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Improvement of asparagine ethylenediamines as anti-malarial *Plasmodium*-selective proteasome inhibitors

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Abstract

The *Plasmodium* proteasome (Pf20S) emerged as a target for antimalarials. Pf20S inhibitors are active at multiple stages of the parasite life cycle and synergize with artemisinins, suggesting that Pf20S inhibitors have potential to be prophylactic, therapeutic, transmission blocking as well as useful for combination therapy. We recently reported asparagine ethylenediamines (AsnEDAs) as immunoproteasome inhibitors and modified AsnEDAs as selective Pf20S inhibitors. Here we report further structure-activity relationship study of AsnEDAs for selective inhibition of Pf20S over human proteasomes. Additionally, we show a new mutation that conferred resistance to AsnEDAs and collateral sensitivity to an inhibitor of the Pf20S $\beta 2$ subunit, same as previously identified resistant mutation. This resistance could be overcome through the use of structure-guided inhibitor design. Collateral sensitivity to inhibitors among respective proteasome subunits

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Author Contributions

G. L., L.A.K. and W.Z. designed the research and wrote the manuscript; W.Z., H.Z. and P.K.S. synthesized the new compounds; W.Z., J.V., T.O. and J.C.H. performed biochemical and cell-based experiments; J.I.O., R.M.S., P.K.T. and R.A.C. performed Ex vivo experiment. R.W. and G.S. carried out the HPLC and HRMS analysis; B.F.C.K performed analysis of whole genome sequencing results. All authors discussed the results and commented on the manuscript.

The authors declare no competing financial interests.

ASSOCIATED CONTENT

Supporting Information. Synthesis and compound characterization of all new compounds, Table S1–2 and Figure S1–2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

underscores the potential value of treating malaria with combinations of inhibitors of different proteasome subunits to minimize the emergence of drug resistance.

Graphical Abstract

Keywords

Malaria proteasome inhibitors; antimalarials; resistance

Introduction

Since the first isolation of quinine from Cinchona bark in 1820,¹ many effective small molecules have been developed and approved for the treatment of malaria. Yet, despite significant advances, morbidity and mortality from malaria remain intolerably high, with increasing incidence in many countries in Africa noted in the most recent World Health Organization (WHO) malaria report.² Malaria is due to infection with the eukaryotic parasite *Plasmodium*, leading to an estimated 219 million malaria cases and 435,000 deaths worldwide in 2017.² *P. falciparum* is the most virulent human malaria parasite, and is responsible for the large majority of deaths. Treatment of malaria with artemisinin-based combination therapy (ACT) and vector control measures are key to lessening the burden of malaria. However, recent reports of emerging resistance to ACTs³⁻⁴ and widespread resistance to other previously standard antimalarials⁵ and some ACT partner drugs⁶ underscores the need to develop drugs with novel targets that have efficacy against multiple stages of the parasite. The *Plasmodium* life cycle includes hepatic, erythrocytic and gametocyte stages in the human host and additional developmental stages in the mosquito vector. Drugs that are effective at various stages are highly desirable for malaria treatment and prevention, yet few of the antimalarials in use are effective throughout the parasite life cycle.

Interference with protein degradation is of interest for development of antimicrobials, beginning with the introduction of species-selective proteasome inhibitors against *Mycobacterium tuberculosis* and followed by reports of species-selective inhibitors of the proteasomes of trypanosomes and *Leishmania*.^{7–10} The *Plasmodium* proteasome (Pf20S) has also been explored as a novel target in vitro and in animal models of malarial infection.^{9–14} Genetic evidence demonstrates the essentiality of the Pf20S, and multiple lines of evidence suggest that inhibitors of Pf20S could be broadly effective as therapeutic, prophylactic and transmission-blocking compounds.^{12, 15} Further, proteasome inhibitors act synergistically with artemisinins against malaria parasites in vitro, and a potential link to the mode of action of artemisinin has been proposed.^{9, 12, 16}

The antimalarial effects of non-selective proteasome inhibitors and the development of inhibitors with selectivity for *Plasmodium* proteasomes have been reported (Figure 1). For example, irreversible active site modifiers compounds 18¹⁰ and 28¹¹ were recently reported to be more cytotoxic to *P. falciparum* than to human cells. However, species selectivity at the enzyme level was limited, with IC₅₀ values against Pf20S only a few-fold lower than against human proteasomes. Humans express two major classes of proteasomes, the constitutive proteasome (c-20S) and immunoproteasome (i-20S), as well as intermediate forms that contain a mixture of subunits characteristic of each. The constitutive proteasome (c-20S) is expressed in all cells, whereas immunoproteasome (i-20S) is predominantly expressed in immune cells and can be upregulated in many types of cells stimulated with interferon γ or at the site of inflammation or infection. Inhibition of the human c-20S leads to cell death by apoptosis, whereas specific inhibition of the i-20S leads to host immune suppression.^{17–19} Thus, to mitigate the toxicity of Pf20S inhibitors, it is vital to establish species selectivity at the enzyme level against both c-20S and i-20S.

Recently, we reported asparagine ethylenediamines (AsnEDAs, Figure 1) as a versatile class of peptidomimetic proteasome inhibitors.¹⁸ The cryo-EM structure of the human i-20S with PKS21004 showed that the P1 biphenyl ring binds to the S1 and S1' side pockets (S1SP), the P3 asparagine *t*-butyl amide binds to the S3 pocket and the P4 phenylpropionate moiety binds to the S4 pocket. A concise structure-activity relationship study of this scaffold revealed that introduction of hydrophilic moieties at both ends enhanced selectivity over the human proteasomes without sacrificing potency against Pf20S or anti-parasite activity against erythrocytic stages of *P. falciparum*.¹²

To gauge target engagement, we selected for parasites resistant to PKS21004 using the Dd2 *P. falciparum* strain, and identified a mutation in Pf20S β 6 (A117D) in these parasites. The β 6A117D mutation appears to induce conformational changes in the adjacent regions of β 6 and β 5, which likely propagates to S1SP and S3 of β 5. In line with this hypothesis, the β 6A117D mutation confers resistance to AsnEDAs, but not to bortezomib or carfilzomib, both of which do not directly bind to the S1SP nor fully occupy the S3 binding pockets as the AsnEDAs.¹² This mutation increases greater susceptibility to a β 2 inhibitor, demonstrating collateral sensitivity between inhibitors that target different active proteasome subunits.¹² Herein we report further SAR studies of AsnEDAs, AsnEDA resistance in *Plasmodium* and structural modifications that minimize AsnEDA cross resistance while retaining potency and selectivity.

Results and Discussion

The Pf20S β 5A49S mutation confers resistance to proteasome inhibitors

In earlier work, we repetitively exposed *Pf*Dd2 to increasing concentrations of PKS21004 and isolated two resistant clonal parasite lines with a 130-fold shift in EC₅₀ compared to the parental Dd2 parasites.¹⁸ Both resistant clones harbor the mutation A117D in β 6 of Pf20S. β 6 A117 is in close proximity to two tyrosines, and this mutation to a charged aspartate is predicted to lead to a steric conflict with the tyrosines and subsequent conformational changes in the S1 side pocket (S1SP) and part of the S3 pocket, enabling resistance to AsnEDAs. This hypothesis is supported by the lack of resistance of Dd2- β 6A117D parasites to bortezomib and carfilzomib as there is no or minimal overlap in binding. Bortezomib does not bind either the S1SP or the S3 and though carfilzomib does bind to the S3, it does so with a smaller P3 Leu residue and thus less affected by conformational changes attributed to the β 6A117D mutation. In fact, the Dd2- β 6A117D mutant parasites are 4-fold more sensitive to bortezomib than are parental Dd2 cells and 12.5-fold more sensitive than the parental parasites to a Pf20S β 2 inhibitor, WLW-VS.

We next chose an alternative AsnEDA and selected for resistance by exposing *Pf* cultures in vitro to multiple high dose pulses of TDI4258 over one week followed by approximately three weeks of culture without the compound to recover normal growth before isolating the resistant clonal parasite lines. Whole-genome sequencing identified a variant in the β 5-encoding *PRE2* gene that resulted in an A49S mutation in β 5 (Table S1).

Amino acid A49 of the 20S β 5 is highly conserved across different 20S cores from bacterial to human proteasomes. It is proposed to participate in binding with substrate and with peptide-based proteasome inhibitors by forming a hydrogen bond with a backbone amide.²⁰ In line with this, several studies established that certain A49 polymorphisms (A49S, A49V or A49T) in cancer cell lines and yeast confer resistance to bortezomib.^{20–21} Similarly, Dd2- β 5A49S is highly resistant to PKS21004 and TDI4258 and demonstrates a 541-fold and >100-fold increases in EC₅₀ values (table 1), respectively. This shift is much greater than those seen with Dd2- β 6A117D resistant parasites. Additionally, the Dd2- β 5A49S mutant was 2.4-fold more resistant to bortezomib than the parental strain. This is in contrast to the Dd2- β 6A117D strain, which was 4-fold more susceptible to bortezomib than the parental strain. However in a similar fashion to the Dd2- β 6A117D mutation, the Dd2- β 5A49S mutation showed 4-fold increased susceptibility to the β 2 inhibitor, WLW-VS. At the enzyme label, the β 5A49S mutation showed increased labeling of the Pf20S β 5 by MV151 comparing to wild type in the presence of TDI4258, suggesting a markedly reduced binding of TDI4258 to the Pf20S β 5A49S (Figure S1).

Structure-activity relationship

We hypothesized that the resistance to AsnEDAs arising from the β 6A117D mutation could be ascribed to conformational changes in the S1SP pocket and that resistance arising from the β 5A49S mutation could be due to interference with hydrogen bonding between Pf20S β 5 and the amide backbone of a peptidomimetic inhibitor. We speculated that modifying the AsnEDAs could circumvent or reduce resistance associated with these mutations. Previous

studies demonstrated that toluene-sulfonyl (tosyl) and 5-methylisoxazol-3-carboxylate are two optimal P4 constituents of the AsnEDAs integral for Pf20S inhibition.¹² P4 p-toluenesulfonamide PKS21221 and its matched pair isoxazolyl carboxamide PKS21208 showed equal activities against Pf20S enzyme (IC₅₀ values: 3.8 nM vs 5.2 nM) and anti-malarial activity in cultured *P. falciparum* (EC₅₀ values: 1.6 nM vs 1.7 nM). However, truncation of the S1SP binding group to a 2-F phenyl in both compounds resulted in differential loss in potency against the Pf20S enzyme and against cultured parasites (Table 2). WHZ-05, with a P4 tosyl, was associated with an 8.4-fold decrease in enzyme inhibition and 4.7-fold decrease in activity against cultured parasites compared to PKS21221, whereas PKS21251 lost over 1000-fold in potency in both the enzymatic assay and antiparasitic activity against Pf 3D7 in red blood cells. These results suggested that protein P4 p-toluenesulfonamide was able to compensate for the more truncated P1 substitution.

To further explore the SAR of AsnEDAs, 24 compounds with diverse P4 p-toluenesulfonamide and P1 groups were synthesized as illustrated in Scheme 1. Structures and purity of all compounds were confirmed by ¹H-NMR, ¹³C-NMR LC-MS and HR-MS (see Supporting Information). Enzyme inhibition IC₅₀ values against Pf20S β5, c-20S β5c and i-20S β5i were determined as reported (Table 3 and Figure 2A).

Replacing the P1 amide with urea, phenylurea WHZ-01 (IC₅₀-Pf20S, 2576 nM) and benzylurea WHZ-02 (2766 nM), respectively, or sulfonamide (WHZ-03, 15135 nM) significantly reduced inhibitory potency against the enzymatic activity of the Pf20S β5, β5c and β5i, and diminished their activity against cultured parasites (Table 3), suggesting a delicate binding mode of the P1 in AsnEDAs at the S1 pocket that is immediately adjacent to the catalytic site. Next, we focused on substituted P1 benzamides. Benzamide WHZ-04 was potent against Pf20S β5 (20.8 nM), with marked selectivity over β5c (68-fold) and modest selectivity over β5i (4.8-fold). Introduction of a 2-F (WHZ-05) or 3-F substituent (WHZ-06) on the phenyl ring of WHZ-04 had little effect on potency and selectivity, but 4-F (WHZ-07) led to 6-fold reduction in potency and 3-fold loss in selectivity over i-20S. Incorporation of 2-Cl led to a 10-fold reduction in potency (WHZ-08, 211 nM), whereas introduction of 2-OH (WHZ-10, 6.1 nM) greatly improved Pf20S potency and maintained selectivity. Installing a hydroxyl group at 3- position yielded the more selective inhibitor WHZ-11 compared to WHZ-05, with 195- and 21- fold selectivity for Pf20S β5 over c-20S β5c and i-20S β5i, respectively. Replacing the 3-OH of WHZ-11 with 3-MeO (WHZ-12, 8.9 nM) and 3-ethynyl group (WHZ-13, 4.7 nM) enhanced potency by 2.5- and 4.8-fold, respectively. In this study, WHZ-13 was the most potent AsnEDA-based Pf20S inhibitor, with good selectivity over c-20S and i-20S (90- and 20- fold, respectively). In contrast, a bulkier substituent, for example isopropyl (WHZ-14), or strong electron withdrawing groups (WHZ-15 and WHZ-16), were poorly tolerated. Replacement of the P1 phenyl with a pyridyl ring resulted in a significant reduction in potency (WHZ-17, 18 and 19). In an effort to extend the SAR to di-substituted P1, WHZ-20, 21, 22, 23 and 24 were synthesized and studied. None of these compounds exhibited improved Pf20S inhibition or species selectivity relative to WHZ-13.

Additionally, the AsnEDAs inhibitors reported above were inactive against $\beta 1$ and $\beta 2$ of the Pf20S, as WHZ-13 only inhibited the labeling of the Pf20S $\beta 5$ by a pan proteasome activity-based fluorescent probe MV151,²² but not $\beta 1$ and $\beta 2$ (Figure 2B).

In a report on the peptide epoxyketone chemotype of proteasome inhibitors, Pf20S showed a much better tolerability for *D*-amino acids at the P3 position than human c-20S and i-20S, and was associated with reduced host cell toxicity.¹⁰ To explore if replacing the *L*-Asn(*t*-Bu) with *D*-Asn(*t*-Bu) in AsnEDAs could retain the potency and/or improve selectivity, WHZ-12D and WHZ-13D were synthesized and characterized, but the potency of both compounds was reduced by >200-fold against Pf20S (Table S2) compared to their *L*-counterparts, suggesting that *D*-Asn(*t*-Bu) AsnEDAs do not fit into enzyme binding sites optimally.

Activity of AsnEDAs against cultured *P. falciparum*

Next, we determined the activity of all synthesized compounds against cultured Pf3D7 (Table 3), including WHZ-12D and WHZ-13D (Table S1). The Pf20S inhibitory activities, IC₅₀ data, correlated well with activity against cultured parasites, EC₅₀ data (Figure S2), suggesting a consistent pharmacology of the series. We also determined the cytotoxicity of synthesized compounds against human HepG2 and Karpas 1106P cells, and found that none of the compounds showed EC₅₀ lower than 11.1 μ M, the highest concentration tested (Table 3). Due to the importance of combination therapy while treating malaria and our previous work demonstrating synergy between inhibitors targeting multiple catalytic proteasome subunits, we tested the in vitro activity of WHZ-04 in the presence of Pf20S $\beta 2$ inhibitor WLW-VS. Consistent with our previous data, isobologram analysis demonstrated synergistic to additive killing of wild-type PfDd2 parasites by the combination of WHZ-04 and WLW-VS in a 72-h dual drug assay (FIC 0.52 ± 0.13 from three independent experiments) (Fig. 3B).

Additionally, WHZ-13 was further tested for *ex vivo* activity against 34 fresh *P. falciparum* isolates collected from malaria patients in Uganda. The EC₅₀ values of WHZ-13 against Ugandan isolates ranged from 4.4 – 16.0 nM, (geometric mean and SD 7.1 ± 1.3 nM) (Fig. 3C). As a control, chloroquine showed an EC₅₀ range from 4.5 – 27 nM (16.9 ± 1.6 nM) against the same isolates.

Resistance profile of AsnEDAs

To see how the change of P1 affected the resistance profile of Dd2- $\beta 6A117D$ and Dd2- $\beta 5A49S$, we determined EC₅₀ values for select AsnEDAs against parental Dd2 and both resistant lines (Table 4). In contrast to high level resistance to PKS21004 (130-fold and 541-fold) and TDI4258 (10-fold and >100-fold), Dd2- $\beta 6A117D$ and Dd2- $\beta 5A49S$ had reduced resistance to WHZ-04, -12, -13. Among them, WHZ-04 with P1 benzamide showed the smallest shift in EC₅₀ against both resistant strains. Furthermore, WHZ-04 showed increased inhibition of labeling the Pf20S $\beta 5A49S$ in comparison to TDI4258 (Figure S1), suggesting that reducing the size of the P1 substituent may offer an opportunity to circumvent resistance.

In vitro pharmacokinetic properties

We determined in vitro pharmacokinetic properties for select compounds (Table 5). All AsnEDAs showed reasonable permeability by PAMPA at pH 7.4. However, WHZ-05 was cleared by liver microsomes (human, rat and mouse) at rates of 141, 124, 141.4 $\mu\text{L}/\text{min}/\text{mg}$, respectively. Change of the P1 2-fluorobenzamide (WHZ-05) to 4-fluorobenzamide (WHZ-07) improved stability in rat and mouse microsomes, but not in human liver microsomes. Change to P1 3-methoxybenzamide (WHZ-12) did not improve stability against human, rat or mouse liver microsomes. However, the P1 benzamide in WHZ-04 and 3-ethynylbenzamide in WHZ-13 markedly improved the microsomal stability in rat and mouse.

Conclusion

Compounds that selectively target proteasomes of microbes have recently been intensely pursued for *Mycobacterium tuberculosis*, *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania donovani*.^{7–8, 23} Several studies have also shown that selective Pf20S inhibitors can be developed.^{9, 11, 13} The synergy observed between proteasome inhibitors and artemisinins suggests that a Pf20S inhibitor drug could provide benefit both on its own and as a partner in ACT.^{9, 12, 16} To mitigate host toxicity from cross-inhibition of human proteasomes, much effort has been made to improve species selectivity while maintaining potency. Furthermore, understanding the potential mechanisms of resistance against proteasome inhibitors is critical for developing anti-microbial proteasome inhibitors.

Several recent studies reported the development of peptide epoxyketones, peptide vinyl sulfones and peptide boronate Pf20S selective inhibitors.^{10–11, 24} However, the energy gained through the reaction of the warheads with the hydroxy group of the active site might offset the differential binding affinity of the space filling moieties, which may consequently reduce selectivity. We recently reported non-covalent species-selective AsnEDAs that are active against *Pf* at multiple stages of the life cycle.¹² In the current study, we extended our studies of this class of inhibitors for Pf20S.

In addition to the reported $\beta 6\text{A}117\text{D}$ mutation, we identified a novel mutation, $\beta 5\text{A}49\text{S}$, that also confers resistance to AsnEDAs. $\beta 5\text{A}49$ is highly conserved across species, and several studies of cancer cells have reported mutations at A49 that confer resistance to bortezomib or carfilzomib.^{20, 25} A49 is part of the S1 pocket of the substrate binding channel and crucial for interactions with substrates and inhibitors.²⁶ Its mutation restricts access to the active site of the $\beta 5$ subunit. How this mutation affects the binding of the AsnEDAs remains to be explored. We are actively pursuing the structure of Pf20S with one of the P4 p-toluenesulfonamide AsnEDA by cryo-EM.

We extended our initial structure-activity relationship study of AsnEDAs for Pf20S by exploring P1 and P4 moieties. We found that a P4 p-toluenesulfonamide, but not a P4 isoxazole-3-carboxamide, allowed the P1 biphenyl group to be replaced with substituted benzamides without forgoing potency and selectivity. In agreement with a structural analysis, replacing the P1 biphenyl with a phenyl markedly reduced resistance without sacrificing potency or species selectivity.

Pharmacokinetic studies demonstrated that metabolic stability was markedly improved by changing the substituents on the P1 phenyl rings, suggesting that further studies of trimming of P1 substituents may improve potency, selectivity and metabolic stability.

Experimental Section

The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consent. Purity of all final compounds were determined on a Waters Acquity Ultra Performance Liquid Chromatography (UPLC/MS) and all were > 95%.

In vitro cultivation

P. falciparum laboratory lines were grown under standard conditions at 5% hematocrit in RPMI 1640 medium, 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate and 0.1 mg/ml gentamicin. Parasites were placed in an incubator under 5% carbon dioxide, 5% oxygen and 90% nitrogen at 37 °C as previously described.

Human constitutive proteasome, immunoproteasome (peripheral blood mononuclear cells), proteasome $\beta 5$ substrate suc-LLVY-AMC, $\beta 5i$ substrate Ac-ANW-AMC,²⁷ were purchased from Boston Biochem. MV151 was synthesized following the reported method.²² WLW-VS was synthesized as reported.⁹

Variant identification in resistant clones

Selection of parasites resistant to TDI4258 required three different attempts. We selected with the EC₉₀ of the compound (60nM) at a starting parasitemia of 0.5%. Parasites were grown for one week at this level of selection and then allowed to recover which took approximately three weeks. Parasites were allowed to recover to a 2% parasitemia and then diluted to 0.5% parasitemia and placed on increased drug pressure (120nM). There was no reduction of parasite growth at this selection pressure and clonal resistant parasites were isolated by limiting dilution as described.¹² Our resistant clones were maintained on 160nM of compound though the level of resistance was stable when drug pressure was removed and parasites were repeatedly challenged. We attempted to induce resistant parasite lines from the 3D7 lab line, Dd2 and IPC4884, a recently described artemisinin resistant parasite, but as previously reported, we were only successful in isolating resistant parasites from the Dd2 parental line.

One clonal isolate from TDI4258 resistant parasite population showing 100-fold shift in EC₅₀ was submitted for whole genome sequencing using the Mi-Seq platform as reported.¹²

Enrichment of Pf20S

Enrichment was performed as reported with modifications to the order of chromatography column.¹² In brief, cell free lysates of *P. falciparum* N54 (TropIQ, Netherland) parasites were first concentrated using 100 kD cutoff spin columns, followed by two column chromatographic separation: a Superose 6 column (10 × 300 mm) and a DEAE FF column

(5 mL). Fractions with LLVY-AMC proteolytic activity were pooled, concentrated, aliquoted and stored at -80°C .

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Pf20S active subunits labeling by MV151

Enriched Pf20S (15 nM) or Pf20S β 5A49S (2 μg) in 20 mM HEPES, 0.5 mM EDTA, PH 7.5 was incubated with test compounds at the indicated concentrations for 1 h at 37°C prior to addition of MV151 (final concentration at 2 μM) and incubated for a further 1 h at 37°C . The samples were then heated with 4X SDS loading buffer at 95°C for 10 min and run on a 12% Novex™ Bis-Tris Protein Gel with MOPS SDS running buffer. The gel was rinsed with double distilled H_2O and scanned at the TAMRA channel on a Typhoon Scanner (GE Healthcare).

IC₅₀ determination

IC₅₀ values against human c-20S β 5c and i-20S β 5i were determined from 11-point serial dilution of inhibitors in DMSO as reported.^{28–29} For the IC₅₀ values against Pf20S β 5 (final concentration 1 nM), we conducted the experiment in the presence of 0.5 μM WLW-VS in enzymatic assays.¹² Substrate suc-LLVY-AMC was used for c-20S and Pf20S at final concentration 25 μM , and Ac-ANW-AMC was used as substrate of i-20S at final concentration 15 μM . Activator PA28 was used for Pf20S, as Pf20S is not stable in the presence of 0.02% SDS, which was used in the assays for c-20S and i-20S.

Anti-malarial activity in erythrocytic stage

Parasite growth inhibition assays were performed on parasites cultured in sterile 96-well plates at a total 200 μL volume per well and a 0.5% initial parasitemia and 2% hematocrit. Plates were placed in an airtight chamber flushed with 5% O_2 , 5% CO_2 and 90% N_2 for 72 hours. Plates were then placed in the -80°C freezer for lysis upon thawing.³⁰ SYBR Green solution (100 μL of 0.2 μL SYBR Green per mL lysis buffer) was then added to each well and the plates were shaken in the dark at room temperature for 1 hour. Fluorescence was then recorded in a SpectraMax Gemini plate reader with excitation 490 nm and emission 530 nm. Fluorescent counts were normalized and plotted by non-linear least square regression to yield EC₅₀ values (PRISM). In vitro dual drug assay and isobologram analysis were completed as previously described.¹² Five volume to volume (1:0, 3:1, 1:1, 1:3 and 0:1) drug combinations were plated onto a 96 well plate and parasite growth at 72 hours was

assayed as described above. This was done three times independently and performed in triplicate each time.

Ex vivo EC₅₀ values against *P. falciparum* field isolates in Uganda

The activity of WHZ-13 was tested, as previously described, against *P. falciparum* isolates using a 72-h growth inhibition assay with parasite DNA readout by Sybr Green detection.^{30–31} These isolates were collected in June–August, 2018 from patients living in the Tororo and Busia Districts, Uganda, who were newly diagnosed with *P. falciparum* malaria and before antimalarial treatment was administered. The relevant clinical trials and analyses of cultured parasites were approved by the Uganda National Council of Science and Technology, the Makerere University Research and Ethics Committee, and the University of California, San Francisco Committee on Human Research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Pf20S	<i>P. falciparum</i> 20S proteasome
c-20S	constitutive proteasome
i-20S	immunoproteasome

REFERENCES

1. Kyle RA; Shampo MA, Discoverers of quinine. JAMA 1974, 229 (4), 462. [PubMed: 4600403]
2. WHO World Malaria Report 2018; World Health Organization: Geneva: World Health Organization, 2018.
3. Ashley EA; Dhorda M; Fairhurst RM; Amaratunga C; Lim P; Suon S; Sreng S; Anderson JM; Mao S; Sam B; Sopha C; Chuor CM; Nguon C; Sovannaroeth S; Pukrittayakamee S; Jittamala P; Chotivanich K; Chutasmit K; Suchatsoonthorn C; Runchaoren R; Hien TT; Thuy-Nhien NT; Thanh NV; Phu NH; Htut Y; Han KT; Aye KH; Mokuolu OA; Olaosebikan RR; Folaranmi OO; Mayxay M; Khantavong M; Hongvanthong B; Newton PN; Onyamboko MA; Fanello CI; Tshefu AK; Mishra N; Valecha N; Phyo AP; Nosten F; Yi P; Tripura R; Borrmann S; Bashraheil M; Peshu J; Faiz MA; Ghose A; Hossain MA; Samad R; Rahman MR; Hasan MM; Islam A; Miotto O; Amato R; MacInnis B; Stalker J; Kwiatkowski DP; Bozdech Z; Jeeyapant A; Cheah PY; Sakulthaew T;

Chalk J; Intharabut B; Silamut K; Lee SJ; Vihokhern B; Kunasol C; Imwong M; Tarning J; Taylor WJ; Yeung S; Woodrow CJ; Flegg JA; Das D; Smith J; Venkatesan M; Plowe CV; Stepniewska K; Guerin PJ; Dondorp AM; Day NP; White NJ; Tracking Resistance to Artemisinin, C., Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 2014, 371 (5), 411–423. [PubMed: 25075834]

4. Yeung S; Socheat D; Moorthy VS; Mills AJ, Artemisinin resistance on the ThaiCambodian border. *Lancet* 2009, 374 (9699), 1418–1419. [PubMed: 19854365]
5. Rosenthal PJ, The interplay between drug resistance and fitness in malaria parasites. *Mol Microbiol* 2013, 89 (6), 1025–1038. [PubMed: 23899091]
6. Ouji M; Augereau JM; Paloque L; Benoit-Vical F, *Plasmodium falciparum* resistance to artemisinin-based combination therapies: A sword of Damocles in the path toward malaria elimination. *Parasite* 2018, 25, 24. [PubMed: 29676250]
7. Lin G; Li D; de Carvalho LP; Deng H; Tao H; Vogt G; Wu K; Schneider J; Chidawanyika T; Warren JD; Li H; Nathan C, Inhibitors selective for mycobacterial versus human proteasomes. *Nature* 2009, 461 (7264), 621–626. [PubMed: 19759536]
8. Lin G; Chidawanyika T; Tsu C; Warriar T; Vaubourgeix J; Blackburn C; Gigstad K; Sintchak M; Dick L; Nathan C, N,C-Capped dipeptides with selectivity for mycobacterial proteasome over human proteasomes: role of S3 and S1 binding pockets. *J Am Chem Soc* 2013, 135 (27), 9968–9971. [PubMed: 23782398]
9. Li H; O'Donoghue AJ; van der Linden WA; Xie SC; Yoo E; Foe IT; Tilley L; Craik CS; da Fonseca PC; Bogoy M, Structure- and function-based design of *Plasmodium*-selective proteasome inhibitors. *Nature* 2016, 530 (7589), 233–236. [PubMed: 26863983]
10. LaMonte GM; Almaliti J; Bibo-Verdugo B; Keller L; Zou BY; Yang J; Antonova-Koch Y; Orjuela-Sanchez P; Boyle CA; Vigil E; Wang L; Goldfod GM; Gerwick L; O'Donoghue AJ; Winzeler EA; Gerwick WH; Otilie S, Development of a Potent Inhibitor of the *Plasmodium* Proteasome with Reduced Mammalian Toxicity. *J Med Chem* 2017, 60 (15), 6721–6732. [PubMed: 28696697]
11. Yoo E; Stokes BH; de Jong H; Vanaerschot M; Kumar T; Lawrence N; Njoroge M; Garcia A; Van der Westhuyzen R; Momper JD; Ng CL; Fidock DA; Bogoy M, Defining the Determinants of Specificity of *Plasmodium* Proteasome Inhibitors. *J Am Chem Soc* 2018, 140 (36), 11424–11437. [PubMed: 30107725]
12. Kirkman LA; Zhan W; Visone J; Dziedzic A; Singh PK; Fan H; Tong X; Bruzual I; Hara R; Kawasaki M; Imaeda T; Okamoto R; Sato K; Michino M; Alvaro EF; Guiang LF; Sanz L; Mota DJ; Govindasamy K; Wang R; Ling Y; Tumwebaze PK; Sukenick G; Shi L; Vendome J; Bhanot P; Rosenthal PJ; Aso K; Foley MA; Cooper RA; Kafsack B; Doggett JS; Nathan CF; Lin G, Antimalarial proteasome inhibitor reveals collateral sensitivity from intersubunit interactions and fitness cost of resistance. *Proc Natl Acad Sci U S A* 2018, 115 (29), E6863–E6870. [PubMed: 29967165]
13. Li H; Tsu C; Blackburn C; Li G; Hales P; Dick L; Bogoy M, Identification of potent and selective non-covalent inhibitors of the *Plasmodium falciparum* proteasome. *J Am Chem Soc* 2014, 136 (39), 13562–13565. [PubMed: 25226494]
14. Li H; Ponder EL; Verdoes M; Asbjornsdottir KH; Deu E; Edgington LE; Lee JT; Kirk CJ; Demo SD; Williamson KC; Bogoy M, Validation of the proteasome as a therapeutic target in *Plasmodium* using an epoxyketone inhibitor with parasite-specific toxicity. *Chem Biol* 2012, 19 (12), 1535–1545. [PubMed: 23142757]
15. Zhang M; Wang C; Otto TD; Oberstaller J; Liao X; Adapa SR; Udenze K; Bronner IF; Casandra D; Mayho M; Brown J; Li S; Swanson J; Rayner JC; Jiang RHY; Adams JH, Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis. *Science* 2018, 360 (6388).
16. Dogovski C; Xie SC; Burgio G; Bridgford J; Mok S; McCaw JM; Chotivanich K; Kenny S; Gnädig N; Straimer J; Bozdech Z; Fidock DA; Simpson JA; Dondorp AM; Foote S; Klonis N; Tilley L, Targeting the cell stress response of *Plasmodium falciparum* to overcome artemisinin resistance. *PLoS Biol* 2015, 13 (4), e1002132. [PubMed: 25901609]
17. Muchamuel T; Basler M; Aujay MA; Suzuki E; Kalim KW; Lauer C; Sylvain C; Ring ER; Shields J; Jiang J; Shwonek P; Parlati F; Demo SD; Bennett MK; Kirk CJ; Groettrup M, A selective

- inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* 2009, 15 (7), 781–787. [PubMed: 19525961]
18. Santos R. d. L. A.; Bai L; Singh PK; Murakami N; Fan H; Zhan W; Zhu Y; Jiang X; Zhang K; Assker JP; Nathan CF; Li H; Azzi J; Lin G, Structure of human immunoproteasome with a reversible and noncompetitive inhibitor that selectively inhibits activated lymphocytes. *Nat Commun* 2017, 8 (1), 1692. [PubMed: 29167449]
19. Bedford L; Lowe J; Dick LR; Mayer RJ; Brownell JE, Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. *Nat Rev Drug Discov* 2011, 10 (1), 29–46. [PubMed: 21151032]
20. Huber EM; Heinemeyer W; Groll M, Bortezomib-resistant mutant proteasomes: structural and biochemical evaluation with carfilzomib and ONX 0914. *Structure* 2015, 23 (2), 407–417. [PubMed: 25599643]
21. Oerlemans R; Franke NE; Assaraf YG; Cloos J; van Zantwijk I; Berkers CR; Scheffer GL; Debipersad K; Vojtekova K; Lemos C; van der Heijden JW; Ylstra B; Peters GJ; Kaspers GL; Dijkmans BA; Schepers RJ; Jansen G, Molecular basis of bortezomib resistance: proteasome subunit beta5 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood* 2008, 112 (6), 2489–2499. [PubMed: 18565852]
22. Verdoes M; Florea BI; Menendez-Benito V; Maynard CJ; Witte MD; van der Linden WA; van den Nieuwendijk AM; Hofmann T; Berkers CR; van Leeuwen FW; Groothuis TA; Leeuwenburgh MA; Ovaa H; Neefjes JJ; Filippov DV; van der Marel GA; Dantuma NP; Overkleeft HS, A fluorescent broad-spectrum proteasome inhibitor for labeling proteasomes in vitro and in vivo. *Chem Biol* 2006, 13 (11), 1217–1226. [PubMed: 17114003]
23. Khare S; Nagle AS; Biggart A; Lai YH; Liang F; Davis LC; Barnes SW; Mathison CJ; Myburgh E; Gao MY; Gillespie JR; Liu X; Tan JL; Stinson M; Rivera IC; Ballard J; Yeh V; Groessl T; Federe G; Koh HX; Venable JD; Bursulaya B; Shapiro M; Mishra PK; Spraggon G; Brock A; Mottram JC; Buckner FS; Rao SP; Wen BG; Walker JR; Tuntland T; Molteni V; Glynn RJ; Supek F, Proteasome inhibition for treatment of leishmaniasis, Chagas disease and sleeping sickness. *Nature* 2016, 537 (7619), 229–233. [PubMed: 27501246]
24. Xie SC; Gillett DL; Spillman NJ; Tsu C; Luth MR; Ottilie S; Duffy S; Gould AE; Hales P; Seager BA; Charron CL; Bruzzese F; Yang X; Zhao X; Huang SC; Hutton CA; Burrows JN; Winzeler EA; Avery VM; Dick LR; Tilley L, Target Validation and Identification of Novel Boronate Inhibitors of the *Plasmodium falciparum* Proteasome. *J Med Chem* 2018, 61 (22), 10053–10066. [PubMed: 30373366]
25. Kale AJ; Moore BS, Molecular mechanisms of acquired proteasome inhibitor resistance. *J Med Chem* 2012, 55 (23), 10317–10327. [PubMed: 22978849]
26. Groll M; Ditzel L; Lowe J; Stock D; Bochtler M; Bartunik HD; Huber R, Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 1997, 386 (6624), 463–471. [PubMed: 9087403]
27. Blackburn C; Barrett C; Blank JL; Bruzzese FJ; Bump N; Dick LR; Fleming P; Garcia K; Hales P; Hu Z; Jones M; Liu JX; Sappal DS; Sintchak MD; Tsu C; Gigstad KM, Optimization of a series of dipeptides with a P3 threonine residue as non-covalent inhibitors of the chymotrypsin-like activity of the human 20S proteasome. *Bioorg Med Chem Lett* 2010, 20 (22), 6581–6586. [PubMed: 20875739]
28. Singh PK; Fan H; Jiang X; Shi L; Nathan CF; Lin G, Immunoproteasome beta5i-Selective Dipeptidomimetic Inhibitors. *ChemMedChem* 2016, 11 (19), 2127–2131. [PubMed: 27561172]
29. Sula Karreci E; Fan H; Uehara M; Mihali AB; Singh PK; Kurdi AT; Solhjoui Z; Riella LV; Ghobrial I; Laragione T; Routray S; Assaker JP; Wang R; Sukenick G; Shi L; Barrat FJ; Nathan CF; Lin G; Azzi J, Brief treatment with a highly selective immunoproteasome inhibitor promotes long-term cardiac allograft acceptance in mice. *Proc Natl Acad Sci U S A* 2016, 113 (52), E8425–E8432. [PubMed: 27956634]
30. Smilkstein M; Sriwilaijaroen N; Kelly JX; Wilairat P; Riscoe M, Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents Chemother* 2004, 48 (5), 1803–1806. [PubMed: 15105138]

31. Rasmussen SA; Ceja FG; Conrad MD; Tumwebaze PK; Byaruhanga O; Katairo T; Nsoby SL; Rosenthal PJ; Cooper RA, Changing antimalarial drug sensitivities in Uganda. *Antimicrob Agents Chemother* 2017.

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Figure 1.

Examples of proteasome inhibitors tested and developed against Pf20S. Presently, no peptide boronate selective for Pf20s over all forms of the human 20S has been reported. Irreversible inhibitor compounds 18¹⁰ and 28¹¹ were developed in the peptide epoxyketone and peptide vinyl sulfone classes, respectively. Noncovalent inhibitor TDI4258¹² was derived from PKS21004, a class of proteasome $\beta 5$ inhibiting asparagine ethylenediamines (AsnEDAs, Figure 1), to impart selectivity over human proteasomes. The cryo-EM structure of PKS21004 with human i-20S demonstrated that moieties denoted as P1, P3 and P4 interact with the S1 and S1SP, S3 and S4 binding pockets of the $\beta 5$ subunits of human i-20S (Figure 1), respectively.¹⁸

Figure 2.

Inhibition of $\beta 5$ subunits of Pf20S, c-20S and i-20S by AsnEDAs. (A) Hydrolysis of suc-LLVY-AMC by $\beta 5$ of Pf20S, c-20S and i-20S was dose-dependently inhibited by WHZ-13 and WHZ-21. The assays were conducted in the presence of a $\beta 2$ specific inhibitor, WLW-VS, to minimize interference from hydrolysis of suc-LLVY-AMC by the $\beta 2$ subunit of Pf20S. Human $\beta 5$ activity was assayed with suc-LLVY-AMC for $\beta 5c$ and Ac-ANW-AMC for $\beta 5i$. Experiments were performed in triplicate and repeated on three separate occasions. (B) WHZ-13 dose-dependently inhibited the labeling of Pf20S $\beta 5$ by an activity-based probe MV-151, but not $\beta 2$ and $\beta 1$.

Figure 3.

Antimalarial activity by AsnEDAs. A) Antimalarial and cytotoxicity of WHZ-04, WHZ-13 and WHZ-21 against *Pf*3D7 and the human cell lines HepG2 and Karpas 1106P. Curves are representative of three independent experiments. Data for all compounds were listed in Table 3. B) Isobologram for WHZ-04 in combination with WLW-VS, individual data points represent FICs at particular compound concentration ratios. The straight lines represent the type of interaction. Data points below the 0.5 line show synergism and data around 1.0 line are additive. Representative of three independent experiments. C) Ex vivo activity of WHZ-13 and chloroquine (CQ) against 34 clinical isolates in Tororo, Uganda. Data are presented as geometric means $\pm$ SD.

Scheme 1.
Synthesis of AsnEDAs.

Table 1.Inhibition of *P. falciparum* Dd2 parental and resistant lines by proteasome inhibitors.

ID	EC ₅₀ (nM)		
	Dd2	Dd2-β6A117D	Dd2-β5A49S
PKS21004	2.5	326 (130) *	1352 (541)
TDI4258	27.6	279 (10) *	> 2770 (> 100)
Bortezomib	419	106 (0.25) *	1024 (2.4)
WLW-VS	91.6	7.3 (0.08) *	26.0 (0.28)

All results are the mean of three independent experiments with the fold change noted in parenthesis.

* data were taken from Ref. 12 for comparison.

Table 2.

Direct comparison of AsnEDAs with biphenyls and monophenyls at P1 position.

Compound	Type	IC ₅₀ (nM)			<i>Pf</i> 3D7 EC ₅₀ (nM)	Note
		Pf20S	β5i	β5c		
PKS21221		3.8	4	110	1.6	Ref. 12
PKS21208		5.2	24	1,130	1.7	Ref. 12
WHZ-05		32	169	1,140	7.5	This study
PKS21251		5,800	6,340	39,000	4,000	This study

Table 3.

Proteasome inhibition and cytotoxicity of compounds. Data shown are the mean of at least three independent experiments with fold of change in parenthesis.

ID	IC ₅₀ (nM) / (fold of selectivity)			EC ₅₀ (nm)	
	Pf20S β5	β5c	β5i	Pf 3D7	HepG2
WHZ-01	2,576 ± 326	> 100,000 (> 3.9)	2,090 ± 29 (0.8)	> 2770	> 11,000
WHZ-02	2,766 ± 314	46,600 ± 9,670 (16.8)	2,350 ± 153 (0.85)	2,140 ± 180	> 11,000
WHZ-03	15,135 ± 4,565	> 100,000 (> 6.6)	2,892 ± 1,004 (0.2)	> 2770	> 11,000
WHZ-04	20.8 ± 9.0	1,410 ± 326 (68)	100 ± 6.7(4.8)	8.4 ± 0.3	> 11,000
WHZ-05	32.0 ± 1.0	1,140 ± 336 (36)	169 ± 32 (5.3)	7.5 ± 2.3	> 11,000
WHZ-06	29.6 ± 2.0	2,200 ± 430 (74)	128 ± 9 (4.3)	11.7 ± 0.5	> 11,000
WHZ-07	121 ± 4.0	6,700 ± 2,600 (55)	179 ± 16 (1.5)	50.9 ± 6.4	> 11,000
WHZ-08	211 ± 34	26,050 ± 4,010 (123)	1,400 ± 130 (6.6)	98 ± 4.3	> 11,000
WHZ-09	113 ± 0.4	5,000 ± 340 (44)	38 ± 3 (0.3)	130 ± 2.9	> 11,000
WHZ-10	6.1 ± 2.9	409 ± 130 (67)	24 ± 4.6 (4)	9.6 ± 1.1	> 11,000
WHZ-11	22.4 ± 4	4,380 ± 1,490 (195)	475 ± 33 (21)	15.4 ± 0.5	> 11,000
WHZ-12	8.9 ± 0.9	506 ± 100 (57)	38 ± 4.5 (4.3)	2.3 ± 0.6	> 11,000
WHZ-13	4.7 ± 1.4	430 ± 130 (91)	112 ± 11 (24)	3.1 ± 0.1	> 11,000
WHZ-14	40.4 ± 3	9,120 ± 600 (225)	200 ± 5 (5.0)	98.5 ± 7.7	> 11,000
WHZ-15	250 ± 7	37,090 ± 14,320 (148)	763 ± 41 (3.1)	167 ± 12	> 11,000
WHZ-16	390 ± 2	40,190 ± 10,460 (103)	4,690 ± 517 (12)	132.4 ± 17	> 11,000
WHZ-17	1,088 ± 206	29,370 ± 19,690 (27)	2,090 ± 470 (1.9)	> 2770	> 11,000
WHZ-18	911 ± 6	> 100,000 (> 110)	3,560 ± 540 (3.9)	283 ± 3.8	> 11,000
WHZ-19	639 ± 113	> 100,000 (> 156)	3,111 ± 200 (4.9)	227 ± 41	> 11,000
WHZ-20	40.5 ± 3.0	540 ± 45 (13)	72.7 ± 27 (1.8)	83.7 ± 5.2	> 11,000
WHZ-21	20.7 ± 0.4	12,030 ± 1,210 (581)	384 ± 30 (19)	13.9 ± 1.3	> 11,000
WHZ-22	126 ± 12	6,040 ± 830 (48)	195 ± 23 (1.5)	68.8 ± 2.0	> 11,000
WHZ-20	4,056 ± 394	> 100,000 (> 24.7)	9,970 ± 734 (2.5)	> 2770	> 11,000
WHZ-24	197 ± 27	> 100,000 (> 507)	5,650 ± 490 (28.7)	117.5 ± 8.9	> 11,000

Table 4.

Growth inhibition of proteasome inhibitors against *P. falciparum* Dd2 and resistant Dd2 lines.

Compound	EC ₅₀ (nM)		
	Dd2	Dd2-β6A117D	Dd2-β5A49S
WHZ-04	7.7 ± 0.8	111.4 ± 14.3 (14)	135.3 ± 17.6 (17)
WHZ-12	2.5 ± 0.2	63.4 ± 13.1 (25)	251.7 ± 29.7 (101)
WHZ-13	3.3 ± 0.2	61.9 ± 20.9 (19)	501.5 ± 1.9 (152)

All results are the mean of three independent experiments. Numbers in parentheses are fold change in resistance.

Table 5.

In vitro pharmacokinetic parameters of select compounds

Compounds	PAMPA @ pH7.4 (nm/sec)	<u>Microsomal stability (μL/min/mg)</u>		
		HLM	RLM	MLM
WHZ-04	137	122	66	64
WHZ-05	105	141.4	124.4	141.4
WHZ-07	NT	149	81	49
WHZ-12	159	202	115	142
WHZ-13	203	61	61	97

NT: not tested. H/R/M LM: human/rat/mice liver microsomes