Title

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Journal

The FEBS journal, 284(10)

ISSN

1742-464X

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Publication Date

2017-05-01

DOI

10.1111/febs.14029

Peer reviewed

Targeting proteasomes in infectious organisms to combat disease

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Keywords: Proteasome, Inhibitor, *Mycobacterium*, *Plasmodium*, *Schistosoma*, kinetoplastid

Abstract

Proteasomes are multisubunit, energy-dependent, proteolytic complexes that play an essential role in intracellular protein turnover. They are present in eukaroytes, archaea and in some actinobacteria species. Inhibition of proteasome activity has emerged as a powerful strategy for anti-cancer therapy and three drugs have been approved for treatment of multiple myeloma. These compounds react covalently with a threonine residue located in the active site of a proteasome subunit to block protein degradation. Proteasomes in pathogenic organisms such as M*ycobacterium tuberculosis* and *Plasmodium falciparum* also have a nucleophilic threonine residue in the proteasome active site and are therefore sensitive to these anti-cancer drugs. This review summarizes efforts to validate the proteasome in pathogenic organisms as a therapeutic target. We describe several strategies that have been used to develop inhibitors with increased potency and selectivity for the pathogen proteasome relative to the human proteasome. In addition, we highlight a cell-based chemical screening approach that identified a potent, allosteric inhibitor of proteasomes found in *Leishmania* and *Trypanosoma* species. Finally, we discuss the development of proteasome inhibitors as anti-infective agents.

Introduction

The proteasome is a large multi-subunit protein complex that regulates numerous cellular functions, including normal protein turnover and degradation of misfolded proteins. It is generally located in the cytoplasm and nucleus of cells [1]. In eukaryotes, proteins destined for degradation, are first conjugated with one or more ubiquitin chains through the side chain of lysine residues [2] whereas bacterial proteins are conjugated with a prokaryotic ubiquitin-like protein (pup) [3]. These ubiquitinated/pupylated proteins are then recognized and unfolded by regulatory proteins that flank the proteolytic core of the proteasome. Once the protein substrate has engaged the proteasome, the ubiquitin and pup chains are removed by deubiqutinases and depupylases,

respectively[4,5]. The unfolded protein substrates are threaded into the barrel-shaped proteolytic core and degraded into short peptide sequences while the released ubiquitin and pup tags are used to label new substrates. The core of the proteasome is formed by two stacked rings of seven β subunits sandwiched between two rings of α subunits. This 28 subunit complex is commonly referred to as the 20S proteasome while the combination of the 20S core and regulatory proteins is referred to as the 26S proteasome. The 20S complex in bacteria is composed of either one α- and one β-type subunit (e.g *Thermoplasma acidophilum*) or two α- and two β-type subunits (e.g *Rhodococcus erythropolis*) [6,7]. In eukaryotes each subunit of the ring is encoded by a separate gene and therefore consist of seven α- and seven β-type subunits [8] (**Fig. 1**).

The subunits responsible for peptide hydrolysis are located in the β-ring. The 14 β subunits of the bacterial proteases are enzymatically active and have broad specificity however, in eukaryotes, only β1, β2 and β5 subunits have hydrolytic activity. Each β subunit has a distinct substrate specificity profile. For example, β1 in the mammalian proteasome has a preference for cleaving on the carboxyl terminal side of acidic residues and therefore substrates that were originally developed for caspases are hydrolyzed efficiently. Thus, the β1 activity is commonly referred to as "caspase-like" [9]. In the same manner, the β2 and β5 subunits are referred to as "trypsin-like" and "chymotrypsin-like", respectively. The most commonly used fluorescent peptidyl substrates to detect activity by these subunits are z-LLE-*AMC* for β1, z-LRR-*AMC* for β2 and suc-LLVY-*AMC* for β5, where *AMC* corresponds to the fluorophore, 7-amino-4-methylcoumarin. In general, natural substrates of the proteasome are longer than the fluorescent substrates and therefore will have extended substrate binding interactions with neighboring β-subunits. For example, a protein that is cleaved by the β5 subunit may interact with β4, β5 and β6 during substrate hydrolysis. A commonly used inhibitor of proteasome activity is MG-132, which consists of z-LLL-*al* where *al* corresponds to an electrophilic aldehyde that reacts with the nucleophilic threonine residue in the active site of the proteasome subunits (**Fig. 2A**). MG-132 preferentially inhibits the β5 subunit in the low nanomolar range and the β1 and β2 subunits in the low micromolar range [10]. Between 1999 and 2012, MG-132 had been used as a research tool in over four thousand scientific studies and was a lead compound for development of several anti-cancer agents [11].

In human cells, short peptides generated by proteasome hydrolysis are a source of antigens presented by major histocompatibility complex class I (MHC-I) molecules. The constitutive proteasome (c20S) is expressed in most cell types, however three additional tissue-specific isoforms are known. Immune cells such as antigen-presenting cells, produce an immunoproteasome (i20S) that has alternative catalytic subunits called β1i, β2i and β5i [12]. These subunits generate peptides for MHC-I that differ from those generated by the constitutive proteasome [13]. The thymoproteasome (t20S), contains β1i, β2i and a β5t subunit that is expressed only in cortical thymic epithelial cells [14]. Finally the spermatoproteasome, expressed in spermatids and mature sperm, is similar to the immunoproteasome except for an alternate α4s subunit [15]. Much attention has focused on the constitutive proteasome when it was revealed that rapidly growing cancer cells were more sensitive to proteasome inhibitors than non-cancerous cells [16]. The cytotoxicity of proteasome inhibitors involves multiple mechanisms that include, inhibition of NF-kB activation, stabilization of cell-cycle regulatory proteins, blocking of

cell-cycle progression at G2-M phase and induction of apoptosis [17,18]. This cytotoxicity formed the basis for development of the drugs, bortezomib (PS-341), carfilzomib (PR-171) and ixazomib (MLN9708) to treat multiple myeloma.

Bortezomib is a dipeptide boronic acid inhibitor that reversibly binds to a nucleophilic threonine residue in the active site of the β5 and β5i subunits [19] (**Fig. 2B**). In 2003, it was approved for intravenous treatment of relapsed or refractory myeloma and eventually as a first-line therapy for patients with multiple myeloma and mantle cell lymphoma. Carfilzomib is an epoxyketone tetrapeptide inhibitor developed from the scaffold of epoxomicin, a natural product isolated from Actinomycetes [20] (**Fig. 2C**). Unlike the boronic acid inhibitors, epoxyketones are irreversible and highly selective for the proteasome. Carfilzomib has demonstrated activity against bortezomib-resistant cell lines [21] and was approved in 2012 for treatment of multiple myeloma patients who had previously received bortezomib. Ixazomib, an orally bioactive dipeptide boronic acid inhibitor with improved pharmacokinetics, pharmacodynamics, and antitumor activity over bortezomib, was approved in 2015 [22]. Ixazomib is a prodrug consisting of a protected cyclic boron that is hydrolyzed to the boronic acid, MLN2238 in plasma and other aqueous solutions (**Fig. 2D**). Each of these drugs preferentially targets the chymotrypsin-type activity of the constitutive (β5) and immunoproteasome (β5i) however co-inhibition of β1/β1i or β2/β2i occurs at higher concentration [23–25].

Pathogens of humans ranging from bacteria to helminths have genomes that encode proteasomes that are structurally and functionally similar to the mammalian complex. Until recently, it was unknown whether inhibitors could be developed that selectively target the parasite proteasome and not the host complex. Several studies have used the approved drugs, bortezomib and carfilzomib, to evaluate whether the parasite proteasome is a possible drug target, however, these studies were performed in culture and toxicity to host cells was not addressed [26–28]. Pharmaceutical companies continue to develop proteasome inhibitors as anti-cancer drugs and more recently to target auto-immune disease [29]. As the number of synthetic compounds increase, the likelihood of discovering analogs with selectivity for a parasite proteasome also rises. Collaborations between pharmaceutical companies and academic researchers studying *Mycobacterium tuberculosis* and *Plasmodium falciparum*, have identified selective inhibitors of the pathogen proteasome that are analogs of bortezomib [30] and carfilzomib [26], respectively. In addition, cell based screening of diverse chemical scaffolds and rational design of inhibitors based on substrate specificity information, have yielded potent compounds with high selectivity for proteasomes of parasitic species and low toxicity to mammalian cells. In this review, we focus on studies in which proteasome inhibitors have been evaluated in cell based assays containing bacteria, protozoa or helminths that cause disease in humans and other mammals. In addition, we summarize the inhibitor development strategies that have been utilized to develop potent and selective antagonists of parasite proteasomes.

1. Bacterial pathogens

M. tuberculosis (Mtb), the causative agent of tuberculosis, is responsible for 1.5 million deaths per year [31]. Mtb

is an intracellular pathogen that is phagocytosed by alveolar macrophages in the lung after being inhaled. It resides in macrophages, resisting innate defense mechanisms. Infected cells can migrate across the alveolar epithelium into the lung tissue and aggregate to form granulomas [32]. If Mtb overcomes the immune system defense, it can multiply and cause the disease soon after primary infection. One third of the world population maintain a latent form of Mtb infection that will produce an active infection in ~5% of the cases. In recent years, the Mtb epidemic has been aggravated by the emergence of drug-resistant strains [33]. Therefore, new drug therapies are badly needed.

Mtb is the only known bacterial pathogen to possess a proteasome, the activity of which is required for Mtb to persist in mice [34,35]. When Mtb is cultured in stress conditions that mimic the acidic (≤ pH.5.5) and nitrosative environment of macrophages, cells are rendered non-replicating and sensitive to proteasome inhibitors [36]. Mtb failed to recover from nitrite-mediated injury when treated with either epoxomicin (**Fig. 3A**) or a boronic inhibitor, MLN-273 (**Fig. 3B**). MLN-273 was developed as a tool to study the mechanism of inhibition of the bacterial proteasome isolated from *Rhodococcus erythropolis* [37] and was subsequently found to inhibit Mtb proteasome (Mtb20S) with an IC_{50} of 1.6 nM [38]. A crystal structure of MLN-273 bound to Mtb20S revealed the mechanism of inhibition [39]. Studies by the same group, determined that bortezomib inhibits the β-subunits of Mtb20S with an IC_{50} value of 300 nM, which is 19-fold less potent when compared to the human β5. Using substrate specificity information, substitution of an *m*-chloro-phenylalanine for leucine in the P1 position of bortezomib reversed the species specificity. The resulting compound, BTZ-16 (**Fig. 3C**), was 8-fold more selective for Mtb20S [30]. These substrate and inhibitor specificity studies determined that there is sufficient difference between the pathogen and host proteasomes to develop compounds with greater selectivity for Mtb proteasome.

Using the chymotrypsin substrate, Suc-LLVY-*AMC*, several plant derived natural products were found to inhibit Mtb20S with IC_{50} values in the range of 25 to 120 μM [40]. In addition, two covalent inhibitors, GL5 (**Fig. 3D**) and HT1171 (**Fig. 3E**) were discovered in a screen of 20,000 compounds [41]. These oxathiazol-2-one compounds had more than 1,000-fold selectivity for Mtb20S over human β5 and were found to irreversibly inhibit the proteasome by cyclocarbonylating the active site threonine. This reaction mechanism shares common features with serine β-lactamases inhibition by O-aryloxycarbonyl hydroxamates and therefore several β-lactamase inhibitors were also found to be potent inhibitors of Mtbs20S [42]. GL5 and HT1171 kill non-replicating Mtb in a dose dependent manner from 12.5 to 50 μM with no toxicity to mammalian cells up to 75 μM. However, they had poor stability in serum and were found to potently inhibit human β5i [43].

Peptide aldehydes, such as, MG-132 are generally poor inhibitors of Mtb20S [38] but potent against the mammalian enzymes (K_i < 25 nM). However, a lipopeptide aldehyde known as fellutamide B (**Fig. 3F**) from the marine fungus, *Penicillium fellutanum* is a potent inhibitor of Mtb20S with a K_i < 10 nM [44]. The alkyl tail likely increases affinity for Mtb20S when compared to short peptide aldehydes. Fellutamide B is also a potent human proteasome inhibitor and therefore insufficient selectivity exists for it to be developed as an anti-microbial compound. Structural studies with this molecule indicated that selectivity may be achieved by substitution of a branched and shorter alkyl tail on the amino terminus as this may interfere with binding to human proteasome while maintaining potency for Mtb20S [44].

Finally, screening of Mtb20S with 1,600 dipeptides containing varying amino- and carboxyl-termini caps identified a reversible inhibitor, DPLG-2 (**Fig. 3G**), with a K_i of 15 nM and greater than 3,600-fold selectivity over human β5 and β5i [45]. This compound was stable in human plasma for more than 22 h and killed non-replicating Mtb in a dose dependent manner. On target efficacy was confirmed using a competition assay with the irreversible inhibitor, HT1171. However, DPLG-2 was rapidly cleared in mice, and the compound was undetectable in animals after 10 minutes (personal communication with Gang Lin).

These studies highlight that specificity differences exist between the proteasomes of Mtb and its obligate human host and that clear progress has been made to develop compounds with increased potency and selectively for the Mtb proteasome. Computational screening of drug-like molecules has yielded several non-covalent compounds that are promising drug candidates [46]. However, a major hurdle in evaluating these inhibitors is the lack of animal models that faithfully mimic the Mtb persistence that occurs in humans [47].

2. Protozoan parasites

Parasitic protozoa are unicellular eukaryotic organisms with varied and complex life cycles, comprising multiple differentiation events, physical environments and hosts. These organisms are the causative agents of several human diseases that combined, cause more than a million deaths annually [48]. The ubiquitin-proteasome system from pathogenic protozoa has been reviewed in detail previously [49,50]; therefore, this section will focus on inhibitor development efforts for Kinetoplastid, Apicomplexa and Amoeba parasites.

a. Kinetoplastid

Trypanosoma & Leishmania

Three kinetoplastid diseases, leishmaniasis, African sleeping sickness and Chagas' disease are caused by Leishmania species, *Trypanosoma brucei* and *Trypanosoma cruzi,* respectively. Together, they affect 20 million people among the world's poorest populations, causing high morbidity and leading to more than 50,000 deaths annually [51]. There is a dire need for drug development to control these diseases, mainly because the emergence of drug resistance and side effects associated with the current therapies [52].

The proteasome was suggested as a drug target for *Trypanosoma* and *Leishmania* when treatment with proteasome inhibitors showed changes in cell growth and development. Specifically, lactacystin (**Fig. 4A**) and MG-132 (**Fig. 2A**) prevented the transformation of *Trypanosoma cruzi* from trypomastigotes into amastigotes in a concentration dependent manner [53]. The purified proteasome from bloodstream and procyclic *Trypanosoma brucei* forms was sensitive to several proteasome inhibitors including lactacystin with IC_{50} of 1 μM. In addition, proliferation of bloodstream and procyclic *T. brucei* was blocking by lactacystin at concentrations greater than 5 μM [54]. *In vitro* assays different *Leishmania* species produced similar results. Promastigotes of *L. donovani* incubated with 2 μM MG-132 for 72 h or 5 μM for 24 h resulted in 50% and 95% cell death, respectively [55]. Surviving cells were shorter with rounded apical ends and had DNA and mitochondrial damage. After six days of treatment of *L. chagasi* with 50 μM lactacystin, a 10-fold reduction in viable cells was recorded; while exposure of promastigotes to lactacystin prior to infection of macrophages decreased survival in the host cell by 98% [56].

Proteins essential to cell cycle were identified as proteasome substrates in these parasites [57,58]. Taken together, these studies reveal the importance of proteasome activity in kinetoplastid parasites.

In a high-throughput phenotypic study performed at the Genomics Institute of the Novartis Research Foundation, a compound termed GNF5343 (**Fig. 4B**) was identified as a hit in proliferation assays of *L. donovani* and *T. brucei* with EC_{50} values of 7.3 and 0.15 μM, respectively. This hit was optimized to improve bioavailability and potency against *L. donovani* resulting in a compound with 400-fold increase in potency (EC_{50} = 18 nM). This compound, GNF6702 (**Fig. 4C**), was also potent against *T. brucei* and *T. cruzi* cultures, with EC_{50} values of 70 and 120 nM, respectively [59]. *T. cruzi* was cultured in the presence of increasing concentration of GNF6702 analogues and two resistant clones were isolated and analyzed by whole genome sequencing. The proteasome was found to be the molecular target of these compounds. This target was subsequently confirmed by the accumulation of ubiquitinylated protein following incubation of *T. cruzi* with the inhibitor. GNF6702 inhibits β5 activity in an unusual non-competitive manner. It does not interact with the threonine residue in the β5 subunits but binds to the β4 subunit at a site near the β4-β5 interface. Mutagenesis studies confirmed that Phe24 and Ile29 in the β4 subunits were important for binding. These residues are conserved in *L. donovani*, *T. cruzi* and *T. brucei* but absent in the β4 subunit of human proteasomes. In fact, the β4 subunit of the drug-resistant *T.cruzi* line and the human proteasomes have methionine at position 29. Consequently, GNF6702 does not inhibit the human proteasome and no accumulation of ubiquitinylated proteins was detected in mouse fibroblast cells following treatment. Using an oral dosing scheme of 10 mg/kg twice daily, parasite burden in liver of mice infected with *L. donovani* was reduced by more than 99.8% after 8 days treatment. In addition, treatment for 7 days resulted in a 5-fold decrease in parasite burden in footpads of mice infected with the dermatotrophic *Leishmania major* strain. In a mouse model of Chagas disease, 20 days of treatment with GNF6702 was sufficient to reduce *T. cruzi* levels in blood, heart and colon to undetectable levels while 100 mg/ml treatment once daily with the same compounds eliminated *T. brucei* in brains of mice modeling stage II sleeping sickness [59].

b. Apicomplexa

The proteasome in *Plasmodium falciparum* has been validated as a therapeutic target following effective killing of the parasite at various life-stages [60] using lactacystin [61,62], marizomib [63], bortezomib [27], MLN-273 [64], MG-132 [62], epoxomicin [62] and carfilzomib [26]. These compounds are generally toxic to mammalian cells, however, MG-132 was found to have more than 225-fold higher selectivity for *P. falciparum* relative to human peripheral blood mononuclear cells [62]. This selectivity was due to its promiscuous inhibition of both the proteasome and the hemoglobin-degrading cysteine proteases. While MG-132 presented an opportunity to develop dual-target inhibitors of malaria parasites, much of the subsequent screening approaches focused solely on proteasome inhibitors that are selective for the *Plasmodium* proteasome active sites.

A library of 670 carfilzomib analogs was screened in a 72 h *P. falciparum* replication assay and a parasite selective compound called PR3 (**Fig. 5A**) was identified. PR3 was 100-fold less potent than carfilzomib against the trophozoite stage but had no toxicity on human fibroblasts up to 50 μM [26]. PR3 was found to preferentially target the β5 subunit of the *Plasmodium* proteasome and treatment of *Plasmodium berghei*-infected mice with

80 mg/kg of inhibitor resulted in a moderate but significant reduction in parasite burden during the treatment period. This is the first demonstration that the *Plasmodium* proteasome could be selectively targeted with low host toxicity. In another study, 1,600 non-covalent N,C-capped peptidic proteasome inhibitors were screened in an *in vitro* assay using purified *P. falciparum* proteasome (Pf20S) and the proteasome β5 selective fluorescent substrate, Suc-LLVY-*AMC* [65]. This library was similar to the set of compounds that was used to identify DPLG-2 (**Fig. 3G**) as a potent and selective inhibitor of Mtb20S proteasome [45]. Nine compounds were identified that had selectivity for Pf20S relative to the human constitutive proteasome. A common feature of the hit compounds was a 4-methylbenzyl group in the P1 position and a bulky homo-phenylalanine in the P3 positon. One compound, cyclic peptide 1 (**Fig. 5B**), had more than 1,450-fold selectivity for *P. falciparum* relative to human fibroblasts after 72 h. This selectivity was due to co-inhibition of the β5 and β2 subunits of *Plasmodium* while only the β5 of the human proteasome was targeted [65]. Whereas dual inhibition of β2 and β5 is lethal to *Plasmodium* viability, pulse inhibition of the mammalian β5 proteasome alone does not result in significant toxicity in most non-transformed cells [66]. In a separate study, a combination treatment of *P. berghei* infected mice with 50 mg/kg of a β5 inhibitor, PR709A (**Fig. 5C**), and 20 mg/kg of a β2 inhibitor, LU102 (**Fig. 5D**), was more effective than treatment with either inhibitor alone [67]. Taken together, these studies suggest that targeting the β5 subunit along with β1 and/or β2 of the *Plasmodium* proteasome may be key for developing potent and selective anti-malarial compounds.

In a follow up study, the *Plasmodium* and constitutive human proteasomes were incubated with a library of diverse synthetic peptides to identify sequences that are preferentially cleaved by each proteasome [68]. Cleavage of bonds within these peptides was detected using LC-MS/MS sequencing. Using this approach, the *Plasmodium* proteasome had a clear preference for tryptophan in the P3 and P1 positions when compared with the human constitutive proteasome [69]. Using a tri-leucine-vinyl sulfone inhibitor as a starting scaffold [70], peptide-vinyl sulfones consisting of tryptophan at P3 or P1 were synthesized. Two compounds, WLL-vs (**Fig. 5E**) and WLW-vs (**Fig. 5F**) targeted the β2 subunit, however, WLL-vs also reacted with the β5 subunit. After a 1 h incubation, WLL-vs potently inhibited P. *falciparum* with 675-fold selectivity over human fibroblasts. This selectivity was due to inhibition of both the β5 and β2 subunits of Pf20S while only the β5 of the human complex was targeted. Subsequently, a single bolus dose of 35 mg/kg WLL-vs was administered to *P. chabaudi* infected mice that resulted in almost complete reduction of parasite burden without toxicity to the host.

Artemisinin derivatives are the first-line drugs used to treat malaria. Resistance to these drugs has emerged in Southeast Asia, resulting in a dire need for alternative treatments [71]. In a study by Tilley and colleagues, co-treatment of *P. falciparum* with dihydroartemisinin (DHA) and either epoxomicin, carfilzomib or bortezomib resulted in strong synergistic killing of both DHA-sensitive and DHA-resistant strains. DHA-resistant parasites have an enhanced cell stress response and therefore are hyper-sensitive to proteasome inhibitors [28]. Epoxomicin, carfilzomib and bortezomib preferentially target the β5 subunit of *Plasmodium* and therefore a similar experiment was performed with the β2 subunit inhibitor, WLW-vs. A combined treatment of DHA and sub-lethal doses of WLW-vs resulted in synergistic killing of a DHA-resistant strain.

In a *P. berghei* mouse model, co-treatment with 1 mg/kg of carfilzomib and 5 mg/kg of DHA significantly reduced parasite burden when compared to treatment with either compound alone [28]. These data suggest that

combination therapy of FDA-approved multiple myeloma drugs with DHA may have clinical use in treatment of artemisinin-resistant strains. However, the well characterized toxicity of these proteasome inhibitors suggests that more work will need to be done to develop safer drugs that could be administered orally for extended periods. To date, the most selective proteasome inhibitors for *Plasmodium* have come from *in vitro* or phenotypic screens of compound libraries or have been rationally designed from substrate specificity information. However, a 3.5 Å structure of the Pf20S bound to WLW-vs has now been elucidated by electron cryo-microscopy and single particle analysis [69]. When this structure was compared to cryo-EM structure of the human constitutive proteasome, the basis for WLW-vs specificity was revealed [72]. The *Plasmodium* β2 ligand binding pocket accommodates the bulky side chains of tryptophan at the P1 and P3 positions, while the human β2 pocket cannot. In fact, the other substrate binding pockets of the human proteasome active subunits also have restricted access for bulky side chains at P1 and P3. Therefore, specificity and structural information now serve as powerful tools to rationally design inhibitors with increased potency towards the *Plasmodium* proteasome and reduced toxicity of the human complex [73].

Babesia is a tick-transmitted protozoan parasite that infects erythrocytes of several wild and economically valuable animal species causing serious economic losses in the livestock industry. Some *Babesia* species can infect humans and human babesiosis is considered an emerging tick-borne zoonotic disease [74]. Available drugs to treat babesiosis in cattle are toxic and the emergence of resistance is likely [75]. The potential of epoxomicin to inhibit parasite growth was tested in several *Babesia* species [76]. In *in vitro* cell cultures, epoxomicin showed an IC_{50} in the nanomolar range for *Babesia bovis*, *B. bigenima*, *B. ovata*, *B. caballi*, and *B. equi*. A combination of epoxomicin and diminazene aceturate potentiates the effect on parasitemia and growth inhibition in *in vitro* cell cultures of *B. bovis*, *B. bigemina*, and *B. caballi*. *In vivo* assays performed in mice infected with *B. microti* indicate that parasitemia is significantly lower in mice treated with 0.05 mg/kg or 0.5 mg/kg epoxomicin ten days when compared to control treatment animals. The proteasome was not validated as the molecular target of epoxomicin in this study, however, *B. bovis* proteasome subunits are expressed during the erythrocyte stage [77] and epoxyketone inhibitors are known to be highly selective for proteasome subunits [78]. Therefore, it is expected that epoxomicin selectively targets the Babesia proteasome.

c. Amoeba

Entamoeba histolytica, a parasite causing intestinal disease, is transmitted in contaminated water or food from one host to the other in the form of a latent cyst. Excystation occurs in the digestive tract to produce a proliferative trophozoite form that can cause amoebic dysentery or liver abscess [79]. Encystation is key to propagating infection and therefore blocking this step is a therapeutic goal [80]. Exposure of *E. histolytica* to low micromolar amounts of lactacystin for three days, resulted in 73 to 100% growth inhibition [81]. In addition, lactacystin prevented encystation by the reptile pathogen *E. invadens* [82], which is commonly used as a model for *E. histolytica* [81,82]. The proteasome was isolated from *E. histolytica* and the chymotrypsin-type, trypsin-type and caspase-type activity were shown to be inhibited by lactacystin. These studies demonstrate that *Entamoeba*

species require proteasome activity for conversion of the disease-causing trophozoite form into the infective cyst form.

Acanthamoeba spp. are widespread free-living opportunistic amoeba that cause granulomatous amebic encephalitis, cutaneous acanthamebiasis, and acanthamoeba keratitis (AK). Treatment of AK infections are difficult due to resistant of the cyst stage to chemical therapies, making encystation a major challenge in the successful treatment of amoebae infections [83]. The ubiquitin-proteasome system and lysosomal proteases are important components of encystation, due to the high levels of protein turnover that occurs during this differentiation stage [84,85]. In culture, incubation of *A. castellanii* with micromolar amounts of lactacystin and bortezomib decreased encystation by 30% to 49% [86].

d. Other Protozoa

Tritrichomonas foetus is an obligate parasite of the bovine reproductive tract that can cause endometritis, infertility and early embryonic death [87]. This sexually transmitted disease is responsible for considerable economic losses to the beef and dairy cattle. In addition, *T. foetus* is a causative agent of chronic diarrhea in domestic cats [88]. Imidazoles have been used to treat cats but are often ineffective [88]. *T. foetus* exists as a trophozoitic form but can transform into a pseudocyst under nutrient and temperature stress [89]. Incubation of *T. foetus* cultures with 20 μM lactacystatin for 18 h resulted in complete inhibition of growth of the trophozoitic form with a general arrest of cell cycle in the G2/mitosis phases and prevented transformation of the trophozoitic form to the pseudocyst form during cold-induction assays [90]. In another study, gliotoxin, a proteasome inhibitor isolated from fungi [91], inhibited *T. foetus* growth at concentrations ≥ 1 μM arresting cell cycle in the G2/mitosis phase and causing DNA fragmentation. When the proteasome was isolated from gliotoxin-treated cells, chymotrypsin-like activity was reduced by 48-67% compared to untreated cells [92].

3. Helminths

Schistosoma

Schistosomiasis is a tropical water-borne disease caused by the *Schistosoma* blood fluke that infects over 240 million people [93]. The disease is chronic and painful as a result of various pathologies associated with the parasite's eggs that become trapped in various visceral organs. There is no vaccine and treatment of schistosomiasis relies on just one drug, praziquantel [94–97]. The risk of resistance and the lack of alternative drugs spurs the identification of new drug options.

The contribution of the ubiquitin-proteasome system in protein hydrolysis in *Schistosoma* has been demonstrated in cercariae (infective larvae) and adult worms [98], and the 20S proteasome was purified, and analyzed by 2-DE/MS [99]. Indirect evidence points to the schistosome proteasome as a drug target. Mice exposed to cercariae that had been pre-treated with MG-132 had 65% less migrating lung parasites than control-treated mice [98]. In addition, exposure *in vitro* of *S. mansoni* post-infective larvae (schistosomula) to short-interfering RNAs targeting

a deubiquitinase subunit of the 19S regulatory particle, resulted in 78% decrease in parasite viability [100]. Preliminary data from our group using WormAssay software [101], shows that incubation of adult *S. mansoni* worms with 1 µM of either bortezomib or carfilzomib, results in more than 90% reduction in parasite motility when compared to a DMSO treated control (**Fig. 6**).

Cestoda

Alveolar echinococcosis is a rare but often fatal disease in humans caused by metacestode larvae of *Echinococcus multilocularis*. This tapeworm is more commonly found in dogs, however rodents act as intermediate hosts. *E. multilocularis* causes parasitic tumors in many human organs and therapies include some benzimidazoles [102]. In one study, *E. multilocularis* metacestodes were incubated with 426 FDA-approved drugs and bortezomib was identified as the most potent drug with EC_{50} of 0.6 µM [103]. Metacestode treatment with bortezomib induced serious morphological alterations resembling apoptotic cells at concentrations as low as 50 nM. The proteasome was validated as the molecular target of bortezomib, because accumulation of ubiquitinated proteins was evident. In addition, chymotrypsin-like proteasome activity in cellular lysates was inhibited in a dose dependent manner. Despite good *in vitro* efficacy against larvae, bortezomib had poor efficacy in a mouse model of secondary alveolar echinococcosis [103]. However, future improvement of the animal model could lead to a better understanding and validation of proteasome inhibitors *in vivo*.

4. Tools and techniques for proteasome inhibitor discovery

For groups involved in research on pathogenic organisms, there is a many tools available to evaluate the proteasome as a drug target. The fluorescent substrates, z-LLE-*AMC*, z-LRR-*AMC* and suc-LLVY-*AMC* appear to be universally applicable for monitoring β1, β2 and β5 activity, respectively, of eukaryotic proteasomes, while suc-LLVY-*AMC* can be used to evaluate proteasome activity in bacteria. These substrates can be purchased from numerous vendors, however it should be noted that serine and cysteine proteases in cellular extracts can also cleave these substrates. Therefore, it is essential to include Pefabloc (1 mM) and E-64 (50 µM) in the lysis buffer prior to performing proteasome activity assays. Proteasome activity in cellular lysates can also be evaluated using activity based probes that target are selective for individual subunits [104] or board acting probes that target all subunits [67].

Most of the proteasome inhibitors that have been investigated as anti-cancer drugs can be purchased from vendors, however, the pathogen specific compounds that have been discussed in this review are not available from commercial sources. Proteasome inhibitor collections that are owned by pharmaceutical companies have been screened by academic groups [26,45,65] and therefore these libraries are likely to be available for testing against other parasites.

X-ray crystallography has been extensively used to study ligand interactions in yeast and mammalian 20S proteasomes. However due to low yield of proteasomes in many pathogens, it can be difficult and often impractical to isolate sufficient proteasome at high purity for crystallography studies. Cryo-EM has recently been used to determine high resolution protein structures of human and plasmodium proteasomes with peptide

inhibitors bound in an active site . With have been used as a test sample for cryo-EM imaging conditions and image processing methods, resulting in structures at resolutions as high as 2.8? However, these are not suitable models to infer detailed ligand selectivity properties of the active sites of eukaryotic proteasomes, which are functionally and structurally more complex.

A

Both X-ray crystallography and cryo-EM studies have been performed on

Irrespective of the pathogenic organism, a common goal of all researchers in this field, is to develop inhibitors that weakly interact with the mammalian proteasomes yet are sufficiently potent to disable the target organism. To date, this has been achieved by rational designed and by Substrate specificity and structural information generated for the human and Plasmodium proteasomes have already been used to design inhibitors that have limited accessibility to the active sites of c20S. These compounds may become a starting point for design of inhibitors that target other pathogen proteasomes. High-resolution cryo-EM showed

For

Conclusion

There is an urgent need for new and more effective drugs to treat parasitic diseases. Many of the diseases discussed in this review affect people, often children, in the developing world; and therefore safe and cost-effective drugs are an essential requirement for drug discovery efforts. Proteases have long been considered as excellent drug targets [105] and protease inhibitors have been used extensively to treat HIV and HCV [106,107], type 2 diabetes [108], cardiovascular diseases [109] and more recently cancer [110,111]. Until recently it was unknown if a proteasome inhibitor targeting a pathogenic organism could be administered orally and have low toxicity towards the mammalian host. In this review, we have highlighted an allosteric inhibitor of kinetoplastid proteasomes that has unprecedented *in vivo* efficacy for clearing parasites following oral administration. This inhibitor is tolerated in mice at daily doses of up to 100 mg/kg and is currently undergoing preclinical toxicity studies [59]. For covalent inhibitors, oral dosing of a peptide vinyl sulfone prevents cardiac damage in dogs due to infection with *T. cruzi* [112]. In this case, the protease inhibitor K777, targets a cysteine protease and not the proteasome in *T. cruzi* [113] but confirms that oral formulation for this class of compound is possible. The anti-malarial compound WLL-vs is chemically similar to K777 and studies are on-going to improve potency and oral bioavailability of WLL-vs [69]. A second generation oral inhibitor of human proteasomes is now an approved drug for treatment of multiple myeloma [114]. This compound, ixazomib, is delivered as a prodrug and hydrolyzes to the biologically active peptide boronic acid form upon exposure to plasma. In addition, a peptide epoxyketone, known as oprozomib, with oral bioavailability is currently in Phase 1b studies for treatment of multiple myeloma [115]. Taken together, these studies reveal that inhibitors targeting the active sites or allosteric sites in the pathogen proteasome can be designed to have good oral bioavailability and low toxicity towards the mammalian host. Therefore proteasome inhibitors offer promise as next-generation anti-parasitic agents.

Acknowledgments

The authors are grateful to Hao Li, A*STAR, Singapore and Gang Lin, Weill Cornell Medical College for comments and advice that were received during the writing of this review.

Author contributions

BBV, CRC and AJO analyzed the literature and co-wrote the review. CRC performed the WormAssay. ZJ and AJO generated the images.

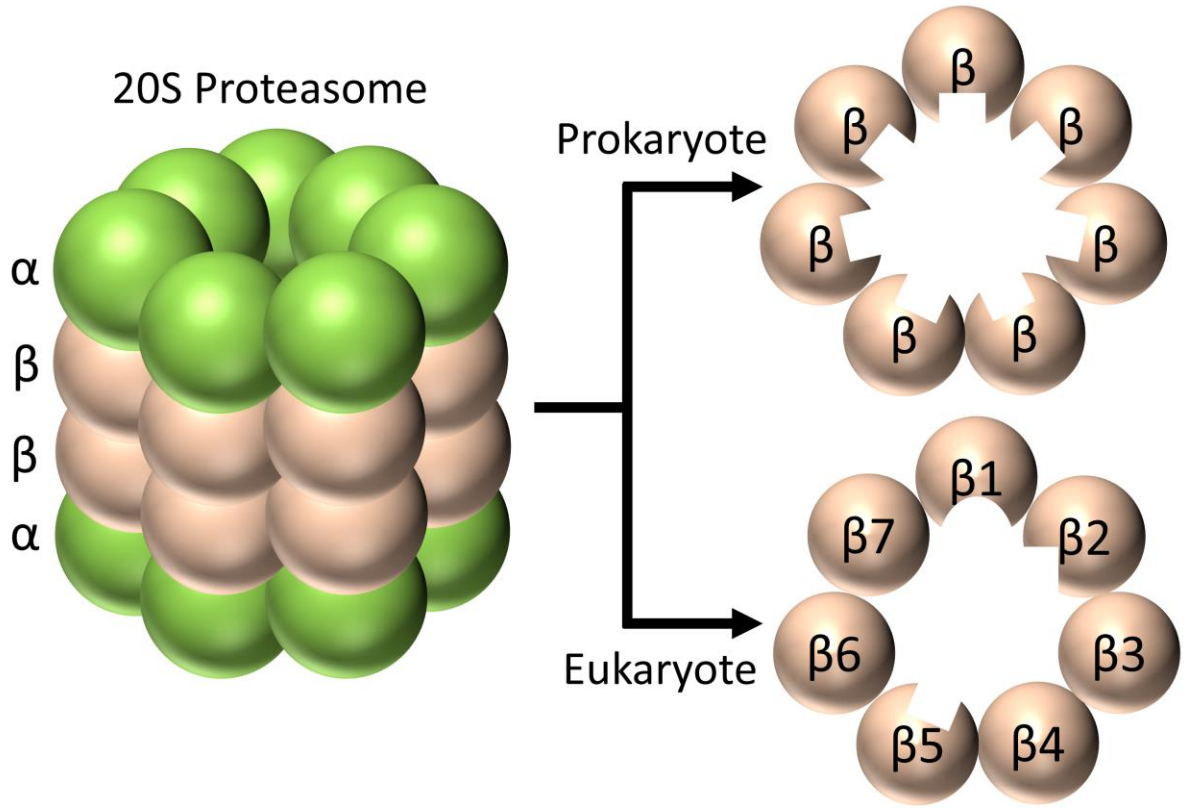

Fig.1 Schematic representation of a 20S proteasome. The 20S proteasomes consists of two outer rings of alpha subunits and two inner rings of beta subunits. The catalytic subunits are located in the beta ring. In prokaryotes such as *M. tuberculosis* all beta subunits are identical and are catalytically active, as indicated by the square substrate binding pocket. In eukaryotes, only the β1, β2 and β5 subunits are active and the differences in substrate specificity is illustrated by the dissimilar shapes of the substrate binding pockets. The substrate specificity of the prokaryotic proteasome is most similar to the β5 specificity of the eukaryotic proteasome.

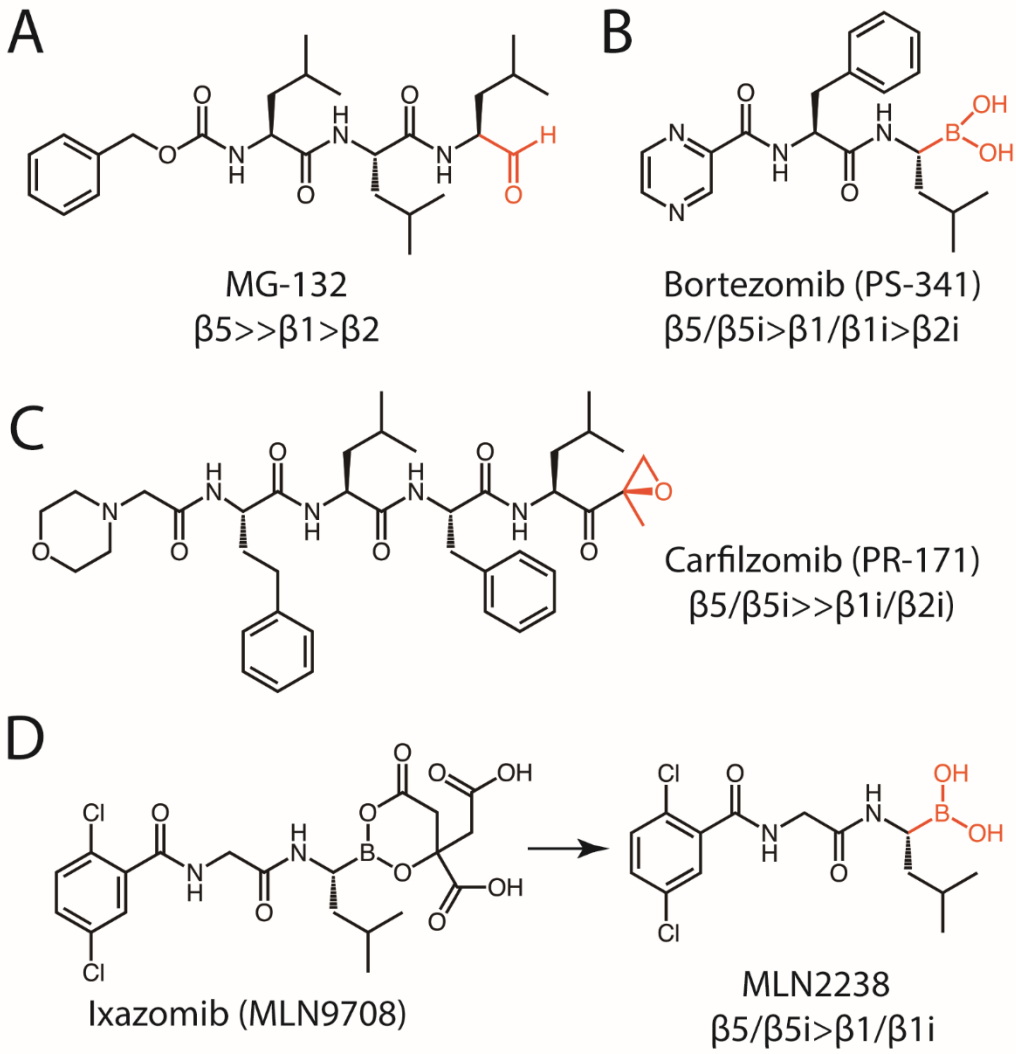

Fig. 2 Structure of MG-132 and approved anti-cancer drugs. **A.** MG-132 is the most commonly used proteasome inhibitor for basic research applications. It is a potent and selective inhibitor of the β5 subunit but will also target the β1 and β2 subunits at high concentration. This selectivity is represented as β5>β1>β2 and the electrophilic aldehyde group is highlighted in red. **B.** Structure of bortezomib, also known as PS-341, with the boronic acid reactive group in red. Bortezomib was the first proteasome inhibitor to be approved as an anti-cancer agent. **C.** Structure of carfilzomib, also known as PR-171 that selectively targets the β5 and β5i subunits of the constitutive proteasome and immunoproteasomes, respectively. The epoxyketone reactive group is highlighted in red. **D.** Structure of the prodrug ixazomib that is hydrolyzed in aqueous solution to the active boronic acid inhibitor MLN2238. Among proteasome inhibitors, ixazomib is the first oral compound to be approved for treatment of multiple myeloma.

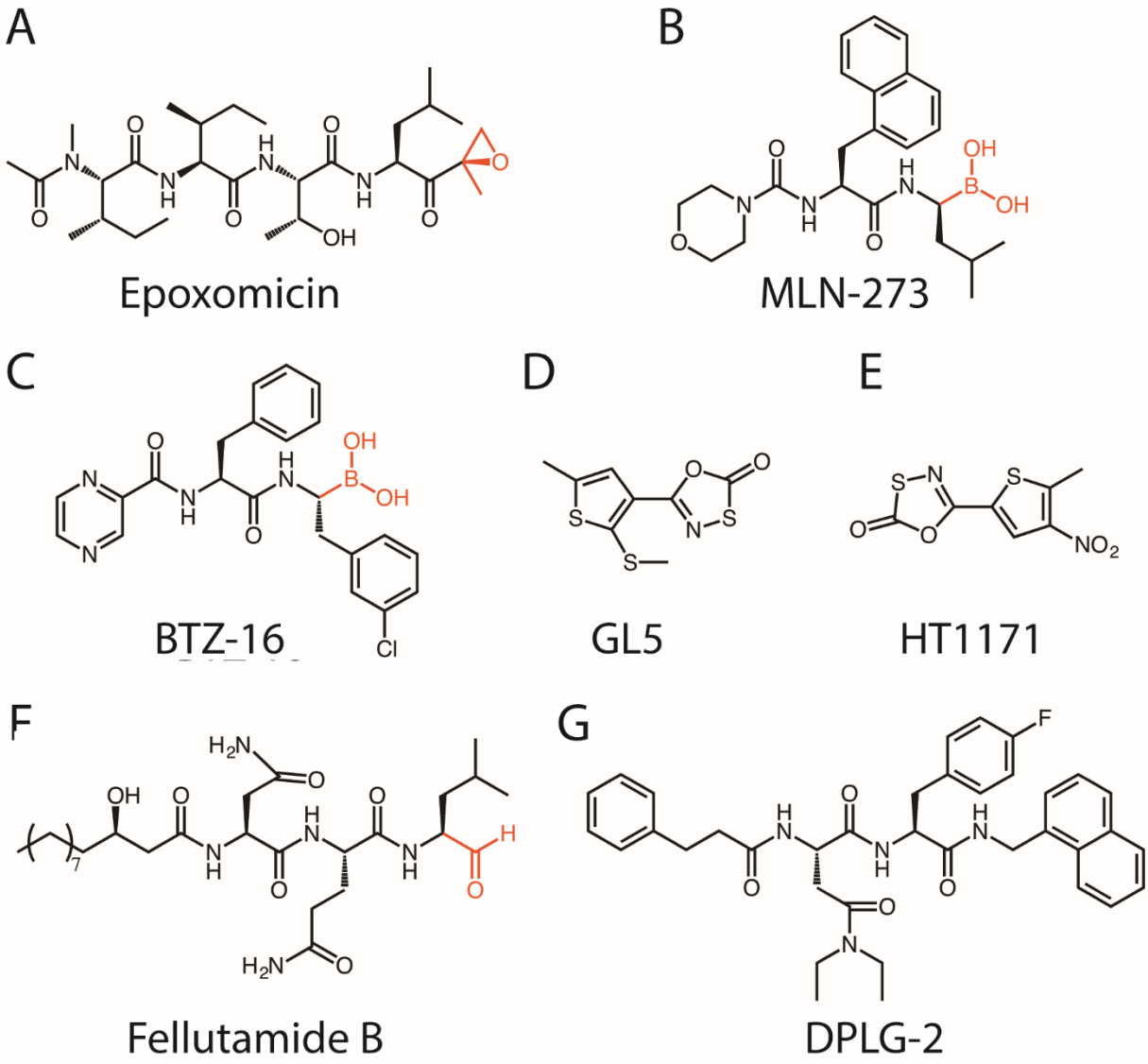

Fig. 3 Structure of Mtb20S inhibitors. Electrophilic groups are colored red. Epoxominin and fellutamide B are natural products. MLN-273 was developed as a tool to study the mechanism of proteasome inhibitor in bacteria and BTZ-16 was rationally designed based of substrate selectivity preferences of Mtb20S. GL5, HT1171 and DPLG-2 were identified as potent and selective inhibitors of Mtb20S in high-throughput screens.

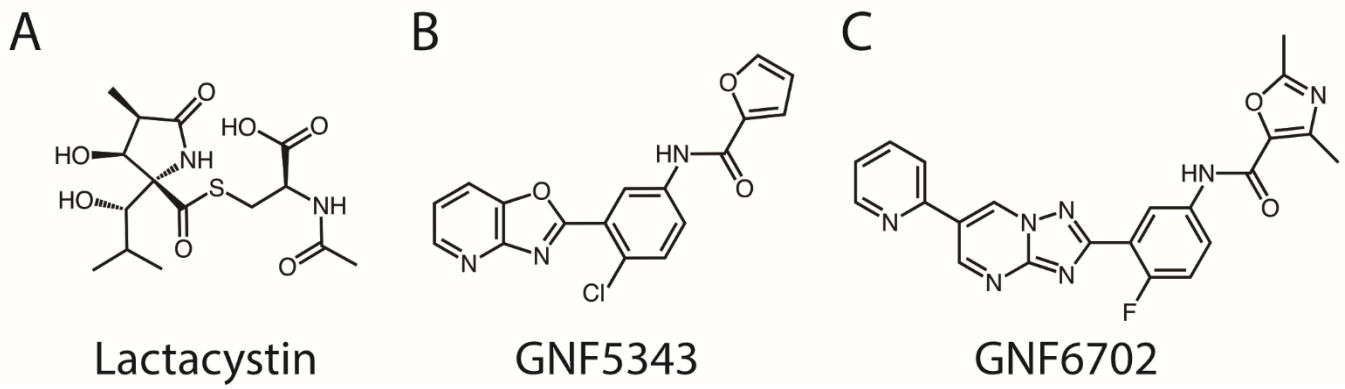

Fig. 4 Structure of kinetoplastid proteasome inhibitors. A. Lactacystin is a natural product synthesized by Streptomyces and has be used extensively in cell-based assays with kinetoplastid parasites. **B.** GNF5343, is an azabenzoxazole that was identified as a potent inhibitor of *L. donovani* and *T. brucei* proliferation from a screen of 3 million compounds. **C.** GNF6702 was optimized from GNF5343 to have improved bioavailability and potency.

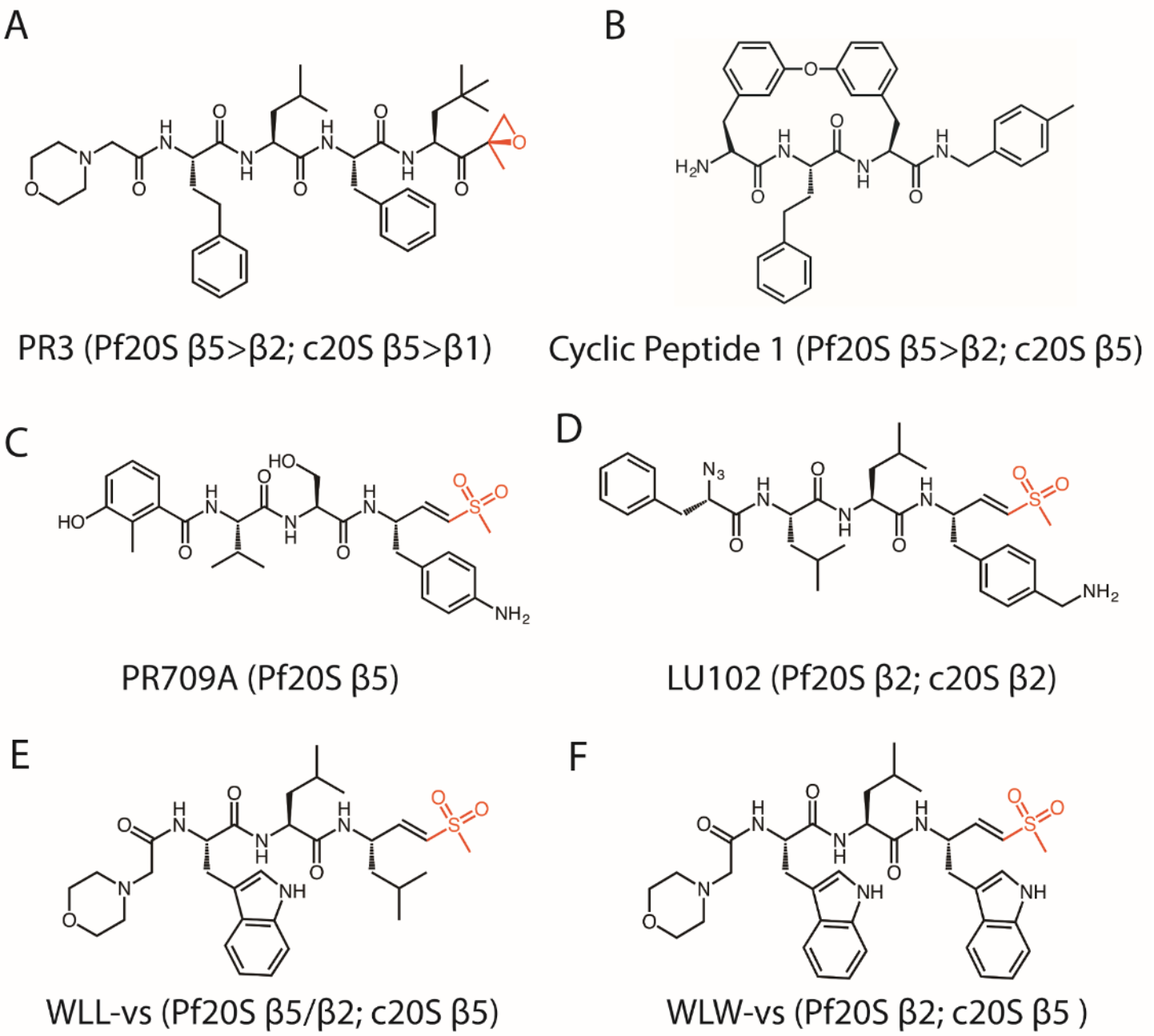

Fig. 5 Structure of Plasmodium proteasome inhibitors. A. PR3 was identified as a selective inhibitor of Pf20S in a screen of carfilzomib analogs. It preferentially targets the Pf20S β5 over β2 while also targeting the β5 of c20S more than β1 (Pf20S β5>β2; c20S β5>β1) **B.** Cyclic peptide 1 was discovered in a screen of non-covalent N,C-capped proteasome inhibitors. **C.** PR709A was found to be a selective inhibitor of Pf20S β5 and **D.** LU102 is selective for the β2 subunits of Pf20S and human c20S. **E.** WLL-vs was designed from substrate specificity differences between the Pf20S and c20S proteasomes and co-inhibits Pf20S β5 and β2. F. WLW-vs inhibits Pf20S β2 and synergizes with dihydroartemisinin to kill artemisinin resistant strains of Plasmodium.

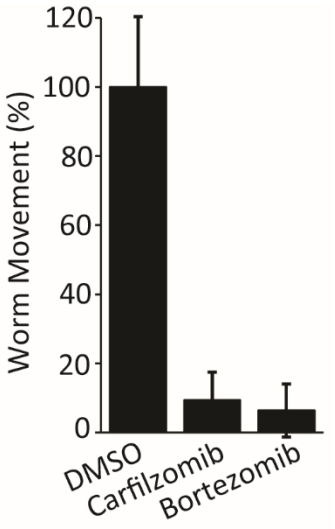

Fig 6. Quantification of worm mobility following proteasome inhibitor treatment. *S. mansoni* worms were exposed to 1 μM of bortezomib, carfilzomib or a DMSO control for 16 h. Movement was then monitored in each well for 1 minute using a digital camera connected to open source software called WormAssay.

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