Drug Design

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# Rational Design of Proteasome Inhibitors as Antimalarial Drugs

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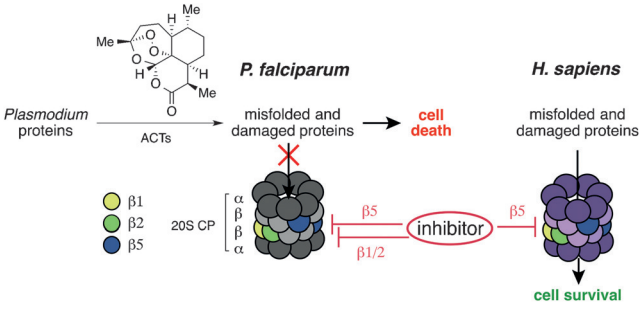
Malaria remains one of the most prevalent infectious disease in the developing world and its most severe form in humans is caused by the parasite *Plasmodium falciparum*. Artemisinin, which is used in combination with drugs with longer half-lives and different modes of action (artemisinin-based combination therapy, ACT), is the treatment of choice for malaria, although its efficacy is hampered by the emergence of resistance to the drug. It has been shown that the stress response to artemisinin-induced cellular damage, measured by the level of ubiquitinated proteins, is enhanced in drug-resistant parasites.[1] Therefore, the ubiquitin-proteasome system, which mediates the degradation and recycling of misfolded and damaged proteins among others, represents an attractive target for combating *P. falciparum*.

The key component of the ubiquitin-mediated degradation pathway, the 20S proteasome (core particle, CP), maintains biological homeostasis and regulates many essential processes in the cell through the cleavage of most intracellular proteins. The CP is formed by two outer α rings and two inner β rings, each composed of seven subunits ($\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$).[2] The proteolytic activity of this N-terminal threonine protease is carried out by 3 of the 14 different subunits: β1, β2, and β5. Each of the catalytic centers displays a distinct substrate specificity dictated by the shape of the selectivity pockets in the non-primed part of the substrate binding channel (referred to as S1, S2…).

For many years, the human proteasome has been regarded as an anticancer target, which has led to the design and discovery of numerous inhibitors, including the FDA-approved dipeptidic boronic acid Velcade and the epoxyketone Kyprolis. More recently, compounds that selectively inhibit the immunoproteasome to reduce chronic inflammation and autoimmune disorders have been developed.[3] Interestingly, a variety of proteasome inhibitors have also been found to be active against *P. falciparum*. This noteworthy observation has validated the proteasome as an attractive target against malaria, since it plays a role at each stage of the parasite lifecycle.[4] Understanding the structural differences between parasite and human CPs could open an avenue for the design of selective inhibitors that are able to kill the protozoa while sparing the host.

Until 2016, no structure of the *Plasmodium* proteasome was available and the discovery of suitable ligands relied solely on screening trials. Nevertheless, several studies identified CP inhibitors with a greater potency towards the parasite proteasome compared to its human counterpart. In a comprehensive analysis, the groups of Bhanot, Overkleeft, and Bogyo provided insight to explain the observed selectivity.[5] They were able to establish that blockage of the *P. falciparum* β5 subunit during the replication stage (schizogony) was sufficient to decrease parasitic growth, however, co-inhibition of β2 and β5 subunits resulted in protozoa killing at all stages of the blood lifecycle. In order to assess the significance of these in vitro findings, in vivo tests were conducted as well. Mice infected with *P. chabaudi* were injected with a combination of two known CP inhibitors specific for either the β2 or the β5 subunits. As a result, parasitic growth was reduced and slowed, while minimally toxic side effects and less severe symptoms were observed in the treated mice compared to the controls.[5] From their investigation, the authors concluded that molecules that inhibit several subunits of *P. falciparum* CP should eliminate the parasite with reduced toxicity for the host, as long as they block only one subunit in humans (Scheme 1).

Shortly thereafter, Bogyo and co-workers identified a series of compounds that exhibit remarkable selectivity for the *Plasmodium* proteasome.[6] These substances were




***Scheme 1.*** A model of the action of selective proteasome inhibitors against malaria.

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selected from a library composed of noncovalent ligands. In a cell-based assay, the best hit, a cyclic tripeptide with a homophenylalanine residue at the P3 position, displayed a half-maximal effective concentration ($EC_{50}$) of 35 nM for *P. falciparum*, while retaining exquisite selectivity over human foreskin fibroblasts (HFF, $EC_{50} > 50$ μM). In order to shed light on the origin of the species selectivity, the ligand was docked into a homology model of *P. falciparum* proteasome and its singular mode of binding was compared to existing structures of yeast and murine proteasomes. Based on this overlay, the authors surmised that the S3 specificity pocket of the parasite is crucial to ensure selective binding. The compound was found to be active in vitro against the three protozoan subunits, but was only potent against the human β5 subunit. Consistent with previous observations, a short pulse of inhibitor caused parasite death at several stages of its blood lifecycle.

In an earlier report, Nathan et al. also emphasized the importance of the S3 pocket in terms of the species selectivity of proteasome inhibitors.[7] They investigated the activity of noncovalent N,C-capped dipeptides against *Mycobacterium tuberculosis*, which possesses a eubacterial proteasome. The known structural preferences of *M. tuberculosis* CP for large aromatic residues at P1 and N-substituted asparagine side chains at P3 were exploited for ligand design. Their optimization resulted in a potent compound that achieved an inhibition constant ($K_i$) of 15 nM in vitro, with great selectivity over both human constitutive and immunoproteasomes. Comparison of the X-ray structures of *M. tuberculosis*, yeast, and mammalian (constitutive and immuno-) proteasomes led to the conclusion that the species selectivity upon ligand binding originates primarily from interactions in the S3 pocket.

In 2016, Bogyo, da Fonseca, and co-workers marked a milestone as they offered a rationale to explain the origin of the specificity of a small peptide-based vinyl sulfone inhibitor for *P. falciparum* proteasome by using a combination of structural and biochemical approaches.[8] They first determined the substrate preference of the parasite proteasome by analyzing the degradation pattern of 228 divergent peptides. Their previous hypothesis that the S1 and S3 specificity pockets are the most different between humans and *Plasmodium* was verified. Since a tryptophan residue at these positions is favored in protozoa, three peptide-based vinyl sulfones (WLW-vs, WLL-vs, and LLW-vs) were employed and their activities evaluated in vitro with purified *P. falciparum* CP. Remarkably, the three compounds predominantly displayed activity towards the parasite β2 subunit, whereas the human β2 site was hardly affected. In order to understand this intriguing observation, da Fonseca and co-workers performed high-resolution cryo-electron microscopy (cryo-EM) of WLW-vs bound to the parasite proteasome. The EM structure of the ligand complexed to β2 (3.6 Å resolution overall) is depicted in Figure 1 A,[8] in parallel with the X-ray structure of Z-LLL-vs bound to yeast β5 (Figure 1 B).[9] Upon comparison with the EM structure of the human constitutive proteasome,[10a] it became clear that the β2 binding pocket is broader and more open in the parasite than in humans, thus enabling an easier fit of the tryptophan at the P3 position. The

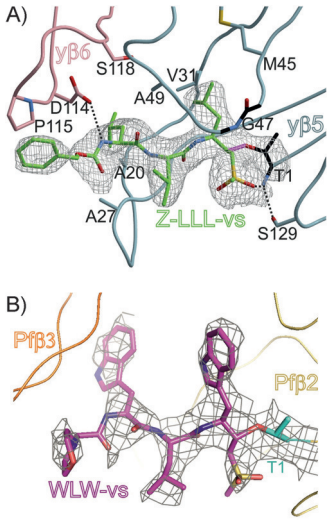


***Figure 1.*** A) The X-ray 2 $F_o - F_c$ electron density map (grey mesh, contoured at 1 $\sigma$, 3.1 Å resolution) depicts Z-LLL-vs (green) bound to the β5 subunit (blue) of yeast CP, with the active-site nucleophile Thr1 shown in black.[9] B) The cryo-EM electron density map (3.6 Å resolution) depicts WLW-vs (magenta) bound to the β2 subunit (yellow) of *P. falciparum* CP, with the active-site nucleophile Thr1 shown in cyan.[8]

β1 subunit of both species is too constrained to allow binding, while the β5 subunits seem more suited for accommodating a tryptophan at P3. WLW-vs was shown to synergize with dihydroartemisinin to kill ART-resistant parasites in early trophozoite *P. falciparum* cultures in a single dose at a concentration at which WLW-vs only blocked parasitic β2 activity and therefore was not expected to affect the growth of the parasite.

By contrast, WLL-vs inhibited the *Plasmodium* β2 and β5 subunits in vitro, as well as the β5 human subunits, and its cell-based activity even extended to the parasite β1 subunit. Significantly higher concentrations of inhibitor were required to observe activity in HFF cells, and no off-target toxicity was observed. Considering these promising findings, experiments on mice infected with *P. chabaudi* were conducted with a single dose of WLL-vs, and the result was almost complete clearance of the parasite load without any significant side effects.

This recent study highlights once more the use of cryo-EM to determine the conformation of a ligand bound to its target protein, which was until now mostly achieved by X-ray crystallography. Another pioneering example has recently been provided by the high-resolution structure of the human 20S proteasome bound to a substrate analogue.[10a] The low yields afforded by the purification of *P. falciparum* CP do not permit crystallization trials to be performed. The rapid technological progress in electron detectors and image processing has offered new possibilities for obtaining structures at resolution comparable with NMR or crystallographic methods.[10b] Cryo-EM is particularly well suited to large molecules (above 100 kDa). Another advantage of this technique is the preservation of the physiological environment of the protein. Protein–ligand interactions are thus maintained and are not subject to crystallization artifacts. The

  

information obtained by cryo-EM can nevertheless be combined with that originating from crystallography to obtain more precise atomic resolution.

The present work by Bogyo, da Fonseca, and co-workers[8] paves the way for the development of new inhibitors against malaria. The structural data obtained by cryo-EM should enable the rational design of ligands with exquisite selectivity towards the *P. falciparum* proteasome and minimal toxicity in humans. The possibility to hit all stages of the parasite lifecycle and the synergistic effects observed with ACT treatment render this therapeutic approach particularly attractive for future research efforts.

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