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Lydia Mata-Cantero, María Jesús Chaparro, Gonzalo Colmenarejo, Concepción Cid, Álvaro Cortés, et al.. Identification of small molecules disrupting the ubiquitin proteasome system in malaria Running title: Inhibition of *P. falciparum* Ubiquitin Proteasome System. *ACS Infectious Diseases*, 2019, 5 (12), pp.2105-2117. 10.1021/acsinfecdis.9b00216 . hal-03023985

HAL Id: hal-03023985

<https://hal.science/hal-03023985>

Submitted on 25 Nov 2020

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Identification of small molecules disrupting the ubiquitin proteasome system in malaria

Running title: Inhibition of *P. falciparum* Ubiquitin Proteasome System

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33

34 **Abstract**

35

36 The Ubiquitin Proteasome System (UPS) is one of the main proteolytic pathways in
37 eukaryotic cells, playing an essential role in key cellular processes such as cell cycling and signal
38 transduction. Changes in some of the components of this pathway have been implicated in
39 various conditions, including cancer and infectious diseases such as malaria. The success of
40 therapies based on proteasome inhibitors has been shown in human clinical trials. In addition to
41 its proven tractability, the essentiality of the *Plasmodium falciparum* UPS underlines its potential
42 as a source of targets to identify new antimalarial treatments. Two assays, previously developed

to quantify the parasite protein ubiquitylation levels in a high throughput format, have been used to identify compounds that inhibit parasite growth by targeting *P. falciparum* UPS. Among the positive hits, specific inhibitors of the *P. falciparum* proteasome have been identified and characterized. Hits identified using this approach may be used as starting points for development of new antimalarial drugs. They may also be used as tools to further understand proteasome function, and to identify new targets in *P. falciparum* UPS.

Abbreviations

UPS, ubiquitin proteasome system; DUB, deubiquitinase; TUBEs, tandem ubiquitin binding entities; UBA, ubiquitin-associated domains; iRBCs, infected red blood cells; HTS, high throughput screening; S/B, signal to background; CV, coefficient of variation; SD, standard deviation; Ub, ubiquitin; TCAMs, Tres Cantos antimalarial Set; PFI, Property Forecast Index; IFI, Inhibition Frequency Index; ACTs, artemisinin combination therapies; NA, non-adjusted

Keywords

Plasmodium falciparum, proteasome, UPS, inhibitor, screening, ubiquitylation

Introduction

The most severe form of malaria is caused by the protozoan parasite *Plasmodium falciparum*. This disease is one of the deadliest infectious diseases in the world, causing more than 400,000 deaths per year (1). Current first-line treatment is based on artemisinin combination therapies (ACTs). However, the malaria parasite has developed resistance against all widely used antimalarials, and ACTs are not an exception. Increasing drug resistance has led to an urgent need for developing new therapies effective not only on acute infection with high parasite loads in blood, but also on different stages of the parasite to block transmission. Thus, identification of drugs targeting different mechanisms in different stages of the parasite is crucial for malaria eradication (2).

In recent years, *P. falciparum* ubiquitin proteasome system (UPS) has been considered a promising target for drug development (3, 4). Ubiquitylation is a post-translational change in which ubiquitin (Ub) is covalently bound to the target protein. This process requires the sequential action of three groups of enzymes, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ligases. E3 ligases are the main responsible for substrate specificity leading to the transfer of ubiquitin from the E2 ligase to the final substrate. This ubiquitylation process can also be reverted by the action of deubiquitylation enzymes (DUBs). The cooperative interplay between the ubiquitylation and deubiquitylation processes determines the prevalence of certain ubiquitylated proteins in the cell and, thus, the activation/deactivation of specific functions (5).

Eukaryotic 26S proteasome consists of a 20S core, responsible for catalytic activity, and two regulatory 19S particles located at both sides that control entry of ubiquitylated proteins into the proteolytic core. 20S comprises four heptameric rings, of which the outer rings are formed by alpha subunits and the inner rings by catalytic beta subunits, β 1 (caspase-like), β 2 (trypsin-like) and β 5 (chymotrypsin-like) (6).

UPS controls key processes in eukaryotic cells including apoptosis, cell cycle and DNA repair. Thus, UPS deregulation has been related to multiple diseases such as cancer, neurological disorders, and pathogen infections (7). In 2003, the proteasome inhibitor bortezomib was the first drug within the UPS approved by the US Food and Drug Administration for the treatment of multiple myeloma. Several UPS inhibitors have subsequently been launched or tested in clinical trials (8, 9).

In addition to its proven tractability as drug target, different studies have revealed the essentiality of *P. falciparum* UPS in its survival and transmission (10, 11). Irreversible inhibition of parasite growth on all parasite stages has been seen using different types of human proteasome inhibitors, some of them within the nanomolar range (3). Moreover, they also show a synergistic behavior in resistant parasites when combined with artemisinin (3, 12). While *P. falciparum* proteasome exhibits a high degree of homology with its human counterpart, some divergences have been found in the β 2 active site by cryo-electron microscopy (12, 13). These divergences are being exploited to develop inhibitors with selectivity for the parasite proteasome to avoid side effects (12-14).

On the other hand, most studies of *P. falciparum* UPS are focused on the *P. falciparum* proteasome remaining the UPS upstream components understudied. Although all the main UPS components have been identified in the parasite genome *in silico*, only some of them have been tested experimentally (15, 16). While E1 and E2 paralogs are highly conserved, E3 ligases and DUBs are highly divergent, thus offering the possibility to develop selective drugs.

The objective of this study was to identify inhibitors with potential to interfere with any of the steps involved in the parasite UPS. For that, two previously reported cellular assays that quantitatively measure changes in ubiquitylated protein levels (17) were used. As a key advantage, these methods do not require extensive or previous knowledge of the targets they are hitting. A high throughput screening (HTS) campaign was run with the Tres Cantos AntiMalarial Set (TCAMS), a GSK compound collection of ca 13K compounds that inhibits *P. falciparum* *in vitro* growth (18). As a result, we were able to identify new chemical entities that inhibit the parasite proteasome, validating the approach used. Moreover, compounds acting through other components of the parasite UPS were also identified. All these hits can be followed up to develop new antimalarial drugs with novel modes of action.

Results and discussion

High throughput screening at single shot with AlphaLISA assay

Two cellular assays previously developed were used to run an HTS campaign (17). Progression cascade of the whole HTS can be found in the graphical abstract. In both assays, the signal measured is proportional to the amount of ubiquitylated proteins amount present in *P. falciparum* cells, allowing the identification of UPS inhibitors that are able to change the total levels of ubiquitylated proteins in cells.

The homogeneous AlphaLISA assay was selected to perform the primary screening campaign, as it can be run in 1536-well plates and requires no washing steps. AlphaLISA is a cellular assay where compounds are incubated with infected red blood cells (iRBCs) for 1 hour. This time of incubation was chosen to rule out that the changes measured in the total pool of

ubiquitylated protein after treatment are the result of the effect of the compound on other pathways instead of on the UPS.

As the assay is cell-based the selected compound collection consisted of the TCAM set (18) and other compounds from the GlaxoSmithKline HTS screening collection which inhibited *P. falciparum in vitro* growth in previous whole cell phenotypic screenings. A total of 14,747 compounds were tested. The aim was to identify compounds that inhibit parasite growth through the UPS, as the mechanisms of action of most compounds in this collection set remain unknown.

Assay was performed in duplicate at a final assay concentration of 10 μ M. Two controls per plate were used: Control 1, samples in absence of compounds (basal levels of ubiquitylated proteins in the cell) and Control 2, samples in presence of proteasome inhibitor MG132 at a concentration that produces complete proteasome inhibition (19). The AlphaLISA signal obtained for each compound was normalized as percent stimulation compared to Control 1, corresponding to 100% stimulation. Control 2 was not used to normalize the percent inhibition of each compound because the levels of ubiquitylated protein accumulation could vary depending on the target affected within the UPS. MG132 increased ubiquitylated protein levels in the cells approximately 1.8 times as compared to Control 1 (Figure 1A) (180% stimulation) and was used to control the assay performance for each plate, that was in the range of previously reported data (17).

Patterns in plate responses were corrected using an in-house developed algorithm (20). The percentage of stimulation found for the compounds followed a normal distribution in both replicates (Figure 1B). Most compounds and Control 1 samples showed a percentage of stimulation centered around 100% (inactive compounds). The right tail in pink correspond to the actives hits of the assay. These compounds have a percentage of stimulation above the robust cut-off in at least one of the replicates. Robust cut-off was estimated through a robust algorithm (21) that considers the mean value of Control 1 plus three times its standard deviation. The resulting cut-offs for both replicates were very similar (132% and 133% for replicates 1 and 2 respectively). Figure 1C shows the correlation of the percentage stimulation between both replicates. In addition, a Pareto ranking was calculated with the two responses, to estimate a round cut-off for this bivariate distribution. Compounds labeled in yellow are the ones rescued in this way, giving a total hit rate of 6.8% of all compounds tested.

Figure 1. AlphaLISA screening campaign. Compounds were tested in the AlphaLISA assay in duplicate at 10 μ M. Two controls per plate were included and normalized as percent stimulation. Control 1 corresponded to the signal obtained in absence of inhibition (basal levels of ubiquitylated proteins corresponding to 100%), while Control 2 was the signal measured in wells treated with the proteasome inhibitor MG132 (1.5 μ M). **A.** Bars represent the average percent stimulation for each control in the replicates. Average signal to background obtained per plate (S/B =Average % stimulation control 2 wells / Average % stimulation control 1 wells) is given in bold for each replicate. **B.** Number of wells for a given percent stimulation. All compounds tested (14,747) plus control 1 wells are represented. Black color corresponds to control 1 wells, while wells treated with compounds appear in grey. Compounds with percent stimulation above the statistical cut-off for each replicate being considered active hits are depicted in pink. **C.** Correlation of the percent stimulation obtained for each replicate. Inactive compounds are colored grey, and active hits in at least one replicate are labeled in pink as in B. Yellow compounds were rescued after calculating a Pareto round cut-off. The line indicates the perfect correlation ($y=x$). **D.** Active hits were then assayed at dose response starting at 100 μ M (1:3 dilutions) per duplicate. Graph shows the correlation of the pEC50s obtained for each replicate. Compounds displaying a maximum percent stimulation higher than 131% were considered active compounds. Actives in two replicates ($n=2$) are represented in black, those active in one replicate and inactive in the other one in pink ($n=1$), and actives in one replicate but whose curves could not be fitted (non-adjusted NA) in the other replicate appear in grey. The black line is the perfect correlation ($y=x$), while dotted lines represent ± 1 log difference of perfect correlation.

A total of 996 compounds were considered actives in at least one of the replicates. They were progressed to dose response experiments to determine their potency. The quality of the assay was assessed with different proteasome inhibitor being in the range of previously reported data (17) (Table 1). The minimum percentage of stimulation considered out of the noise of the assay was 131%, because this corresponded to the statistical cut-off in both replicates. Compounds displaying curves with a maximal asymptote above this percent stimulation in at least one replicate were considered active compounds. Thus, 544 compounds met these criteria, giving a confirmation rate of 54.6%.

Figure 1D depicts the correlation between pEC50s obtained per replicate. 438 compounds were positive in both replicates, showing a pEC50 correlation within the range of $pEC50_{replicateX} - pEC50_{replicateZ} < 1$, represented by grey dotted lines. The other 106 compounds were active in one of the replicates, while in the other replicate they were inactive ($pEC50 < 4$, pink) or could not be fitted with the analysis software due to variability (grey, non-adjusted, NA).

Hit confirmation with DELFIA assay

Hits from the AlphaLISA assay were further tested in the orthogonal DELFIA assay (17). This heterogeneous assay was performed to confirm the effect of the hits on the UPS and to rule out non-specific interference in the AlphaLISA assay as washes are performed between each addition step.

Hits from the AlphaLISA assay (544 compounds) were tested at dose response per duplicate (Figure 2). Assay behavior was similar to that of AlphaLISA in terms of signal to background and cut-off. The results of the DELFIA assay showed a greater variability in replicates due to the extensive washing steps but, at the same time, the stringent conditions allowed for robust validation of the inhibitors found in the primary screening with the AlphaLISA assay.

Presence of the proteasome inhibitor MG132 led to increase ubiquitinated protein levels up to 185%, (Figure 2A). Compounds with a maximum asymptote higher than 131% were active (robust cut-off). Figure 2B shows the correlation of the pEC50s obtained between DELFIA replicates. Only 106 hits were active in both replicates. Curves from many compounds could not

211 be fitted in one of the replicates due to assay variability or because they were close to the
 212 response background (labeled as NA in Figure 2B).

213

214 **Figure 2. Hit confirmation in DELFIA assay.** Hits from the AlphaLISA campaign were tested
 215 in the DELFIA assay in duplicate in dose response (first point 100 μ M). Two controls were
 216 included per plate and normalized as percent stimulation. Control 1 corresponds to the signal
 217 obtained in the absence of inhibition (basal levels of ubiquitylated proteins corresponding to
 218 100%), while Control 2 is the signal measured in wells treated with the proteasome inhibitor
 219 MG132 (2 μ M). **A.** Bars represent the average percent stimulation per replicate. Average of signal
 220 to background obtained per plate (S/B =average % stimulation Control 2 wells/average %
 221 stimulation Control 1 wells) is indicated in bold for each replicate. **B.** Correlation of the pEC50s
 222 recorded for each replicate. Compounds displaying a maximum percent stimulation higher than
 223 131% were actives. In black, actives in two replicates ($n=2$); in pink, actives in only one replicate
 224 ($n=1$); and in grey, compounds active in one replicate but whose curves could not be fitted in the
 225 other replicate (non-adjusted NA). **C.** Correlation of the average pEC50s recorded in the
 226 AlphaLISA and DELFIA assays per compound. Compounds labeled in black were active in more
 227 than three replicates ($n=2$ in AlphaLISA and/or DELFIA) and had a pEC50>4.5 in all replicates.
 228 The black line indicates the perfect correlation ($y=x$), while dotted lines represent ± 1 log difference
 229 of perfect correlation. **D.** Correlation of the average pEC50s recorded in the AlphaLISA and whole
 230 cell assays per compound. Labels are the same than in C.

231
232

			UPS activity assays		Proteasome activity assays					
Compound	Structure	Reported pEC50 whole cell 3D7A	pEC50 AlphaLISA	pEC50 DELFIA	pIC50 THP1			pIC50 <i>P. falciparum</i>		
					β5	β1	β2	β5	β1	β2
MG132		7.65	7.17 ± 0.04	7.41 ± 0.21	6.22 ± 0.01	5.25 ± 0.11	5.22 ± 0.11	6.86 ± 0.18	6.11 ± 0.02	6.72 ± 0.02
Bortezomib		7.05	7.95 ± 0.08	8.16 ± 0.05	7.29 ± 0.03	6.43 ± 0.20	5.56 ± 0.16	7.33 ± 0.26	<4*	<4

233 * Some inhibition observed (~50% activity)

234 Table 1. Results for tool compound proteasome inhibitors in AMC assay for human THP1 and *P. falciparum* samples.

Figure 2C shows the correlation of the pEC50s found in the AlphaLISA and DELFIA assays. 311 compounds were confirmed in the DELFIA assay in one or both replicates, revealing a confirmation rate of 57.1% with the AlphaLISA assay. Compounds active in more than two replicates in total (more than two active replicates in either AlphaLISA or DELFIA), and with a pEC50 higher than 4.5 (black) showed the best correlation, as they were the most reliable hits. Comparison of UPS and whole cell assays (Figure 2D) indicate that compounds are less potent in UPS assays, probably due to differences in the incubation times with the compounds, 1 hour vs. 48 hours respectively.

The results of both assays were used to select compounds for further studies. Thus, the 311 compounds considered hits were tested in proteolytic activity assays of the proteasome which clearly differentiated two groups: compounds whose target is the proteasome (26S) and compounds that probably affect other UPS components upstream the proteasome (see graphical abstract).

Proteasome inhibitors

Proteasome activity assays based on fluorescence readout were performed as described (22). Compounds showing autofluorescence at 460 nm were also tested in the Proteasome-Glo assay whose readout is luminescence. Proteasome-enriched fractions from *P. falciparum* extracts were used as proteasome source as previously described (22). All compounds were tested at single shot at 50 μ M in the chymotrypsin-like, caspase-like, and trypsin-like proteasome activity assays. Positive compounds were then subjected to dose response assays to assess their potency, in duplicate. Sixteen compounds were active in at least one of the activities tested and with an inhibition rate higher than 50%. Proteasome activity assays were also performed on human proteasome-enriched fractions from THP1 cells to assess selectivity. Proteasome inhibitors MG132 and bortezomib were included as controls in all experiments (Table 1). The identification of proteasome inhibitors was expected and validated the screening rational.

Most of the proteasome inhibitors found were not selective for the *P. falciparum* proteasome, which hampers further interest from a drug discovery point of view in malaria. However, results

showed that 6 compounds belonging to the same cluster with a cyanamide group were selective for the *P. falciparum* proteasome. Three of them belong to the TCAM set: TCMD-133787, TCMD-133806, and TCMD-138496. All compounds inhibited the trypsin-like and caspase-like proteasome activities in *P. falciparum*, but not the chymotrypsin-like activity, while they had no effect on any human proteasome activities (Table 2). These compounds showed no cytotoxicity in the HepG2 assay. The Property Forecast Index (PFI) was calculated as a predictor of developability of compounds based on their physicochemical properties (23). Compounds with a PFI < 8 are considered that met the “drug-like” criteria. PFI of cyanamide cluster is less than 8 having a MW < 500, cLogP < 5, and a number of aromatic rings < 4.

A selection of analogs of these compounds were tested in the three proteasome activities of the parasite to better understand their structure-activity relationship (SAR) (data not shown). Compounds without the cyanamide group were inactive in the proteasome activity assays, which strongly suggests that this group is an essential component of the pharmacophore. SAR results also underlined the importance of other parts of the molecule, such as the sulfone group. The least potent compounds in the proteasome, such as GSK’459, do not have this group (Table 2), although in this case the decrease in potency is not reflected in whole cell activity. Activity improved when the position of the heteroatoms or the chiral center were changed, as in compound TCMD-138496.

The subunit specificity of the proteasome inhibitors identified was assessed using the human active-site probe Me4BodipyFL-Ahx3Leu3VS (Figure 3A). This probe covalently reacts with all three β catalytic subunits in human proteasomes (24), but only with the trypsin-like (β 2) and chymotrypsin-like (β 5) in *P. falciparum* (11). The β subunits not bound to the inhibitors are labeled with the probe and then visualized by fluorescence. Treatment with our compounds caused the disappearance of the band corresponding to trypsin-like activity in *P. falciparum* but not in human fractions (Figure 3A). The human proteasome inhibitor bortezomib led to disappearance of all bands in human fractions, but only of the chymotrypsin-like band in *P. falciparum*, in agreement with the results reported in activity assays (Table 1). All bands in the human and parasite proteasomes were lost after treatment with the non-specific inhibitor MG132 (Figure 3A and Table 1). These results confirmed the selectivity of our compounds against trypsin-like activity (β 2) in *P.*

294 *falciparum*, previously shown in the proteasome activity assays. Caspase-like activity could not
295 be confirmed, as it is not labeled by the probe in the *P. falciparum* proteasome (11, 13).

Figure 3. Validation of proteasome inhibitors binding. A. Proteasome active-site probes. The proteasome active-site probe Me4BodipyFL-Ahx3Leu3VS was used reacting with the trypsin-like ($\beta 2$) and chymotrypsin-like ($\beta 5$) proteasome activities in *P. falciparum* and with the three β catalytic subunits in THP1 in the absence of compounds. Proteasome-enriched fractions from *P. falciparum* and THP1 cells were treated with compounds for 1 hour at 100 μ M. The probe was then added labelling the proteasome active sites not previously bound to the compounds. **B. Modelling of GSK'737 binding to *P. falciparum* and human proteasomes.** Proposed binding mode of compound GSK'737 to *P. falciparum* $\beta 2$ unit (trypsin-like activity). The compound is predicted to bind covalently to the catalytic threonine residue, and to establish hydrogen bonds with the backbone of residues Thr-21 and Ala-49. **C. Comparison of the binding modes in human and *P. falciparum*** P1 and P3 sub pockets are highlighted.

Compound	PhysChem properties					Whole cell assays		UPS activity assays			Proteasome activity assays					
	Structure	cLogP	MW	Permeability	PFI	pEC50 3D7A	pEC50 Cytotox HepG2	pEC50 AlphaLISA	pEC50 DELFIA	n	pIC50 THP1			pIC50 <i>P. falciparum</i>		
											β5	β1	β2	β5	β1*	β2
GSK'737		2.4	329.4		6.4	6 ± 0.2	4.2 ± 0.0	5.2 ± 0.2	5.2 ± 0.1	4	<4	<4	<4	<4	6.1 ± 0.2	6.3 ± 0.1
TCMDC-133787		2.1	334.2	349	5.1	6.3 ± 0.1	4.3 ± 0.0	5.7 ± 0.2	4.6	3	<4	<4	<4	<4	6 ± 0.1	6.2 ± 0.2
GSK'057		3.5	384.5	480	7.3	6.3 ± 0.1	4.4 ± 0.2	5.0 ± 0.3	4.8 ± 0.3	4	<4	<4	<4	<4	6.4 ± 0.2	6.3 ± 0.1
TCMDC-133806		1.2	329.2	370	4.9	5.8 ± 0.1	<4	5.2 ± 0.1	4.6 ± 0.1	4	<4	<4	<4	<4	6.9 ± 0.3	6.8 ± 0.2
GSK'459		1.3	275.3		4	6.1 ± 0.2	<4	4.9	4.9 ± 0.5	3	<4	<4	<4	<4	5.3 ± 0.0	5.3 ± 0.1
TCMDC-138496		3.2	279.4		5.3	6.2 ± 0.1	4.1 ± 0.2	4.7 ± 0.1	4.5	3	<4	<4	<4	<4	5.9 ± 0.2	6.4 ± 0.3

* No 100% inhibition (60%-80%)

	Proteasome/Whole Cell Parasite	Proteasome/Cytotoxicity Human	cLogP	MW	Permeability	PFI
Green	> 6	< 4	≤ 3	≤ 350	≥ 100	≤ 6
Yellow	5-6	4-5	3-5	350-450	10-100	6-9
Red	< 4-5	> 5	≥ 5	≥ 450	≤ 10	≥ 9

Table 2. Summary of data for cyanamide proteasome inhibitors

To get some insight into the selectivity profile and mechanism of action of these compounds, molecular modeling studies were performed exploiting the recently published structural information about *P. falciparum* proteasome (12) and internal crystallographic complexes with other cyanamides and different serine and cysteine proteases (25). According to this information, these compounds could be acting as covalent ligands with the nitrile warhead. However, their selectivity towards $\beta 1$ and $\beta 2$ activities appears to point to a specific recognition mechanism. Based on this hypothesis, we modeled complexes of GSK'737 and TCMD-133787 with the $\beta 1$, $\beta 2$ and $\beta 5$ units of the human and *P. falciparum* proteasomes using a covalent docking workflow followed by manual refinement and energy minimization. In our proposed binding mode (Figure 3B), the compounds are attached to the catalytic threonine residue after a reaction with the cyano group of the ligand, that is then interacting with the terminal amine group of the protein, similarly to other available complexes for cysteine proteases (25), in which the nitrogen atom adopts a role as a hydrogen bond acceptor. In our model, the P1 cavity would be partially occupied by the pyrrolidine ring as a proline analogue. This might explain the negligible activity of these compounds towards $\beta 5$ for both species, due to a possible requirement for a small hydrophobic residue (e.g. leucine) in that position for this catalytic unit (26). The different linkers (sulfonamide, methyl sulfone or carbamate) would establish hydrogen bond interactions with the backbone atoms of residues Ala-49 and Thr-21 (*P. falciparum* $\beta 2$ unit numbering scheme) in a similar manner to other known inhibitors such as WLW-vs derivatives (PDB ID 5FMG) (12) or bortezomib (PDB ID 5LF3) (27). The geometry imposed by the threonine-warhead complex would explain the different hydrogen bond patterns that the linkers could establish and, possibly, the potency drop for GSK'459. Finally, all compounds have a bulky, hydrophobic group (i.e. naphthalene derivatives, aryl halides, and 1,3 benzodioxol) that resembles, in different degrees, a tryptophan residue and that would be located in the P3 sub pocket. Recently, it has been discovered that the P3 sub pocket in *P. falciparum* β catalytic units, especially $\beta 2$, is significantly larger than that in their human homologues (Figure 3C), opening new opportunities to achieve selective inhibitors by introducing bulky groups into that position (e.g. tryptophan in WLL-vs inhibitor (12, 28)). According to our proposed binding mode, the presence of these groups in the reported compounds could be an example of such strategy and one of the reasons for their selectivity against the *P. falciparum* proteasome

342

343 **Figure 4. Effect of proteasome inhibitors identified on the intraerythrocytic parasite**
344 **stage.** Synchronized iRBCs with rings or mature trophozoites/schizonts were treated for 24 and
345 48 hours with compounds. Photographs of Giemsa-stained thin blood smears were taken to
346 observe the phenotype and the effect on parasite *in vitro* growth. Chloroquine and atovaquone
347 were included as controls of fast- and slow-acting compounds respectively with modes of action
348 unrelated to UPS inhibition.
349

350 To determine the effect on parasite growth of this family of trypsin-like and caspase-like
351 proteasome inhibitors, iRBCs synchronized in rings or mature trophozoites/schizonts were treated
352 for 24 and 48 hours with the compounds TCMDC-133787 and GSK'459. Bortezomib, chloroquine
353 and atovaquone were used as controls (Figure 4). Bortezomib is a human proteasome inhibitor
354 that inhibits the chymotrypsin-like activity. Treatment of parasites with bortezomib resulted in
355 parasite growth arrest in a similar way to chloroquine either in rings or mature forms. However,
356 treatment with our trypsin-like and caspase-like proteasome inhibitors, TCMDC-133787 and
357 GSK'459 did not prevent progression to the next parasite stage (Figure 4). In humans, *in*
358 *vitro* and *in vivo* protein degradation is significantly reduced only when either the trypsin-like or
359 caspase-like sites are inhibited together with the chymotrypsin-like sites (29). The same may
360 occur in the parasite. It has been reported that the most significant parasite killing results from co-
361 inhibition of the chymotrypsin-like activity with the trypsin-like or caspase-like proteasome

activities (12, 13). Co-inhibition allows for attenuating parasite growth at any point in the intraerythrocytic cycle. Chymotrypsin-like activity is essential for fast inhibition of growth being implicated in parasite schizogony, while inhibition of trypsin-like activity alone does not significantly affect parasite growth as it has been confirmed with our cyanamides. Parasitemia remained constant during the 48 hours tested, while in the control culture parasitemia increased more than 6-fold. They seemed to have no effect on trophozoites or early schizonts, but prevented increase in parasitemia, maybe because they affected merozoite egress, the invasion process, or early ring stages. The fact that not all parasite stages are equally sensitive to inhibition of the different proteolytic activities of the 20S core may suggest that each proteasome activity might be regulating a specific pool of ubiquitylated proteins (29).

On the other hand, some proteasome inhibitors can also react with other serine or cysteine proteases because the proteasome has a hydrolytic mechanism similar to those proteases (30). In fact, although cyanamides were selective for *P. falciparum* proteasome, it has previously been reported that they are inhibitors of different kinds of cathepsins, which are cysteine proteases. A cyanamide-based inhibitor analogue to TCMDC-133787 was reported by our colleagues at GSK as a novel human cathepsin C inhibitor for the treatment of neutrophil-dominated inflammatory diseases such as chronic obstructive pulmonary disease and cystic fibrosis (25). Selectivity for different cathepsins (cathepsins C, B, K, L, and S), all cysteine proteases, was achieved by changing different parts of the molecules. In the case of cyanamides identified here, the selectivity window in terms of cytotoxicity was higher than 100-fold in most cases. Further studies of the action of these compounds on different human cathepsins should be done to assess a good toxicological profile and to ensure the non-toxic effects *in vivo*.

As regards activity against other cysteine proteases of the parasite, it is known that *P. falciparum* has different families of cysteine proteases such as falcipains, which have been considered potential drug targets because they are involved in the hemoglobin degradation process. Treatment of cultured *P. falciparum* parasites with broadly active cysteine protease inhibitors such as leupeptin or E-64 causes the food vacuole to swell and fill with dark-staining material (31) due to accumulation of large amounts of undegraded hemoglobin, with maximum activity achieved in mature trophozoites and schizonts. Absence of a food vacuole defect in Figure

4 suggests that inhibition of cysteine proteases in this digestive organelle is not causing the inhibition of parasite growth.

All these results suggest that compounds belonging to the cyanamide cluster are good candidates to be administered in combination with other antimalarials such as artemisinin. Selective inhibitors acting upon the parasite trypsin-like activity have previously been shown to enhance killing by artemisinin of both sensitive and resistant parasites without host toxicity (12). In contrast to previously reported selective proteasome inhibitors, cyanamides are small molecules that are not peptide mimetics and have good PhysChem properties.

Non-proteasome inhibitors

In addition to proteasome inhibitors, our strategy has allowed the identification of compounds that increased ubiquitylated protein levels through other mechanisms that could be directly associated with parasite growth inhibition. This confirms published results showing that accumulation of ubiquitylated proteins can lead to parasite death (32).

295 compounds were inactive in all proteasome activity assays, forming the group of non-proteasome inhibitors (graphical abstract). Among them, 169 hits were active in the AlphaLISA and DELFIA assays in at least three replicates and with a pEC50 higher than 4.5 (Figure 2C, black dots). This restrictive selection criteria was applied to choose the best compounds of this group. In a first stage, the structures were clustered using a complete linkage hierarchical algorithm (21), which resulted in 64 chemical clusters, 36 of them singletons, with a wide variety of chemotypes. Further inspection revealed that most of them, irrespective of the cluster, contained a phenol group (such as phenol benzylamines), and/or were amino alcohols, including quinine and amino-phenyl-ethanol derivatives. Six were the most populated (82 compounds in total) clusters, with more than 3 members per cluster (Figure 5). The numbers of compounds within each cluster ranged from 38 in cluster 1 as the most populated and 4 in cluster 6 as the less populated. Clusters 1, 3 and 5 had with amino alcohols (pink dots). Most compounds in cluster 1 had a hydroxy group in benzyl position. The substitution of the amine could be aliphatic chains or aromatic rings.

Figure 5. Non-proteasome inhibitors. The six more populated clusters with their scaffolds that share the compounds belonging to each cluster are presented. Graphs show the correlation between the average pEC50 found in the UPS AlphaLISA assay and the pIC50 that inhibits parasite *in vitro* growth (whole cell). The black line is the perfect correlation ($y=x$), the pink and orange dotted lines represent the ± 0.5 log and ± 1 log difference of perfect correlation respectively. Compounds in grey have a phenol group, those in pink an amino alcohol group, and those in orange both kinds of groups in their structure. Dose response curves for one of the compounds belonging to each cluster are shown as examples. Percent stimulations recorded in the AlphaLISA (black) and DELFIA (pink) assays for a representative of the cluster are plotted *versus* compound concentration.

Clusters 3 and 5 shared a quinine scaffold. Moreover, cluster 5 had a long chain and was highly substituted in the right-hand side. The mechanism of action of quinine remains unclear, but the drug has been attributed an inhibitory role of hemozoin biocrystallization like chloroquine. However, some mutations have been found in the HECT E3 ligase *PfUT* (33) in quinine-resistant parasites (34), connecting the resistance with the UPS. The lower correlation seen with the whole cell assay here could point to an additional mechanism involving the UPS system of the parasite.

Clusters 2 and 4 contained compounds with the phenol group (Figure 5, grey dots). In addition, cluster 2 included structures similar to described protease inhibitors with benzamides substituted and an additional hydroxy group in para position, validating our approach. The interplay of phenols with the UPS has previously been reported in human cells. Natural phenolic compounds regulate the UPS during oxidative stress (35) and induce growth arrest or apoptosis probably by regulating the UPS. For example, compounds such as methyl gallate, gallic acid, and tannic acid increase ubiquitinated protein levels in treated human cells. They have an effect on the E1 conjugating enzyme activity and the 26S proteasome through their OH group, and also interact with other proteins such as protein S5a and DUBs USP47 and USP15 (36). The correlation of the pIC50s obtained in the UPS and in the whole cell assays may suggest that phenols present in the compounds could be affecting one or more components of the UPS.

Regarding the physicochemical profile, more than half the compounds showed a good PFI index and had no historical annotations of liabilities found in GSK internal databases (redox, promiscuity, reactivity, etc.), especially compounds belonging to clusters 1 (34/38), 3 (10/10), and 4 (7/9). All compounds had less than 4 aromatic rings and most of them had a MW < 500 and a cLogP < 5. Regarding cytotoxicity, data in HepG2 showed that 77 of the 82 compounds were non-cytotoxic.

Figure 5 shows the correlation between AlphaLISA and *P. falciparum* 3D7A whole-cell assays pEC50s. The difference in pEC50 values was less than one for all of them, exhibiting almost the same potency in both assays despite the fact that treatment with compounds lasted only one hour. Phenols belonging to clusters 2 and 4 were near to perfect correlation, strongly suggesting that parasite growth and UPS inhibitions are related. Amino alcohols present in clusters 3 and 5 also showed a good correlation but, in all cases, compounds were more potent in the whole cell assay, possibly indicating that there are other targets involved in their mode of

action or that more time is needed for compounds to exert their action. Some dose response curves are presented in Figure 5 as an example of the results found for each cluster.

Little is known about components of the UPS in *P. falciparum*. Future identification of the proteins targeted by these non-proteasome inhibitor compounds could reveal novel, validated, tractable and selective targets which could be used in new drug discovery programs. Further studies, like chemogenomic or proteomic methods (e.g. global shift thermal profiling) among others, are required to identify which targets are being hit by the inhibitors found.

Conclusion

Results from the screening campaign shown here revealed compounds hitting different targets within the UPS pathway. They represent excellent starting points for drug discovery programs, particularly compounds belonging to the proteasome inhibitors, as the target they are hitting has been identified. Moreover, non-proteasome inhibitors described could be used as tools to identify new tractable antimalarial targets.

Materials and methods

***P. falciparum* cultures**

P. falciparum 3D7A strain from the Malaria Research and Reference Reagent Resource Center (MR4) was used to perform all assays reported. Parasites were grown in T150 flasks with fresh red blood cells (RBCs) at hematocrit 1% (volume percentage of RBCs) as described in (31).

Preparation of assay plates

Compounds were dissolved in 100% DMSO. Final DMSO concentration in the assays was 1%. AlphaLISA assay plates (Greiner 1536-well white plates) were prepared with compounds by adding 20 nL of each compound per well with an Echo dispenser, except for columns 11 and 12, which were filled with 20 nL of DMSO, and columns 35 and 36, which were left empty. 200 nL of each compound were added to 384-well flat bottom black plates for DELFIA assays, while 40 nL were dispensed to 384-well low volume black plates for proteasome assays. Columns 6 and 18

were left empty. For single shot assays, final compound concentration in the assay was 10 µM in AlphaLISA and 50 µM in the proteasome assays. For dose response determinations in any of the assays described, the highest concentration was 100 µM, and 11 points diluted 1:3 in DMSO were tested in all the assays performed by duplicate.

Purification of iRBCs with mature trophozoites/schizonts

Purified, highly synchronized *P. falciparum*-infected red blood cells (iRBCs) with mature forms were required to perform the HTS campaign. The previously described protocol was used for this purpose (37). As control of inhibition, part of the culture was treated with 1.5 µM of MG132 dissolved in DMSO. Culture was dispensed into AlphaLISA and DELFIA plates as described in each section. All this experimental work was carried out under biosafety level 3 procedures.

TUBE-AlphaLISA and DELFIA assays

A Multidrop Combi liquid handler (Thermo Electron Corporation) was used for each reagent addition to assay plates. The protocol was followed as described by Mata-Cantero et al (17).

Data analysis

Data were normalized to percentage of stimulation using the following equation:

$$\% \text{ Stimulation}(x) = \frac{R_x}{|R_{Ctrl1}|} * 100$$

R_x is the assay response measured for compound X, while R_{Ctrl1} is calculated as the average of control 1 (columns with 1% DMSO) in the same plate of compound X. Data and assay performance statistics, such as signal to background ratio (S/B), robust standard deviation (SD), and coefficient of variation ($CV = (SD/average) * 100$) were calculated using ActivityBase XE (IDBS) templates. Robust cut-offs at robust mean + 3*SD of Control 1 (estimated through a robust algorithm) (38) were used to mark the hits. Before that, patterns present in the plates were detected and removed using an in-house developed algorithm (20). pEC50 values ($pEC50 = -\log_{10}(EC50)$) were obtained with ActivityBase XE non-linear regression bundle. Data visualization and further analysis were performed using TIBCO Spotfire 3.2 software and Microsoft Excel.

Selection criteria and data mining

For single shot experiments, hits had a response above the statistical cut-off (robust mean + 3*SD response of Control 1) of each replicate. In AlphaLISA and DELFIA dose response assays, compounds with a maximum asymptote higher than 131% were considered active compounds (actives). This is based on the response and standard deviation obtained in the experiments calculated as stated above. Compounds with pEC50 values lower than 4 (> 100 µM) were considered inactive. GSK proprietary compound screening databases were queried for historical data of the hits obtained. For proteasome activity assays described in a following section, compounds showing a maximum percentage of inhibition higher than 50% in at least one proteasome activity were considered actives.

Proteasome-enriched fractions

The protocol described by Kisselev et al (22) was adapted to obtain proteasome-enriched fractions present in purified *P. falciparum* cells and THP1 crude extracts. Briefly, *P. falciparum* cultures were synchronized with 5% sorbitol (w/v) and scaled up for five days. Culture was harvested by centrifugation at 700 g for 5 minutes, and parasites were isolated by breaking RBCs with 0.01% saponin (w/v) as previously described. At this point, parasite and THP1 cell were equally processed. Cells were resuspended in sucrose buffer (50 mM Tris buffer pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 50 mM NaCl, 250 mM sucrose, 2 mM ATP, 1 mM DTT) and lysed by nitrogen cavitation for 15 minutes (110 bar or 1500 psi). Lysates were centrifuged at 20,000 g for 30 minutes at 4°C, and supernatants containing soluble protein were subjected to an ultracentrifugation step at 300,000 g for 2 hours at 4°C. The resulting proteasome-containing pellets were resuspended in sucrose buffer and left on ice for 30 minutes to complete solubilization. Samples were centrifuged for 10 minutes at 20,000 g to remove insoluble material. Proteins contained in supernatant, the proteasome-enriched fraction, were measured using the Bradford method.

Proteasome activity assays

Proteasome activities were assayed with fluorogenic or luminescence peptide substrates using previously described methods (22, 39) or the Proteasome-Glo assays (Promega)

respectively. In fluorescence assays, proteasome-enriched fractions from *P. falciparum* cells were dissolved in sucrose buffer at 0.1 mg/mL to measure the three proteasome activities. THP1 proteasome-enriched fractions were adjusted to 0.1 mg/mL for the chymotrypsin-like activity and to 0.2 mg/mL for the caspase-like and trypsin-like activities. 4 μ L of proteasome-enriched fractions at the required concentration were added to compounds and incubated for 1 hour (384-well, low volume plates). Then, 4 μ L of peptides 100 μ M Suc-LLVY-AMC (Sigma), 200 μ M Z-Leu-Leu-Glu-AMC (Sigma) or 200 μ M Ac-RLR-AMC (Boston) in assay buffer (50 mM Tris buffer pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 2 mM ATP, BSA 0.1 mg/ml) were added to the plates to measure the chymotrypsin-like, caspase-like or trypsin-like proteasome activities respectively. Proteasome inhibitors epoxomicin and MG132 at 10 μ M were included as controls. In the Proteasome-Glo assay, proteasome-enriched fractions were assayed in HEPES buffer (10 mM HEPES, 1 mM DTT, 2 mM ATP, BSA 0.1 mg/ml) following manufacturer protocols. Proteasome concentration for *P. falciparum* was kept at 0.1 mg/mL, while THP1 proteasome was used at 0.05 mg/mL for chymotrypsin-like and trypsin-like activities, and at 0.1 mg/mL for caspase-like activity. Volumes and order of addition were the same as in the fluorescence assay. Fluorescence ($\lambda_{ex/em}$ = 380/460 nm) or luminescence were measured in the Envision plate reader (Perkin Elmer). For dose response experiments, the first compound concentration tested was 100 μ M diluting the compounds 1:3 in DMSO (final DMSO concentration, 1%).

Proteasome active-site probes

For all proteasome labeling experiments, the active-site probe Me4BodipyFL-Ahx3Leu3VS (UbiQ) was used at a final concentration of 500 nM in sucrose buffer. THP1 (20 μ g) or *P. falciparum* (10 μ g) proteasome-enriched fractions were incubated with compounds at 100 μ M, or DMSO as control, for 1 hour in sucrose buffer. The active-site probe was added to proteasome enriched fractions previously treated with DMSO or compounds and incubated for 2 hours at RT. Samples were then denatured by addition of Laemmli sample buffer, boiled for 10 minutes and run on a 15% SDS-PAGE gel. Bands were visualized using the fluorescein channel in a Chemidoc reader (Biorad) ($\lambda_{ex/em}$ = 480/530 nm).

Whole cell inhibition assay

The parasite growth inhibition assay was performed using the standard ³H-hypoxanthine incorporation method. This assay relies on parasite incorporation of labeled hypoxanthine that is proportional to *P. falciparum* growth (40).

Parasite inhibition phenotype

P. falciparum cultures were synchronized in schizonts by performing 70% Percoll gradient centrifugation. Next day, culture in rings at 2% parasitemia and 2% hematocrit was treated with compounds at 10 times their IC₅₀s in the whole cell assay, 0.01 μM atovaquone, 0.25 μM chloroquine, 1 μM bortezomib (Selleckchem), 5 μM TCMDC-133787, and 15 μM GSK'459, at a final DMSO concentration of 0.5%. After 24 hours, parasitemia was quantified and photographs were taken of Giemsa-stained thin blood smears. The same experiment was carried out treating the parasites in mature trophozoites/schizonts.

HepG2 cytotoxicity assay

The acute cytotoxic effect of compounds administered to growing human liver-derived HepG2 cells (ATCC HB-8065) was determined using the luminescence assay previously described (41).

Molecular modeling

The structure of the three catalytic β1, β2, and β5 subunits of the *P. falciparum* proteasome were obtained from the cryo-EM derived structure (PDB ID 5FMG) (12, 28), while human subunits were obtained from the apo X-ray crystal (4R3O) (42). The Schrodinger 2017-3 suite was used to pre-process protein structure, model the missing side chains, and assign optimal protonation states. 3D models for the compounds were generated with Ligprep, followed by a conformational analysis to generate low-energy states for input in the docking protocol. The covalent docking module in Maestro was used to generate and score different poses with a covalent bond between the cyano group and the oxygen in the Thr-1 side chain (nucleophilic addition to a triple bond). The resulting poses for the 6 systems (β1, β2, and β5 for human and *P. falciparum*) were manually analyzed and corrected based on information from internal crystal complexes with other cysteine

proteases, and the final complexes were energy-minimized to a final RMSD from the original structure of 0.3 Å.

Acknowledgements

Human RBCs were supplied by the Blood Transfusion Center of CAM (Madrid, Spain) and Banc de Sang i Teixits (Barcelona, Spain). Human biological samples were sourced ethically, and their research use complied with the terms of the informed consent under an IRB/EC approved protocol. We also thank Jim Brown for critically review of the manuscript. GSK has funded this work.

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