasomes.

Inhibitors that are selective for each of the catalytic subunits of the mammalian
proteasome have been developed. Therefore, we started by evaluating these previously characterized inhibitors in the hopes that they could be used for selective chemical knockdown of the Plasmodium proteasome catalytic subunits. In mammalian cells, NC001, LU102, and NC005-mvs show highly selective inhibition of the β1, β2, and β5 subunits, respectively. We treated live P. falciparum cultures with these inhibitors for 1 h and assessed the remaining proteasome activity by labeling the lysates with BMV037 (Figure 2). Interestingly, we find that only LU102 has a small degree of selectivity for the same β subunit that it targets in the human proteasome. Although the selective β2-targeting window for LU102 is narrow, it is the only inhibitor we have identified that can selectively inhibit β2 activity when used in live P. falciparum cultures. NC005-mvs exclusively inhibits the mammalian β5 subunit up to 100 μM, but surprisingly blocks all proteasome catalytic sites in P. falciparum at 40 μM (Figure 2, see also ref 22). NC001 does not inhibit any of the parasite proteasome subunits (Figure 2) at concentrations where it is capable of complete and selective inhibition of the mammalian β1 activity. We observed similar effects upon direct treatment of purified proteasomes with NC001 (Supporting Information Figure 3). We also tested WL407, a recently reported epoxketone inhibitor that selectively inhibits the β1 subunit of the mammalian proteasome. Again, we observed no inhibition of the parasite β1 subunit (Supporting Information Figure 3). We have so far been unable to identify a proteasome inhibitor that can selectively inhibit the Plasmodium β1 subunit in culture. β1 selective inhibitors are usually designed with a P3 proline or more recently, by a valine-urea-valine motif. We predict that a kink or bend in the substrate pocket of the human proteasome is missing in the S3 position of the β1 subunit in Plasmodium and hence inhibitors with this design are not accessible to the Plasmodium active site. Future work will focus on designing inhibitors that can target the Plasmodium β1 site selectively.

Differences in the S1 pocket of the host and parasite proteasome in all three catalytic subunits have been identified by sequence alignment. Our data here suggests that the substrate binding pockets of each of the catalytic subunits are significantly different from the host. We find that inhibitors that are highly selective for a particular subunit in mammalian proteasome often do not retain the same exquisite subunit selectivity in the parasite proteasome. This difference in cross-species subunit selectivity can be exploited for design of proteasome inhibitors with selective parasite killing effects. Inhibitors that can coinhibit all the catalytic subunits in Plasmodium, but only target one catalytic site in the host proteasome would be highly potent antimalarials with low toxicity.

**Selective β5 Inhibition during Schizogony Blocks Parasite Replication.** Since none of the mammalian proteasome inhibitors tested so far showed selective inhibition of the parasite chymotrypsin-like site, we assessed the proteasome inhibition profile of some recently synthesized proteasome inhibitors containing non-natural amino acids and a urea motif in their structures. We identified the vinyl sulfone PR709A that inhibited the β5 subunit in purified P. falciparum 20S proteasome without significant inhibition of the other subunits (Figure 3A).

In order to directly determine the effect of proteasome inhibition on parasite growth, we treated infected erythrocytes with inhibitors for 1 h, washed out the inhibitor, and then used the same compound treated parasites to determine proteasome inhibition and parasite growth. To assess effect on parasite growth, the culture is left to grow for another cycle (~48 h) before parasitemia is determined. We treated Plasmodium cultures at a late stage in the intraerythrocytic cycle (schizont; 36~48 h post invasion) to ensure sufficient parasite numbers for proteasome labeling. We first determined the effect of selective β5 inhibition in intact parasites using the β5 selective

**Figure 2.** Subunit selective mammalian proteasome inhibitors do not retain subunit selectivity in P. falciparum. (A) Structures of the proteasome inhibitors. Intact human foreskin fibroblasts (HFF; B) or intact P. falciparum schizonts (C) were treated for 1 h with each inhibitor at the indicated final concentrations at 37 °C followed by postlysis labeling with 10 μM BMV037 (for P. falciparum) or 2 μM MV151 (for HFF). Samples were analyzed by SDS-PAGE followed by scanning of the gels for fluorescence using a flatbed laser scanner. The position of each of the labeled active β subunits is indicated.
inhibitor PR709A (Figure 3A). To our surprise, we found that the extent of \( \beta_5 \) subunit inhibition directly correlated with a decrease in parasite growth suggesting that this subunit activity is essential for the parasite (Figure 3B). To ensure that the sensitivity to \( \beta_5 \) inhibition was not compound specific, we performed the same treatment using WL588, another \( \beta_5 \) selective compound that we identified with a smaller window of selectivity for \( \beta_5 \) than PR709A (Supporting Information Figure 4). WL588 is an epoxyketone, which has been shown to be highly specific for the proteasome.24,25 We observed a similar reduction in schizont replication that corresponded with the inhibition of the \( \beta_5 \) activity (Supporting Information Figure 4). This strongly suggests that the effect on parasite growth upon inhibitor treatment is due to direct inhibition of the \( \beta_5 \) subunit.

To assess the effect of PR709A in mammalian cells, we performed the same treatment as described above in human foreskin fibroblast (HFFs). To our surprise, PR709A did not inhibit any proteasome subunits and showed no cell toxicity up to 50 \( \mu \)M (Figure 3C). Extended treatment of nonconfluent HFFs with PR709A revealed that this inhibitor is nontoxic to host cells even with continuous 72 h treatment (Table 1).

Table 1. EC50 of Prolonged Treatment of LU102 and PR709A in \( P. falciparum \) and HFF

|    | treatment time (h) | \( P. falciparum \) EC50 (\( \mu \)M) | HFF EC50 (\( \mu \)M) | selectivity (HFF/Pf) |
|----|-------------------|---------------------------------|----------------------|---------------------|
| LU102 | 24                | 0.252 ± 0.006                    | 11.47 ± 0.033        | 46                  |
|      | 72                | 0.170 ± 0.004                    | 5.12 ± 0.12          | 30                  |
| PR709A | 24                | 0.50 ± 0.02                      | 245 ± 13             | 490                 |
|      | 72                | 0.29 ± 0.01                      | 165 ± 3              | 569                 |

*24 and 72 h treatment of ring stage parasites are shown. For 24 h treatment, cells are treated, washed, and placed in new media for a further 48 h of growth. EC50s are expressed as mean ± standard deviation. Selectivity is determined as the ratio of EC50 in HFF over EC50 in \( P. falciparum \).

To investigate whether the observed sensitivity of parasites to \( \beta_5 \) subunit inhibition occurs at all stages in the intraerythrocytic cycle of \( P. falciparum \), we performed 1 h treatments on synchronized cultures of rings (6–8 h post invasion (h.p.i.)), trophozoites (28–30 h.p.i.), and schizonts (38–40 h.p.i.). Although PR709A is highly selective for \( \beta_5 \) subunit, we found that this inhibitor has differential inhibition at different stages of replication.
the blood cycle to assay the effects of stage specific β2 inhibition. We observed that each of the catalytic subunit was inhibited to a similar extent at all 3 stages of the P. falciparum blood cycle (Supporting Information Table 1). At concentrations of WLS88 that inhibited 30% of the β5 subunit with no inhibition of the other subunits, we found that only schizont viability was correspondingly reduced. At concentrations that inhibited 50–60% of the β5 activity, the compound reduced growth of parasites at all three stages, with schizonts showing the greatest sensitivity and trophozoites being least sensitive to the compound (Supporting Information Table 1).

Selective β2 Inhibition Does Not Affect Plasmodium Growth. To selectively inhibit the β2 subunit of the Plasmodium proteasome, we used LU102, which is highly selective for the human trypsin-like active site at concentrations up to 10 μM (Figure 2). In Plasmodium, LU102 treatment for 1 h selectively inhibits β2 when used at concentrations up to 2 μM but the compound coinduces β5 and β1 at higher concentrations (Figure 2). We find that with a short treatment of LU102 in live P. falciparum schizont cultures, we achieve 50% inhibition of the β2 activity with no significant inhibition of β5 or β1, yet parasite growth was not affected (Figure 4A). This is similar to the effect of the compound on HFF cells, where selective (and complete) block of β2 activity does not affect cell viability (Figure 4B). From this, we concluded that P. falciparum is not sensitive to a short-term inhibition of β2 alone.

Sensitivity to Proteasome Inhibition Is Stage Specific. Given that the sensitivity to selective β5 inhibition is only prominent during Plasmodium schizogony, we hypothesized that coinhibition of β5 with β2 and/or β1 should attenuate parasite growth at other points in the parasite blood stage life cycle. When P. falciparum culture was treated with a concentration of LU102 that blocks all β subunits (25 μM), we found that parasites at all stages of the blood cycle were effectively killed (Figure 4C). This complements our previous report where we observed complete attenuation of ring and trophozoite growth at high concentrations of proteasome inhibitors. Furthermore, we observed a similar trend of stage-specific sensitivity to inhibition of the proteasome subunits, where schizonts were more susceptible than rings, which were more susceptible than trophozoites. Higher doses of LU102 also completely blocked Plasmodium berghei sporozoite development in HepG2 liver cells (Figure 4C). Most importantly, HFF viability was only slightly reduced by 1 h treatment with 25 μM of LU102 (Figure 4B), as LU102 preferentially targets β2 in mammalian cells.

It is important to note that there have been conflicting reports on schizont sensitivity to proteasome inhibition. Some groups have observed that parasite growth is only blocked when Plasmodium cultures are treated at ring and trophozoite stages,27 while others have noted that proteasome inhibitors can attenuate growth at all stages of the asexual life cycle.28 We find that a short pulse of proteasome inhibition is able to attenuate Plasmodium growth at all stages of the blood cycle, although the different stages have different sensitivity to proteasome inhibition. In our experiments, we used a short pulse of inhibition to assess effects on parasite growth at different stages instead of continuous treatment as was reported by other groups. This is important, as most reported proteasome inhibitors are covalent, irreversible inhibitors and potency of inhibitors increases with time of incubation. As such, the best way to compare effectiveness of proteasome inhibition on a specific life stage of the parasite is to administer the same short, noncontinuous treatment.

Inhibition of Multiple β Subunits Results in Synergistic Effects In Vivo. Given the promising initial in vitro culture studies of the proteasome inhibitors that revealed a substantial therapeutic window (Table 1), we wanted to assess
the effects of these compounds on parasite growth in vivo. We used the rodent parasite *Plasmodium chabaudi* which possesses many traits similar to *P. falciparum*, such as its synchronous growth in the host and preference to invade mature red blood cells. We found that this treatment had a small, but statistically significant, effect on parasite replication in the host. Importantly, the inhibitor was nontoxic to the host, even when used at the relatively high dose of 50 mg/kg (Supporting Information Figure 6).

Mice were dosed during early schizont stage of the parasite. We found that this treatment had a small, but statistically significant, effect on parasite replication in the host. Importantly, the inhibitor was nontoxic to the host, even when used at the relatively high dose of 50 mg/kg (Supporting Information Figure 6).

We then assessed the effect of LU102, which has reduced toxicity to the host due to its preferential targeting of the mammalian β2 subunit (Figure 4B). As described above, LU102 has a small window of selective β2 targeting in *Plasmodium* and coinhibits β5 and β1 at higher concentrations. We infected Balb/c female mice with 1 million *P. chabaudi* parasites and treated them with either vehicle (n = 4) or 20 mg/kg of LU102 (n = 4) via intravenous injection for four consecutive days from day 2 to day 5 postinfection (Figure 5B). LU102 treatment significantly inhibited *P. chabaudi* growth with no detectable host toxicity (Supporting Information Figure 6). This treatment was also substantially more effective at slowing parasite growth than treatment with 50 mg/kg of PR709A.

As both PR709A and LU102 treatments did not cause apparent host toxicity, we wanted to determine the effects of coinhibition of both β5 and β2 subunits on *P. chabaudi* infection. Since we believed that the enhanced parasite killing observed for LU102 compared to PR709A was the result of its coinhibition of multiple subunits, we reasoned that it might be more effective to use a combination of LU102 and PR709A to ensure more complete inhibition of both β5 and β2 subunits. Therefore, we treated mice with PR709A (50 mg/kg) and LU102 (20 mg/kg) as a single bolus dose via tail-vein injection 1 day after infection. We observed a strong decrease in parasite replication with this treatment even with just a single dose (Figure 5C and D). This treatment caused a small amount of toxicity in the host as we observed reduced activity of the mice immediately following the injections. However, the mice recovered normal activity in less than a day. To reduce this apparent toxicity, we performed the treatment at 50 mg/kg of PR709A and 10 mg/kg of LU102 as a single dose that was injected i.v. on day 1, 3, and 5 postinfection. This dose was able to reduce parasite replication in vivo with overall low toxicity as assessed by good general activity of mice and insignificant weight loss over the period of treatment (Figure 5C and D; Supporting Information Figure 6). For both cotreatment regimes, we observed close to complete clearance of parasite load over the period of treatment (Figure 5D). Moreover, we observed that the mice that received either of the cotreatments had a much less severe form of the infection, where the maximum parasitemia of the treated mice never reached levels found in the vehicle treated animals (Figure 5C). Furthermore, the mice that received the cotreatments lost substantially less weight over the course of infection (Supporting Information Figure 6). To our knowledge, this is the first demonstration of proteasome inhibitors that can dramatically reduce parasite load with no host morbidity and at minimal toxicity.

**Conclusion.** The *Plasmodium* proteasome has been validated as a potential antimalarial target, however, the main obstacle to further drug development efforts is the significant host toxicity caused by proteasome inhibitors that cotarget the mammalian proteasome. In the work presented here, we first demonstrate that proteasome inhibitors often do not have the same subunit-targeting profiles for the host and parasite proteasomes. Furthermore, *Plasmodium* parasites undergoing schizogony are highly sensitive to selective inhibition of the chymotrypsin-like activity of the proteasome, a phenomenon that is not observed in mammalian cells. However, not all stages of the parasite are equally sensitive to proteasome inhibition, and the most significant parasite killing results from coinhibition of multiple *Plasmodium* catalytic proteasome subunits.

We find *P. falciparum’s* sensitivity to proteasome inhibition during schizogony especially intriguing. We predict that this is due to a number of different factors. This includes rapid nuclear division in schizogony, which makes parasites especially

**Figure 5.** In vivo assessment of subunit selective inhibitors in *Plasmodium chabaudi* mouse model of malaria. (A) Infected Balb/c mice were treated with 50 mg/kg of PR709A (n = 5) or vehicle (n = 4) for 3 consecutive days via tail vein injections starting from day 2 postinfection. Treatment was administered at night. (B) *P. chabaudi* infected Balb/c mice were treated with 20 mg/kg of LU102 (n = 4) or vehicle (n = 4) for 4 consecutive days via i.v. injections. (C) Infected mice were treated with vehicle or combination of LU102 and PR709A (n = 4 for each group), at the indicated amounts. Compounds were administered at night. The dosing schedule is indicated by the dots below the day. Percent parasitemia was measured by blood smear counts and FACS analysis. (D) Plot of data from panels A–C expressed as a percentage of the vehicle treated mice (Color of each bar corresponds to the same color used in A–C). Error bars represent SEM.
susceptible to a block of proteasome-mediated regulation of cell cycle factors. Synthesis of short-lived proteins involved in nuclear division and subsequent cell division and egress from the host cell may also increase the burden on the proteasome. This effect of increased proteasome burden is one of the underlying reasons for the heightened sensitivity to proteasome inhibition in multiple myeloma cells. Finally, since proteasome transcription reaches a maximum during late-trophozoite stage, it is possible that proteasomes that are inhibited during the schizont stage are not readily repurposed thus causing difficulties for parasite egress from the host cell.

With an understanding of the catalytic subunits that are important for parasite survival, we can now devise an optimal strategy for designing highly potent Plasmodium proteasome inhibitors that are able to selectively kill parasites. Schizont growth can be selectively attenuated with inhibition of β5 alone. For inhibition at all stages in the blood cycle, coinhibition of β5 with β2 and/or β1 is required for maximum killing. We have shown here that mammalian proteasome inhibitors that target just one catalytic proteasome site often can coinhibit several catalytic subunits of the Plasmodium proteasome at the same concentration. This, together with nontransformed and endogenous nonreplicating mammalian cells’ high resistance to short-term proteasome inhibition, allow us to use proteasome inhibitors as antimalarial therapeutic at low host toxicity.

In conclusion, we have used subunit selective proteasome inhibitors and a proteasome activity based probe to determine subunit dependency of the Plasmodium proteasome. With this new roadmap for how to effectively target parasites with proteasome inhibitors, our future work will focus on optimizing inhibitors that target multiple catalytic subunits in the parasite proteasome while showing weak binding to the host proteasome.

**METHODS**

Additional details and sections are available in Supporting Information. Synthetic procedures for NC001, LU102, NC005-mvs, PR709A, and WL40723 have been previously reported. Activity-Based Probe Labeling of Mammalian and Parasite Lysates. Synthesis of BMV037 is described in Supporting Information. For all lysates or purified proteasome labeling experiments, MV151 was used at a final concentration of 2 μM and BMV037 was used at a final concentration of 10 μM. Lysates or purified proteasome were incubated with MV151 at 37 °C for 1 h, or BMV037 for 3 h at 37 °C. Samples were denatured by addition of SDS sample buffer, boiled briefly, and run on a 12% SDS PAGE. Gels were scanned at the TAMRA channel (for MV151) or Cy5 channel (for BMV037) on a Typhoon Scanner (GE Healthcare). Quantification of the intensity of the labeled proteins was done using ImageJ. A positive control was included in all experiments to allow background subtraction of the labeled subunits.

Parasite Culture, Harvesting of Life Cycle Stages, and Lysate Preparation. P. falciparum D10 cultures were maintained, synchronized, and lysed, as previously described. Tightly synchronized parasites for stage specificity experiments were obtained by enriching for mature schizonts on a 70% Percoll gradient followed by sorbitol treatment to a ~2 h window of synchrony.

Correlation of P. falciparum Proteasome Activity to Viability. P. falciparum was cultured at around 15–25% parasitemia at 1% hematocrit to ensure sufficient parasite was available for proteasome labeling. P. falciparum culture (500 μL per well) was first treated for the indicated amount of time, and spun down at 3200 rpm for 3 min and supernatant aspirated to remove inhibitor. Culture was then washed 2 times in fresh media, and resuspended in 500 μL of media. This culture (20 μL) was added to 180 μL of 1% hematocrit, thus diluting the parasitemia 10-fold to allow for the parasites to reinfect. Parasitemia was assessed after 48–60 h when parasites were at late trophozoites/early schizonts. The remaining inhibitor-treated culture was spun down, and parasite lysate was prepared and labeled with activity-based probe, as described above.

**In Vivo Assessment of Proteasome Inhibitors.** All mouse experiments were approved by the Stanford Administration Panel on Laboratory Animal Care and we strictly followed their specific guidelines. For each drug test, Balb/c mice (~20 g) were infected by intraperitoneal (i.p.) injection with 1 × 10⁶ P. chabaudi parasites isolated from an infected mouse on Day 0. Drug dose and formulation are described in Supporting Information. All treatment groups were closely monitored for drug-induced toxicity by observing physical appearance and activities of mice. Weight of mice was also monitored daily after infection. Parasitemia was monitored daily by thin blood smear obtained from the tail vein and quantified by light microscope counting and FACS.

**ASSOCIATED CONTENT**

6 Supporting information
Additional methods, table, and figures. 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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