Proteasome Inhibition in P. falciparum: MG132 as a tool compound and the generation of MG132-tolerant parasites

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PROTEASOME INHIBITION IN *P. FALCIPARUM*: MG132 AS A TOOL COMPOUND AND THE GENERATION OF MG132-TOLERANT PARASITES

Dissertation

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Abstract

The ubiquitin-proteasome system (UPS), composed of classes of proteins central to the process of cellular protein turnover in eukaryotes, is essential to the life cycle of the malaria parasite, *Plasmodium falciparum*. Although the UPS has been well characterized in other organisms, the extent of its involvement in different stages of *P. falciparum* growth and development has not been investigated in depth. MG132, a small-molecule proteasome inhibitor known to target the 20S proteasome core (part of the catalytic center for selective protein degradation), has been used successfully in many research studies that require proteasome inhibition. We present data supportive of the conclusion that MG132 is highly effective as a tool for *P. falciparum* research. In this thesis, I describe the effects of partial and complete proteasome inhibition on parasite growth and development by the use of variable concentrations of MG132. I also assess the effects of MG132 on 20S *P. falciparum* proteasome enzymatic activities. I have generated parasite lines that exhibit tolerance, or low-level resistance, to MG132, through intermittent compound exposure. Sequencing of the catalytic β-5 subunit of the MG132-tolerant parasites reveals non-synonymous point mutations in three tolerant parasite lines. The use of MG132 as a tool compound for study of the UPS in *P. falciparum* facilitates research into detailed roles of the proteasome using reversible partial and complete inhibition. MG132-tolerant lines are also valuable tools for studying the genesis of different levels of drug resistance and cross-resistance in parasite evolution.

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Chapter I:

Introduction to malaria research and the ubiquitin-proteasome system

Malaria

Malaria is a vector-borne, parasitic illness that affects hundreds of millions of people each year, worldwide. It is caused by single-celled apicomplexan parasites from the genus *Plasmodium*, which are spread among human populations by mosquito vectors [1-2]. Although the global malaria prevalence has been reduced and even eliminated in some areas through control efforts, around 200 million people still suffer from malaria infection every year, and around 500,000 die as a result, including many young children [1-3]. Tropical areas in Africa, Asia, and the Americas, ideal environments for *Anopheles* mosquitos (the primary vector), are the most affected [2-4]. *Plasmodium falciparum* is the species responsible for the most severe forms of malaria and nearly all human deaths from the disease [1-4]. The high morbidity and mortality observed in some parts of Africa is largely due to the fact that many cases of infection there are caused by *P. falciparum* [1-4]. Other species that can cause human disease are *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium knowlesi,* and *Plasmodium ovale* [1-2].

Malaria control and eradication efforts have been a priority in endemic areas because of the heavy public health burden and crippling morbidity and mortality associated with the disease [1-5]. Malaria was problematic in the United States until the early 1950s, when comprehensive elimination efforts were successful [1-3]. A current map of areas affected by malaria is shown in Figure 1 [6].

The malaria parasite is spread through female *Anopheles* mosquito vectors, which transmit the infection during blood meals [1-3]. The parasite undergoes many cycles of

replication in the human blood stream, destroying red blood cells in the process, which results in the pathologies characteristic of malaria, such as anemia and periodic fever [1-3]. Cerebral vascular complications, coma and death can also result from cerebral malaria, which can be the result of a severe case of infection by *P. falciparum* [1-2]. A schematic representation of the full *P. falciparum* life cycle, including the sexual stages in the mosquito and the asexual stages in the human host, is shown in Figure 2 [7].

Prevention and treatment of patients for malaria is central to public health in endemic areas and has implications for patient care. Anti-malarial prophylaxis and chemotherapy are highly effective, but can be compromised by the spread of resistant parasites [1-3, 8-10]. Depending on geographic area, the CDC recommends atovaquone/proguanil (Malarone), primaquine, mefloquine, doxycycline, or chloroquine in endemic areas, for malaria prophylaxis [2]. Atovaquone/proguanil is economically burdensome, but well-tolerated with very few side effects; primaquine is relatively fast-acting and highly effective against *P. vivax*, but its use is compromised by the presence of glucose-6-phosphatase dehydrogenase mutations, particularly in African populations [2,3]; chloroquine and mefloquine are inexpensive and can be taken by children and pregnant women; and doxycycline is very inexpensive and can prevent other infections in addition to malaria [2,3].

Resistance selection is always a concern with the use of antimalarial drugs used for chemotherapy, so the World Health Organization (WHO) recommends combination therapy [1,3,8-10]. The standard in malaria treatment in most highly endemic areas is artemisinin-based combination therapy (ACT) [1,3, 8-10]. Chloroquine and mefloquine

can be used in areas without high incidence of resistance [2-3]. Information about malaria transmission and chemotherapeutic drug regimens by region is presented in Figure 1 and Table I [3,6].

P. falciparum Research

The *P. falciparum* knowledge base has increased significantly over the past 40 years, mainly due to the crossing of significant milestones in laboratory research capabilities [11-16]. Culturing of *P. falciparum* parasites has been possible in the laboratory since 1976, when the first successful attempt at long-term continuous culture was reported [11]. Although there are differences between the parasite life cycle *in vivo* and *in vitro*, cultivation of *P. falciparum* has led to considerable advances in research [12-13]. *Plasmodium* culture has allowed for increased insight into the biology of the parasite and the development of tools for combatting malaria disease [12]. Research areas that have been benefitted significantly from this milestone include drug development and characterization, evolutionary bioinformatics, malaria immunology, drug resistance, host-pathogen interactions, vaccines, gene expression, parasite-mosquito dynamics, parasite sexual stages, transmission, and many other areas [8,12-13]. The publication of other methods then followed, such as the stage-synchronization of cultured parasites a few years later [13]. Another major milestone in *P. falciparum* research was the complete sequencing of the parasite genome in 2002 [14]. Access to complete genomic information paved the way for examination of parasite genome on a global scale, including the sequences of open reading frames and putative genes not previously identified [7,14]. The fact that the parasite genome is very A/T rich (the genome is over 80% A/T, and over

90% A/T when introns and non-coding regions are excluded) was confirmed with examination of the full genome [7,14]. The subsequent publication of the parasite transcriptome and proteome expanded the core knowledge of *P. falciparum* even more, most notably by drawing attention to the fact that gene expression and protein content are tightly regulated and highly periodic, with very little overlap between stages [15-16]. A heat map of *P. falciparum* microarray data that shows transcript levels with respect to developmental stage is shown in Figure 3 [15].

Other high-impact topics in recent *P. falciparum* research include the identification of single-nucleotide polymorphisms (SNPs) through genomic analysis of laboratory and field strains, use of SNP databases in molecular barcoding for the tracking of parasite evolution and the spread of a variety of traits, the use of Genome-Wide Association Studies (GWAS), which can be used to identify the genetic roots of a variety of phenotypic traits, and detailed tracking and molecular study of drug resistance (including emerging ACT resistance) [8,17-22].

The Ubiquitin-Proteasome System (UPS)

The proteasome is a large (~ 2.5 MDa), multi-subunit protein complex that is present in nearly all eukaryotic cells [23-27]. It is a central component of the broader ubiquitin proteasome system (UPS), which includes a large set of enzymes and structural proteins that facilitate the designation and degradation of many intracellular proteins [23-27]. The pathway leading to degradation of a protein by the catalytic 20S proteasome core is shown in Figure 4 [27]. This multi-step cascade begins with the attachment of a ubiquitin

(Ub) peptide to an E1 ubiquitin-activating enzyme, in a process that requires ATP. Next, the Ub is transferred to an E2 ubiquitin conjugating enzyme, and the Ub is finally moved from the E2 enzyme to the substrate protein by an E3 ubiquitin ligase [23-27]. This process happens to the same substrate multiple times, building a polyubiquitin (pUb) chain, the signal that is recognized by regulatory complexes as of the 19S proteasome [23-27].

The 26S proteasome particle is made up of the 19S regulatory complex, which controls substrate entry, and the 20S proteasome core, which includes proteases for the dismantling of substrate proteins [23-27]. The basic structure of the 26S proteasome is shown in Figure 4 [27]. The 19S proteasome consists of the regulatory particle non-ATPase (RPN) and regulatory particle triple-A ATPase (RPT) subunits (the "lid and base"), which recognize, bind to, and draw polyubiquitinated proteins into the catalytic 20S proteasome core (shown in dark red in the proteasome complex in Figure 4) [23-27]. The 20S proteasome is composed of seven alpha subunits (designated α-1 through α-7), which are primarily structural and make up the "outer" ring of the complex, and seven beta subunits (designated β-1 through β-7), which make up the "inner" ring of the complex and include both structural and catalytic subunits. The β-1, β-2, β-5 subunits are active threonine proteases, and constitute the enzymes that actually degrade substrate proteins [23-27].

Substrate proteins degraded by the UPS can include those that originate from

transcription or translation errors such as misfolded or truncated proteins, cellular signals that are no longer needed at a given time, such as cyclins or cytokines, transcription factors, or any protein with a cellular presence that is limited to one or more discrete temporal intervals during the parasite life cycle [23-27]. Disruption of proteasome function can hinder the cell's ability to rid itself of protein waste, causing the lingering presence of poly-ubiquitinated proteins [23,28-29]. If the cell is unable to recycle these proteins, the accumulation of this cellular "garbage," or cellular proteins that are unnecessary and possibly toxic, can interfere with many cellular functions [23, 28-29]. Progression of the cell cycle, gene expression, actin remodeling, protein transport, and apoptosis pathways are all known to be affected by proteasome inhibition [23,28-29].

A number of compounds interfere with UPS function, including those that target the 20S proteasome core directly, inhibiting the catalytic activities of its proteolytic subunits [28, 30]. Chemical structures of some of these compounds are shown in Figure 5 [29]. Many of these small-molecule proteasome inhibitors have been found to have potent anti-malarial activity against cultured *P. falciparum* parasites, motivating exploration of the UPS as a target for chemotherapeutic intervention in malaria treatment [29, 31-32].

Evidence for the importance of the UPS in *P. falciparum*

The UPS has been well studied and characterized in human cells, and has been shown be involved in the cell cycle, the immune response, apoptosis, and regulation of transcription

factors [23, 28-32]. Many questions remain about the specific roles of the proteasome and the consequences of its inhibition in *P. falciparum* [31-32]. Over 100 components of the UPS have been identified in *P. falciparum* by genomic studies, including nine ubiquitin or ubiquitin-like proteins and over thirty components of the 26S proteasome: eight E1 or E1-like activating enzymes, fourteen E2 or E2-like conjugating enzymes, over fifty E3 or E3-like ubiquitin ligases and twenty-nine de-ubiquitinating (DUB) or DUB-like proteins [33-35]. In addition to the demonstrated antimalarial activity of proteasome inhibitors in culture, another obvious reason for parasite biologists' interest in the UPS is the fact that *P. falciparum* depends heavily on protein turnover throughout its life cycle [16]. The *P. falciparum* transcriptome and proteome reveal proteasome expression throughout the parasite life cycle, consistent with its importance [15,16]. Many protein components of the UPS are encoded by genes that contain SNPs that are under selection in parasite populations isolated from patients in disease-endemic areas, suggesting a possible role in drug resistance for sequence variation in UPS proteins [15, 18].

Overall, the occurrence of malaria creates significant individual and societal burdens in many regions of the world and continued efforts are necessary for eradication of the disease [1-6, 9]. Concerted public health efforts, including vector-targeted strategies and malaria chemotherapy, have been highly successful at lessening the impact of malaria and reducing morbidity and mortality [4, 6, 9]. Malaria research has provided many additional tools for treatment and prevention, and there is a need for these efforts to be continued [12-16]. In the area of chemotherapy, these efforts must include the development of new malaria drugs with diverse cellular targets in order to limit the spread of drug resistance. The UPS contains many possible targets for new drug

compounds, and more research is necessary to reveal the roles and functions of UPS subunits in parasite growth and development (7-10, 29, 31). The UPS is known to have a role in many mammalian cellular pathways, but the specific roles of the UPS in *P. falciparum* that have yet to be fully defined (23-33). More research is required to clarify the roles of the proteasome in *P. falciparum*, as well as the effects of its inhibition (31-32). Proteasome inhibition with small-molecule inhibitors like MG132 has proven effective in studying the mammalian UPS, and such compounds could be similarly useful in the study of the parasite UPS (28-30). In addition to its examination as a possible drug target, the UPS is also currently of significant interest in the field of parasite biology because it could be a central regulator of many cellular processes, and advancing our understanding of UPS functions can advance research in other areas of parasite cell biology, as well [29,31-33].

Figures and Tables

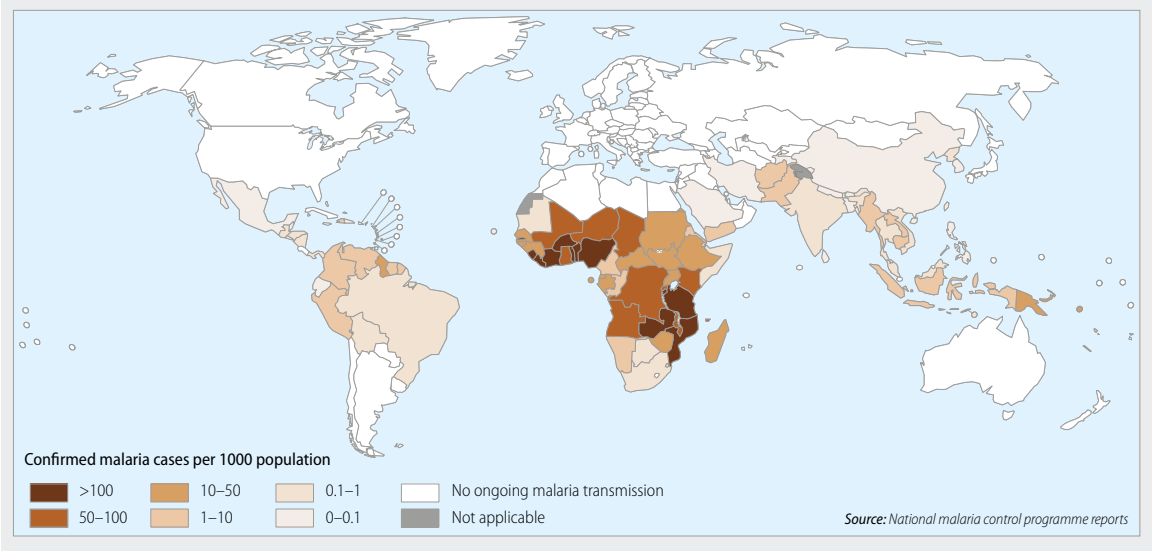

Figure 1: Global distribution of malaria transmission as reported by the World Health Organization in 2014 [3,6], reflecting the aggregated incidence of infection by the five species of Plasmodium known to infect humans (*Plasmodium falciparum, Plasmodium vivax, Plasmodium knowlesi, Plasmodium ovale and Plasmodium malariae*).

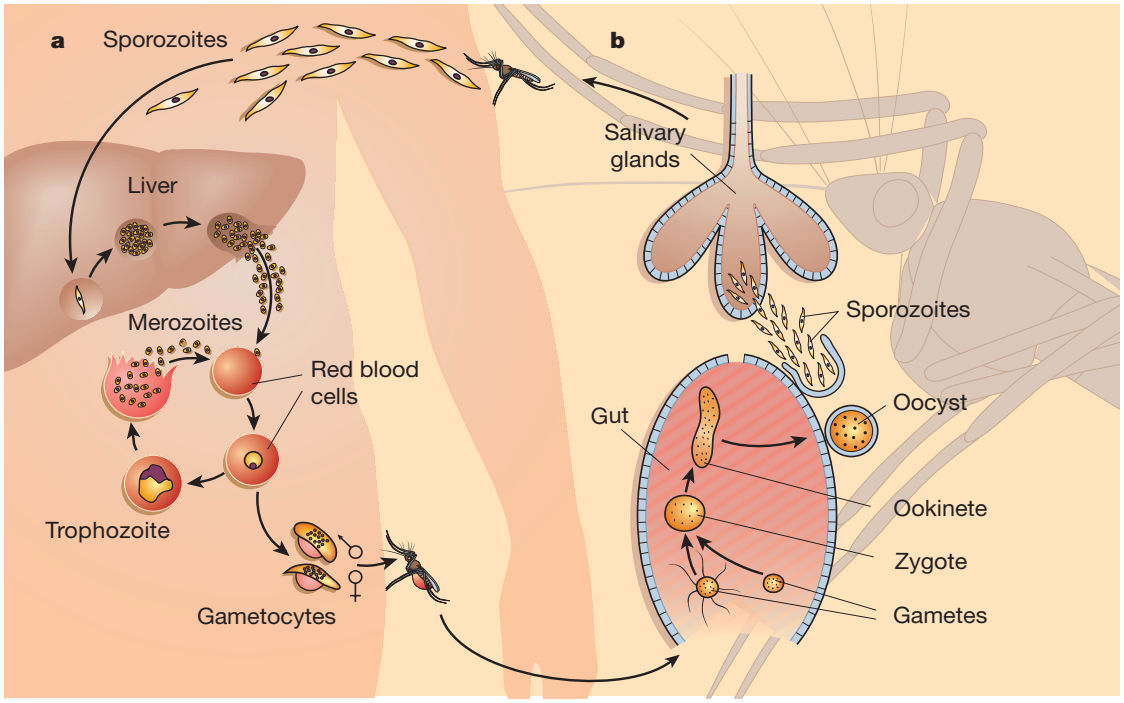

Figure 2: Life cycle of *P. falciparum* [7]. Sexual reproduction occurs in the mosquito midgut, resulting in the presence of sporozoites in mosquito saliva. Sporozoites enter the bloodstream of a human host during blood feeding. The sporozoites infect the liver, replicate, and are released into the bloodstream in a form allowing for the infection of red blood cells. The parasites then enter the asexual reproduction cycle in the host bloodstream. After significant disease progression, gametocytes can form in the blood stream of an infected person, and they can be taken up by another mosquito during a blood meal. The life cycle can then continue for another round via the initiation of sexual reproduction in the mosquito host.

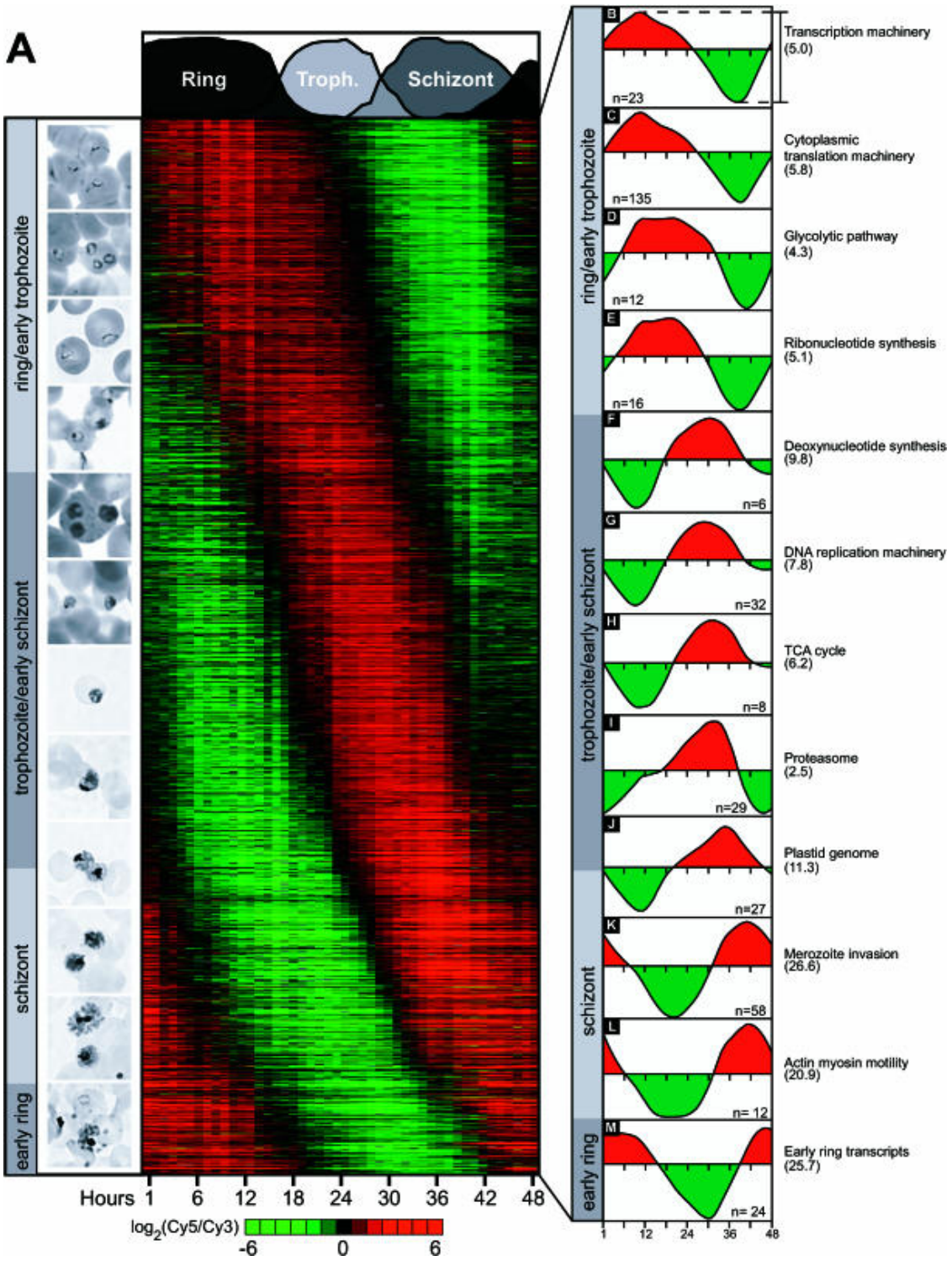

Figure 3: Transcriptome of *P. falciparum.* Transcripts are shown with respect to stage [15]. As indicated in rightward panel I, expression of the proteasome is prominent during trophozoite stage.

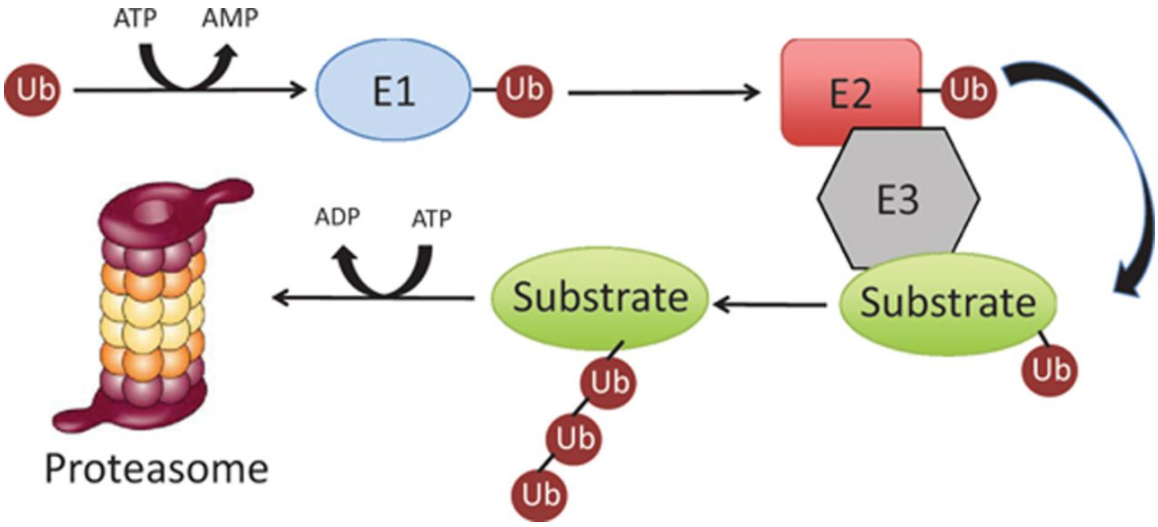

Figure 4: Overview of the ubiquitin-proteasome system [27]. A ubiquitin peptide is attached to an E1 ubiquitin-activating protein by a process that requires ATP, then transferred to an E2 ubiquitin-conjugating enzyme. And E3 ubiquitin ligase then transfers the ubiquitin peptide to a substrate protein. This process happens many times, adding more ubiquitin molecules to create a polyubiquitin tag, designating the substrate protein for degradation by the proteasome.

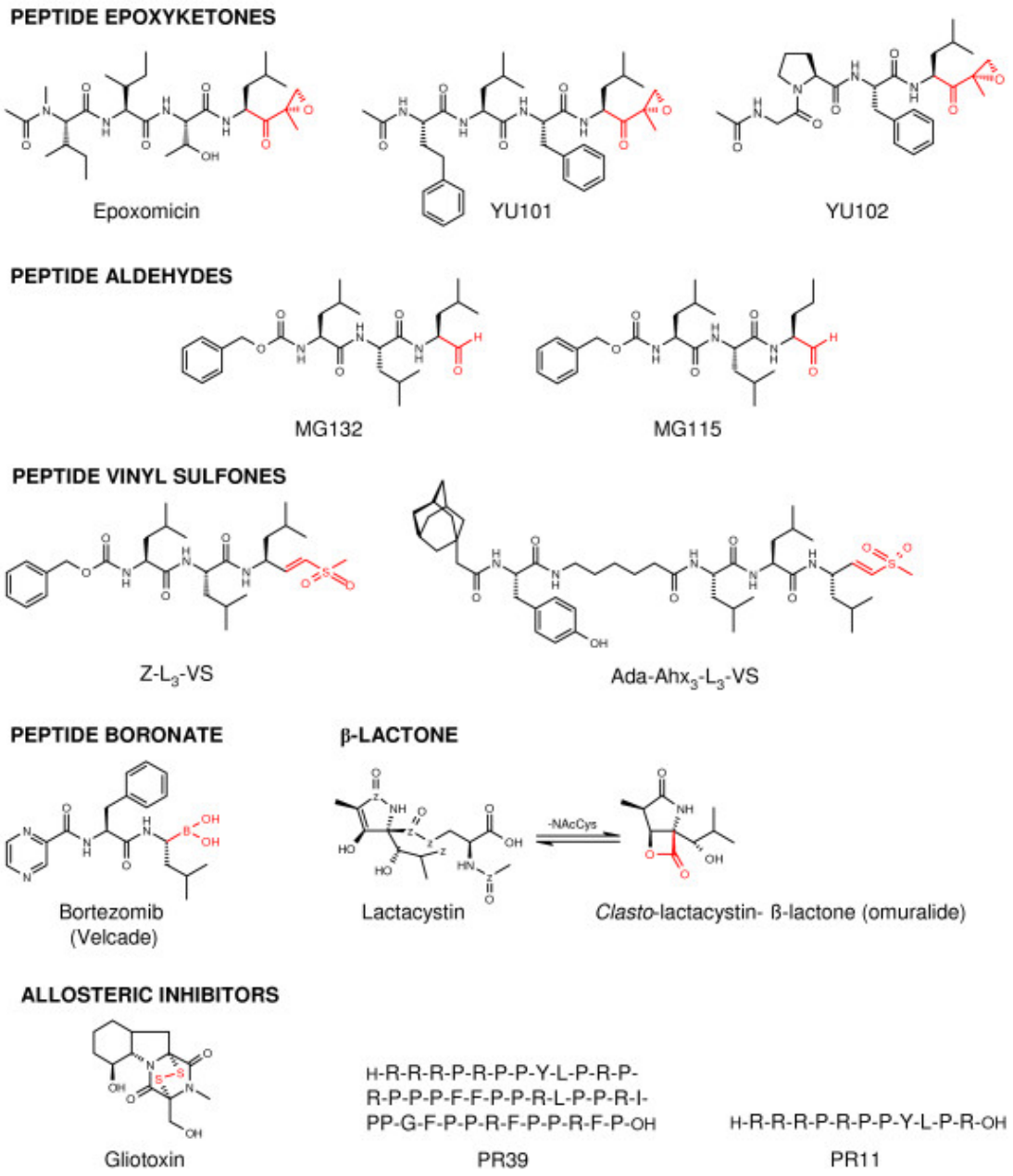

Figure 5: Chemical structures of canonical proteasome inhibitor compounds. Pharmacophores are highlighted in red. For PR39 and PR11, amino acid sequences are shown. Binding sites within the UPS are unknown [29]. All compounds, except allosteric inhibitors PR11 and PR39, have been shown to be active against *P. falciparum* [29].

Policy	AFR	AMR	EMR	EUR	SEAR	WPR	Total
ACT is used for treatment of *P. falciparum*	43	9	8	1	9	9	79
Pre-referral treatment with quinine/artemether IM/artesunate suppositories	40	4	5		5	3	57
Single dose primaquine used as gametocidal for *P. falciparum*	3	19	4	3	7	3	39
Primaquine is used for radical treatment of *P. vivax* cases	7	21	7	3	10	9	57
Directly observed treatment with primaquine is undertaken	4	12	2	5	3	4	30
G6PD test is recommended before treatment with primaquine	5		4		2	6	17
Number of countries/areas with ongoing malaria transmission	**45**	**21**	**8**	**3**	**10**	**10**	**97**
Number of *P. falciparum* endemic countries/areas	**44**	**17**	**8**	**0**	**9**	**9**	**87**
Number of *P. vivax* endemic countries/areas	**7**	**19**	**6**	**3**	**10**	**10**	**55**
Number of countries/areas endemic for both *P. falciparum* and *P. vivax*	**6**	**17**	**6**	**0**	**9**	**9**	**47**

ACT, artemisinin-based combination therapy; AFR, African Region; AMR, Region of the Americas; EMR, Eastern Mediterranean Region; EUR, European Region; G6PD, glucose-6-phosphate dehydrogenase; IM, intramuscular; SEAR, South-East Asia Region; WPR, Western Pacific Region

Table I: Malaria transmission and treatment information by region [3].

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Chapter II:

MG132 functions as an effective small-molecule proteasome inhibitor for partial or complete inhibition of the *P. falciparum* proteasome

J. Marisha Collins and Marc A.T. Muskavitch

Abstract

The proteasome is a highly conserved multi-subunit protein complex that serves as a protein "recycling bin" for eukaryotic cells. It is part of the ubiquitin-proteasome system, which consists of 30 to 60 proteins that facilitate the modification and degradation of cellular proteins. The proteasome is essential for the growth of rapidly dividing cells, which has led to the use of proteasome inhibitors such as bortezomib in the chemotherapeutic treatment of cancer. Proteasome inhibitors are currently of interest to infectious disease biologists because they also arrest the growth of pathogens, such as the malaria parasite *Plasmodium falciparum*. Although previous studies of the parasite proteasome have been pursued, little is currently known about the biology and roles of the ubiquitin-proteasome system in *P. falciparum*.

This study addresses the abilities of proteasome inhibitor compounds to hinder growth and development of parasites in culture and to impair the proteolytic activities of the *P. falciparum* proteasome core. MG132, a small-molecule peptide aldehyde proteasome inhibitor, was identified as an effective tool compound for studies requiring partial and complete proteasome inhibition. Exposure to lower concentrations of MG132 resulted in partial proteasome inhibition and developmental delays. Exposure to higher concentrations of MG132 led to lethality after 24 hours, but proved reversible when incubation time was shortened. This reversibility could allow for study of recovery and downstream consequences of temporary proteasome inhibition in live parasites. Overall, MG132 is ideal for effective partial or complete inhibition of the *P. falciparum* proteasome.

Introduction

Malaria is a vector-borne parasitic infection for which a large portion of the world population is at risk. Although the global impact of the disease has been greatly reduced in the past several hundred years, it remains a fixture in many parts of Africa, Asia and the Americas, and it is responsible for over 200 million symptomatic cases and over 600,000 deaths, annually [1,2]. Malaria is caused by infection with parasites from the genus *Plasmodium*, which are intracellular eukaryotic organisms that replicate in human hosts and in mosquito vectors [3].

Parasite genetic diversity makes conventional vaccination against malaria very difficult and largely ineffective for protecting human populations [4,5]. Rapid evolution enables the rise of genetic resistance to malaria chemotherapy, making reliable drug treatment a challenge [5-7]. Despite the general success of modern treatments, there remains a need to develop new drugs directed against diverse targets. Cases of resistance to the current standard for chemotherapy, artemisinin-based combination therapy (ACT), have been reported [1, 8-11].

The biology of *Plasmodium* parasites is complex. Although similar to that of other eukaryotes in many ways, the genome of *Plasmodium falciparum*, the species responsible for the most severe human malaria cases, contains many genes that have not been well-studied and lack evident homology to genes with known functions [12,13]. The percentages of *P. falciparum* genes with homologs in other well-studied organisms is shown in Table I. Even those parasite genes and gene products with sequence homology

to those found in other organisms often do not exhibit similar expression patterns, subcellular localization or post-translational modifications, and so they could function by mechanisms very different from their homologous counterparts [12,13].

The ubiquitin-proteasome system (UPS) is a eukaryotic subcellular "recycling bin" responsible for the breakdown of proteins that are no longer needed by the cell [14-16]. A diagram of the 26S proteasome structure, including the 19S proteasome regulatory particle and the 20S proteasome core, is shown in Figure 1. The UPS is central to the process of clearing misfolded, truncated, or mistranslated proteins from the cell [14-17]. It also breaks down stage-specific proteins after they are no longer needed by the cell, and so it has a vital role in processes such as cell cycle progression [16,17]. Recent studies have shown that the UPS participates in apoptosis, cellular import/export, gene expression, actin remodeling, and stress responses [14-20]. The UPS can also affect intercellular dynamics such as host-pathogen interactions and inflammation [20-22].

Increased knowledge of the *P. falciparum* UPS could have many practical applications. The pathology and symptomology of parasite infection depends critically on stage progression in the liver and in erythrocytes [3]. Frequent, rapid changes in proteome composition accompany stage shifting, which suggests that protein degradation by the parasite UPS could play a central role in parasite infection [23]. In addition, *Plasmodium* parasites depend on the metabolic resources of a host during every life-cycle stage, making host-pathogen interactions critical to the infection processes [3].

A variety of small-molecule proteasome inhibitors (SMPIs) have been identified and utilized in research and clinical settings [24-28]. SMPIs have been utilized clinically in the chemotherapeutic treatment of cancer because of their ability to arrest rapid cell division [26-28]. The critical role of the UPS in the cell cycle has also led infectious disease biologists to explore SMPIs as tools for the control of pathogens [28-32]. Furthermore, UPS function has been shown to have direct impacts on viral and bacterial pathogenesis, and could have a significant role in malaria pathogenesis [21-22, 28-32].

Known SMPIs are numerous, and vary in mechanism of action, specificity, and toxicity to various cell types [24, 31-37]. In previous studies of the biology of the proteasome in *P. falciparum*, researchers have made use of several SMPIs such as MG132, bortezomib, lacatacystin, epoxomicin and chemical structural variants synthesized by individual labs for optimization [31-36]. When choosing an SMPI for a particular research study or clinical purpose, the first step is to identify the SMPI that is appropriate for the work at hand, knowing that the desired attributes of an ideal SMPI will vary for each type of study.

Some SMPIs are reversible inhibitors, meaning proteasome function can be restored with removal of the soluble compound [24]. Others are irreversible inhibitors that alter proteasome function permanently by covalent molecular interactions [24]. Because the activities of many separate components and subunits are required for effective UPS function, the specific UPS element(s) with which an SMPI interacts would be another important consideration. Some SMPIs target one of the three types of protease activity

associated with the 20S proteasome core: trypsin-like (TPL), chymotrypsin-like (CTL), and caspase-like (CPL) activities; while others inhibit more than one activity [24,25]. Some inhibitors target UPS regulatory subunits [24,25]. Hence, a given compound's range of target(s) within the UPS should be considered, in addition to any off-target effects outside of the UPS [24,28,33].

We directly compare the anti-malarial action and inhibition of UPS enzymatic activities of several SMPIs in *P. falciparum*. We find that MG132, a reversible SMPI [24], proves to be an effective UPS-directed tool compound in parasite culture over a range of concentrations that corresponds with partial or complete proteasome inhibition. Sub-lethal concentrations of MG132 result in partial proteasome inhibition and a delay in stage progression without total arrest of cell proliferation. The inhibitory effects of high doses of MG132 are rapid and prove to be reversible following short periods of exposure, as affected cultures are able to recover and grow normally after SMPI pressure is removed. We demonstrate that MG132 is a rapid and effective SMPI for partial or complete inhibition of the *P. falciparum* proteasome.

Materials and Methods

Bioinformatic Analysis

Plasmodium gene sequences were obtained from the PlasmoDB website: http://plasmodb.org/plasmo/ (EuPathDB Project Team, Athens, GA). Homology comparisons between *P. falciparum* genes and those from other species were conducted using the PlasmoDB "orthology phylogenetic profile" search algorithm. Sequences from other species and "e values" for sequence comparisons were obtained from the NCBI/BLAST website: http://blast.ncbi.nlm.nih.gov/Blast.cgi (The National Library of Medicine, Bethesda, MD). Multiple sequence alignment was performed using Geneious® software (Biomatters Ltd., Auckland, New Zealand).

Strains and Cells

All strains were obtained from the laboratory of D.F. Wirth (Harvard School of Public Health, Boston MA). The strains 3D7 (MRA-102, line P2G12) and Dd2 (MRA-156) were used for experiments [38,39]. Hematocrit (packed human red blood cells in CPDA-1) used for parasite culture was obtained from Research Blood Components (Boston, MA). Jurkat cells were obtained from the laboratory of W. Johnson (Boston College, Chestnut Hill, MA) and were originally Clone E6-1, TIB-152™ from ATCC (Manassas, Virginia).

Proteasome Inhibitors and Control Compounds

MG132, mefloquine, and azithromycin were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO). Lactacystin was obtained from Cayman Chemical (Ann Arbor, MI).

Bortezomib was obtained from LC Laboratories (Woburn, MA). Stock solutions were made by dissolving compounds in DMSO at a concentration of 10 mM.

Parasite Culture Conditions and Stage Synchronization

All parasite culturing was done under the following standard conditions unless otherwise noted: RPMI cell culture medium (Life Technologies Corp., Grand Island, NY) supplemented with 5.94 g/L HEPES, 0.05 g/L Hypoxanthine, 2.016 g/L Sodium Bicarbonate, and 0.025 mg/mL gentamicin was used for culture of *P. falciparum*. Either 0.5% Albumaxx II® (Life Technologies Corp., Grand Island, NY) or 10% Human Serum (Interstate Blood Bank, Memphis, TN) were also added to supplement media used for parasite culture. All cultures included 1-5% hematocrit.

For parasite growth under standard conditions, cultures were incubated, in a stationary manner, at 37^{o}C, in the presence of a gaseous mixture consisting of 5% CO_2, 1% O_2, 94% N_2. Cultures were diluted with uninfected red blood cells, and fresh complete medium when parasitemia exceeded 1% or at the appropriate time for experimental requirements.

For parasite stage synchronization, culture hematicrit, including infected red blood cells (iRBCs), was isolated by centrifugation at approximately 500 x g, and supernatant media were discarded. Cells were then incubated in 5% sorbitol at 37^{o}C for 5 minutes. Centrifugation was then repeated, and the sorbitol-containing supernatant discarded. Cells were then returned to culture with fresh medium.

Culture Growth Assay with SYBR Green®

Cultures for growth assays were incubated in 384-well plates, under standard conditions, for 72 hours. Each sample replicate consisted of 40uL culture with 1% hematocrit at 1% parasitemia (unless otherwise noted), 0.5% Albumaxx II, and the appropriate concentration of compound or drug being studied. Assays were initialized when synchronous parasite cultures were at ring stage, unless otherwise noted. After a defined incubation period, sample cultures were lysed and stained by the addition of 10uL lysis buffer (0.16% saponin, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1.6% Triton X-100) with SYBR Green® (Life Technologies Corp., Grand Island, NY) diluted 1:1,000. Plates were read with emission/excitation settings at 494 nm/520 nm, respectively. Growth was assessed by calculating ratio of the fluorescence readings of small molecule-treated sample cultures to those of untreated control cultures incubated in the same plate. Assay curves were generated and data analyzed using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA). The non-linear regression curve algorithm (variable slope) with IC_{50} calculation was used.

Delayed-Death Assay

To assess any delayed-death effects of compounds, growth assays were performed with modifications. In addition to the standard 72-hour incubation, assays were performed using 24- and 120-hour incubation times for comparison. For samples incubated for 120 hours, media was changed and fresh compound added once during incubation (at 72 hours). At the end of incubation period, samples were lysed and stained in the same manner as SYBR Green®-based growth assay. Sample reading and analysis were also

conducted according to the protocol for SYBR Green®-based growth assay. Statistical significance of observed differences was determined by comparing the IC_{50} values for each incubation time to the values for other incubation times for the same compound with Turkey's multiple comparison test. Statistical tests were conducted using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA).

20S Proteasome Enzymatic Activity Assays

Enzymatic activities were assessed using SUC-LLVY-AMC, Z-LLE-AMC, and Boc-LLR-AMC fluorogenic substrates (Boston Biochem, Cambridge, MA). Stock solutions of each of the substrates were made at 100 μM concentration in DMSO. Enzymes were prepared from whole-cell lysate obtained from either cultured parasites or Jurkat cells. To prepare parasite lysates, synchronous parasites were grown to schizont stage and iRBCs were collected by centrifugation at 500 x g. The iRBCs were resuspended in PBS with 0.15% saponin and incubated at room temperature until RBC lysis (<5 min). Free parasites were collected by centrifugation and washed with PBS, then stored at -80^{o}C. On the day of the activity assay, parasites were thawed and resuspended in lysis buffer (50 mM Tris HCl, 25 mM KCl, 10 mM NaCl, 1 mM MgCl, 1 mM ATP, 2% glycerol) and 0.1mm glass disruption beads. Cells were lysed mechanically by repeated agitation (vortex), and lysates were clarified by centrifugation. Supernatant was removed and kept on ice to be used as enzyme for assays. Jurkat cells were grown in RPMI supplemented with 10% human serum and lysed by agitation with mechanical disruption beads in the same manner as isolated parasites. Assay samples were 40 μL total volume and included 2 μL substrate stock (5 μM final concentration), 4 μL 10x inhibitor compound or plain

DMSO, and 5 µL enzyme (lysate), in assay buffer (50 mM Tris HCl, 25 mM KCl, 10 mM NaCl, 1 mM MgCl, 1 mM ATP). Assays were incubated for 90 minutes at 37^{o}C and measured for fluorescence at excitation/emission 380 nm/460 nm, respectively. Activity inhibition curves were generated and data analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Non-linear regression curve algorithm (variable slope) with IC_{50} calculation was used.

Stage Delay Assessment

Parasite stage progression during exposure to sub-lethal levels of MG132 was assessed via a 48-hour time course during which 3D7 parasites were counted for parasitemia and stage by microscopy at separate time points. A set of 200 µL synchronous sample cultures, with 0.5% Albumaxx II, 2% hematocrit, and 1% starting parasitemia, were incubated in 96-well plates under standard culture conditions for 48 hours. Cultures were diluted 1:5 at ring stage in fresh media, maintaining the same concentrations of hematocrit and respective compound. Parasites were examined at each time point by fixed-cell light microscopy / visual counting, blind to sample identification, to assess developmental stages and parasitemia. For each time point, over 2500 red blood cells were counted.

SDS PAGE and Western Blot Analysis

A set of 25 mL 3D7 cultures at 2% hematocrit and approximately 5% parasitemia were exposed to MG132 (or plain DMSO) during early/mid trophozoite stage. One culture was harvested for t=0 sample and remaining cultures were supplemented with 50 nM – 1 µM

of MG132 or plain DMSO, then incubated under standard conditions for 6 hours, and harvested using the same saponin lysis protocol as that described above, for enzymatic activity assays. Small aliquots (200 uL) of cultures exposed to 0 (control), 500 nM, and 1 μM MG132 were saved and diluted 20-fold in MG132-free complete medium and 3% hematocrit and incubated under standard culture conditions for 7 days to assess parasite recovery. Protein was extracted from harvested parasites by agitation with 2 mm disruption beads in modified T-NET lysis buffer (50 nM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 100 nM MG132, 2 mM Ubiquitin Aldehyde). Samples were normalized by keeping lysate volume proportional to original culture volume, with the goal of all samples having the same number of cells per unit volume. SDS PAGE gel-based protein fractionation was conducted using 4–15% Mini-PROTEAN® TGX™ gels (BioRad, Hercules, CA 94547) and Tris-glycine buffer (25 mM Tris, pH 8.3, and 192 mM glycine) with 0.1% SDS. Western blots were generated using Tris-glycine buffer containing 15% methanol. An anti-ubiquitin primary antibody (Catalog #P4D1, Santa Cruz Biotechnlogy, Dallas, TX), previously shown to detect ubiquitylated proteins in *P. falciparum*, was used to detect the presence of ubiquitin-modified proteins [36].

Results

Bioinformatic Analysis

Sequence analysis of individual components of the *P. falciparum* UPS was conducted in order to reveal similarities and differences between parasite UPS subunits and those of other organisms. Comparison of "e values" generated by protein sequence alignment of subunits from the 26S proteasome complex of *P. falciparum* and orthologous sequences in other organisms indicates a high degree of overall conservation (Table II). The most highly conserved subunits, with several comparisons showing an "e value" of 0, indicating no significant difference, are the proteasome regulatory subunits (e.g., RPN and RPT genes of the 19S proteasome complex). These subunits are responsible for substrate recognition, the opening of the proteasome pore, removal of polyubiquitin tags bound to substrate, and unfolding the substrate peptide to prepare it for degradation inside the 20S proteasome "barrel" [14-17].

The "alpha" and "beta" subunits of the 20S proteasome core demonstrate sequence similarity between species, but to a lesser degree than that we observe for the regulatory 19S proteasome complex (Table II). A multiple-sequence alignment of one of these units, ß5 (an active protease, responsible for the chymotrypsin-like proteolytic activity of the proteasome core) is presented in Figure 2. Several residues that demonstrate high conservation between the organisms included are also key residues of the active site [14]. The protein sequences upstream of the catalytic threonine (Figure 2, first active site arrow) exhibit very low conservation between species. Cleavage of this portion of the polypeptide, leaving the active threonine at the N-terminus, is necessary for the enzyme

to become active after translation [14,16]. Downstream of the catalytic threonine, the N-terminal region of the mature protein demonstrates a higher degree of conservation than the C-terminal region. Overall, the β5 subunit (a common target of inhibitors of 20S proteasome proteolytic activity) shows higher sequence conservation between species in comparison to the other subunits of the 20S proteasome core, including the well-conserved catalytic subunits, β1 and β2, but significantly lower conservation than other members of the 26S proteasome, such as the regulatory subunits of the 19S proteasome regulatory particle (Table II).

P. falciparum Growth Inhibition by Proteasome Inhibitors

Growth/inhibition curves were generated in order to calculate half-maximal inhibitory concentration (IC_{50}) values for each compound. Inhibition of parasite proliferation in culture was measured by growing 3D7 and Dd2 parasites for 72 hours (1.5 growth cycles) in the presence of varying concentrations of proteasome inhibitors MG132 (0 – 2.5 μM), bortezomib (0 – 5 μM) and lactacystin (0 – 50 μM) and mefloquine (0 – 625 nM), a known anti-malarial drug used as a control [42]. Relative DNA content was measured by SYBR Green® assay at the end of incubation period (Figure 3). Growth IC_{50} concentrations for each compound were calculated and the averages are indicated in Table III. For all compounds tested, IC_{50} values for the two strains used, 3D7 and Dd2 (a chloroquine-resistant strain [39]) were similar, Lactacystin had the highest IC_{50} value, which was in the three-digit nanomolar range (Table III). Bortezomib was the second highest, near 100 nM for both strains, and exhibited the highest variability between replicates. MG132 had the lowest IC_{50} values (34 nM and 40 nM for 3D7 and Dd2,

respectively) and the lowest standard error among replicate assays. MG132 was favored for further proteasome inhibition work because it exhibited the lowest IC_{50} values and consistent antimalarial activity against cultured parasites.

Delayed Death Assay

A delayed death assay was conducted to assess whether prolonged exposure to MG132, which could be required in studies involving partial proteasome inhibition, could result in parasite lethality at significantly lower MG132 concentrations. To check for any delayed death effects on parasites resulting from MG132 exposure, SYBR Green® assays were used to compare growth rates over varied exposure times (Figure 4). Delayed death is defined as a greater than 10-fold reduction in IC_{50} when compound exposure time is extended for an additional growth cycle [43]. This effect could indicate targeting of the parasite apicoplast [43]. Mefloquine, which should not affect the apicoplast, and azithromycin, which should affect the apicoplast, were included as control compounds. Mefloquine is known to be a fast-acting anti-malarial compound, with potent anti-malarial activity within the first 24 hours of exposure [42]. Azithromycin is known to target the apicoplast and to have a pronounced delayed death effect when exposure time is extended from 72 to 120 hours (1.5 to 2.5 cycles of growth) [43].

As seen in Figure 4, growth inhibition curves were generated by comparing to control cultures, the cultures incubated for 24-, 72- and 120-hours in the presence of the compound indicated. IC_{50} values were calculated and summarized in Table IV. All assays began with parasites at ring stage. As predicted, the mefloquine IC_{50} value did not change

notably between 24, 72, and 120 hours of incubation, while the IC_{50} for Azithromycin dropped significantly between 72 and 120 hours. Although there was a slight decrease in the MG132 IC_{50} value when exposure time was extended, the change was less than 2-fold (Table IV). Neither the IC_{50} for MG132 nor the shape of inhibition curve changed significantly with respect to incubation time, following a similar pattern to that we observe for mefloquine. We conclude that there is no delayed death effect associated with prolonged MG132 exposure, so the compound could be used in studies requiring long-term partial proteasome inhibition without any drastic changes in the concentration required to achieve lethality.

Stage Delay Assessment

In order to assess the ability and speed of parasite progression through growth stages while in the presence of sub-lethal MG132 concentrations, cultures were incubated in the presence of 25 nM and 50 nM MG132 and monitored for 48 hours. Synchronous 3D7 parasite cultures at ring stage or mid-trophozoite stage were exposed to MG132, mefloquine, or vehicle (DMSO), and parasitemia and stage distribution were quantified at 12-hour intervals for each culture (Figure 5). Significant delay in stage progression is observed at 50 nM MG132, and this delay is similar to that we observe for mefloquine, a compound known to cause a delay in parasite stage progression [42]. This delay entails an elongation of trophozoite maturation, resulting in inhibitor-treated parasites lagging behind control culture stage progression by about 12 hours. This occurred whether compound was added during the trophozoite stage or during the ring stage. Although growth is slowed, parasites do exhibit continuing developmental progression throughout

the 48-hour incubation period in the presence of up to 50 nM MG132. The continued, but slowed, progression of parasites in the presence of 50 nM MG132 indicates that this concentration of MG132 can be used for partial proteasome inhibition in live parasites.

Inhibition of Proteasome Enzymatic Activities

The proteolytic activities associated with functioning proteasomes were measured using fluorogenic substrates [25] designed to individually measure chymotrypsin-like (CTL), trypsin-like (TPL), or caspase-like (CPL) proteolytic activities. Assay curves were generated to measure the abilities of MG132, bortezomib, and lactacystin to inhibit each type of activity (Figure 6). Inhibition of activity in the parasite proteasome (3D7 cell lysate) was tested and compared to that of the human proteasome (Jurkat cell lysate). Activity IC_{50} values for all compounds were calculated and are summarized in Table V. MG132 completely inhibited CTL activity with an IC_{50} of 129 nM in the parasite proteasome, without reducing TPL or CPL activities significantly below 50% at the maximum concentration used, 250 μM. This specificity is more apparent for the parasite proteasome than the human proteasome, for which all three types of activity are fully inhibited by MG132. Bortezomib inhibits CTL activity with an IC_{50} of 56 nM and CPL activity with an IC_{50} of 1285 nM without reducing TPL activity significantly below 50% in the parasite proteasome. Lactacystin reduces CTL activity to minimal levels with an IC_{50} of 3429 nM and lowers CPL activity to moderate levels (not to baseline), but does not significantly reduce TPL activity in the parasite lysate. All compounds exhibited higher IC_{50} values for the parasite proteasome than for the human proteasome.

Proteasome Inhibition Effects of Various Concentrations of MG132 and Parasite Recovery

Accumulation of ubiquitin-tagged proteins, as evidence of proteasome inhibition, was examined by western blot in parasites exposed to varying concentrations of MG132 for 6 hours (Figure 7). This accumulation is visible in all parasite cultures exposed to MG132, and it increases notably between 0 and 50 nM, then steadily increases as the concentration of MG132 increases, appearing to plateau when 500 nM or greater is added. (Figure 7A). Even the lowest MG132 concentration used, 50 nM, is sufficient to cause detectable inhibition of the proteasome after 6 hours of exposure.

In order to determine whether cultures exposed to high concentrations of MG132 for short periods of time were able to recover following removal of inhibitor, cultures exposed to 500 nM or 1,000 nM MG132 for 6 hours were incubated under standard culture conditions for seven days following the removal of drug pressure. Growth and progression of these cultures was observed and compared to unexposed control cultures (Figures 7C and 7D). Although slow growth is apparent for the first cycle following the 6 hour MG132 exposure (2-3 days), parasites recovered and were growing normally by day 7. We conclude that MG132 would be a candidate compound for studies seeking to assess the downstream after-effects of temporary proteasome inhibition in live parasites.

Discussion

The UPS has numerous roles in the life cycles of eukaryotic cells, and the immediate and downstream effects of partial or complete proteasome inhibition vary between species [14-17]. Novel roles for the UPS are likely in *P. falciparum*; an obligate intracellular parasite that depends heavily on regular periodic shifts in gene expression that accompany stage progression [12-13, 23]. Protein degradation is one of the most important functions of the UPS, and the need for constant protein turnover is a hallmark of *P. falciparum* [14-17, 23]. In fact, only 6% of its proteome is common between all stages [23]. However, the proteasome and essential components of the UPS are present during all parasite developmental stages [16,23]. It is likely that the proteasome, and UPS-dependent protein degradation, plays significant roles in stage progression. Study of the UPS and the effects of differing levels of proteasome inhibition are desirable for better understanding of *P. falciparum* biology as a whole.

We chose three canonical small-molecule proteasome inhibitors (SMPIs) at the start of this work because of their abilities to act as anti-malarials in *P. falciparum* culture [31-33]. Enzymatic activity assays were used to examine the ability of each SMPI to hinder specific types of catalytic activities in the proteasome core. These comparative studies revealed that exposure to MG132, lactacystin or bortezomib each result in complete inhibition of culture growth with half-maximal inhibitory concentration (IC_{50}) values below 1 μM, comparably to previous studies [31-33]. The compounds demonstrated differing levels of inhibition of enzymatic activity. The lower growth IC_{50} value observed

for MG132 in parasite culture and specificity in enzymatic activity assays led us to choose the use of MG132 for further examination as a tool compound for partial and complete proteasome inhibition studies.

Lactacystin is a commonly used SMPI that has been utilized previously in proteasome studies, including experiments with *P. falciparum* [31-32,44]. It is an irreversible inhibitor that is known to covalently modify active sites inside the 20S proteasome core [24, 44]. We found lactacystin to have the highest growth IC_{50} value of the three compounds studied, with low variation between independent experiments. In regard to enzymatic activity, lactacystin was able to completely inhibit chymotrypsin-like (CTL) activity in the *P. falciparum* proteasome, but did not completely inhibit the trypsin-like (TPL) or caspase-like (CPL) activities. However, the compound required a very high concentration for inhibition of CTL activity, with an IC_{50} of over 3,000 nM. Although lactacystin is a canonical SMPI and does arrest parasite growth completely, the relatively high IC_{50} values for inhibition of both growth and enzymatic activity raise concerns about the efficiency of its use as a tool compound. Furthermore, the nearly 10-fold difference in the concentration required for inhibition of enzymatic activity compared to the concentration that arrests culture growth could make it difficult to study partial proteasome inhibition in live parasites. The large difference in growth IC_{50} and activity IC_{50} could also be the result of off-target effects overshadowing proteasome inhibition in culture, which is also a concern [24, 31-33].

Bortezomib is a reversible SMPI that has previously been used in laboratory-based proteasome studies, as well as clinical treatments [24,26,31-33,45]. In our hands, this compound demonstrated a desirably low growth IC_{50}, but with high variation among replicate assays, meaning that its activity in culture is less predictable and consistent. This could be due to compound instability problems, as encountered by others when using bortezomib in culture medium of neutral or higher pH [45]. In this study, bortezomib demonstrated the ability to inhibit CTL and CPL activities in assays, but did not reduce TPL activity below 50%. The IC_{50} for inhibition of CPL activity is higher than that of CTL, so experiments aimed at specific inhibition of CTL activity may be feasible using lower concentrations. The fact that bortezomib is a commercially available for clinical treatment approved for cancer chemotherapy could make it preferable for experiments aimed at developing new anti-malarial treatments [24-27]. The similarly low IC_{50} values for inhibition of growth and enzymatic activity might appear to make it an attractive candidate for experiments examining proteasome activity in culture. However, based on our assessment, it is not ideal for use with *P. falciparum* in culture because of the relatively high variation observed between separate growth assays and persistent problems with compound instability [45].

MG132 is a reversible inhibitor that is commonly used in cell culture and *in vivo* proteasome inhibition studies [24, 31-33, 46]. MG132 is also known to act directly on 20S proteasome catalytic activity, although it does have some off-target effects [24]. In this study, MG132 exhibited far less variation in IC_{50} values between individual growth assay replicates, suggesting that there were no problems with compound stability. It also

has a relatively low commercial cost. The inhibition of enzymatic activity of the proteasome core subunits by MG132, as shown by our *in vitro* activity assays, seemed to be specific to the chymotrypsin-like activity in *P. falciparum* parasites. Neither the trypsin-like nor the caspase-like proteolytic activities of the proteasome are significantly affected, in contrast to the impacts of MG132 on human proteasome activities. This is a surprising result, and it could make MG132 of interest when precise targeting of CTL activity within the UPS is desired, with the caveat that MG132 has been shown to exhibit some off-target effects outside of the UPS [24].

Upon further study, we find that MG132 is an effective and fast-acting anti-malarial in parasite culture. We do not observe the delayed death effect seen with some anti-malarial compounds [43]. Azithromycin, for example, has very little potency unless the parasites are incubated for longer than 3 days [43]. In delayed-death assays, MG132 growth curves exhibited similar forms to those for mefloquine, a compound known to inhibit growth within the first 24 hours of exposure [42]. Thus, MG132 can be relied upon to induce similar lethality (or lack thereof) at a given concentration when exposure time is extended. This could be of use in longer-term proteasome inhibition experiments.

The ability to induce partial inhibition of proteasome activity with low doses of MG132 also recommends it as a SMPI tool compound [40-41]. The delay (but not complete arrest) seen in developmental stage progression during 48 hours of exposure demonstrates the ability of low concentrations of MG132 to create cellular stress without causing immediate global lethality. This suggests that any critical processes controlled by

the proteasome can be studied using MG132-mediated partial proteasome inhibition in live cells by addition of sub-lethal concentrations of MG132; and that experiment duration could span at least one complete growth cycle.

MG132 is highly effective for low-level, selective, partial proteasome inhibition in live *P. falciparum* cultures when administered at sub-lethal concentrations. In experiments conducted by others, MG132 has been shown to have a dual effect in parasites by targeting both the proteasome and falcipain cysteine proteases in *P. falciparum* [46]. However, MG132 has only been shown to inhibit *P. falciparum* cysteine proteases at higher concentrations [46]. Therefore, the cellular stress and inhibition of parasite development observed during exposure to low concentrations of MG132 is likely primarily due to proteasome inhibition, as demonstrated by the accumulation of ubiquitylated cellular proteins in parasites exposed to only 50 nM MG132 [Figure 7]. Ubiquitylated proteins accumulate in cells exposed to levels ranging from 50 nM to 1μM, in amounts that increase with respect to MG132 concentration [Figure 7]. This suggests that the degree of MG132-associated proteasome inhibition in cultured *P. falciparum* cells is dose-dependent. Our demonstration of the ability of low concentrations of MG132 to inhibit the proteasome implies that MG132 is an effective compound for the study of partial proteasome inhibition.

We show that MG132 is effective for short-term inhibition of UPS function, as evidenced by observable proteasome inhibition after only 6 hours of treatment with varying concentrations of MG132. We also find that negative effects of proteasome inhibition on

parasite growth are reversible after this short exposure time, even at high MG132 concentrations. Parasites exposed to concentrations as high as 1 μM MG132 for 6 hours were able to recover and proliferate normally within 7 days following removal of drug pressure. Therefore, transient effects and downstream consequences of short-term proteasome inhibition in *P. falciparum* can be studied using MG132.

From our experiments, and those of others, it appears that the UPS in *P. falciparum* may be particularly crucial to trophozoite development [31-33,46]. The stage delay due to proteasome inhibition is most pronounced during the trophozoite stages, in agreement with previous studies that have observed progression stalling prior to DNA replication [31-33, 46]. Accumulation of ubiquitylated proteins during trophozoite stage is also substantial, as we have shown [Figure 7], which may make the roles of the proteasome during this stage attractive as a focus in future studies of the biology of the *P. falciparum* UPS.

Overall, MG132 is a cost-effective small molecule proteasome inhibitor that has a lengthy history of use in the investigation of UPS function [37, 41,46]. We find that MG132 specifically inhibits *P. falciparum* proteasome CTL activity, within a relatively short period of time. Prasad et al. showed that MG132 targets both the 20S proteasome and falcipains in *P. falciparum* [46]. The effects of MG132 on falcipain activity were demonstrated at concentrations of 100 nM and higher, but the extent of inhibition of falcipains (and other cysteine proteases) by lower MG132 concentrations has not been established. We have presented data that show clear evidence of proteasome inhibition at

the lower concentration of 50 nM MG132, but more research would be required to assess any off-target effects associated with exposure to lower MG132 concentrations. We also demonstrate the ability to utilize MG132 reversibly in low-dose and high-dose experimental treatments, making it a desirable compound for studying of partial or complete inhibition of the proteasome in *P. falciparum*.

Figures and Tables

Table I: Orthologous elements common between *P. falciparum* and other species[a]			
Species:	**Common Orthologous Groups:**	**Common Distinct Genes**	**% P.f. Genes with Orthologues**
Toxoplasma gondii	2308	2474	41.9%
Homo sapiens	1590	1731	29.3%
Mus musculus	1587	1728	29.2%
Danio rerio	1567	1705	28.9%
Anopheles gambiae	1470	1597	27.0%
Saccharomyces cerevisiae	1296	1416	21.9%
Escherichia coli	360	430	7.2%

[a] Numbers reported on PlasmoDB website (http://plasmodb.org/plasmo/)

Table II: *P. falciparum* proteasomal subunits and homologous subunits in other organisms						
PlasmoDB Accession[a]	**Gene[a]**	**Description[a]**	**e-values[b]: *H. sapiens***	***S. cerevisiae***	***D. melanogaster***	***A. gambiae***
PF3D7_1474800	Alpha 1	Alpha Subunit, type 1	9.00E-44	3.00E-53	2.00E-55	2.00E-56
PF3D7_0608500	Alpha 2	Alpha Subunit, type 2	8.00E-59	9.00E-71	2.00E-69	7.00E-75
PF3D7_0317000	Alpha 3	Alpha Subunit, type 3	2.00E-33	6.00E-35	5.00E-35	4.00E-46
PF3D7_1353800	Alpha 4	Alpha Subunit, type 4	4.00E-68	6.00E-67	1.00E-68	2.00E-70
PF3D7_0727400	Alpha 5	Alpha Subunit, type 5	6.00E-66	3.00E-75	1.00E-72	2.00E-71
PF3D7_0807500	Alpha 6	Alpha Subunit, type 6	6.00E-40	1.00E-52	3.00E-59	1.00E-60
PF3D7_1353900	Alpha 7	Alpha Subunit, type 7	4.00E-65	3.00E-58	2.00E-59	1.00E-64
PF3D7_0518300	Beta 1	Threonine Hydrolase	2.00E-38	4.00E-43	5.00E-48	6.00E-48
PF3D7_1470900	Beta 2	Threonine Hydrolase	6.00E-29	7.00E-40	3.00E-41	3.00E-36
PF3D7_0108000	Beta 3	Beta Subunit, type 3	1.00E-51	1.00E-46	1.00E-52	5.00E-47
PF3D7_0803800	Beta 4	Beta Subunit, type 4	8.00E-26	1.00E-25	5.00E-28	1.00E-39
PF3D7_1011400	Beta 5	Threonine Hydrolase	6.00E-56	4.00E-64	5.00E-64	4.00E-62
PF3D7_0931800	Beta 6	Beta Subunit, type 6	4.00E-25	2.00E-27	1.00E-26	8.00E-30
PF3D7_1328100	Beta 7	Beta Subunit, type 7	2.00E-62	2.00E-68	7.00E-71	1.00E-75
PF3D7_1311500	RPT 1	ATPase	1.00E-175	0	0	0
PF3D7_1008400	RPT 2	ATPase (opens pore)	3.00E-165	0	0	0
PF3D7_0413600	RPT 3	ATPase	1.00E-155	8.00E-168	5.00E-165	7.00E-161
PF3D7_1306400	RPT 4	ATPase	1.00E-148	2.00E-163	2.00E-165	4.00E-163
PF3D7_1130400	RPT 5	ATPase (substrate recognition)	6.00E-167	3.00E-176	2.00E-171	4.00E-176
PF3D7_1248900	RPT 6	ATPase	6.00E-169	3.00E-180	0	1.00E-177
PF3D7_0205900	RPN 1	26S Regulatory	3.00E-144	6.00E-77	0	3.00E-117
PF3D7_1466300	RPN 2	Ubiquitin Recognition	4.00E-114	0	4.00E-144	0
PF3D7_1402300	RPN 6	Non-APTase Regulatory	2.00E-28	5.00E-27	1.00E-27	3.00E-26
PF3D7_1030500	RPN 9	Non-APTase Regulatory	5.00E-17	3.00E-30	5.00E-31	2.00E-32
PF3D7_0807800	RPN 10	26S Regulatory	5.00E-28	3.00E-29	4.00E-25	3.00E-31

[a] Subunit information was obtained from PlasmoDB website (EuPathDB Project Team, Athens, GA) and
[b] e-values were generated through BLAST comparison (NCBI, The National Library of Medicine, Bethesda, MD)

Table III: IC_{50} values[a] for inhibition of parasite growth by SMPIs		
	3D7	**Dd2**
Lactacystin (nM)	492 ± 36.7	468 ± 116.1
Bortezomib (nM)	93 ± 22.9	98 ± 41.8
MG132 (nM)	40 ± 4.7	34 ± 6.9

[a] IC_{50} mean and standard error for three independent experiments
Strains: 3D7 is sensitive to most anti-malarial drugs, Dd2 is a chloroquine-resistant strain [38,39]

Table IV: IC_{50} Values for Delayed Death Assay[a]			
	MG132 (nM)	Mefloquine (nM)	Azithromycin (nM)
0.5 Cycles (1 Day)	43 ± 8.6	11 ± 3.0	2331 ± 593
1.5 Cycles (3 Days)	35 ± 2.7	11 ± 2.2	2371 ± 491
2.5 Cycles (5 Days)	27 ± 4.1	8 ± 4.1	*150 ± 81

[a] IC_{50} mean and standard error for three independent experiments
*Mean IC_{50} value is significantly different from the other values in the same column, $p \leq 0.05$, Turkey's multiple comparison test

Table V: IC50 values for inhibition of proteasome enzymatic activity by SMPIs[a]				
			Inhibition of Enzymatic Activity: IC50 Vaule(s) [a] (nM)	
Inhibitor	Substrate:	Activity Type	PF	HS
MG132	SUC-LLVY-AMC	Chymotrypsin-like	129 (±5.4)	71 (±1.9)
	Z-LLE-AMC	Caspase-like	N/A	2,237 (±341)
	Boc-LLR-AMC	Trypsin-like	N/A	798 (±146)
Bortezomib	SUC-LLVY-AMC	Chymotrypsin-like	56 (±33)	6.5 (±1.4)
	Z-LLE-AMC	Caspase-like	1285 (±162)	120 (±17.4)
	Boc-LLR-AMC	Trypsin-like	N/A	1,430 (±102)
Lactacystin	SUC-LLVY-AMC	Chymotrypsin-like	3429 (±1202)	760 (±165)
	Z-LLE-AMC	Caspase-like	N/A	78,437 (±27,871)
	Boc-LLR-AMC	Trypsin-like	N/A	15,883 (±1642)

[a] IC_{50} values are the mean and standard error of three independent experiments

[a] IC_{50} mean and standard error for three independent experiments
PF = *Plasmodium falciparum* (parasite lysate)
HS = *Homo sapiens* (Jurkat cell lysate)
N/A = IC_{50} was not calculated because compound did not substantially reduce activity
Activity Types = Chymotrypsin-like activity is associated with the β5 subunit, caspase-like activity is associated with the β1 subunit, and trypsin-like activity is associated with the β2 subunit [14,24,25].

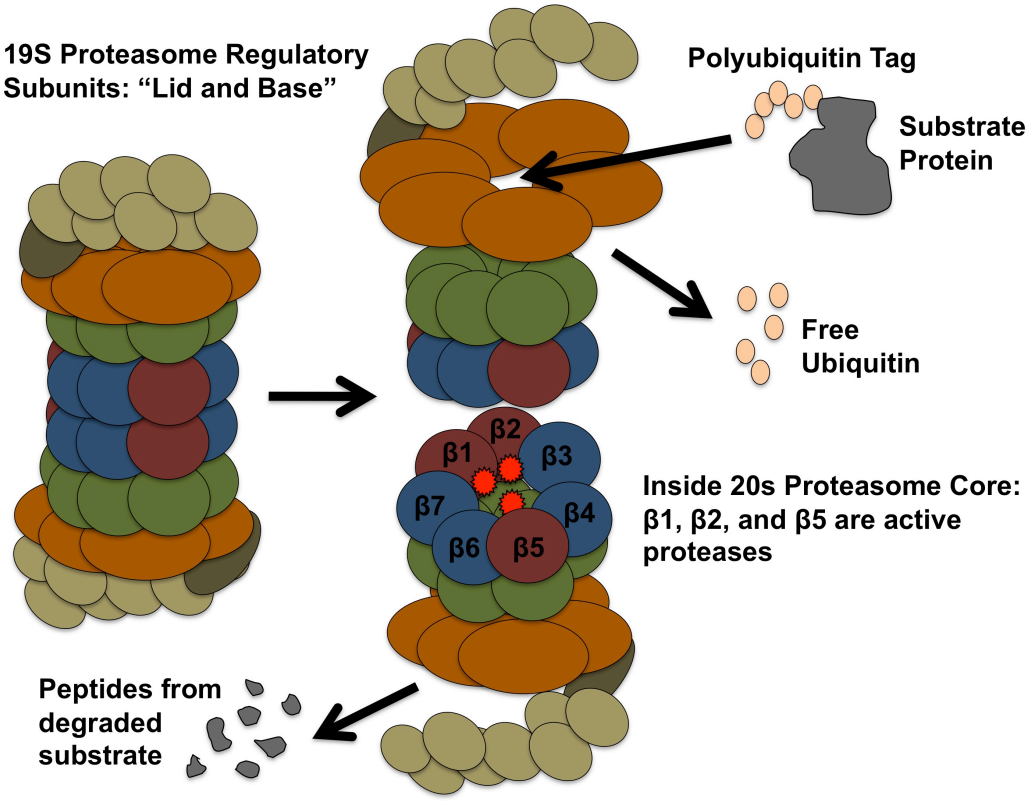

Figure 1: Diagram of the 26S proteasome complex. Shown are regulatory subunits of the 19S proteasome "lid and base" (brown and orange, respectively) and structural α subunits, structural β subunits, and active β subunits of the 20S proteasome core (green, blue, and mauve, respectively). Active sites of the β1, β2, and β5 proteases, facing in the interior of the proteasome core, are shown in red. During the degradation process, protein/peptide substrates that bear a polyubiquitin tag are modified by upstream UPS components, including proteasome regulatory subunits that recognize the polyubiquitin tag. The pore of the complex is opened by ATPases and the substrate is drawn in, where the polyubiquitin tag is removed, releasing free ubiquitin. Substrate is drawn further into the complex, where the active proteases of the 20S proteasome core degrade it and release small peptides that will be "recycled" by the cell.

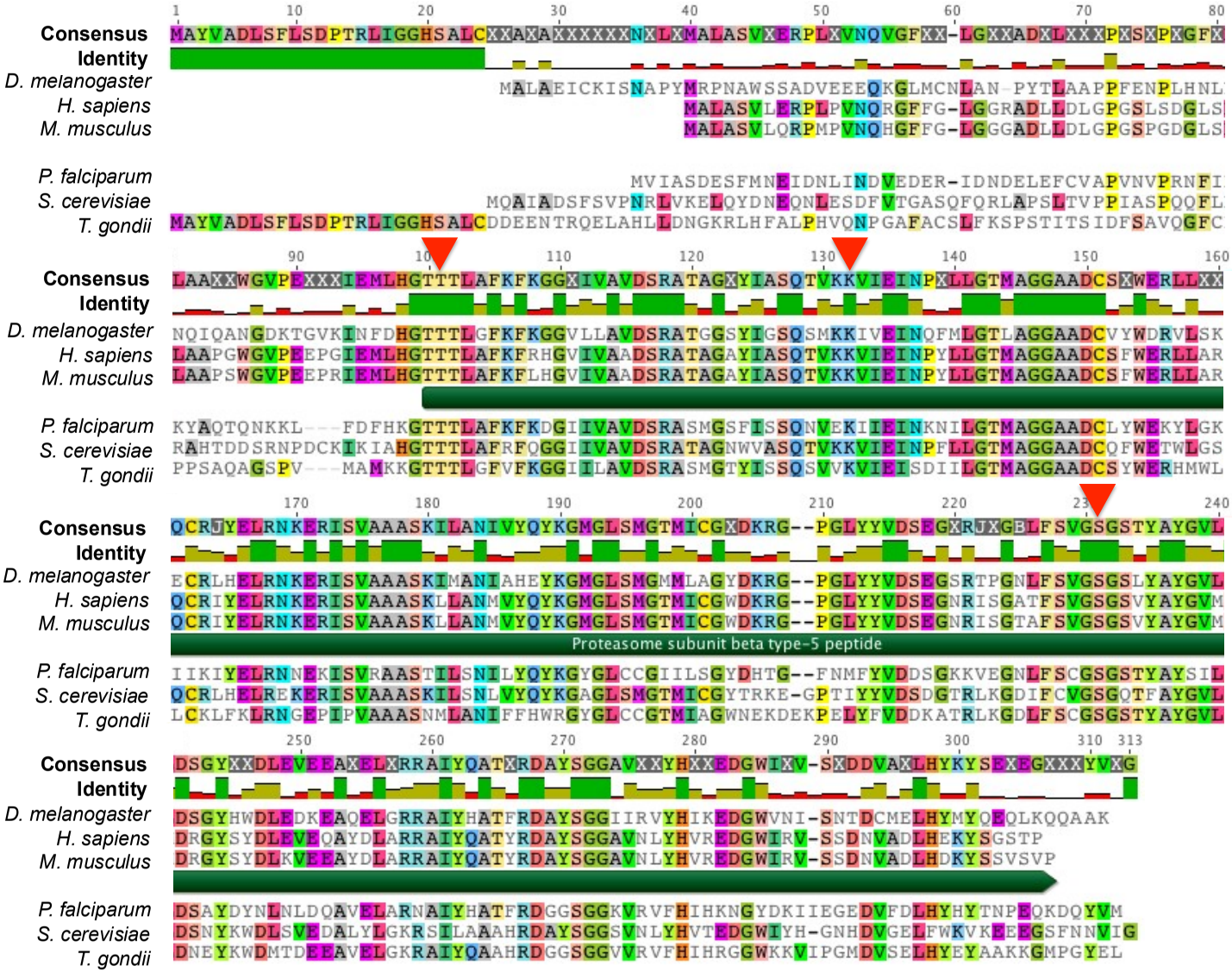
Consensus
Identity
D. melanogaster
H. sapiens
M. musculus
P. falciparum
S. cerevisiae
T. gondii
Proteasome subunit beta type-5 peptide

Figure 2: Multiple sequence alignment of the Beta5 subunit of the 20S proteasome. Protein sequence of *P. falciparum* is compared to species indicated. Key active site residues are indicated by red arrows, including the N-terminal threonine (first red arrow) [14, NCBI protein database: http://www.ncbi.nlm.nih.gov/protein/]. A histogram along the top of the sequence represents the level of identity of each region, and a consensus sequence is shown above. The dark green bar traces the universal consensus sequence for the 20S proteasome Beta5 subunit as retrieved from the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd/).

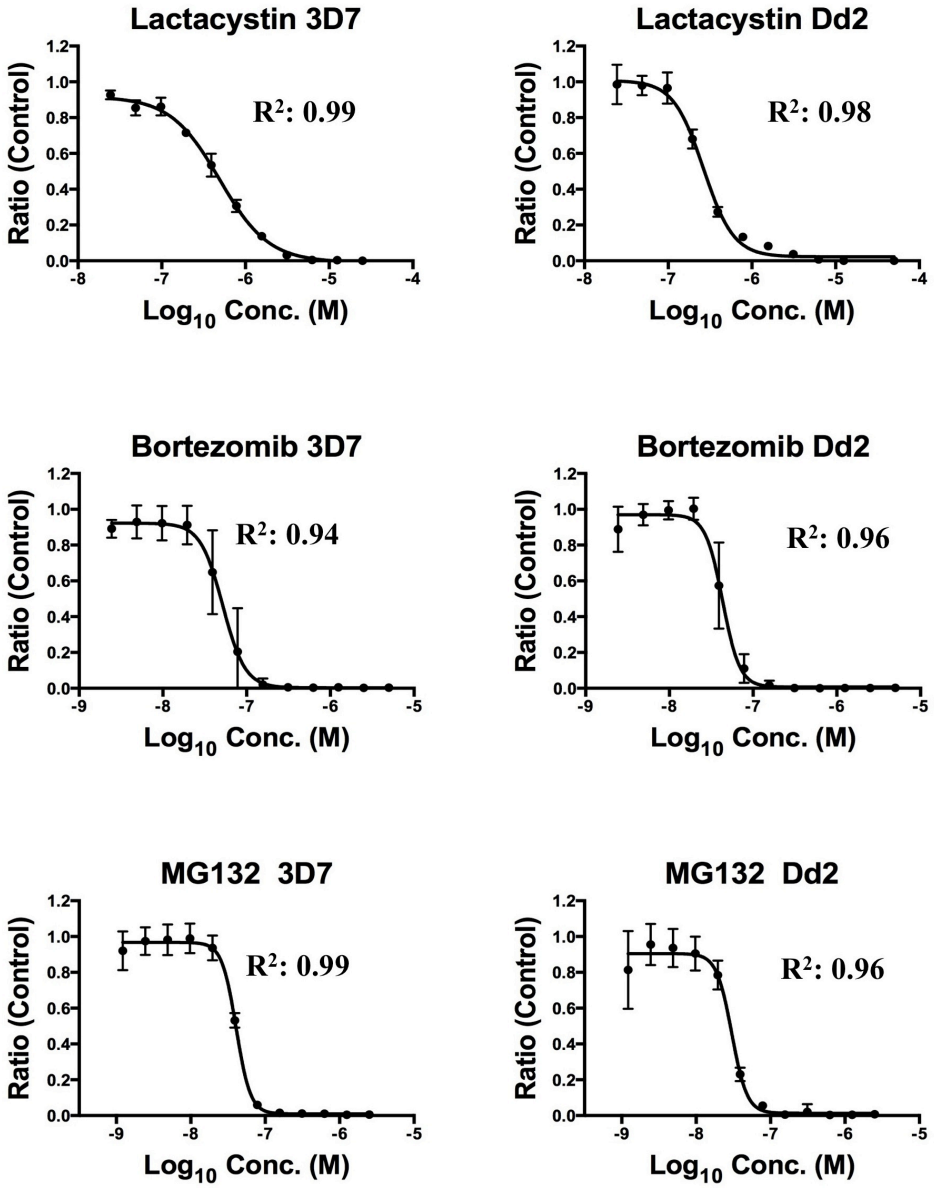

Figure 3: Growth inhibition of cultured *P. falciparum* by small molecule proteasome inhibitors. Non-linear regression curves are shown for 3-day incubation of 3D7 and Dd2 parasite cultures in the presence of three proteasome inhibitors: lactacystin, bortezomib, and MG132. Growth ratios were generated by comparing final DNA content (assessed via SYBR Green® assay) to that of control cultures incubated for the same period of time, but in the absence of inhibitors.

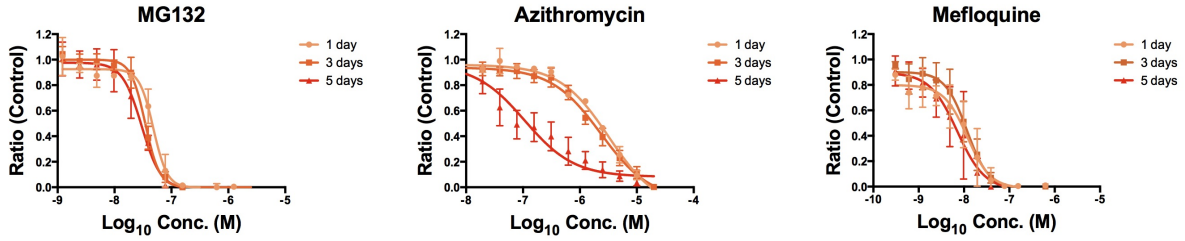

Figure 4: Assessment of delayed death effects of MG132, as compared to mefloquine and azithromycin, for growth of 3D7. Non-linear regression curves were generated by SYBR Green® assay and growth relative to 3D7 was compared after 1, 3 and 5 days of incubation.

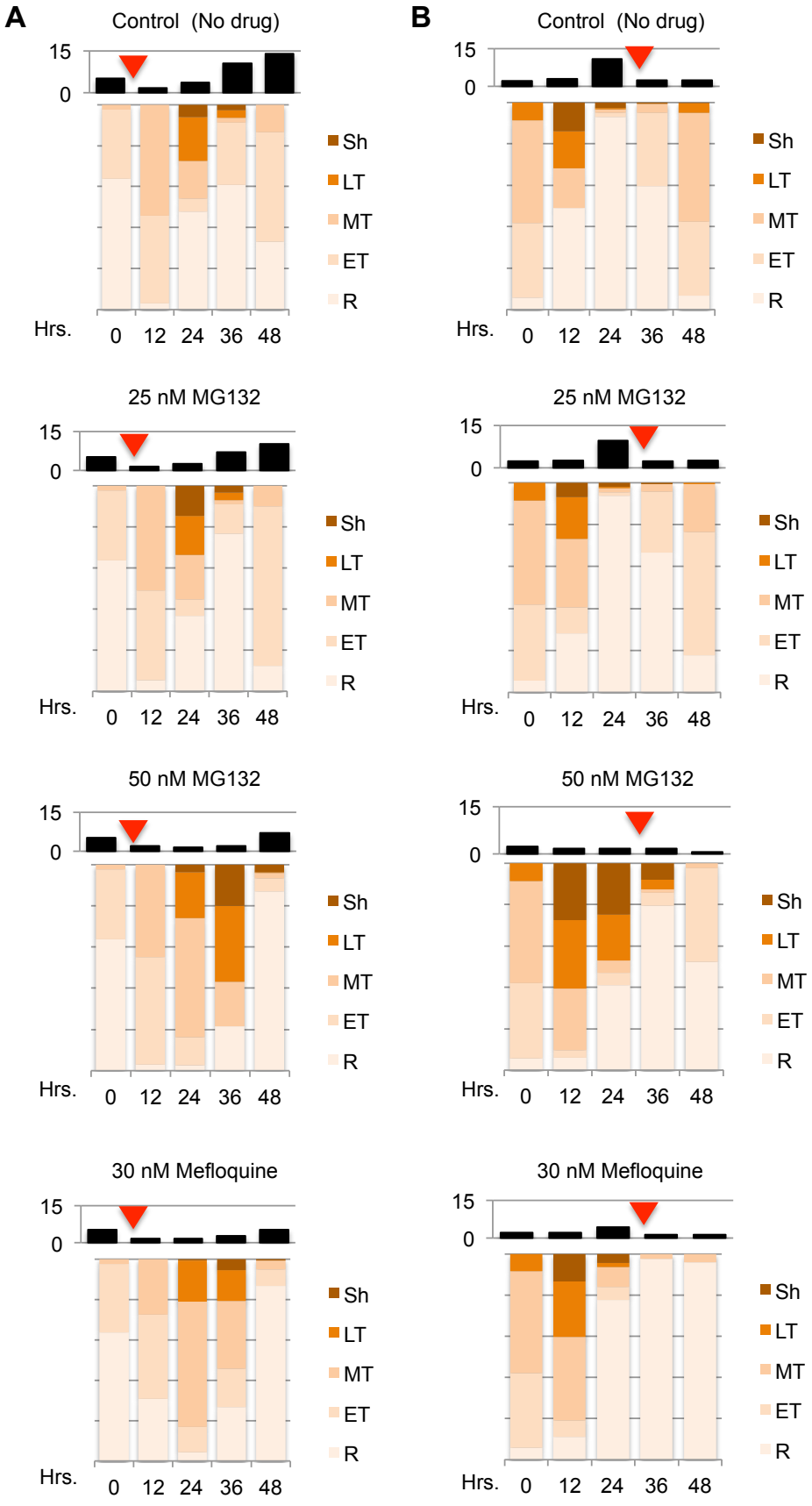
A
B
Control (No drug)
25 nM MG132
50 nM MG132
30 nM Mefloquine
15
0
Hrs.
0 12 24 36 48
Sh
LT
MT
ET
R

Figure 5: Delay in developmental stage progression caused by MG132 exposure. MG132, mefloquine, or vehicle (DMSO) was added to synchronous parasite cultures during either ring stage (A) or mid-trophozoite stage (B) at concentrations indicated. In each chart, parasitemia (top, in black) and stage distribution (bottom, in color) at each time point over a 48-hour period are shown. Arrows mark instances of 1:5 culture dilution with fresh media and uninfected red blood cells. Stage abbreviations: R = ring, ET = early trophozoite, MT = mid-trophozoite, LT = late trophozoite, Sh = Schizont

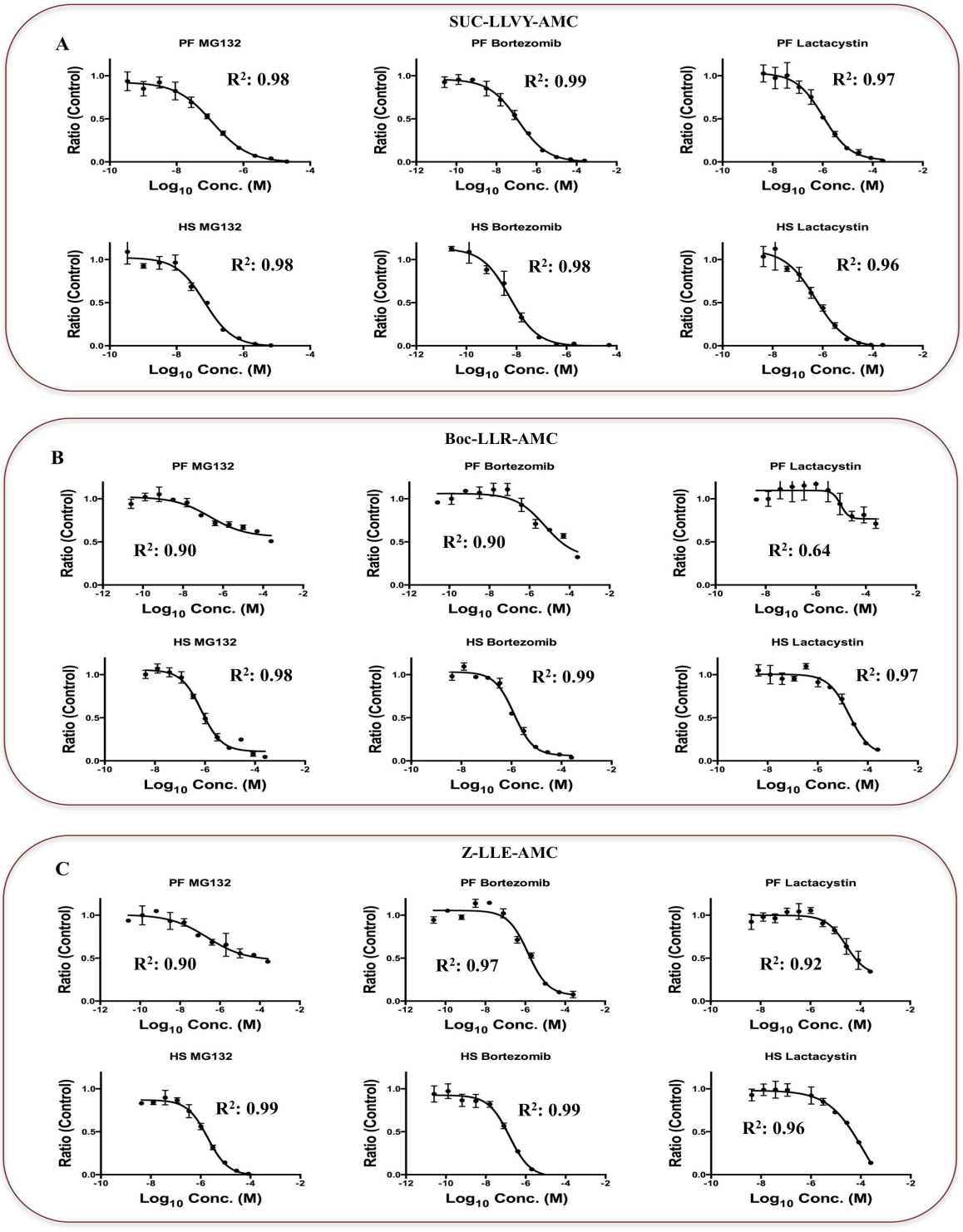
SUC-LLVY-AMC
A
PF MG132
R²: 0.98
PF Bortezomib
R²: 0.99
PF Lactacystin
R²: 0.97
HS MG132
R²: 0.98
HS Bortezomib
R²: 0.98
HS Lactacystin
R²: 0.96
Boc-LLR-AMC
B
PF MG132
R²: 0.90
PF Bortezomib
R²: 0.90
PF Lactacystin
R²: 0.64
HS MG132
R²: 0.98
HS Bortezomib
R²: 0.99
HS Lactacystin
R²: 0.97
Z-LLE-AMC
C
PF MG132
R²: 0.90
PF Bortezomib
R²: 0.97
PF Lactacystin
R²: 0.92
HS MG132
R²: 0.99
HS Bortezomib
R²: 0.99
HS Lactacystin
R²: 0.96
Ratio (Control)
Log10 Conc. (M)

Figure 6: Inhibition of *P. falciparum* proteasome enzymatic activities by small molecule proteasome inhibitors. Non-linear regression curves are shown for incubation of 3D7 parasite (PF) and Jurkat human (HS) cell lysates in the presence of three proteasome inhibitors: lactacystin, bortezomib, and MG132. A) Chymotrypsin-like activity assayed using substrate SUC-LLVY-AMC. B) Trypsin-like activity assayed using substrate Boc-LLR-AMC. C) Caspase-like activity assayed using substrate Z-LLE-AMC.

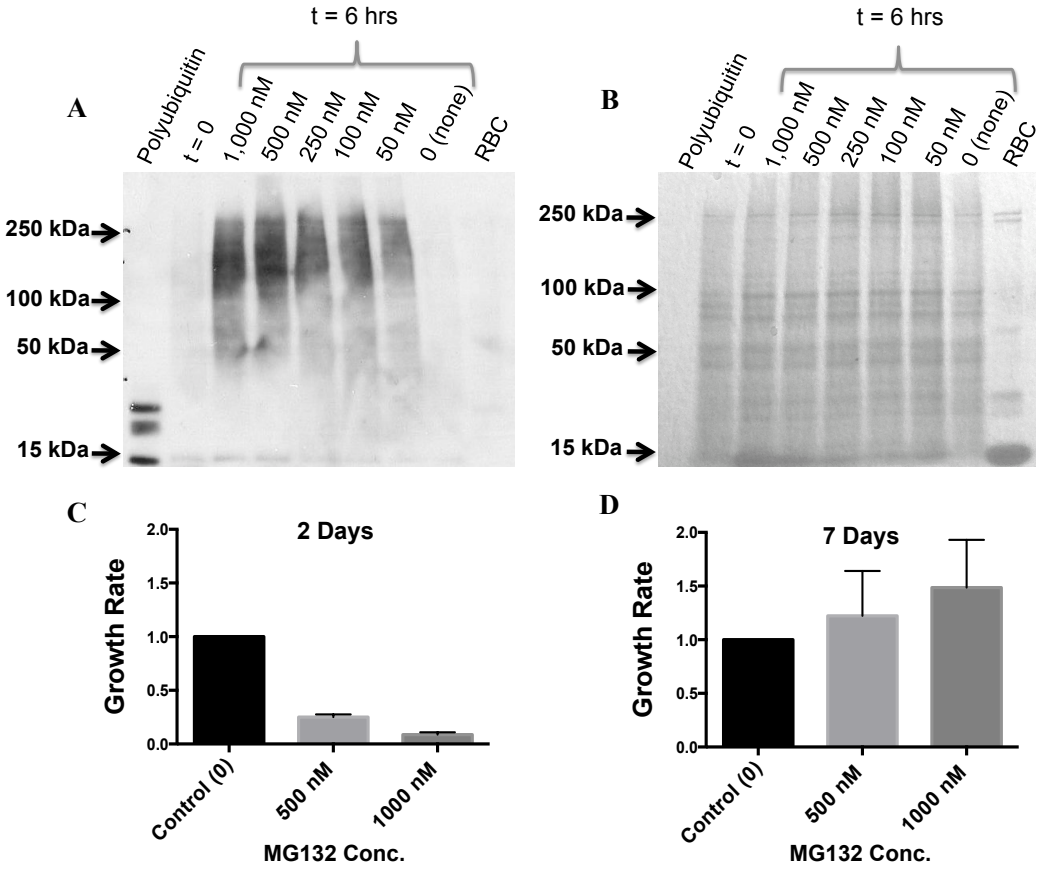

Figure 7: Accumulation of ubiquitylated proteins and parasite recovery of cultures exposed to MG132 for 6 hours. 3D7 cultures were exposed to varying concentrations of MG132 for 6 hours. Accumulation of ubiquitylated proteins in whole-cell parasite lysates was detected by western blot using an anti-ubiquitin primary antibody (A) [36]. Total protein content of lysates was visualized by Coomassie stain (B). RBC = uninfected red blood cell lysate used as a control. After the 6-hour period, cultures exposed to 1,000 nM or 500 nM MG132 (and unexposed control cultures) were diluted 20-fold in medium without MG132, and allowed to resume growth under standard culture conditions. Cultures were monitored over 7 days and growth rates (fold-change in parasitemia) at 2 days (C) and 7 days post-exposure (D) were compared.

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Chapter III:

MG132 resistance in *P. falciparum*: generation and characterization of parasite strains with increased tolerance of the to proteasome inhibitor MG132

Abstract

The ubiquitin-proteasome system (UPS) is a large protein "recycling" system present in eukaryotic cells and inhibition of the UPS by small-molecule proteasome inhibitors is toxic to *Plasmodium falciparum* parasite growth. The proteasome inhibitor MG132 is known to target the ß-5 subunit of the 20S proteasome complex, an active threonine hydrolase that participates in protein degradation. *P. falciparum* strains that demonstrate resistance to MG132 were developed through intermittent compound exposure. Growth inhibition assays were conducted to assess levels of resistance to MG132, and varying levels of tolerance were observed. These lines demonstrated low-level resistance, or tolerance, to MG132 with IC_{50} values of about 2-5 times that of the wild-type parent strain. Sequencing revealed non-synonymous point mutations in the β5 subunit of the 20S proteasome in all resistant lines. Tolerance to anti-malarial compounds is a critical precursor to clinical resistance and total failure of drug efficacy in clinical settings. A deeper understanding of the development of tolerance to proteasome inhibitors like MG132 could offer insights into the biology underlying development of resistance and the role of the UPS in malaria pathogenesis.

Introduction

The evolution of drug resistance in pathogenic organisms is a major concern in the control of infectious diseases. Malaria, which is caused by apicomplexan parasites within the genus *Plasmodia*, is no exception [1-9]. *Plasmodium falciparum*, the species that causes the most acute cases of malaria, has developed resistance to many anti-malarial drugs [3, 5-9]. Historically, many drugs that had been highly effective for chemotherapy when first developed were subsequently rendered obsolete because of the development of resistance within parasite populations [1-13]. The appearance and spread of anti-malarial resistance within parasite populations soon after widespread deployment of many anti-malarial drugs is largely due to the rapid evolution that is characteristic of *Plasmodium* species [1-4, 10-13]. Historical survey data highlighting dates that resistance became a hindrance to drug efficacy in malaria treatment worldwide [1-4] are shown in Figure 1. Although previous clinical and research studies have successfully identified the genetic sources of resistance in several cases, insight into the biology of how resistance commonly arises in *Plasmodium falciparum* remains incomplete [1-5, 7-8].

One reason that *Plasmodium* parasites are highly adaptable is that these species possess the ability to generate substantial genetic polymorphisms over time [10-12]. The complexity of the parasite life cycle, which includes a single round of sexual reproduction within the mosquito vector and multiple rounds of asexual reproduction in the human host [13], provides opportunities for the generation of millions of polymorphic variants within each species, every year. Outcrossing, or recombination between genomes of parasites with differing genotypes, and inbreeding, or recombination between

genomes of parasites with the same genotype, can occur during the sexual recombination stage in the mosquito midgut before parasites are transmitted to humans [13]. This is especially common in malaria-endemic areas where dense human populations coincide with abundant mosquito populations, and single mosquitoes frequently carry multiple parasite genomes [6, 8]. Drug pressure from any chemotherapy that infected individuals are undergoing will select for parasites bearing resistance alleles, which can arise randomly during the many cycles of asexual reproduction in the human bloodstream [10-12]. Overall, the complex biology of the parasite contributes in many ways to the rapid evolution of *Plasmodium* species, and there is a need for continued research to better understand it [14-15].

Tolerance, or low-level resistance, to anti-malarial compounds is an important component of the biological dynamics of resistance [16-19]. Although "complete" resistance to an antimalarial drug or drug cocktail can sometimes be associated with single genetic variants that arise suddenly, this is not always the case [16-20]. Resistance often develops in steps, beginning with an intermediate ability to survive higher concentrations of a compound for longer periods of time than a sensitive strain [16-17]. Although tolerant parasites may not survive exposure to a drug at the concentration used for chemotherapeutic treatment, they may survive lower concentrations that may remain in the bloodstream during the days or weeks following treatment [16-19]. If a person is infected just after completing a chemotherapy regimen, tolerant parasites introduced into their bloodstream have a selective advantage [16-18]. The prominence of tolerance traits in a localized parasite population can give rise to higher-level resistance when subsequent

mutations occur, which could compromise clinical efficacy of the anti-malarial treatment [16-19].

Public health officials have approached the challenge of the spread of drug-resistant parasites in a variety of ways [2, 4, 13]. The World Health Organization (WHO) endorses combination therapy, as opposed to "monotherapy" (the use of single-drug treatment regimes), as the standard treatment for malaria because the use of a cocktail consisting of several compounds with diverse targets is less likely to result in resistance selection [2, 14-15,21-22]. Artemisinin combination therapy (ACT) is currently the most common standard malaria treatment worldwide [1-2, 22]. While ACT continues to be highly successful in long-term clinical use, recent cases of resistance and reduced sensitivity have been reported [23-25].

Parasite physiological systems that facilitate stress tolerance, such as the ubiquitin/proteasome system (UPS), could be of importance to the development of resistance to anti-malarial compounds [16-20]. The UPS is a complex system used by eukaryotic cells for the selective degradation of proteins [26-28]. It consists of a highly diverse set of enzymes and structural proteins that identify, tag, and degrade protein substrates [26-28]. The UPS is present in the apicomplexan parasite that causes the most severe cases of malaria, *P. falciparum*, and plays a vital role in its life cycle [29-31]. Genomic-wide association studies have revealed that the UPS is strongly associated with drug response and resistance in *P. falciparum* field strains [20]. Proteasome inhibition

effectively arrests the growth of parasites in culture, leading researchers to identify the UPS as a possible target for chemotherapeutic treatment of malaria [29-31].

The proteolytic machinery of the UPS, the "26S proteasome complex," consists of a multi-subunit 19S proteasome regulatory "cap" that controls substrate entry, and a 20S proteasome "barrel" that degrades proteins that enter the complex [26-28, 32-33]. The 20S proteasome particle is composed of stacked seven-subunit rings of peptides called alpha (α) and beta (β) subunits. Three of the seven types of β subunits, β1, β2, and β5, are active threonine proteases responsible for the catalytic activity of the complex [26-27, 30]. These proteolytic enzymes feature three types of activity, chymotrypsin-like (CTL), trypsin-like (TPL) and caspase-like (CPL), and function as a group [32-33]. Many common proteasome inhibitors target these enzymes, particularly the β5 subunit, which is responsible for CTL activity [32-34]. Proteasome inhibition mediated by small-molecule proteasome inhibitors (SMPIs) has been utilized clinically for cancer chemotherapy, and a number of cases of clinical resistance have been documented [34-41]. In cases in which the genetic basis of the resistance has been discovered, nearly all resistance involves mutations in the β5 subunit of the proteasome, at or near residues associated with enzymatic active sites or inhibitor binding sites [35-41]. Mutations in residues M45, A49, A50, C52, and C63 have been linked to SMPI resistance in human cell lines [35-41].

One of the first-identified SMPIs, MG132, targets the N-terminal threonine of the β5 subunit active site, and has potent anti-malarial activity in vitro [29-33]. We have generated parasite lines with increased tolerance to MG132. These parasites, called

"MGR" lines, survive high concentrations of MG132 for several days longer than wild-type (WT) lines and even proliferate under drug pressure. This increased tolerance developed after many rounds of intermittent MG132 exposure. After observing parasite survival during MG132 treatment, growth inhibition was measured using a SYBR assay and IC_{50} values were calculated. The increased ability to survive in the presence of MG132 arose in each culture suddenly, but the observable IC_{50} values of individual MG132-tolerant lines varied. Sequencing of the β5 subunit revealed single non-synonymous point mutations in each MGR line that were neither present in the parental WT population nor previously identified as single-nucleotide polymorphisms in other drug resistant parasites strains [42]. We conclude that these mutations arose during the course of MG132 selection, and may contribute to MG132 tolerance and/or to the MG132-resistance phenotype.

There are several lines of experimentation could be followed if one were to extend the work described in this thesis. MGR lines could be subjected to further rounds of selective MG132 pressure to develop fully resistant lines, and resistance to other anti-malarial compounds could be assessed in those lines. This would extend the work beyond the realm of tolerance into the realm of full resistance, and enable exploration of the rate at which parasites become completely unresponsive to MG132. Cross-resistance studies could be conducted with lines that develop complete MG132 resistance, which could elucidate the role(s) that the UPS plays in stress tolerance and general drug resistance in *P. falciparum*. The genome(s) of resistant lines could be sequenced to determine the nature and number of mutations necessary to confer complete resistance. Overall, the

generation of tolerance to MG132 in *P. falciparum*, demonstrated by prolonged survival I observe in the presence of the compound, is a significant finding that could enable further studies of the UPS, drug resistance, and the genetic "bridge" between anti-malarial drug sensitivity and resistance.

Materials and Methods

Parasite Strains and Cells

The WT parental strain used for selections was P2G12, a substrain of the *P. falciparum* 3D7 strain, obtained from the laboratory of Dr. Dyann Wirth of the Department of Immunology and Infectious Disease at the T.H. Chan Harvard School of Public Health (Boston, MA USA) [43]. Packed human red blood cells in CPDA-1 used for parasite culture were obtained from Research Blood Components (Boston, MA). All mutant lines were developed via selection for MG132 resistance in our laboratory.

Compounds and Reagents

MG132 and mefloquine were obtained from Sigma-Aldrich Co. LLC. (St. Louis, MO). Stocks were made by dissolving compounds in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. Stock solutions were stored at -80^{o}C.

Parasite Culture Conditions

All parasite culturing was conducted using the following standard conditions unless otherwise noted. Parasites were cultured in RPMI cell culture medium (Life Technologies Corp., Grand Island, NY), supplemented with 5.94 g/L HEPES, 0.05 g/L hypoxanthine, 2.016 g/L sodium bicarbonate, and 0.025 mg/mL gentamicin. Prior to use, 0.5% Albumaxx II® (Life Technologies Corp., Grand Island, NY) was added to produce "complete" media. All cultures were maintained at 1-5% hematocrit. Cultures were incubated at 37°C in the presence of a gaseous mixture consisting of 1% O_2, 5% CO_2, and

94% N_2. Cultures were diluted with uninfected red blood cells and fresh complete medium when parasitemia exceeded 1% (as measured by microscopic examination of blood smears) or as required for experimental conditions.

Parasite Stage Synchronization

For parasite stage synchronization, infected red blood cells (iRBCs) were incubated in 5% sorbitol at 37°C for five minutes. Cells were isolated by centrifugation at ~500xg and the supernatant discarded. Cells were then returned to culture with fresh complete medium.

Resistance Selection

Parasite drug resistance was generated by intermittent compound exposure, or repeated cycles of drug exposure followed by parasite recovery [25, 44]. Initial selections consisted of 100 mL 3D7 culture in complete medium, 3% hematocrit and 1% parasitemia, mixed stage. Cultures were exposed to either 50 nM or 250 nM MG132 and incubated for at least four days, or two days after signs of slowed or arrested growth. Every two to three days, parasites were fed with media containing fresh drug at the same concentration or greater. After incubation, drug pressure was removed by replacement of culture media with fresh media lacking MG132. Cultures were permitted to recover under standard culture conditions for up to four weeks, during which recovering cultures received replacement RBC and media and parasites were monitored by microscopy every two to three days. A culture was considered "recovered" when parasitemia returned to 1% or higher. The cycle of drug pressure followed by recovery was repeated with the

same or increased drug concentrations until parasites showed an ability to proliferate in the presence of MG132 at a concentration lethal to the parental strain (over five times the IC_{50}, as measured by SYBR Green assays).

SYBR Green Assay for Growth Inhibition

Growth/inhibition curves were generated by growth of cultures in the presence of MG132 concentrations ranging from 0-2.5 μM. Mefloquine was also used as an anti-malarial kill control compound [45], at a range of 0-625 nM, and cultures without drug (DMSO vehicle only) were used as a growth control. For each assay,180 uL cultures containing 1% hematocrit at 1% parasitemia were grown at each concentration. Cultures were grown in 96-well plates for 72 hours, and four samples of 40 μL of each culture were transferred to 384-well clear-bottom, black assay plates for analysis. Each 40 μL sample was processed by the addition of 10 μL lysis buffer (0.16% saponin, 20mM Tris-HCl (pH 7.5), 5mM EDTA, 1.6% Triton X-100) and SYBR Green® (Life Technologies Corp., Grand Island, NY) at 1:1,000 dilution. Samples were incubated at room temperature, protected from light, for at least one hour to allow for complete cell and parasite lysis. Fluorescence was measured using emission/excitation settings of 494 nm/520 nm, respectively. Culture growth was assessed by calculating the ratio of fluorescence readings of compound-treated cultures as compared to untreated control cultures incubated in the same plate. Baseline, which was established by the highest concentration of mefloquine (the kill control), was subtracted from readings and ratio of each sample culture growth to no-drug control was measured. Growth curves were generated and IC_{50} values calculated using the non-linear regression curve algorithm in GraphPad Prism® 6 software (GraphPad Software, Inc., La Jolla, CA). Statistical significance of mean IC^{50} values were

determined by application of the Dunnett's multiple comparisons test, $\alpha = 0.05$, calculated with the GraphPad Prism® 6 software (GraphPad Software, Inc., La Jolla, CA), one-way ANOVA algorithm.

Dilutional Cloning

Clonal lines were generated from bulk selection cultures by limiting dilution. All cultures were synchronous and at ring stage. Using culture cell density measured using a hemacytometer and parasitemia as counted visually by microscopy, calculations were made in terms of iRBC/ μL. Culture was diluted in fresh media and 3% uninfected hematocrit, with a goal of 10 parasites per 96-well plate, for a Poisson probability of generating clonal lines that is greater than 0.99. Diluted sample cultures were then divided into the wells of a 96-well plate and grown under standard culture conditions and fed every two to four days for four weeks. After 2 weeks, cultures were diluted 1:2 with fresh media and hematocrit and plates were checked for parasite growth by lysing and analyzing 40 μL of discarded culture as described above. Wells were checked for parasite growth in the same manner after three weeks. Any wells without evidence of growth after 4 weeks were considered clear of parasites. Wells with stable parasite cultures were scaled up to 25mL cultures, given an individual line number, and used in subsequent experiments.

In order to separate bulk cultures into many reduced-diversity subcultures that can be screened for MG132 tolerance, each originating from three or fewer parasites, limiting dilution was performed as above with modifications. Reduced diversity subcultures were

isolated based on calculations that anticipated thirty-two parasites per culture volume or 1/3 parasites per well in a 96-well plate. The Poisson probability of obtaining cultures that originated from three or fewer parasites using these calculations was predicted to be greater than 0.99.

Screening for Increased Drug Tolerance

Partially clonal/reduced diversity lines were screened for MG132 tolerance by growing cultures in the presence of 100 nM, 250 nM, and 500 nM MG132, respectively, and comparing growth to control cultures incubated without MG132 and cultures grown in the presence of 100 nM mefloquine. For comparison, the WT parental 3D7 strain was exposed to the same compound concentrations.

Sequencing and Sequence Analysis

P. falciparum gene reference sequences were obtained from the PlasmoDB website: http://plasmodb.org/plasmo/ (EuPathDB Project Team, Athens, GA) and NCBI/GenBank website: http://www.ncbi.nlm.nih.gov/genbank/ (The National Library of Medicine, Bethesda, MD). A fragment of the β5 subunit was amplified from each sample by polymerase chain reaction (PCR) for sequencing, which was conducted by Eton Bioscience (Boston Branch, Charlestown, MA 02129). Primers used in sequencing are: Beta5 AmpFwd: 5'-CTCAAGTTAATCATTAAAATATATTATAC-3'; Beta5SeqFwd: 5'-GGAGGAGCTGCTGATTGCTTATATTGG-3'; Beta5Fwd: 5'-ATGGTAATAGCAAGTGATGAAAGC-3'; and Beta5Rev: 5'-

TCACATAACATATTGATCCTTTTG-3'. Analysis of sequence data was conducted using Finch TV® software (Geospiza, Inc., Seattle, WA 98119), The European Bioinformatics Institute website: http://www.ebi.ac.uk/Tools/emboss/ (EMBL-EBI, Hinxton, Cambridge, UK), and ClustalW Alignment Software (Conway Institute, UCD Dublin, Ireland).

Results

Selection of MG132-resistant parasites

We subjected sensitive WT 3D7 cultures to repeated rounds of selection via intermittent MG132 drug pressure in order to obtain parasite cultures resistant to MG132 [25,44]. Cycles of drug pressure, drug removal, and recovery were repeated until an observable shift in the ability of the parasites to survive and grow in the presence of MG132 was detected. These rounds of drug exposure and recovery were carried out by two methods: gradual increase, where the concentration of MG132 in the initial exposure was sub-lethal and increased in subsequent cycles; and rapid selection, where a lethal concentration was used for every round of selection, beginning with the first round. Selection rounds were repeated until parasites were able to proliferate in the presence of at least 250 nM MG132 (Table I), compared to the initial MG132 IC_{50} value of 34 nM in WT parasites (Table II).

To assess the differences in changes in IC_{50} values for MG132 responses that could result from the different methods of selection, three separate cultures from the 3D7 parent strain were used. These cultures were designated MG132 resistance selection cultures #1, #2, and #3. Origin, generations, and numbers of cycles are shown in Figure 2. Selection culture #0 was subjected to gradually increasing selection, with the first exposure cycle involving the addition of 50 nM MG132, a sub-lethal concentration, to culture medium over a six day period. During subsequent cycles, the concentration was increased to 100 nM, 150 nM, 250 nM, and 500 nM MG132. Before resistance was observed (that is, before live parasites were readily visible in smears after two or more days of drug

pressure), selection culture #1 (SC1) was split into culture MGR-1 and culture MGR-2, and MG132 exposure cycles continued for both cultures separately. Culture MGR-1 was eventually separated again into cultures MGR-1A and MGR-1B and subjected to one last round of selection. The rapid selection method was employed for selection culture #3 (SC3), with all selection cycles conducted using 250 nM MG132 or higher, to yield culture MGR-3. Cultures MGR-1A, MGR-1B, and MGR-3 needed eight, eight, and seven rounds of selection, respectively, to develop increased ability to survive under drug pressure. Culture MGR-2 did not develop detectible resistance after nine cycles, so it was dropped from the study.

When an observable number of parasites (>0.2% parasitemia) survived in cultures incubated for more than two days in the presence of 250 nM or greater concentration of MG132, they were considered "tolerant" of the compound and advanced to further study (Figure 2). Bulk selection cultures demonstrating increased tolerance to MG132 were designated MGR-1A, MGR-1B, and MGR-3.

MG132-sensitive parasite cultures (such as our 3D7 WT parent line) respond to drug pressure with a rapid decrease in parasitemia within two days of the addition of at least 250 nM MG132 (Figure 3). Surviving cultures were considered tolerant when parasites grew in number during the first two days of drug pressure, living parasites were still detected after four days under drug pressure, and normal growth resumed within one week after removal of drug pressure. Selection cultures MGR-1A, MGR-1B, and MGR-3 all eventually demonstrated the ability to survive longer than 3D7-WT in the presence of

high concentrations of MG132, as shown in Figure 3. Parasitemia for all three cultures increased during the first two days of exposure to 250nM MG132, indicating the ability proliferate under drug concentrations that would be lethal to an MG132-sensitive strain. Culture growth slowed and began to decrease after four days, indicating that parasites had acquired the ability to survive longer, but not indefinitely, under MG132 drug pressure (Figure 3, right panels).

Growth Inhibition Curves and IC_{50} Values for Bulk Resistant Cultures

To assess the level of MG132 resistance of each selection culture, growth/inhibition curves were generated and half-maximal inhibitory concentrations (IC_{50} values) calculated for MG132 and mefloquine, an anti-malarial compound used as a control [45]. Curves were compared to those of the parent WT 3D7 strain. The growth/inhibition curves for the effect of MG132 and mefloquine on bulk cultures WT 3D7, MGR-1A, MGR-1B, and MGR-3 are shown in Figure 4A and 4C, respectively. Histograms showing the shifts in calculated MG132 and mefloquine IC_{50} values in relation to parental line are shown in Figure 4B and 4D, respectively. A notable increase in MG132 IC_{50} value of least two-fold over WT was observed in all selection cultures (Figure 4A and 4B). Likewise, notable shifts in mefloquine in IC_{50} values were also observed in selection cultures (Figure 4C and 4D).

Establishment of Reduced-Diversity Subcultures and Clonal Lines From MG132-Resistant Cultures

After completion of selection cycles and confirmation of increased tolerance to MG132 in parasite cultures, individual tolerant lines were separated from bulk resistance cultures by limiting dilution cloning. Two strategies were employed to generate genetically distinct parasite lines. For establishment of lines with reduced genetic diversity, or lines that might be clonal but were not assumed to be clonal, limiting dilution was used to generate many subcultures that were screened for MG132 resistance. For establishment of clonal lines, limiting dilution was employed with a goal of generating clonal parasite lines. MG132 concentrations used for selection, number of cycles, and lines isolated by each method are listed in Table I.

For the separation of reduced-diversity subcultures, limiting dilution was used with a dilution factor that should have corresponded to 0.33 parasites per well in a 96-well plate format. Parasite densities of bulk cultures were estimated by counting parasitemia by microscopy and measuring cell density with a hemocytometer. Cultures were then diluted and divided into smaller cultures to allow individual (or a few) parasites to grow into separate subcultures. MGR-1A and MGR-1B (the first of the bulk selection cultures to develop resistance) diluted cultures were separated into a 96-well culture plate for growth. A total of 52 wells from MGR-1A and 47 wells from MGR-1B showed parasite growth (Table I). Twelve subcultures from each were chosen and screened for MG132 and mefloquine tolerance (Figure 5). Screening consisted of determining parasite growth ratios of cultures incubated under drug pressure for three days to the same cultures grown without drug pressure over the same time period. All screened cultures showed increased parasite survival, to varying degrees. Two lines from bulk culture MGR-1A: MGR-1As-1

and MGR-1As-22; and two from MGR-1B: MGR-1Bs-13 and MGR-1Bs-21, were chosen for further examination.

Limiting dilution was conducted for all MGR cultures to establish clonal lines. Limiting dilution was performed using the same protocol as above except that dilution factor was calculated with a goal of 10 parasites from each culture to be divided into a 96-well plate to allow individual parasites to form clonal lines. All wells that demonstrated stable growth within four weeks of limiting dilution were expanded into clonal lines. Clonal lines were isolated from MGR-1A (designated MGR_1Ac-1, MGR_1Ac-2, and MGR_1Ac-3), five from MGR-1B, from which three were chosen for study (MGR_1Bc-1, MGR_1Bc-2, and MGR_1Bc-3), and two from MGR-3 (MGR_3c-1, MGR_3c-2).

IC_{50} Calculation for Reduced-Diversity Subcultures and Clonal Lines

IC_{50} values were calculated from growth/inhibition curves for all clonal lines and reduced diversity cultures, and the results are shown in Table II. As expected, MG132 IC_{50} values for all MGR bulk cultures were greater than for those for 3D7-WT. IC_{50} values revealed increased tolerance to MG132 by a factor between 1.5- and 5-fold for all cultures tested, with the exception of MGR-3c-2, a clonal line that did not demonstrate increased MG132 tolerance. However, with the exception of the MGR-1A-Bulk culture, IC_{50} values of tolerant lines were not high enough to be reflect complete resistance, as determined by statistical significance with a P-value < 0.05. Although separated for only one round of selection, IC_{50} values for reduced diversity subcultures derived from MGR-1A are notably higher than those for reduced diversity subcultures derived from MGR-1B.

MG132 tolerance in the clonal lines was generally lower than reduced-diversity culture and bulk cultures, indicating that parasite population diversity could be a contributing factor to overall resistance [7,9-10]. Slight increases in mefloquine IC_{50} values were observed in most of the tolerant cultures, as well (Figure 6, Table II).

Sequencing of the β5 subunit of MGR and WT lines

Sequencing was performed to check for mutations in the β5 subunit of the 20S proteasome in the MGR and WT cultures as a possible genetic basis for resistance. Modifications to the β5 subunit have been linked to proteasome inhibitor resistance in humans in previous studies [35-41]. All MGR lines featured single point mutations in the β5 subunit, as listed in Table III. Neither the WT nor the MGR bulk cultures appear to have a mixed genotype at either of the codons in question, as illustrated by chromatograms from the WT and MGR bulk culture PCR-targeted β5 sequencing results shown in Figure 7. Figure 7A shows the DNA sequence flanking the WT codon in question and Figures 7B and 7C show the same gene segment in the MGR strains, highlighting the base pair change from A to G in MGR-1A and MGR-1B at position 244 (Table III). This mutation is non-synonymous and results in the amino acid change M22V. Figure 7D and 7E show WT and MGR-3 sequencing chromatograms for the area flanking the point mutation in culture MGR-3, A695G. Only a single peak exists for base pair 695 in MGR-3, indicating that it is the predominant genotype. The A/G mutation at position 695 is non-synonymous and results in the amino acid change G172E (Table III). Sequencing revealed that all reduced-diversity and clonal lines contained the same mutations found in their respective bulk MGR parental cultures [Table III].

Discussion

Intermittent compound exposure is a canonical method used to generate *P. falciparum* populations resistant to anti-malarial compounds [25,44]. This method has been used to generate resistant parasite lines to a variety of compounds, including artemisinin [22-25]. Artemisinin resistance was developed over several years and many selection cycles, and arose in steps beginning with tolerance, and eventually progressing to high-level resistance [25]. Drug tolerance, often a significant precursor to the development of full resistance, is currently of interest to researchers for understanding the evolution of resistance and for early detection of emerging resistance [16-21].

We have generated parasites with increased tolerance to proteasome inhibitor MG132 in fewer than ten rounds of intermittent drug pressure. In previous studies conducted by others, increased tolerance to canonical antimalarial compounds has been attained through similar methods [25,44]. According to authors, tolerance to mefloquine arose rather quickly, within two weeks of exposure to sub-lethal levels of mefloquine [25]. In contrast, tolerance to artemisinin arose more slowly, requiring ten rounds of drug exposure before measurable changes in drug sensitivity were observed [44]. In this study, tolerance to MG132 arose more slowly, in a similar manner to artemisinin, requiring at least seven rounds of drug exposure before a decrease in sensitivity to MG132 was observed.

We used two selection strategies of intermittent compound exposure to encourage the emergence of resistance. The first method involved an initial period of prolonged exposure to 50 nM MG132, a sub-lethal concentration, similar to a precedential method used to obtain mefloquine resistance [25]. Parasites grew under this mild drug pressure for six days, creating cellular stress, and then drug was removed before the complete loss of detectable growing parasites from the culture. During subsequent rounds of selection, the MG132 concentration was increased incrementally to levels lethal for WT parasites. The second method involved using a lethal concentration of MG132 for the first round of selection and using the same concentration in subsequent rounds, similar to the method employed in the previously mentioned study involving the generation of artemisinin resistance [44]. Beginning with a lower concentration of MG132 for selection mimicked the low levels of anti-malarial compounds that can be present in the bloodstream of patients during and after chemotherapeutic treatment regimens, a condition known to facilitate resistance development [2,3,16-18,24]. Reduced bloodstream drug concentrations can occur as a result of sub-therapeutic dosing or longer drug half-lives that allow residual, sub-therapeutic concentrations to linger in body for an extended time after treatment [2,3,16-18,24]. Beginning with a higher concentration of MG132 in rounds of selection mimicked therapeutic doses of antimalarial compounds that would likely be used in chemotherapy, and selection using this method has been successful for the development of drug resistant lines *in vitro* [2,3,15-18,22,44].

During growth under drug exposure, parasites were observed by microscopy to ascertain their ability to survive in the presence of high concentrations of MG132. All MGR lines

demonstrated increased survival with exposure to at least 250 nM MG132 for four days, a remarkable difference from the parental strain. During cycles of exposure to concentrations of 250 nM MG132 or greater, visible parasite growth diminished significantly in sensitive strains within two days of exposure. Survival of selection cultures was the first evidence of MG132 tolerance [16-19,25], and IC_{50} values were calculated following its appearance.

With the exception of MGR-1A Bulk culture and MGR-1As-22 reduced diversity subculture, MG132 IC_{50} values for selection cultures were not much greater than than that for 3D7-WT, so most cultures were not completely resistant to MG132 based on the statistical criterion of $P < 0.05$. Mean IC_{50} values were usually between two- and six-fold higher than for the WT parental line, and parasites survived several days longer while exposed to high concentrations of MG132, identifying the lines as MG132-tolerant [21-24]. In these lines, low-level tolerance to mefloquine, an anti-malarial compound [29,50] that was used to check for cross-resistance, was also observed. Although the changes in tolerance level for mefloquine in MGR cultures were slight, the apparent correlation between increases in tolerance to MG132 and mefloquine ($r = 0.83$, $P = 0.0001$, Figure 6) implies that parasites resistant to proteasome inhibitors could be resistant to other anti-malarial drugs. The observation of increased mefloquine IC_{50} values in MGR lines could be due to an increased ability to withstand cellular stress – conferred by mutations in the UPS β5 subunit – even if that stress is induced by exposure to a compound that does not target the UPS directly.

The MGR cultures contained point mutations in the β5 subunit of the 20S proteasome. These mutations do not appear at a comparable level in chromatograms for the WT cultures, nor does the WT nucleotide sequence appear in chromatograms for MGR lines. In other words, neither the MGR lines nor the WT parental line exhibit a mixed genotype. In addition, the nucleotide variants in question are not listed in the PlasmoDB database as previously observed single-nucleotide polymorphisms (SNPs) in the 3D7 strain of *P. falciparum* [42], so likely arose during our selections. The emergence of these traits to predominance in cultures subjected to selection implies that these mutations result from MG132 resistance selection, as *P. falciparum* does not readily retain mutations without selective pressure [18,20, 49-51]. This assertion is further supported by the fact that many studies have linked point mutations in the β5 subunit of the 20S proteasome to SMPI resistance in other systems [35-41].

A diagram showing the locations of the point mutations in the β-5 subunit protein sequence in MGR lines is presented in Figure 8. The mutations are shown in relation to several important features of the 20S proteasome β-5 subunit. Amino acids known to be part of the active site and the S1 binding pocket [46-48] are indicated. As indicated in Figure 8A, the mutation in MGR-1A and MGR-1B, M22V, is adjacent to the amino acid Ser21, a residue of the S1 binding pocket, a cavity in the tertiary protein structure known to bind small-molecule proteasome inhibitor compounds that affect β-5 subunit catalytic function [46-48]. The mutation in MGR-3, G172E, is in close proximity to a cluster of residues that are part of the active site, Asp166, Ser169, Gly170 [46-48]. This substitution could result in changes in proteasome catalytic function, as the replacement

of glycine with glutamic acid could cause a change in either protein conformation or enzymatic activity. Increased proteasome activity, either by enhancement of catalytic function or overexpression of proteasomes, has previously been linked to proteasome inhibitor resistance [36-38, 40-41]. Locations of mutations in the β5 subunit that have been linked to proteasome inhibitor resistance (human proteasome β5 subunit residues M45, A49, A50, C52, and C63) [35-41] are also indicated in Figure 8. As shown in the model of the homologous *S. cerevisiae* β5 subunit (Figure 8B), both mutations are predicted to be in the same general region within the protein tertiary structure, suggesting the importance of this region for the action of MG132 [32].

We used two methods to separate genetically distinct lines derived from the original bulk selection culture. One method involved the use of dilution cloning to generate "reduced diversity" subcultures derived from one or a few individual parasites. These parasite populations were then analyzed by two rounds of screening for MG132 tolerance before selecting a few sublines to be used for full growth/inhibition curves. The other method involved the use of dilution cloning with a greater dilution factor in order to establish several clonal lines for further examination. Although the point mutations observed in the parental resistant populations were present in all reduced diversity cultures and clonal lines derived from them (Table II), the IC_{50} values we observed for reduced diversity cultures and clonal lines were not equal to those observed for MGR parental bulk cultures. Tolerance levels differed, suggesting that secondary genetic variation elsewhere in the genome, influencing either MG132 tolerance or overall parasite fitness, was likely present. This question could be addressed more rigorously by whole-genome sequencing of reduced diversity cultures and clonal lines, to determine whether such mutations could

be identified, in the future. The fitness cost of MG132 tolerance in *P. falciparum* is unknown, so these two methods were employed to ensure that stable cultures could be established by at least one method. Generating many subcultures and screening them proved to be valuable. The reduced-diversity subcultures of MGR-1A and MGR-1B demonstrated differing tolerance levels, and the ability to select from over 40 lines for robust growth and higher tolerance resulted in less risk of resistant culture failure in subsequent experiments. Overall, the reduced diversity lines had higher MG132 IC_{50} values than the clonal lines. One of the MGR-3 lines, MGR-3c-2, did not demonstrate tolerance to MG132 as assessed by IC_{50}, which could mean that the overall fitness of that parasite line was low, or that secondary mutations that resulted in MG132 sensitivity could have occurred in that line.

Our studies have shown that resistance to the SMPI compound MG132 arises quickly and predictably in *P. falciparum*. Several parasite lines were generated with single, stable point mutations in the β5 subunit that were retained through several rounds of selection, cloning, experimentation, and isolation of DNA for sequencing. Whether proteasome inhibitors could be utilized in the chemotherapeutic treatment of malaria is unclear, even though they are used as drugs for treatment of other conditions [34-41]. Historically, tolerance and resistance mutations selected for by anti-malarial drugs have arisen rapidly in the field, which has led to failure of some anti-malarial drugs very soon after their introduction [1-3,15,17]. However, these were instances in which anti-malarial compounds were administered as monotherapies, rather than as components of combination therapies [2-4,18,19]. The distinct mechanisms of SMPI action, as compared

to anti-malarial drugs currently used for chemotherapy, could make them effective drug cocktail components, although toxicity studies are necessary [2-4,18,19]. In previous studies, cases of multi-drug resistance have been observed and traced to mutations in genes such as pfMDR (*P. falciparum* multi-drug resistant locus), and pfCRT (*P. falciparum* chloroquine resistance transporter) [3,5,8,44]. The observed correlation between slight increases in MG132 and mefloquine tolerance would need to be verified through further study to determine whether the correlation is specific to these tolerance-selected parasite lines, or indicative of a connection between UPS function and the action of quinolone chemotype anti-malarial compounds [20,49]. This idea is worth exploring in the future, especially since genome-wide association studies (GWAS) of *P. falciparum* field strains have revealed that several components of the UPS have undergone positive selection related to drug resistance [20,49]. This may be due to the role of the UPS in cellular stress responses [20,26,27]. The mutations generated in this study add to the catalogue of tolerance-associated genotypes, information that may prove useful eventually for predicting and tracking resistance to anti-malarial compounds.

Figures and Tables

Table I: Selection of MG132 Resistance in *P. falciparum* cultures[a]			
Selection Culture:	**MG132 Concentration (x number of cycles):**	**Reduced Diversity Cultures:[b]**	**Clonal Lines Isolated:[b]**
MGR-1A	50 nM (1x)	52 Lines total	3 Lines Total
	75 nM (1x)	MGR-1As-1	MGR-1Ac-1
	150 nM (2x)	MGR-1As-22	MGR-1Ac-2
	250 nM (4x)		MGR-1Ac-3
MGR-1B	50 nM (1x)	47 lines total	5 Lines Total
	75 nM (1x)	MGR-1Bs-13	MGR-1Bc-1
	150 nM (2x)	MGR-1Bs-21	MGR-1Bc-2
	250 nM (4x)		MGR-1Bc-3
MGR-3	250 nM (5x)		2 Lines Total
	500 nM (1x)		MGR-3c-1
	1,000 nM (1x)		MGR-3c-2

[a] MG132 concentrations shown are those that were used for selection cycles, in order listed.

[b] Numbers of total lines resulting from culture growth after limiting dilution are indicated in the first line. Parasite lines (with names) listed below were used in further study. Poisson probabilities of lines being clonal are as follows. “Clonal” is defined as a culture or line that originated from one parasite, seeded during limiting dilution. Poisson probability calculation is based on the number of wells, among all wells potentially inoculated, that ultimately yielded viable parasite subcultures. For all “clonal” lines: $P > 0.99$. For “reduced diversity” cultures: $P = 0.90$ and $P = 0.91$ for MGR-1A and MGR-1B, respectively. For the MGR-1B parental culture, five clonal populations arose in 96-well plates, but only three wells were expanded into clonal lines.

Table II: IC_{50} Values of Reduced Diversity Cultures and Clonal Lines [a]					
Type:		**MG132 IC_{50} (nM)**	**Fold Change**	**Mefloquine IC_{50} (nM)**	**Fold Change**
WT 3D7 Parent Line	3D7 WT	34 ± 6.5		7.3 ± 1.3	
Bulk Selection Cultures:	MGR-1A-Bulk	158 ± 35**	4.6	17.4 ± 3.9	2.4
	MGR-1B-Bulk	127 ± 59	3.7	15.0 ± 5.4	2.0
	MGR-3-Bulk	76 ± 17	2.3	13.0 ± 2.9	1.8
Reduced Diversity Subcultures:	MGR-1As-1	115 ± 65	3.4	21.2 ± 8.8	2.9
	MGR-1As-22	152 ± 52*	4.5	22.7 ± 5.6	3.1
	MGR-1Bs-13	64 ± 18	1.9	12.9 ± 3.0	1.8
	MGR-1Bs-21	92 ± 12	2.7	17.3 ± 2.0	2.4
Clonal Lines:	MGR-1Ac-1	56 ± 12	1.7	10.0 ± 2.2	1.4
	MGR-1Ac-2	70 ± 25	2.1	12.0 ± 4.0	1.6
	MGR-1Ac-3	68 ± 37	2.0	14.0 ± 6.5	1.9
	MGR-1Bc-1	80 ± 20	2.4	15.9 ± 3.6	2.2
	MGR-1Bc-2	56 ± 10	1.6	11.0 ± 2.3	1.5
	MGR-1Bc-3	76 ± 4	2.3	14.8 ± 1.3	2.0
	MGR-3c-1	69 ± 20	2.0	14.1 ± 3.1	1.9
	MGR-3c-2	33 ± 2	1.0	8.5 ± 0.1	1.2

[a] IC_{50} values are the mean and standard error of at least two independent experiments
* Mean IC_{50} value is significantly different than wild type, $P \leq 0.05$
** Mean IC_{50} value is significantly different than wild type, $P \leq 0.01$

Table III: Point Mutations in the β5 Subunit of the 20S Proteasome[a]		
Selection Culture:	**Base Pair Change:**	**Amino Acid Change:**
MGR-1A	A244G	M22V
MGR-1B	A244G	M22V
MGR-3	G695A	G172E

[a] Non-synonymous mutations as detected by sequencing of the β5 subunit of the wild type 3D7 parent line and MGR resistance lines. Mutations listed were confirmed in all clonal lines and reduced diversity cultures associated with selection cultures listed, by PCR-targeted resequencing.

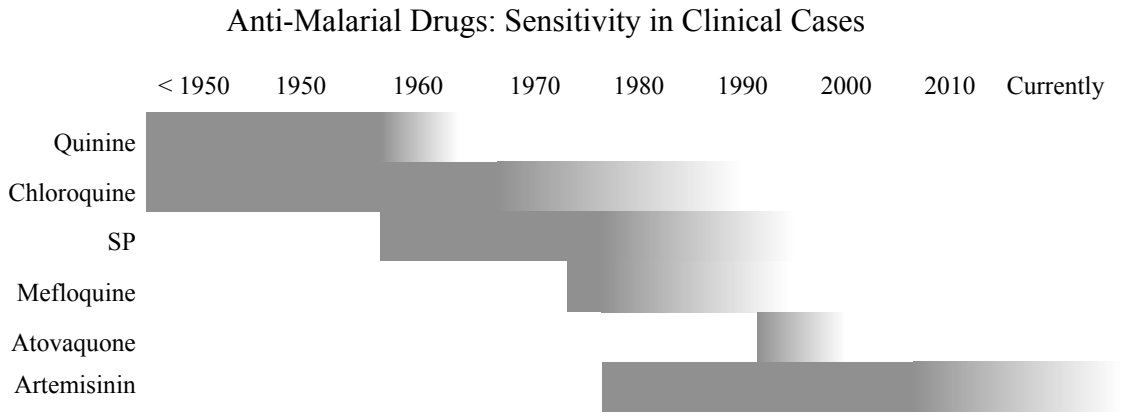


Figure 1: Spread of resistance to some common anti-malarial drugs by year [1-4]. Overall sensitivity to anti-malarial compounds in historical clinical cases is shown. Shading indicates initiation of widespread clinical use of each compound, and fading indicates waning of overall potency for treatment of symptomatic malaria cases. SP: sulfadoxine-pyrimethamine

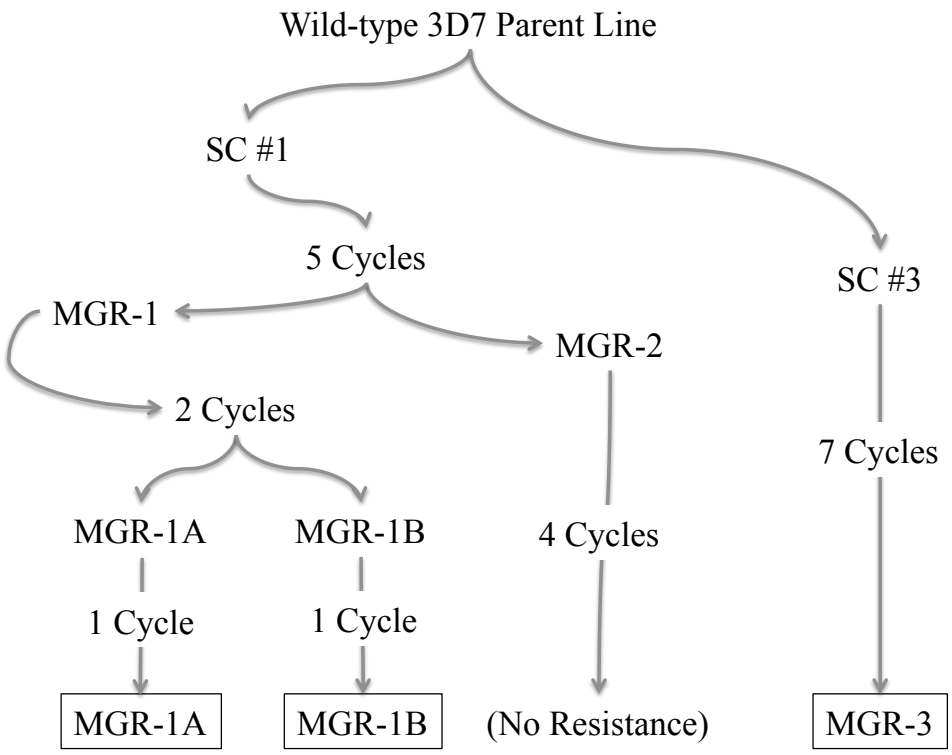


Figure 2: Development of MG132-resistant cultures through cycles of intermittent parasite exposure to MG132. Origin and generations of "MGR" resistant lines are shown, as well as number of selection cycles between steps. Selection culture #1 (SC #1) was exposed to five cycles of selection before being split into MGR-1 and MGR-2 before tolerance was detected; then MGR-1 was split into MGR-1A and MGR-1B. Selection culture #3 (SC #3) was an independent line that was never divided into separate selections. MGR-2 did not acquire observable tolerance and was eliminated from the study.

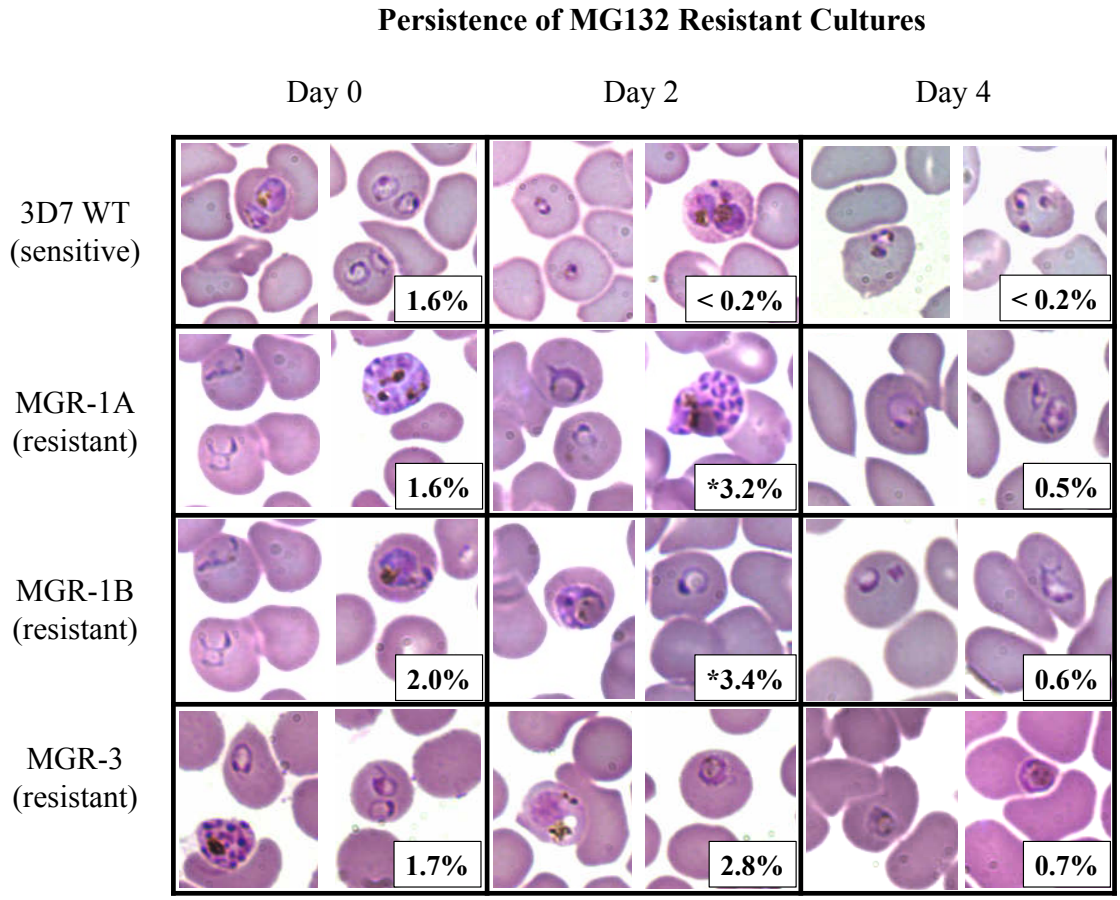


Figure 3: MG132 tolerance of cultures subjected to resistance selection, assessed by observed persistence. During cycles of MG132 selection, cultures were monitored by microscopy to check for phenotypic changes in response to drug pressure. Photos shown compare the persistence of wild type parasites to MGR lines after 0, 2 and 4 days of drug pressure (250 nM MG132). Parasitemia of cultures shown is indicated in the lower right corner of each photo. Those marked with an asterisk (*) were diluted 1:5 with fresh media and drug on the day the photo was taken, before continuing incubation.

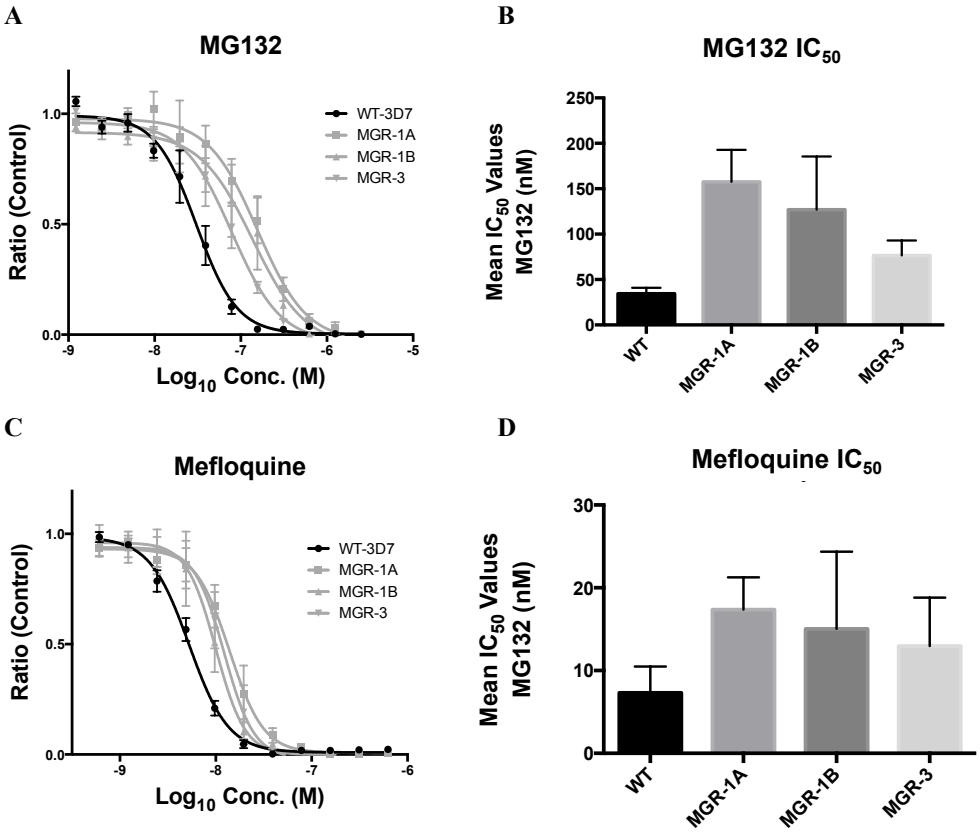
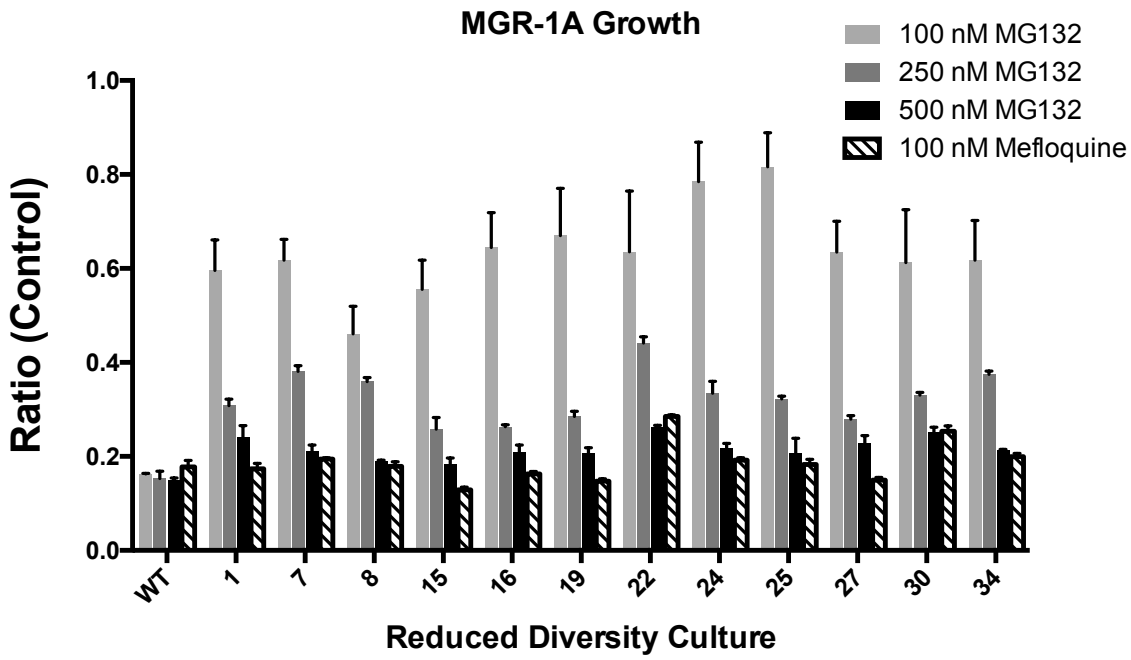


Figure 4: Resistance levels of MGR-1A, MGR-1B, and MGR-3. IC_{50} curves for bulk resistance cultures were compared to WT-3D7. Comparative growth/inhibition curves for MG132 and mefloquine are shown in A and C, respectively. Mean IC_{50} values are shown in panels B and D.


MGR-1A Growth
100 nM MG132
250 nM MG132
500 nM MG132
100 nM Mefloquine
Ratio (Control)
1.0
0.8
0.6
0.4
0.2
0.0
WT
1
7
8
15
16
19
22
24
25
27
30
34
Reduced Diversity Culture

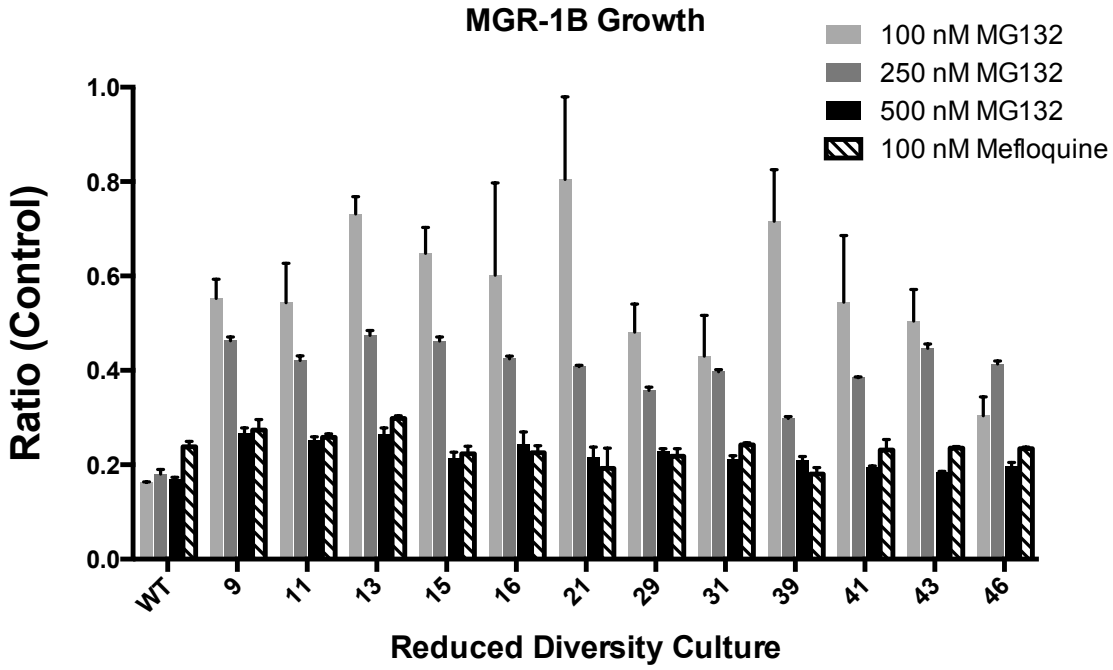

MGR-1B Growth
100 nM MG132
250 nM MG132
500 nM MG132
100 nM Mefloquine
Ratio (Control)
1.0
0.8
0.6
0.4
0.2
0.0
WT
9
11
13
15
16
21
29
31
39
41
43
46
Reduced Diversity Culture

Figure 5: Reduced-diversity subcultures were screened for tolerance to MG132 by growing each culture under drug pressure for three days. Each culture was grown in the presence of varying concentrations of MG132 or 100 nM mefloquine (anti-malarial control) and ratios of parasite presence (DNA content of culture) after growth period compared to unexposed control cultures are shown.

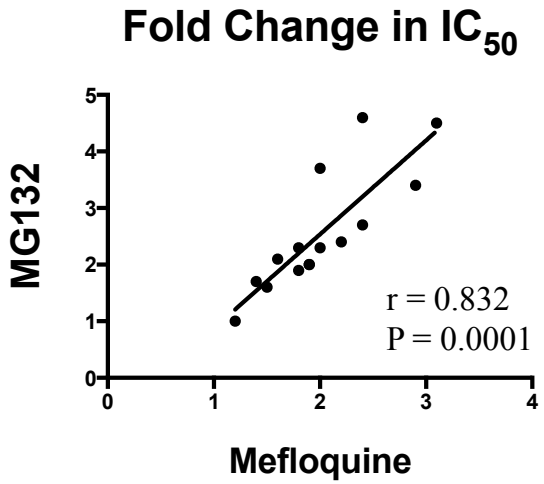


Figure 6: The correlation between fold changes in MG132 and mefloquine IC_{50} values observed in MGR lines is shown. Each point represents one individual MGR line (numerical values for individual lines are listed in Table III), X and Y axes represent fold changes in mefloquine and MG132 IC_{50} values, respectively. Plot was generated with GraphPad Prism® 6 software linear regression algorithm with Pearson's correlation calculation (GraphPad Software, Inc., La Jolla, CA).

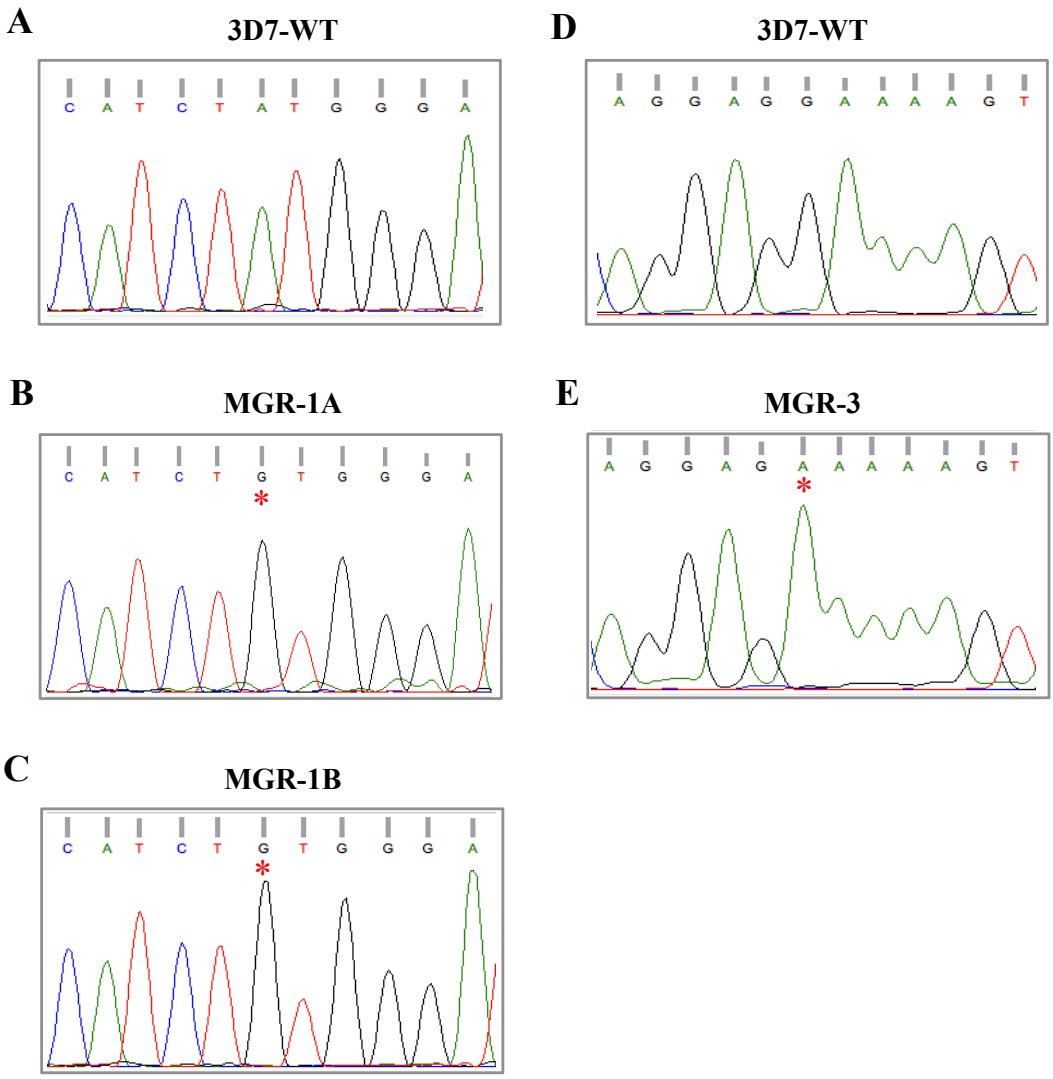
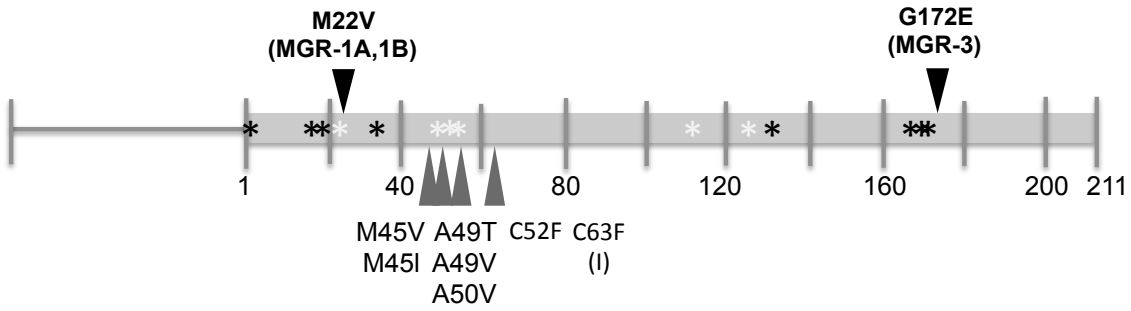


Figure 7: Sequencing chromatograms of genomic segments within which non-synonymous mutations were detected in resistant lines, with WT 3D7 chromatograms for comparison. WT-3D7, MGR-1A, MGR-1B chromatograms for the genomic segment flanking base pair 244 are shown in sections A, B, and C, respectively. WT-3D7 and MGR-3 chromatograms for the genomic segment flanking base pair 695 are shown in D and E, respectively. Each changed base pair is indicated by a red asterisk.

A.


M22V
(MGR-1A,1B)
G172E
(MGR-3)
1
40
80
120
160
200
211
M45V
M45I
A49T
A49V
A50V
C52F
C63F
(I)

B.

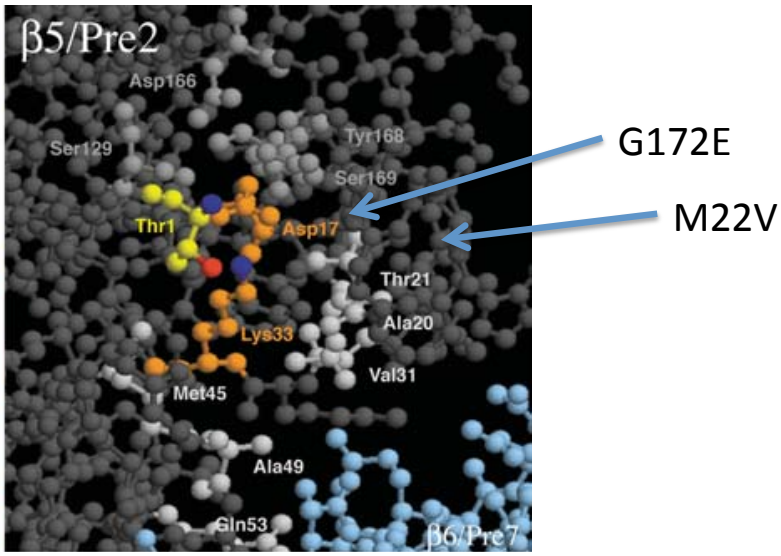

β5/Pre2
Asp166
Ser129
Tyr168
Ser169
Thr1
Asp17
Thr21
Ala20
Lys33
Val31
Met45
Ala49
Gln53
β6/Pre7
G172E
M22V

Figure 8: A) Diagram of the *P. falciparum* 20S proteasome β5 subunit mature protein sequence. Tick marks begin at the N-terminal threonine (Thr1), since the sequence upstream of it is cleaved to produce the mature, active protein. Active site residues (Thr1, Glu17, Arg19, Lys33, Ser129, Asp166, Ser169, Gly170) are each marked with a black asterisk, and key residues of the S1 binding pocket (Thr1, Thr21, Gly47, Ala49, Ala50, Asp114) are each marked with a white asterisk. Black arrows at the top of the diagram point to the amino acid changes in MGR cultures generated in this study. Gray arrows below the diagram point to mutations known to be associated with proteasome inhibitor resistance, published previously by others [29-34]. Amino acid #63 is cysteine in the human proteasome (precedent for proteasome inhibitor resistance in published literature, add REFs), but is isoleucine in *P. falciparum*, and the distinction is noted parentheses. **B)** Three-dimensional model of the homologous *S. cerevisiae* proteasome β5 subunit, highlighting locations of key residues within the active site and the S1 binding pocket. Positions at which mutant residues of MGR lines (M22V and G172E) are predicted to be located are indicated by arrows [32]. Explanation of coloring is taken from Reference 32: "The hydroxyl oxygen of Thr1 is red and the nitrogen of its free amino group is dark blue; other parts are yellow. Asp17 and Lys33 are colored orange except the epsilon-amino group of Lys33, which is also dark blue. The conserved residues Ser129, Asp166 and Ser169 (as well as the variable residue 168) are shown in slightly brighter tone of the subunit color. Together with Lys33 and Asp17, these conserved residues contribute to the charge relay system surrounding Thr1. The variable residues at positions 20, 21, 31, 45, 49 and 53 are each colored in a still brighter tone and form the surface of the substrate binding pocket."

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Chapter IV:

Summary and Conclusions

Study of the ubiquitin proteasome system (UPS) has proven valuable in virtually all well-studied eukaryotic organisms [1-5]. Protein turnover and ubiquitin modification of cellular proteins are of critical importance to general cell biology, and research conducted in these areas has increased the knowledge not only of the UPS itself, but also of many other cellular processes [5-11]. The eukaryotic UPS has been shown to directly affect the cell cycle, transcription, translation, post-translational modification, protein trafficking, actin remodeling, secretion, and cell-cell interactions [1-6]. The components of this study of proteasome inhibition in *Plasmodium falciparum*: the use of low concentrations of MG132 for incomplete proteasome inhibition, the use of high concentrations for rapid, reversible UPS inhibition, parasite recovery following short-term MG132 exposure, and the generation of MG132 tolerance in *P. falciparum* lines, support the importance of the UPS in the malaria parasite life cycle. The data that I have generated in this study, taken together with those of others, suggest a significant role for the parasite UPS in *P. falciparum* stage progression and drug resistance [12-14, 17-21].

It has been known for years that the genome of *P. falciparum* contains homologs of canonical components of the UPS, and that complete UPS inhibition halts parasite replication and division *in vitro* [11-14]. Although the essentiality of the UPS for parasite proliferation has been well established, knowledge gaps remain regarding its function during the complex life cycle of *P. falciparum* [11-14]. Precedents in other systems have shown that the UPS plays a central role in many cellular processes, which is likely true of *P. falciparum*, as well [1-11]. Manipulation of *P. falciparum* UPS function could further elucidate the dynamics of other processes such as the cell cycle regulation, stage

progression, transcription, protein homeostasis, host-pathogen interactions, and general malaria pathogenesis [1-11]. My work, in conjunction with that of others, has shown that the UPS likely plays a significant role in parasite development during all erythrocytic stages [10, 12].

Chemical inhibition of the UPS, via the use of compounds that directly interfere with UPS function, is highly valuable as a research tool for studying the biology of complex cellular systems [5-10]. Targeting of the proteolytic activity of the 20S proteasome core particle, the protein-degrading component of the UPS, with small-molecule proteasome inhibitor compounds (SMPIs) has been utilized by biologists for study of the UPS and the systems affected by the UPS [5-12]. Proteasome inhibition has also been explored clinically as a treatment strategy for disease, leading to the use of bortezomib as an anti-cancer chemotherapeutic drug [15-17]. Infectious disease biologists have also explored the prospect of using proteasome inhibition for improved control of pathogens [12, 14, 17-22].

One of the goals of this study was to better understand the dynamics of the *P. falciparum* UPS through proteasome inhibition by SMPIs. In this work and that done by others, SMPIs have been shown to arrest the proliferation of *P. falciparum* in culture [12, 14, 20-22]. Commercially available SMPIs vary widely in efficacy, stability, specificity, toxicity, and reversibility; so data regarding compound performance in particular systems is required to evaluate their usefulness in experimental applications [6-12]. Three compounds – MG132, lactacystin, and bortezomib – were chosen for this work because

of their previously described ability to inhibit the growth of *P. falciparum* in culture [10, 12, 20-22]. MG132 performed arguably better for efficient low-dose proteasome inhibition than the other two compounds tested, causing a measurable stage delay without causing complete lethality in culture. Although MG132 targets cysteine proteases in *P. falciparum* in addition to the proteasome (e.g., falcipains), this has only been shown to occur at higher concentrations than those required for UPS inhibition [22]. However, further study would need to be completed to assess the precise compound levels that result in off-target (non-UPS) effects [22].

The delay in stage progression observed in *P. falciparum* due to exposure to sub-lethal MG132 concentrations is most pronounced during the trophozoite stage. In addition, ubiquitylated proteins accumulate in maturing trophozoites within six hours of exposure, suggesting that proteasome-related protein turnover is critical during that stage. From these data, it can be inferred that the UPS likely plays a crucial role in trophozoite developmental progression. This information could lead to further study of parasite proteome maintenance and turnover during trophozoite stage, via the UPS. The UPS also has been shown to have critical roles in global transcription, translation integrity, and proteome maintenance in other systems, which supports the likelihood of a central role in *P. falciparum* proteome homeostasis and gene expression [5, 6, 8, 10, 23-26].

The dynamics of drug resistance are also important for the study of parasite biology [25-32]. One canonical function of the UPS is cellular stress tolerance, which is a critical component of the early stages of drug resistance development [5, 27-32]. Furthermore,

various components of the UPS have been identified in genome-wide association studies as being under selection in malaria endemic areas, which implies that the UPS could have critical roles in drug resistance and/or the fitness of parasite populations in the field [25-26]. Therefore, another valuable contribution of this study is the generation of MG132 tolerance in parasite selection cultures following repeated intermittent exposure to MG132.

The generation of MG132 tolerance after fewer than 10 rounds of selection is a significant result of this work. Although the measured IC_{50} values of the MGR lines are not much higher than wild type, these lines were able to survive several days longer in the presence of concentrations of MG132 that are toxic to the parental wild type line. The increased ability of parasites to survive exposure to high levels of anti-malarial compounds, even if proliferation is slow, has been identified as a sign of emerging resistance and predictor of the possible reductions in clinical efficacy [27-32]. The observation that tolerance to an anti-malarial compound does not always result in an increased IC_{50} value has also been made in regard to artemisinin resistance [27-28]. In the field, tolerance would likely manifest as parasites that exhibit prolonged clearance times *in vivo*, or that are slower to respond to clinical anti-malarial drug therapy [27-32]. These parasites tend to linger in the bloodstream of patients longer during malaria chemotherapy; a precursor to the development and eventual spread of fully resistant parasites [27-32]. Drug tolerance, as seen in the MGR lines generated by this study, is a common bridge between sensitivity and resistance in parasites and is of critical importance in the understanding of the emergence of resistance [27-32].

In conclusion, study of proteasome inhibition in *P. falciparum* has the potential to expand in many directions. The development of proteasome inhibitors as components of drug cocktails in the chemotherapeutic treatment of malaria is a strong possibility [12-14, 22, 29]. The fact that SMPIs significantly affect parasites at concentrations much lower than those that affect the human proteasome *in vivo* indicates the potential use of proteasome inhibition in anti-malarial chemotherapy in humans [12-14, 22, 27, 29]. It is also known that even low-level interference in proteasome function can increase the clinical efficacy of other drugs and overcome resistance [17, 27, 29]. The MG132-tolerant parasite lines generated by this study could be used for further study of drug resistance mechanisms resulting from UPS-related stress tolerance, subjected to more compound exposure cycles for the generation of higher levels of MG132 resistance, or tested for cross-resistance to other proteasome inhibitors or anti-malarial compounds.

Studies like this one are important for filling knowledge gaps related to general parasite biology and the genesis of drug resistance. The next steps fort advancing our understanding the *P. falciparum* UPS and its roles in parasite biology could be numerous. The MG132-tolerant lines generated by this work, in parallel with other drug-resistant lines, could be used to measure cross-resistance to a panel of anti-malarial compounds. MG132 could be used to study short- and long-term effects of low-level proteasome inhibition, such as impacts on gene expression, stage progression, or protein trafficking. Overall, the study of the roles of the UPS, UPS inhibition, and SMPI resistance in *P. falciparum* have far-reaching and significant implications for our understanding of

parasite cell biology, malaria infection dynamics, anti-malarial drug development, and anti-malarial drug resistance – critical areas in which increased knowledge will advance our understanding of parasite biology and enhance our ability to control malaria infections in humans.

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Chapter V:
Appendices

Appendix I:
Protocols

Protocol: Proteasome Activity Assay

Substrates:

1) Trypsin-like activity: Boc-LRR-AMC (FW 773.76)

   (Catalog # S-300, Boston Biochem, Inc., Cambridge, MA)

   a. DMSO stock = 2 mM (20x) = add 3,231 uL DMSO to 5 mg

2) Chymotrypsin-like activity: Suc-LLVY-AMC (FW 763.9)

   (Catalog # S-280, Boston Biochem, Inc., Cambridge, MA)

   a. DMSO stock = 2 mM (20x) = add 3,190 uL

3) Caspase-like activity: Z-LLE-AMC (FW 664.8)

   (Catalog # S-230, Boston Biochem, Inc., Cambridge, MA)

   a. DMSO stock = 2 mM (20x) = add 2,776 uL

Buffers:

Assay/Lysis Buffer, pH 8

50mM Tris HCl

25 mM KCl

10 mM NaCl

1 mM MgCl

1 mM ATP

*2% Glycerol in lysis buffer only

Add to 1 L

Concentration:	**Add to 1 L:**	**Add to 100 mL (1x)**	**Add to 100 mL (10x)**
50 mM Tris HCl	50 mL of 1 M Stock	5 mL of 1 M Stock	50 mL of 1 M Stock
25 mM KCl (FW 74.55)	1.864 g	0.186 g	1.864 g
10 mM NaCl (FW 58.44)	0.584 g	0.058 g	0.584 g
1 mM $MgCl_2$ (FW 95.21)	0.095 g	0.010 g	0.095 g
1.0 mM ATP (FW 551.14)	0.552 g	0.055 g	0.552 g
*2% Glycerol (Lysis Buffer)		*2ml	
H_2O	950 ml	95 ml or 93ml	50 ml

Lysate:

Add ~5x volume lysate with agitation beads, vortex, alternate with ice 6x

Assay, each 3x sample:

15 uL Lysate

87 uL Assay Buffer

12 uL 10x Inhibitor or DMSO

6 uL 20x Substrate

Divide into 3x 35 uL aliquots for tech reps.

Incubate at 37^{o}C 90 min in the dark, read at excitation/emission 380/460 nm λ_{em}

Activity Assay Test: Capacities of Assay Substrates

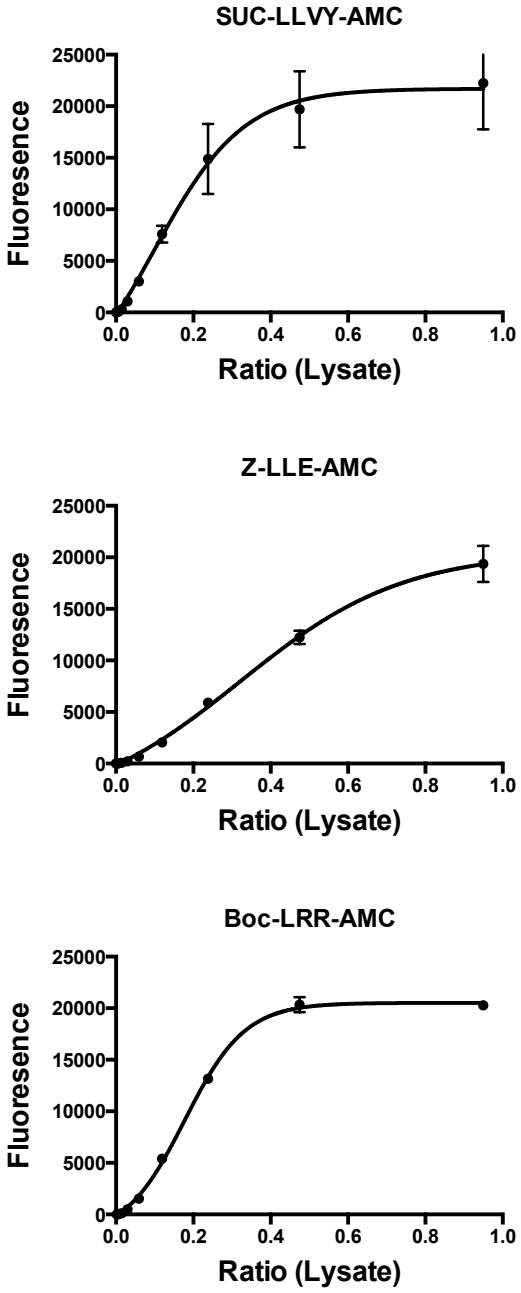

Figure S1: Enzymatic activity assay linear range assessment in Jurkat cells. X axis = lysate concentration.Y axis = fluorescence.

Protocol: Parasite Delayed Death Assay (Using SYBR Green)

Cultures: Culture medium RPMI with 0.5% Albumaxx II, 1% hematocrit, synchronized ring stage parasites. Incubation is done under standard conditions (provide details).

Compounds (kept in 10 mM DMSO stock), highest concentration used in assay:

MG132 (Sigma), 2.5 μM

Azithromycin (Sigma), 20 μM

Mefloquine (Sigma), 0.625 μM

24 hour incubation: begin with cultures at 1.5-2.0% parasitemia

72 hour incubation: begin with cultures at 1% parasitemia

120 hour incubation: begin with cultures at 0.2% parasitemia

Preparation of drug master plates ("source plates"):

1) Prepare drug plates as "source plates" for addition to culture:
    a. Dilute compound stock solution (10 mM or 25 mM in DMSO) in culture medium to a final volume of 800 uL at concentration of 4x appropriate highest assay concentration (e.g., 10 uM for 2.5 uM final treatment concentration)

b. Take 400 uL from first well, add that volume to 400 uL of culture medium in the next well and mix, perform successive serial 2x dilutions in culture medium to obtain 12 concentrations of each drug, one drug per row of source plate

Initial addition of drugs to parasite cultures

2) Dispense parasite source culture and drug into wells of a 96-well culture plate:
    a. Add 150 uL culture medium and 2 uL of infected RBCs at appropriate parasitemia to each well. (Resulting in 1% hematocrit after addition of 50 uL of drug from the source plate.)
    b. Make at least four replicate wells for each drug concentration assayed (1 plate per drug, with four rows of drug assay cultures and two rows of control cultures with no drug)
    c. Dispense 50 uL of 4x concentrated drug or plain media into each well from the source plate, mix wells

Incubation with drugs

3) Incubate all assay plates under standard conditions (37 °C, 5% CO_2 + 1% O_2 + balance N_2), using secondary containers (plastic bins with open water reservoirs) to assure humidity and minimize well drying

Harvest incubation cultures

4) Harvest after incubation period*

a. **Develop and read cultures:**

i. Mix cultures to distribute cells uniformly in medium, then transfer 40 uL of each culture into a black, clear-bottom, 384-well plate (Greiner Bio-One, Inc., Monroe, North Carolina, USA)

ii. Add 10 uL of SYBR Green buffer (0.16% saponin, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1.6% (w/v) Triton X-100) with 1/1000 SYBR Green to each well of 96 well plate

iii. Seal plate with foil and keep at room temperature for 1-24 hours

iv. Read plates using SYBR standard protocol (see Chapter 2, Methods)

*** Feed 120-hour cultures after 72 hours of incubation:**

v. Remove 160 uL of culture medium from each well of 120-hour culture plate, being careful not to disturb cells in the bottom

vi. Add 120 uL of fresh culture medium

vii. Add 40 uL of 4x concentrated drug, diluted the same way as before, from fresh source plate, mix

viii. Incubate plates under the same growth conditions for an additional 48 hours

ix. After incubation, develop and read cultures using SYBR buffer as described above

Parasite Minimal Medium (Export Media for Recover of Secreted Proteins)

*These components are for incomplete media. Final concentrations of 0.5% Albumaxx II or 5-10% human serum should be added for complete media preparation.

Inorganic salts (mg/L)

$Ca(NO_3)_2 \cdot 4H_2O$	100.0
KCl	400.0
$MgSO_4$ (anhydrous)	48.8
NaCl	5,300
$NaHCO_3$	2,000
Na_2HPO_4 (anhydrous)	800

Amino acids (essential amino acids in bold) (mg/L)

Adenine A	18
Alanine A	57
Arginine	57
Asparagine	57
Aspartic acid	57
Cysteine	**57**
Glutamic acid	**57**
Glutamine	**300**
Glycine	57
Histidine	57

myo-Inositol I	57
Isoleucine	**57**
Leucine L	285
Lysine	57
Methionine	**57**
p-Aminobenzoic	6
Phenylalanine	57
Proline	**57**
Serine	57
Threonine	57
Tryptophan	57
Tyrosine	**57**
Valine	57

Other components (mg/L)

d-**Ca pantothenate**	0.25
d-**Glucose**	2,000.0
HEPES	5,958.0
Hypoxanthine	50

References for Minimal Medium Recipe:

Divo, A. A., T. G. Geary, N. L. Davis, and J. B. Jensen**. (1984)** Nutritional requirements of *Plasmodium falciparum* in culture. I. Exogenously supplied dialyzable components necessary for continuous growth. **J. Protozool.** 32**:**59-64.

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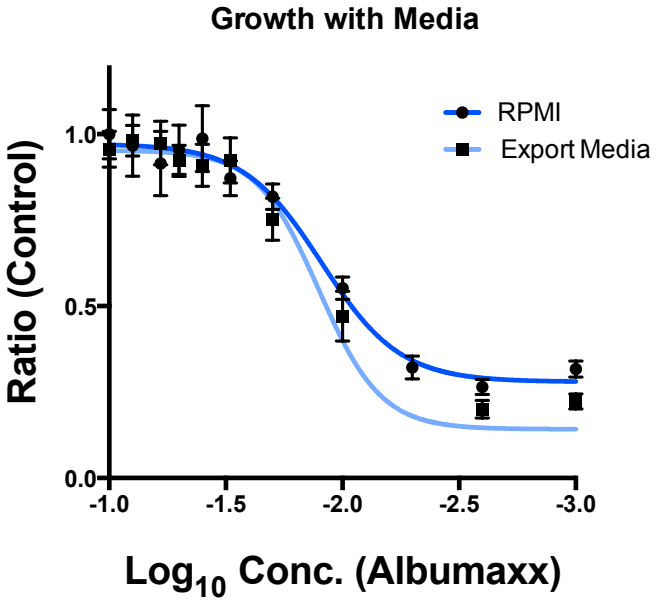

Figure S2: Growth of parasites in minimal media compared to standard RPMI. X axis = Log_{10} (concentration of Albumaxx II stock); Y axis = growth of parasites as measured by SYBR Green analysis, compared to growth in complete RPMI medium.

Appendix II:
Additional Figures

Proteasome inhibition and HRP II in *P. falciparum* parasites

The ubiquitin-proteasome system (UPS) has wide-ranging effects on the biology of *Plasmodium falciparum* [1-3]. Interference in UPS function by chemical proteasome inhibition has been explored as both a method for understanding *P. falciparum* biology and as a potential strategy for anti-malarial chemotherapeutic treatment [1-3]. During the course of my experiments in proteasome inhibition in *P. falciparum*, I observed that exposure to MG132, a small-molecule proteasome inhibitor, resulted in the accumulation of histidine-rich protein II (HRP II) in cultured parasites. In order to assess the scope of this effect, experiments were performed to visualize HRP II levels in parasites or culture medium following incubation of cultures in the presence of MG132.

HRPII is a *P. falciparum* parasite protein that is secreted from infected red blood cells of malaria-infected individuals [4-7]. It accumulates in large quantities and is readily detectible in the blood of infected persons; and its presence is often used as an indicator of malaria infection in rapid diagnostic tests [4-7]. While the details of HRP II function in the *P. falciparum* life cycle have not been fully investigated, the protein likely plays a role in malaria pathology [4-7].

In order to detect HRP II secretion from *P. falciparum*-infected red blood cells, early ring-stage 3D7 cultures were incubated in the presence of 0 nM – 50 nM MG132 for 12 and 24 hours, and HRP II present in culture medium was assessed. A western blot of relative amounts of HRP II captured from culture media over time is shown in Figure S3.

A visible reduction in HRP II secretion is apparent in cultures exposed to 50 nM MG132 for 12 and 24 hours. No visible reduction in HRP II secretion is apparent in cultures grown without drug or in cultures exposed to 100 nM mefloquine, used as an anti-malarial control compound [8].

In order to assess HRP II accumulation in *P. falciparum* parasites due to exposure to MG132, parasites from the experiment above were isolated and cellular protein assayed by western blot. Figure S4 shows a western blot of HRP II in cell lysates of cultures exposed to 0 – 50 nM MG132 or 0 – 60 nM mefloquine. Heightened levels of HRP II are detected in parasites incubated in the presence of 12.5 nM – 100 nM MG132. Very little accumulation of HRP II is detected in parasites incubated without drug or with mefloquine, an anti-malarial compound known to arrest *P. falciparum* growth within 24 hours [8].

The experiments above suggest that proteasome inhibition by MG132 interferes with secretion of HRP II from malaria-infected red blood cells. More research would be required in order to fully understand the mechanism of this possible interference. The inability of mefloquine to cause the same level of HRP II accumulation in the growth medium suggests that this effect is specific to the action of MG132, and not simply a consequence of arrested parasite growth. Although MG132 has been shown to target both the 20S proteasome and falcipains (cysteine proteases) in *P. falciparum,* these data suggest proteasome inhibition is most likely the cause of HRP II accumulation [9]. The ability of low concentrations of MG132 to cause accumulation of HRP II points to

proteasome inhibition as the primary cause of this accumulation, since MG132 has only been shown to inhibit falcipains at concentrations above 100 nM [9].

In order to fully assess the relationship between HRP II and proteasome inhibition, more research would be required. Because HRP II has a possible role in malarial pathology, thiese data obviously support the notion of using low-level proteasome inhibition in chemotherapeutic drug cocktails [4-7]. Exploration of the UPS and HRP II could also lead to increased knowledge of the biology of protein export/secretion in *P. falciparum*, which is not fully understood [6-7]. Overall, the fact that proteasome inhibition by MG132 impacts HRP II secretion is an important finding, but more research would be required to fully understand the implications.

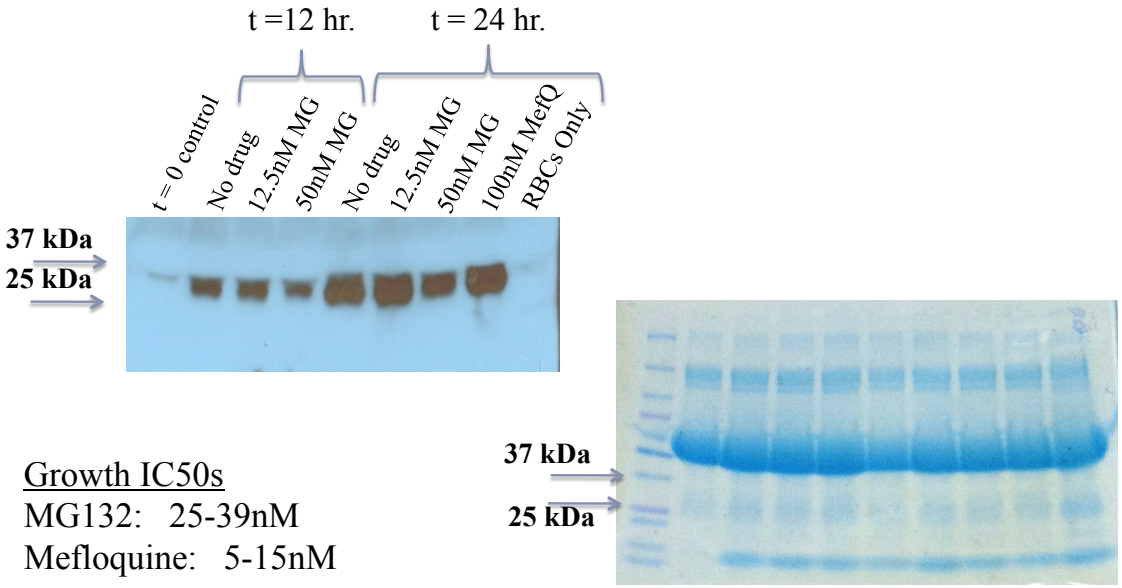

Figure S3: HRPII secretion. Western of HRPII in parasite growth media after 12 or 24 hr drug pressure. Western blot and probing of concentrated media with HRPII primary antibody (left); Coomassie stain of identical gel with same samples (right).

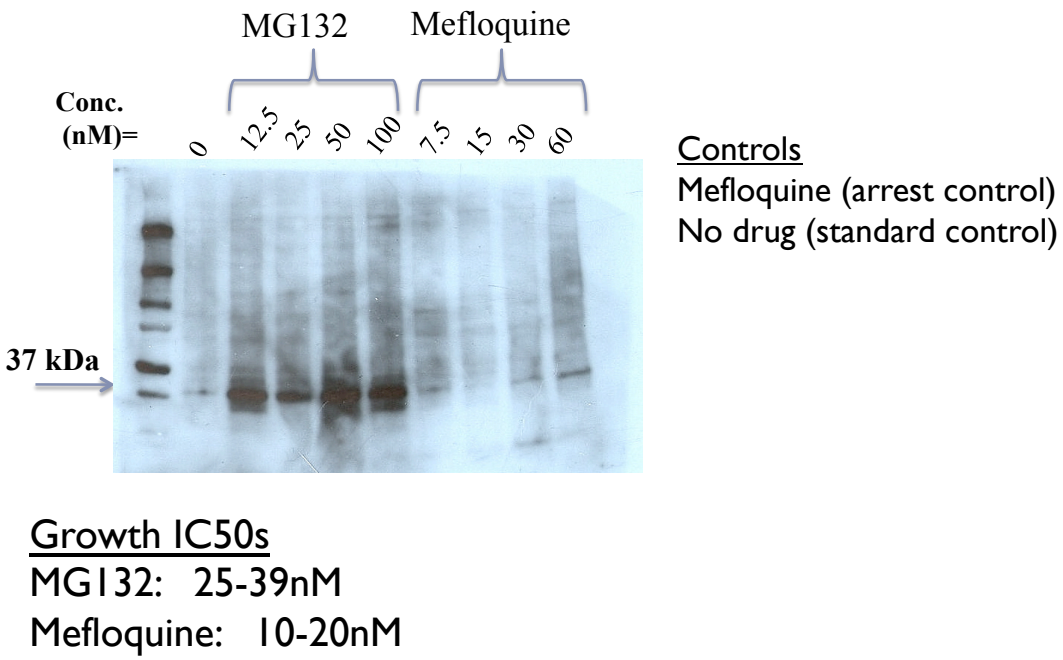

Figure S4: HRPII accumulation in parasites due to MG132 exposure. Western blot and probing of HRPII in ring/early trophozoite stage parasites after 12 hr drug pressure from MG132 or mefloquine.

References:

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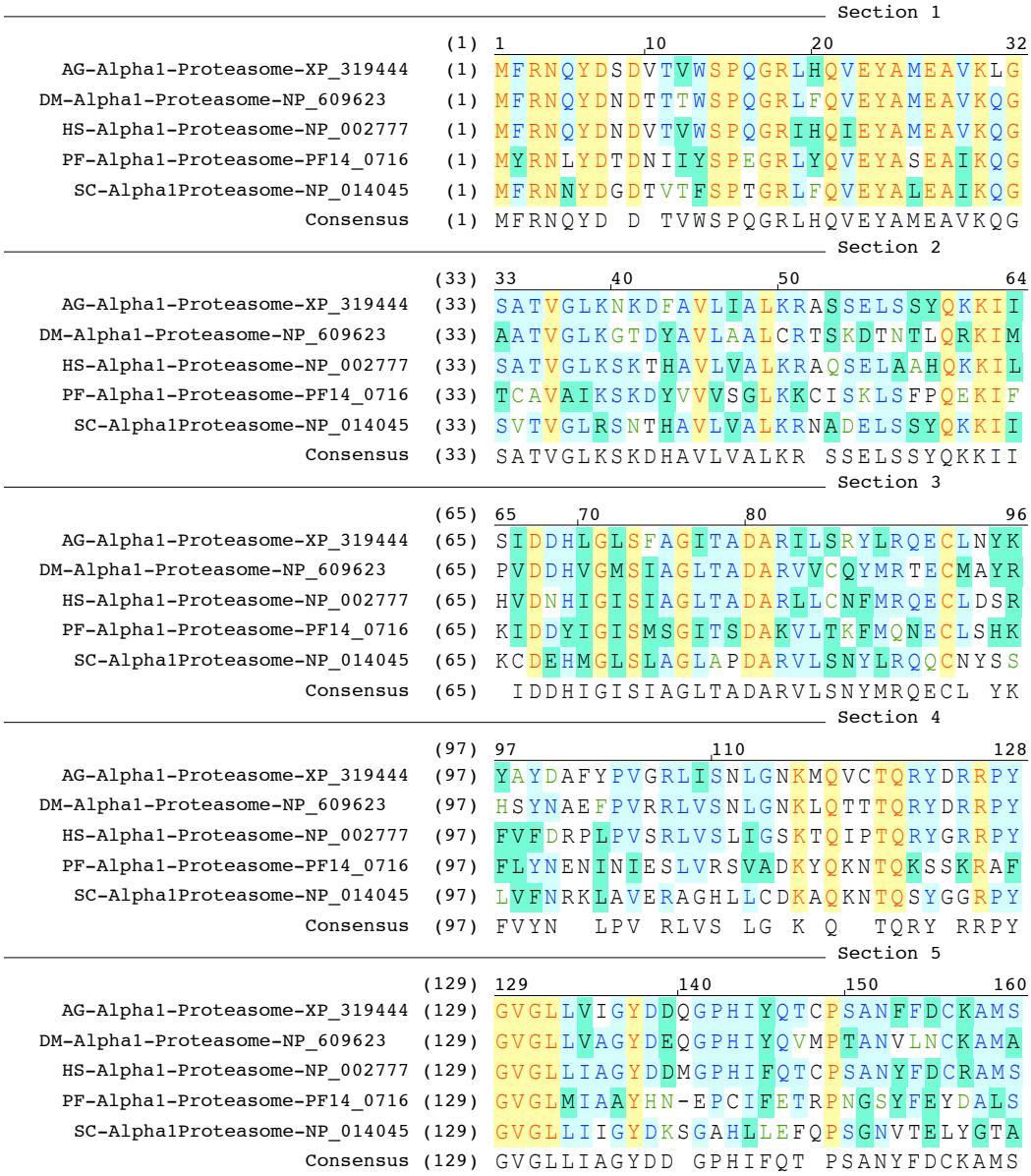
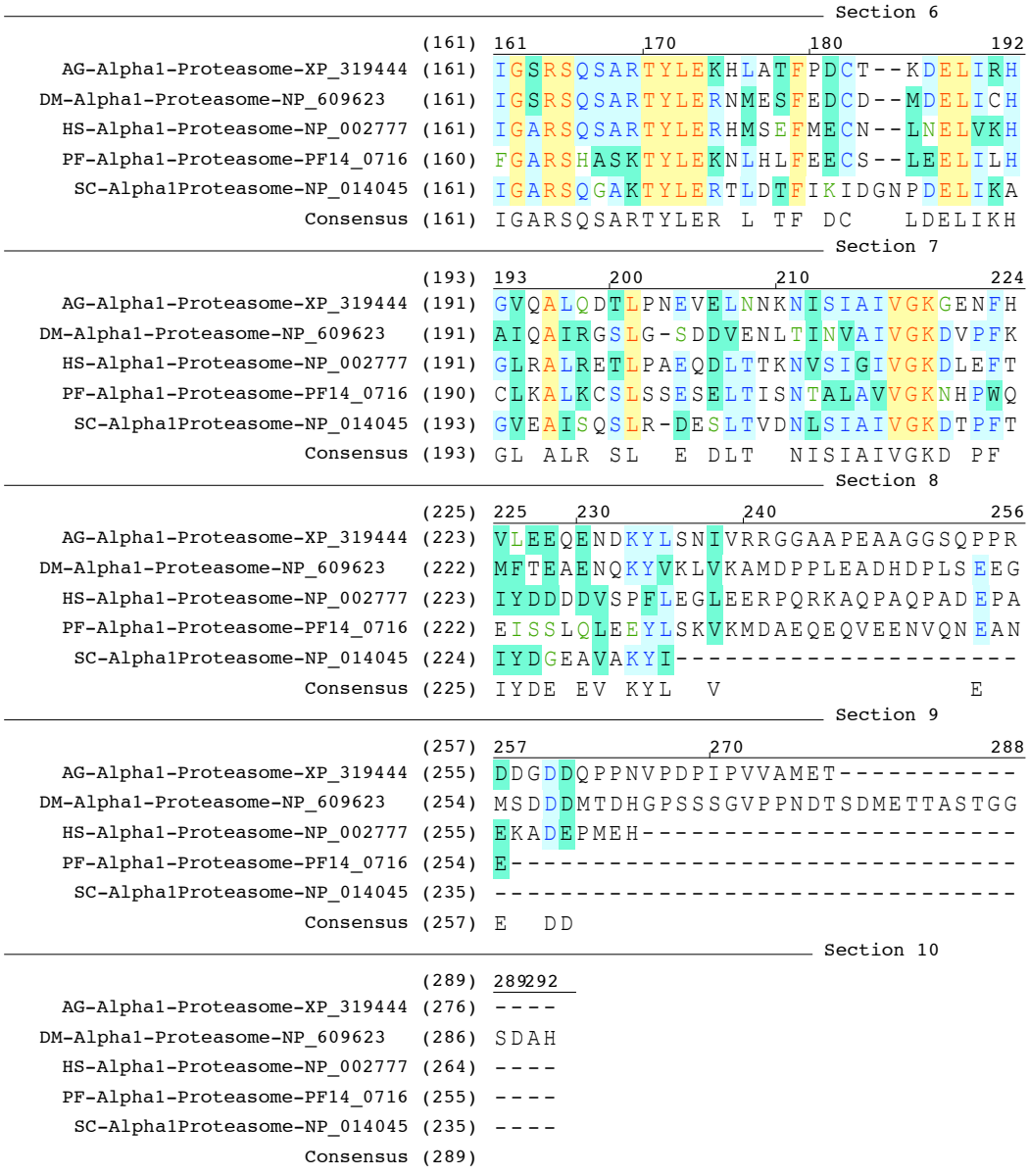
Appendix III:
Multiple Sequence Alignments

Multiple Sequence Alignment: Proteasome α-1 Subunit




Section 6
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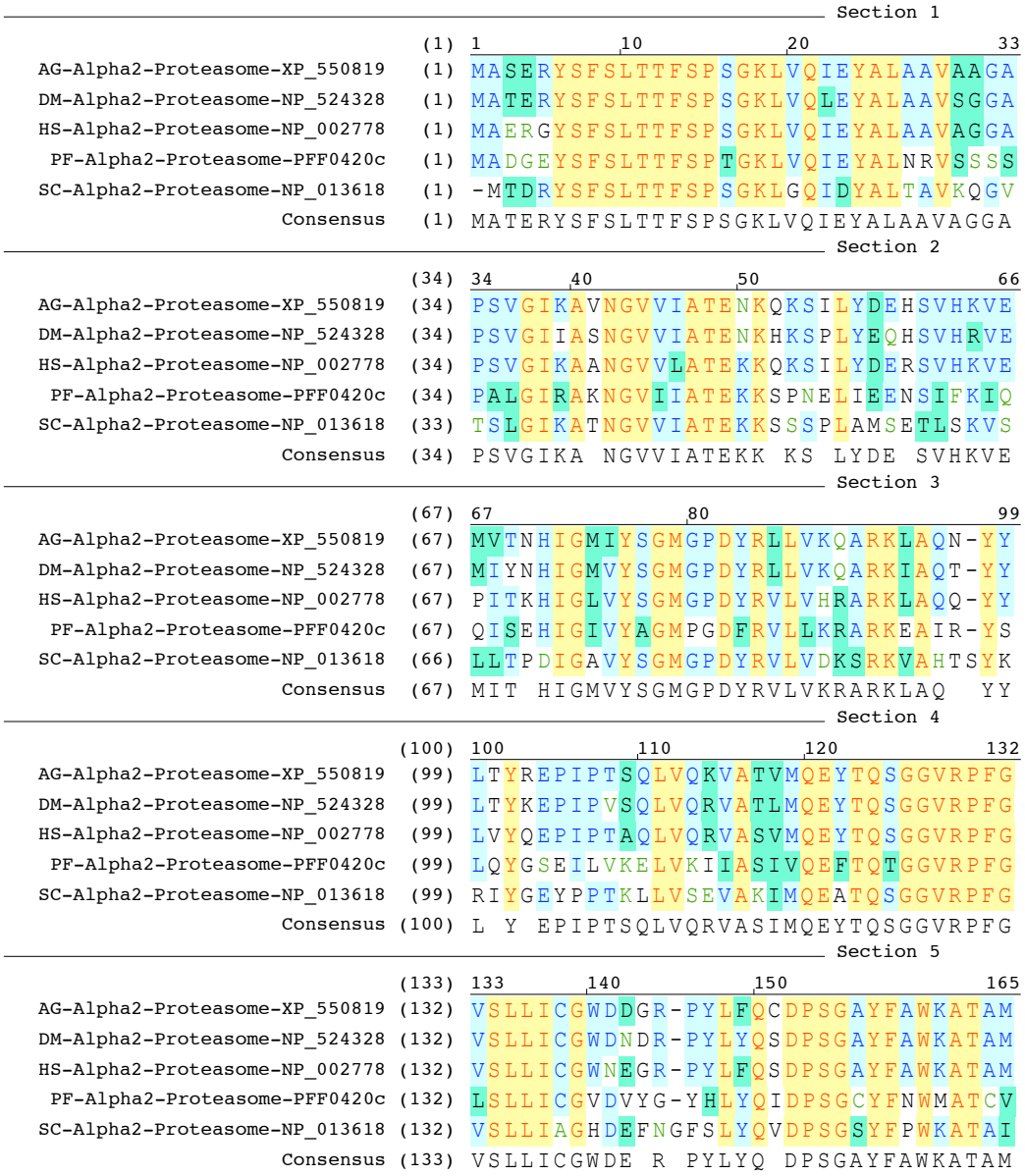
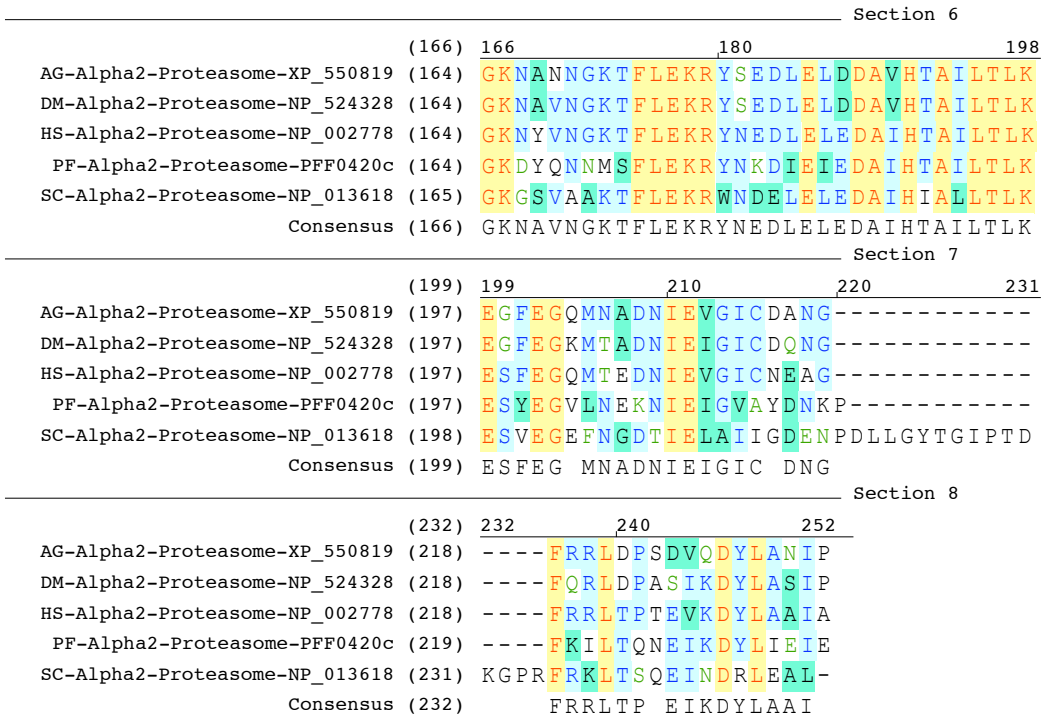
DM-Alpha1-Proteasome-NP_609623 (161) IGSRSQSARTYLERNMESFEDCD--MDELICH
HS-Alpha1-Proteasome-NP_002777 (161) IGARSQSARTYLERHMSEFMECN--LNELVKH
PF-Alpha1-Proteasome-PF14_0716 (160) FGARSHASKTYLEKNLHLFEECS--LEELILH
SC-Alpha1Proteasome-NP_014045 (161) IGARSQGAKTYLERTLDTFIKIDGNPDELIKA
Consensus (161) IGARSQSARTYLER L TF DC LDELIKH
Section 7
(193) 193 200 210 224
AG-Alpha1-Proteasome-XP_319444 (191) GVQALQDTLPNEVELNNKNISIAIVGKGENFH
DM-Alpha1-Proteasome-NP_609623 (191) AIQAIRGSLG-SDDVENLTINVAIVGKDVPFK
HS-Alpha1-Proteasome-NP_002777 (191) GLRALRETLPAEQDLTTKNVSIGIVGKDLEFT
PF-Alpha1-Proteasome-PF14_0716 (190) CLKALKCSLSSESELTISNTALAVVGKNHPWQ
SC-Alpha1Proteasome-NP_014045 (193) GVEAISQSLR-DESLTVDNLSIAIVGKDTPFT
Consensus (193) GL ALR SL E DLT NISIAIVGKD PF
Section 8
(225) 225 230 240 256
AG-Alpha1-Proteasome-XP_319444 (223) VLEEQENDKYLSNIVRRGGAAPEAAGGSQPPR
DM-Alpha1-Proteasome-NP_609623 (222) MFTEAENQKYVKLVKAMDPPLEADHDPLSEEG
HS-Alpha1-Proteasome-NP_002777 (223) IYDDDDVSPFLEGLEERPQRKAQPAQPADEPA
PF-Alpha1-Proteasome-PF14_0716 (222) EISSLQLEEYLSKVKMDAEQEQVEENVQNEAN
SC-Alpha1Proteasome-NP_014045 (224) IYDGEAVAKYI----------
Consensus (225) IYDE EV KYL V E
Section 9
(257) 257 270 288
AG-Alpha1-Proteasome-XP_319444 (255) DDGDDQPPNVPDPIPVVAMET----------
DM-Alpha1-Proteasome-NP_609623 (254) MSDDDMTDHGPSSSGVPPNDTSDMETTASTGG
HS-Alpha1-Proteasome-NP_002777 (255) EKADEPMEH----------
PF-Alpha1-Proteasome-PF14_0716 (254) E----------
SC-Alpha1Proteasome-NP_014045 (235) ----------
Consensus (257) E DD
Section 10
(289) 289 292
AG-Alpha1-Proteasome-XP_319444 (276) ----
DM-Alpha1-Proteasome-NP_609623 (286) SDAH
HS-Alpha1-Proteasome-NP_002777 (264) ----
PF-Alpha1-Proteasome-PF14_0716 (255) ----
SC-Alpha1Proteasome-NP_014045 (235) ----
Consensus (289)

Multiple Sequence Alignment: Proteasome α-2 Subunit




Section 6
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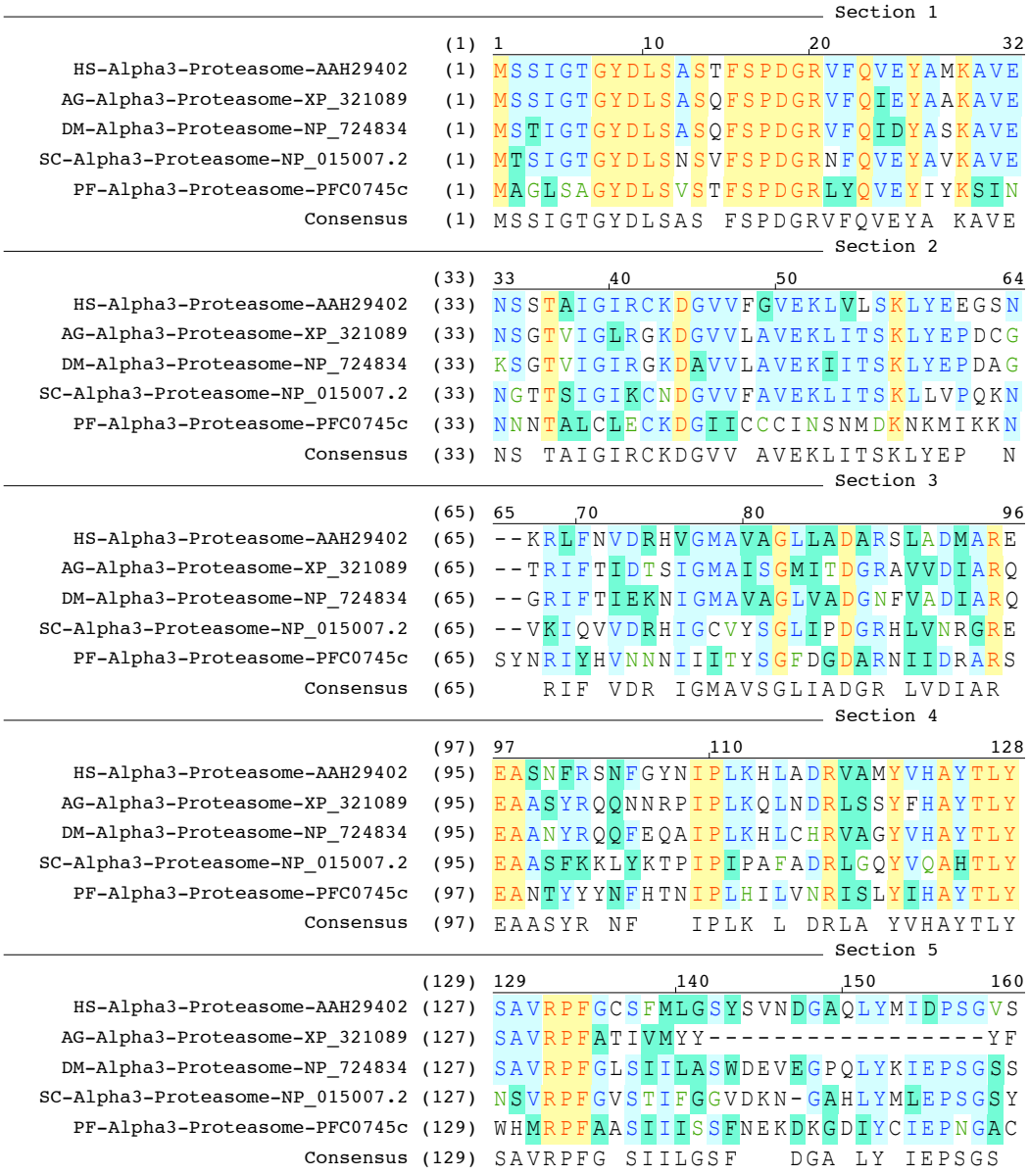
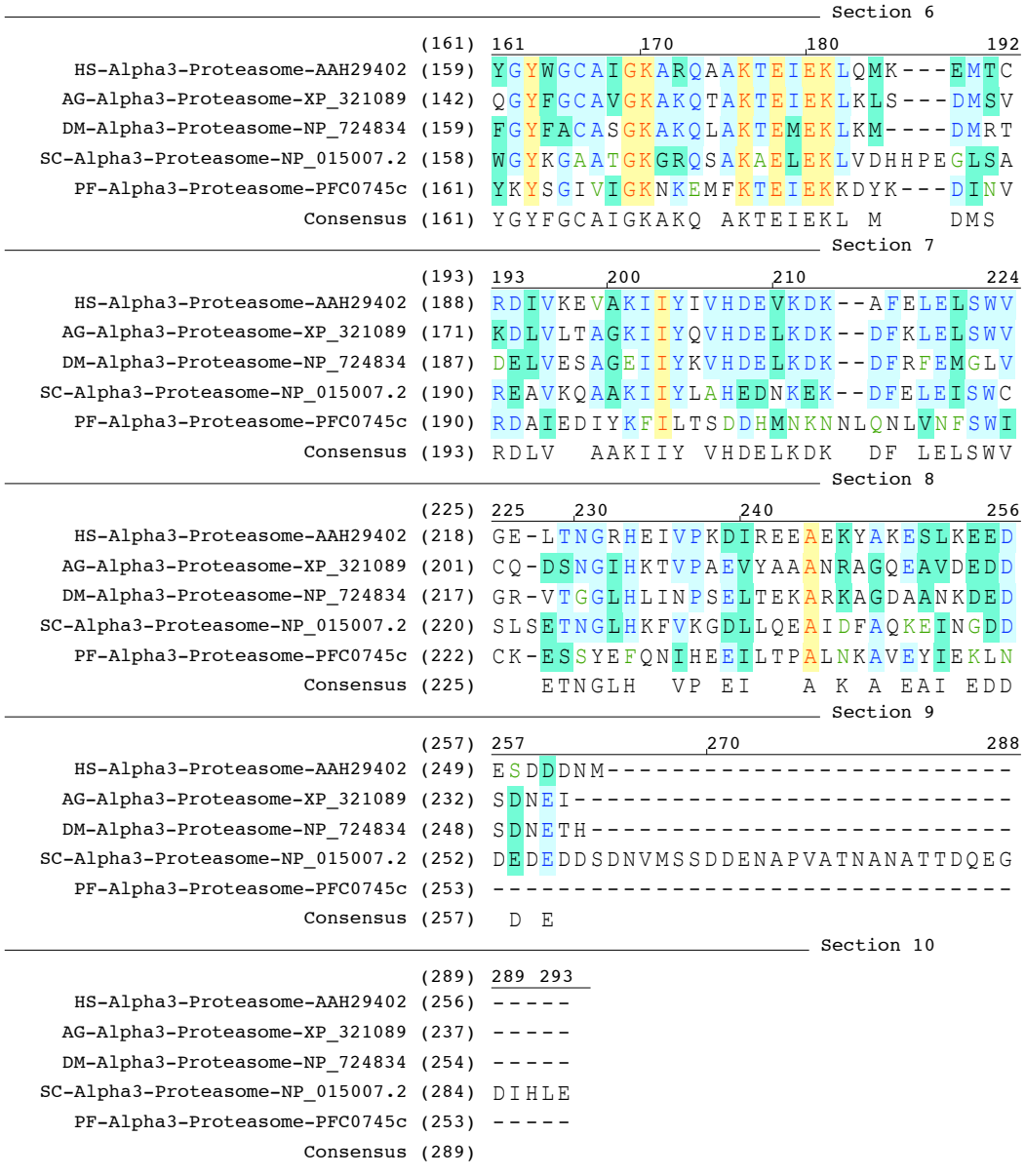
DM-Alpha2-Proteasome-NP_524328 (164) GKNAVNGKTFLEKRYSEDLELDDAVHTAILTLK
HS-Alpha2-Proteasome-NP_002778 (164) GKNYVNGKTFLEKRYNEDLELEDAIHTAILTLK
PF-Alpha2-Proteasome-PFF0420c (164) GKDYQNNMSFLEKRYNKDIEIEDAIHTAILTLK
SC-Alpha2-Proteasome-NP_013618 (165) GKGSVAAKTFLEKRWNDELELEDAIHIALLTLK
Consensus (166) GKNAVNGKTFLEKRYNEDLELEDAIHTAILTLK
Section 7
(199) 199 210 220 231
AG-Alpha2-Proteasome-XP_550819 (197) EGFEGQMNADNIEVGICDANG----------
DM-Alpha2-Proteasome-NP_524328 (197) EGFEGKMTADNIEIGICDQNG----------
HS-Alpha2-Proteasome-NP_002778 (197) ESFEGQMTEDNIEVGICNEAG----------
PF-Alpha2-Proteasome-PFF0420c (197) ESYEGVLNEKNIEIGVAYDNKP----------
SC-Alpha2-Proteasome-NP_013618 (198) ESVEGEFNGDTIELAIIGDENPDLLGYTGIPTD
Consensus (199) ESFEG MNADNIEIGIC DNG
Section 8
(232) 232 240 252
AG-Alpha2-Proteasome-XP_550819 (218) ----FRRLDPSDVQDYLANIP
DM-Alpha2-Proteasome-NP_524328 (218) ----FQRLDPASIKDYLASIP
HS-Alpha2-Proteasome-NP_002778 (218) ----FRRLTPTEVKDYLAAIA
PF-Alpha2-Proteasome-PFF0420c (219) ----FKILTQNEIKDYLIEIE
SC-Alpha2-Proteasome-NP_013618 (231) KGPRFRKLTSQEINDRLEAL-
Consensus (232) FRRLTP EIKDYLAAI

Multiple Sequence Alignment: Proteasome α-3 Subunit

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AG-Alpha3-Proteasome-XP_321089 (142) QGYFGCAVGKAKQTAKTEIEKLKLS---DMSV
DM-Alpha3-Proteasome-NP_724834 (159) FGYFACASGKAKQLAKTEMEKLKM----DMRT
SC-Alpha3-Proteasome-NP_015007.2 (158) WGYKGAATGKGRQSAKAELEKLVDHHPEGLSA
PF-Alpha3-Proteasome-PFC0745c (161) YKYSGIVIGKNKEMFKTEIEKKDYK---DINV
Consensus (161) YGYFGCAIGKAKQ AKTEIEKL M DMS
Section 7
(193) 193 200 210 224
HS-Alpha3-Proteasome-AAH29402 (188) RDIVKEVAKIIYIVHDEVKDK--AFELELSWV
AG-Alpha3-Proteasome-XP_321089 (171) KDLVLTAGKIIYQVHDELKDK--DFKLELSWV
DM-Alpha3-Proteasome-NP_724834 (187) DELVESAGEIIYKVHDELKDK--DFRFEMGLV
SC-Alpha3-Proteasome-NP_015007.2 (190) REAVKQAAKIIYLAHEDNKEK--DFELEISWC
PF-Alpha3-Proteasome-PFC0745c (190) RDAIEDIYKFILTSDDHMNKNNLQNLVNFSWI
Consensus (193) RDLV AAKIIY VHDELKDK DF LELSWV
Section 8
(225) 225 230 240 256
HS-Alpha3-Proteasome-AAH29402 (218) GE-LTNGRHEIVPKDIREEAEKYAKESLKEED
AG-Alpha3-Proteasome-XP_321089 (201) CQ-DSNGIHKTVPAEVYAAANRAGQEAVDEDD
DM-Alpha3-Proteasome-NP_724834 (217) GR-VTGGLHLINPSELTEKARKAGDAANKDED
SC-Alpha3-Proteasome-NP_015007.2 (220) SLSETNGLHKFVKGDLLQEAIDFAQKEINGDD
PF-Alpha3-Proteasome-PFC0745c (222) CK-ESSYEFQNIHEEILTPALNKAVEYIEKLN
Consensus (225) ETNGLH VP EI A K A EAI EDD
Section 9
(257) 257 270 288
HS-Alpha3-Proteasome-AAH29402 (249) ESDDDNM-------------------------
AG-Alpha3-Proteasome-XP_321089 (232) SDNEI-------------------------
DM-Alpha3-Proteasome-NP_724834 (248) SDNETH-------------------------
SC-Alpha3-Proteasome-NP_015007.2 (252) DEDEDDSDNVMSSDDENAPVATNANATTDQEG
PF-Alpha3-Proteasome-PFC0745c (253) -------------------------------
Consensus (257) D E
Section 10
(289) 289 293
HS-Alpha3-Proteasome-AAH29402 (256) -----
AG-Alpha3-Proteasome-XP_321089 (237) -----
DM-Alpha3-Proteasome-NP_724834 (254) -----
SC-Alpha3-Proteasome-NP_015007.2 (284) DIHLE
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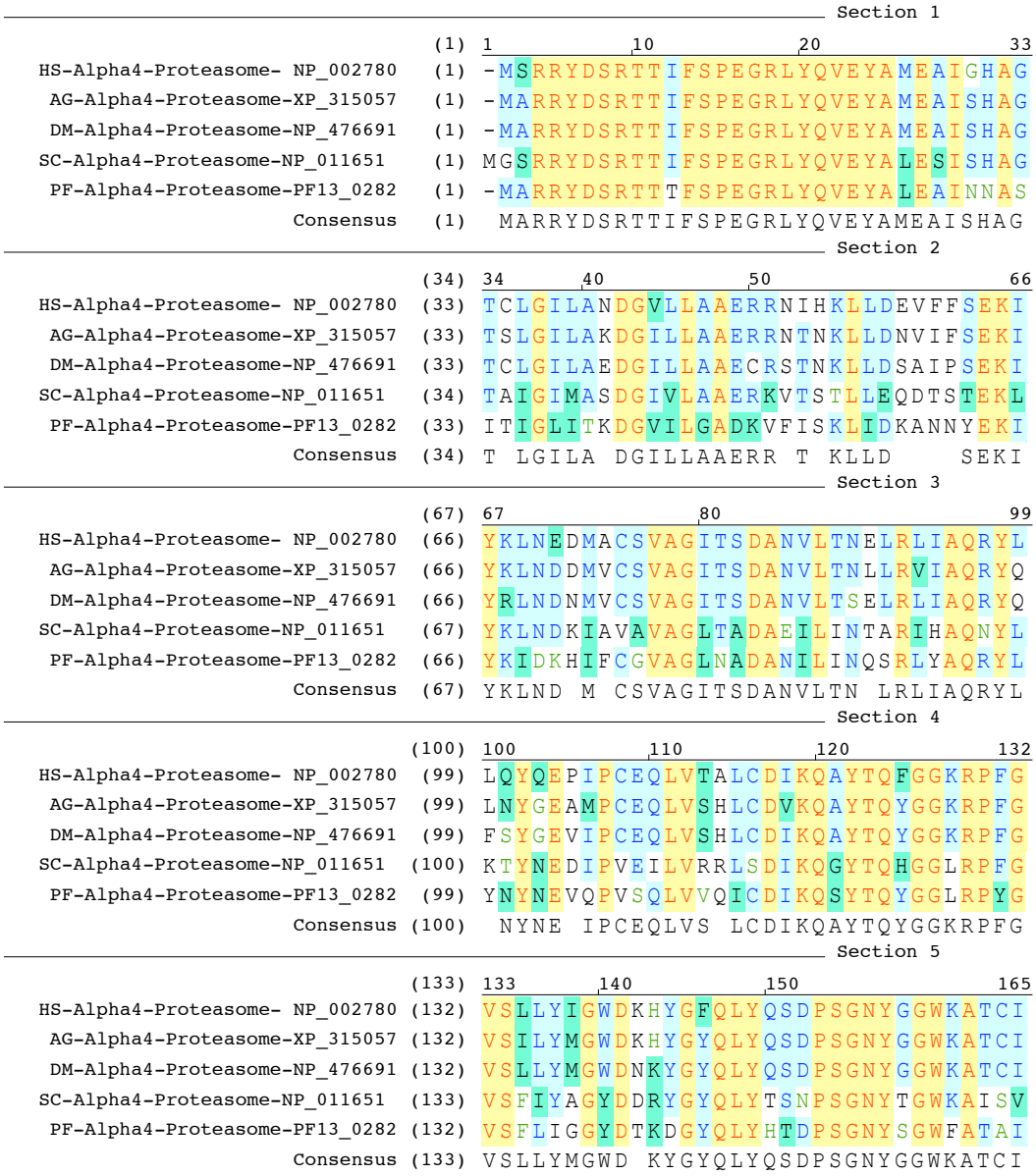
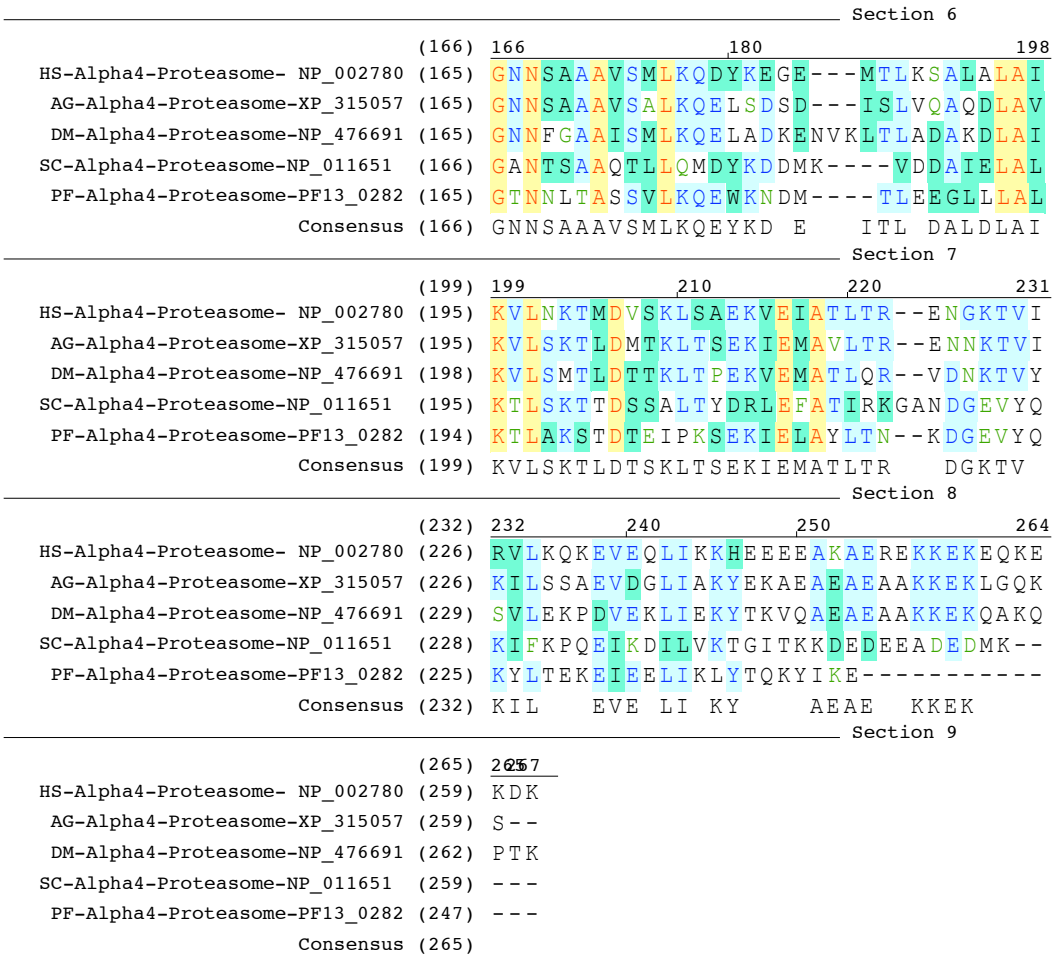
Consensus (289)

Multiple Sequence Alignment: Proteasome α-4 Subunit

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AG-Alpha4-Proteasome-XP_315057 (165) GNNSAAAVSALKQELSDSD---ISLVQAQDLAV
DM-Alpha4-Proteasome-NP_476691 (165) GNNFGAAISMLKQELADKENVKLTLADAKDLAI
SC-Alpha4-Proteasome-NP_011651 (166) GANTSAAQTLLQMDYKDDMK----VDDAIELAL
PF-Alpha4-Proteasome-PF13_0282 (165) GTNNLTASSVLKQEWKNDM----TLEEGLLLAL
Consensus (166) GNNSAAAVSMLKQEYKD E ITL DALDLAI
Section 7
(199) 199 210 220 231
HS-Alpha4-Proteasome- NP_002780 (195) KVLNKTMDVSKLSAEKVEIATLTR--ENGKTVI
AG-Alpha4-Proteasome-XP_315057 (195) KVLSKTLDMTKLTSEKIEMAVLTR--ENNKTVI
DM-Alpha4-Proteasome-NP_476691 (198) KVLSMTLDTTKLTPEKVEMATLQR--VDNKTVY
SC-Alpha4-Proteasome-NP_011651 (195) KTLSKTTDSSALTYDRLEFATIRKGANDGEVYQ
PF-Alpha4-Proteasome-PF13_0282 (194) KTLAKSTDTEIPKSEKIELAYLTN--KDGEVYQ
Consensus (199) KVLSKTLDTSKLTSEKIEMATLTR DGKTV
Section 8
(232) 232 240 250 264
HS-Alpha4-Proteasome- NP_002780 (226) RVLKQKEVEQLIKKHEEEEAKAEREKKEKEQKE
AG-Alpha4-Proteasome-XP_315057 (226) KILSSAEVDGLIAKYEKAEAEAEAAKKEKLGQK
DM-Alpha4-Proteasome-NP_476691 (229) SVLEKPDVEKLIEKYTKVQAEAEAAKKEKQAKQ
SC-Alpha4-Proteasome-NP_011651 (228) KIFKPQEIKDILVKTGITKKDEDEEADEDMK--
PF-Alpha4-Proteasome-PF13_0282 (225) KYLTEKEIEELIKLYTQKYIKE----------
Consensus (232) KIL EVE LI KY AEAE KKEK
Section 9
(265) 265 267
HS-Alpha4-Proteasome- NP_002780 (259) KDK
AG-Alpha4-Proteasome-XP_315057 (259) S--
DM-Alpha4-Proteasome-NP_476691 (262) PTK
SC-Alpha4-Proteasome-NP_011651 (259) ---
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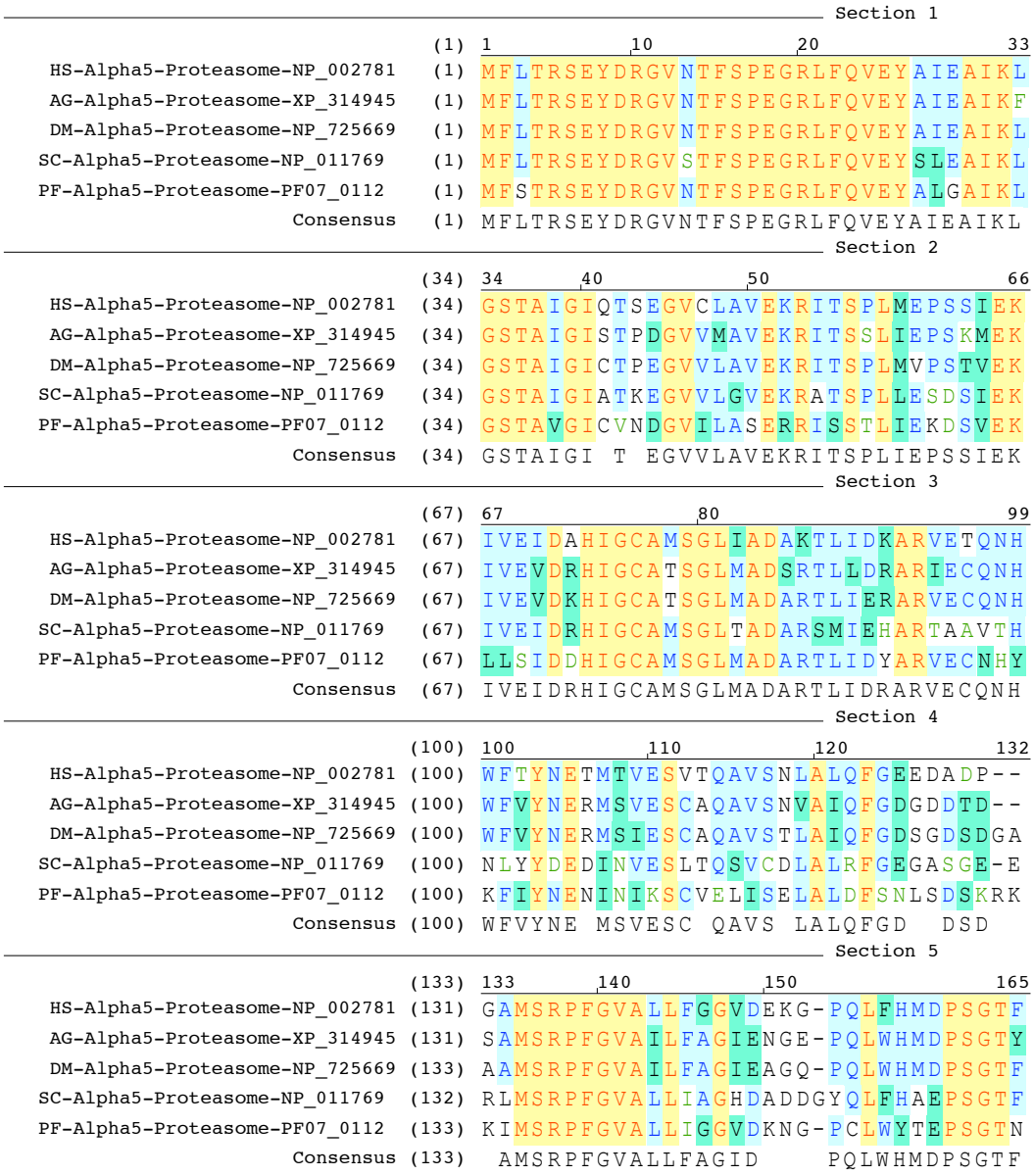
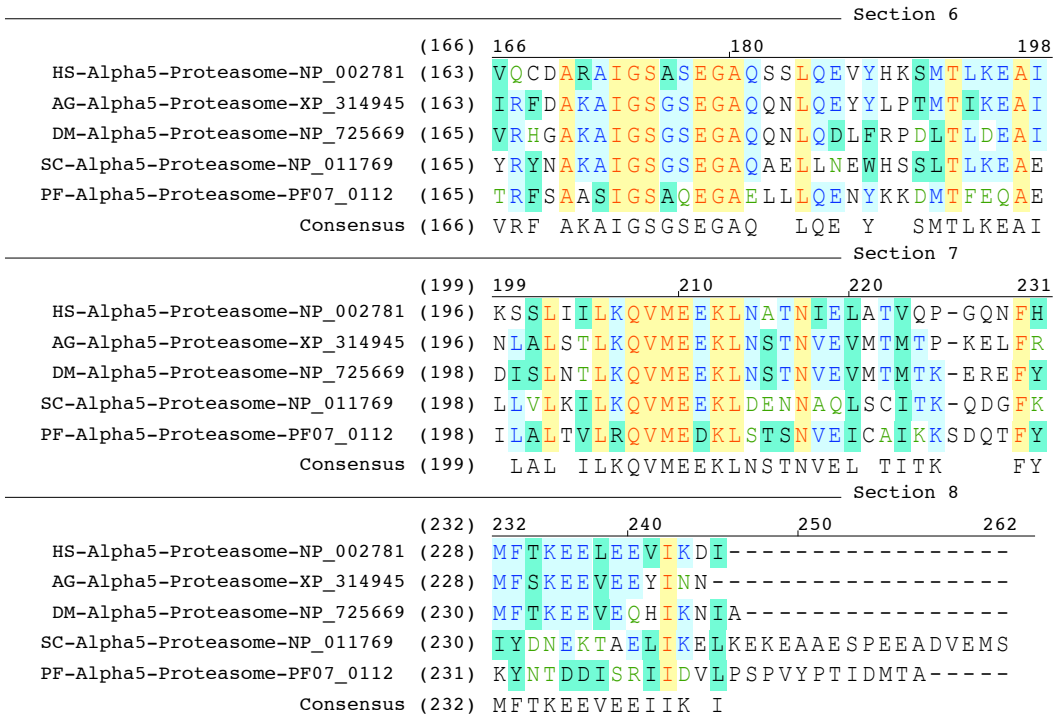
Consensus (265)

Multiple Sequence Alignment: Proteasome α-5 Subunit

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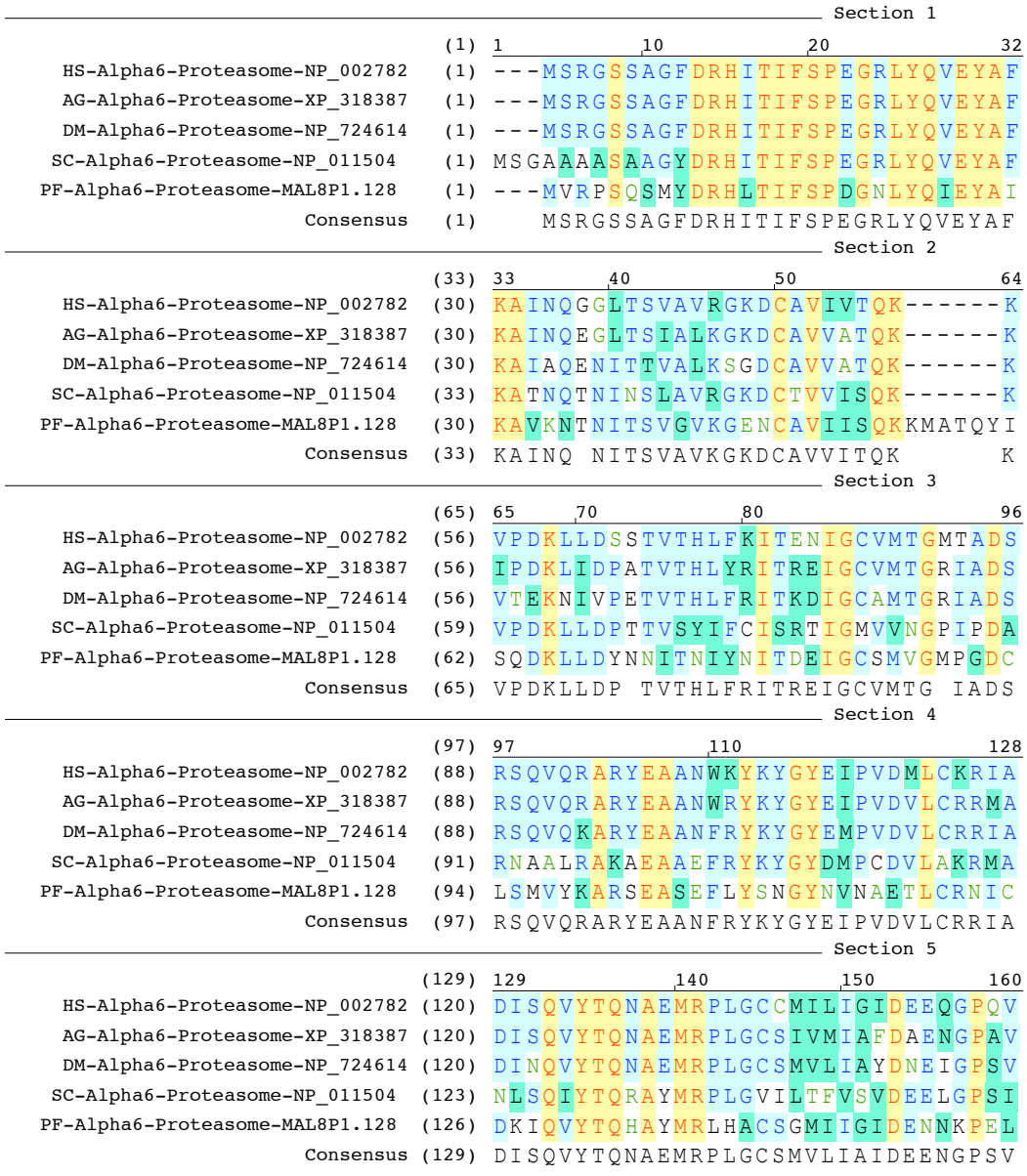
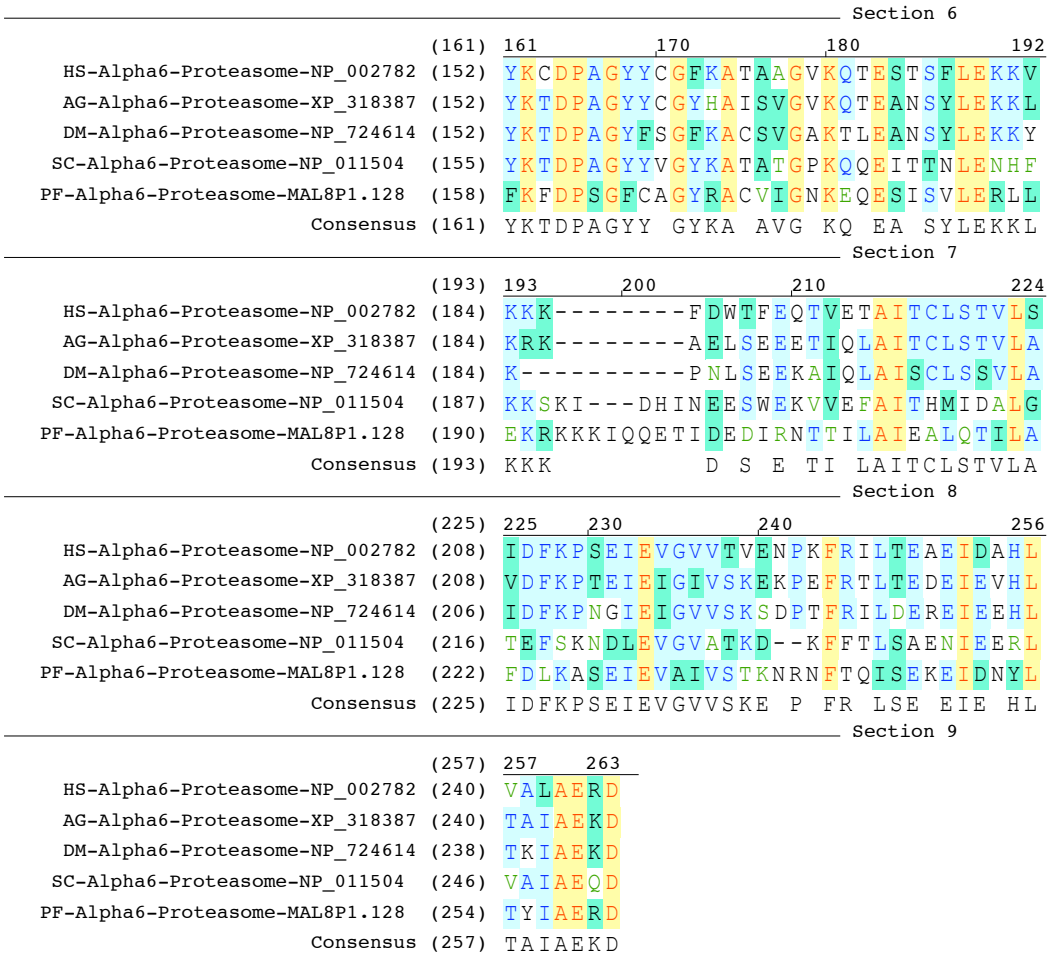
AG-Alpha5-Proteasome-XP_314945 (163) IRFDAKAIGSGSEGAQQNLQEYYLPTMTIKEAI
DM-Alpha5-Proteasome-NP_725669 (165) VRHGAKAIGSGSEGAQQNLQDLFRPDLTLDEAI
SC-Alpha5-Proteasome-NP_011769 (165) YRYNAKAIGSGSEGAQAELLNEWHSSLTLKEAE
PF-Alpha5-Proteasome-PF07_0112 (165) TRFSAASIGSAQEGAELLLQENYKKDMTFEQAE
Consensus (166) VRF AKAIGSGSEGAQ LQE Y SMTLKEAI
Section 7
(199) 199 210 220 231
HS-Alpha5-Proteasome-NP_002781 (196) KSSLIILKQVMEEKLNATNIELATVQP-GQNFH
AG-Alpha5-Proteasome-XP_314945 (196) NLALSTLKQVMEEKLNSTNVEVMTMTP-KELFR
DM-Alpha5-Proteasome-NP_725669 (198) DISLNTLKQVMEEKLNSTNVEVMTMTK-EREFY
SC-Alpha5-Proteasome-NP_011769 (198) LLVLKILKQVMEEKLDENNAQLSCITK-QDGFK
PF-Alpha5-Proteasome-PF07_0112 (198) ILALTVLRQVMEDKLSTSNVEICAIKKSDQTFY
Consensus (199) LAL ILKQVMEEKLNSTNVEL TITK FY
Section 8
(232) 232 240 250 262
HS-Alpha5-Proteasome-NP_002781 (228) MFTKEELEEVIKDI----------
AG-Alpha5-Proteasome-XP_314945 (228) MFSKEEVEEYINN----------
DM-Alpha5-Proteasome-NP_725669 (230) MFTKEEVEQHIKNIA----------
SC-Alpha5-Proteasome-NP_011769 (230) IYDNEKTAELIKELKEKEAAESPEEADVEMS
PF-Alpha5-Proteasome-PF07_0112 (231) KYNTDDISRIIDVLPSPVYPTIDMTA-----
Consensus (232) MFTKEEVEEIIK I

Multiple Sequence Alignment: Proteasome α-6 Subunit

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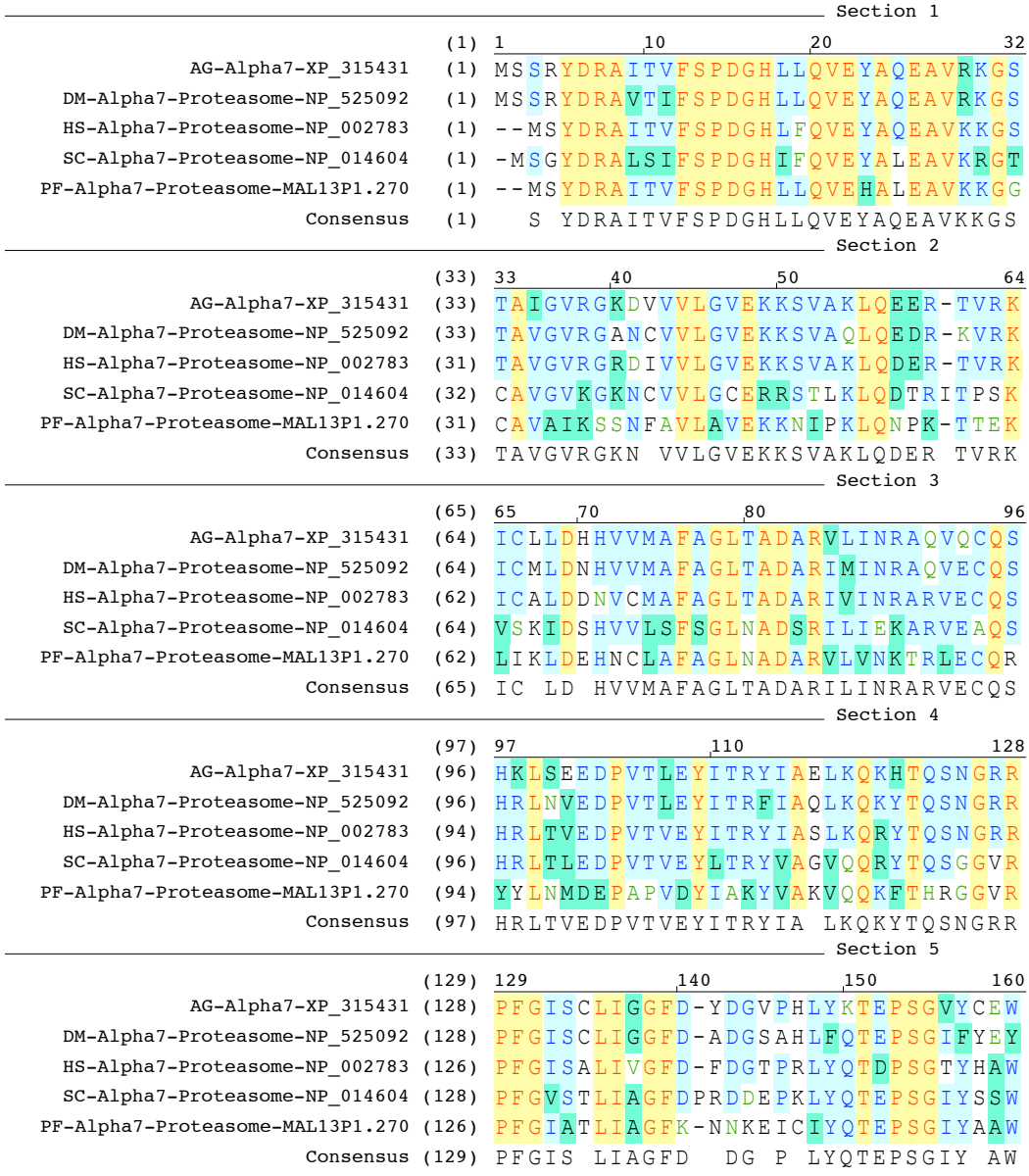
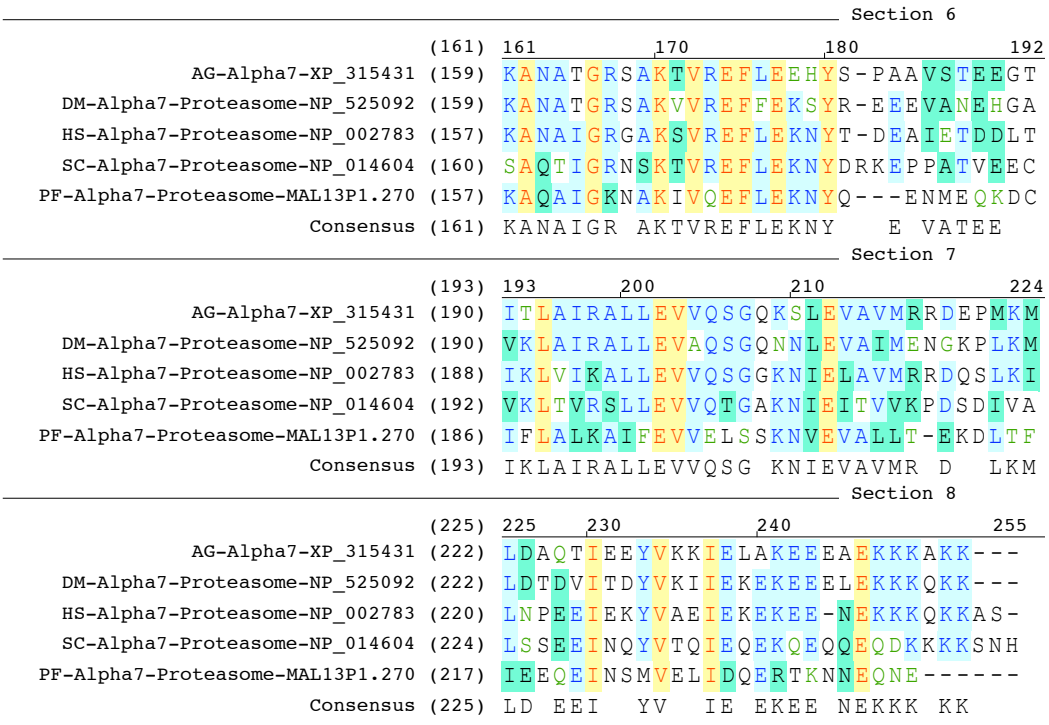
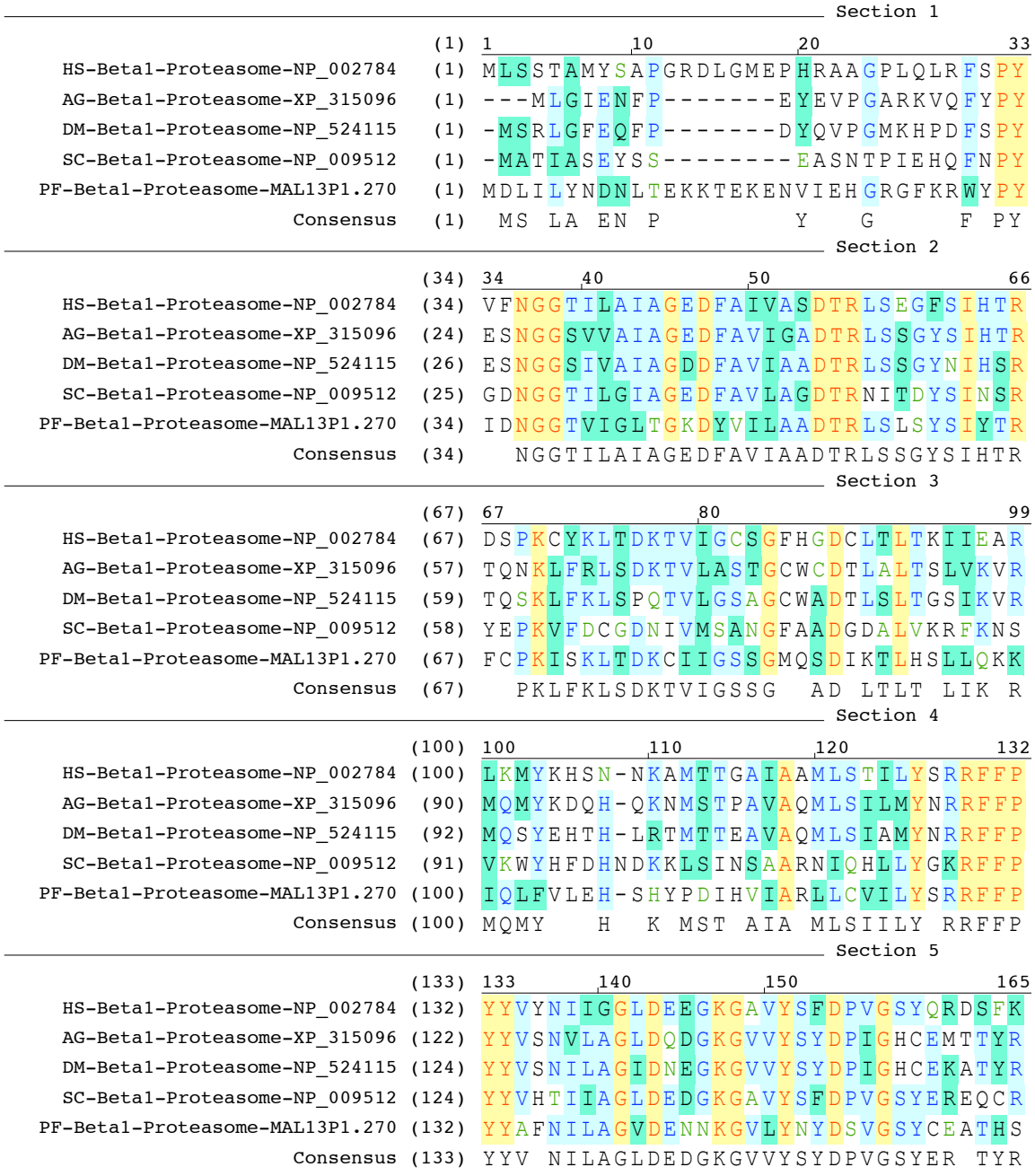
AG-Alpha6-Proteasome-XP_318387 (152) YKTDPAGYYCGYHAISVGVKQTEANSYLEKKL
DM-Alpha6-Proteasome-NP_724614 (152) YKTDPAGYFSGFKACSVGAKTLEANSYLEKKY
SC-Alpha6-Proteasome-NP_011504 (155) YKTDPAGYYVGYKATATGPKQQEITTNLENHF
PF-Alpha6-Proteasome-MAL8P1.128 (158) FKFDPSGFCAGYRACVIGNKEQESISVLERLL
Consensus (161) YKTDPAGYY GYKA AVG KQ EA SYLEKKL
Section 7
(193) 193 200 210 224
HS-Alpha6-Proteasome-NP_002782 (184) KKK--------FDWTFEQTVETAITCLSTVLS
AG-Alpha6-Proteasome-XP_318387 (184) KRK--------AELSEEETIQLAITCLSTVLA
DM-Alpha6-Proteasome-NP_724614 (184) K----------PNLSEEKAIQLAISCLSSVLA
SC-Alpha6-Proteasome-NP_011504 (187) KKSKI---DHINEESWEKVVEFAITHMIDALG
PF-Alpha6-Proteasome-MAL8P1.128 (190) EKRKKKIQQETIDEDIRNTTILAIEALQTILA
Consensus (193) KKK D S E TI LAITCLSTVLA
Section 8
(225) 225 230 240 256
HS-Alpha6-Proteasome-NP_002782 (208) IDFKPSEIEVGVVTVENPKFRILTEAEIDAHL
AG-Alpha6-Proteasome-XP_318387 (208) VDFKPTEIEIGIVSKEKPEFRTLTEDEIEVHL
DM-Alpha6-Proteasome-NP_724614 (206) IDFKPNGIEIGVVSKSDPTFRILDEREIEEHL
SC-Alpha6-Proteasome-NP_011504 (216) TEFSKNDLEVGVATKD--KFFTLSAENIEERL
PF-Alpha6-Proteasome-MAL8P1.128 (222) FDLKASEIEVAIVSTKNRNFTQISEKEIDNYL
Consensus (225) IDFKPSEIEVGVVSKE P FR LSE EIE HL
Section 9
(257) 257 263
HS-Alpha6-Proteasome-NP_002782 (240) VALAERD
AG-Alpha6-Proteasome-XP_318387 (240) TAIAEKD
DM-Alpha6-Proteasome-NP_724614 (238) TKIAEKD
SC-Alpha6-Proteasome-NP_011504 (246) VAIAEQD
PF-Alpha6-Proteasome-MAL8P1.128 (254) TYIAERD
Consensus (257) TAIAEKD

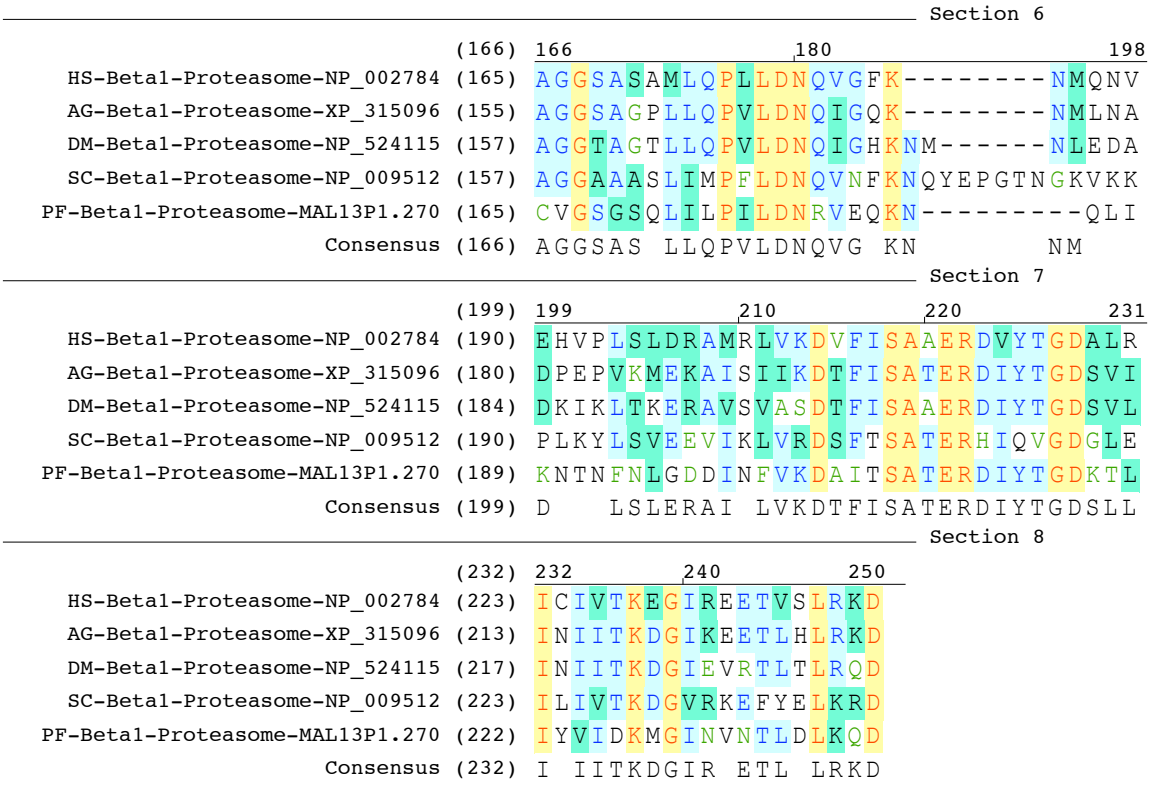
Multiple Sequence Alignment: Proteasome α-7 Subunit



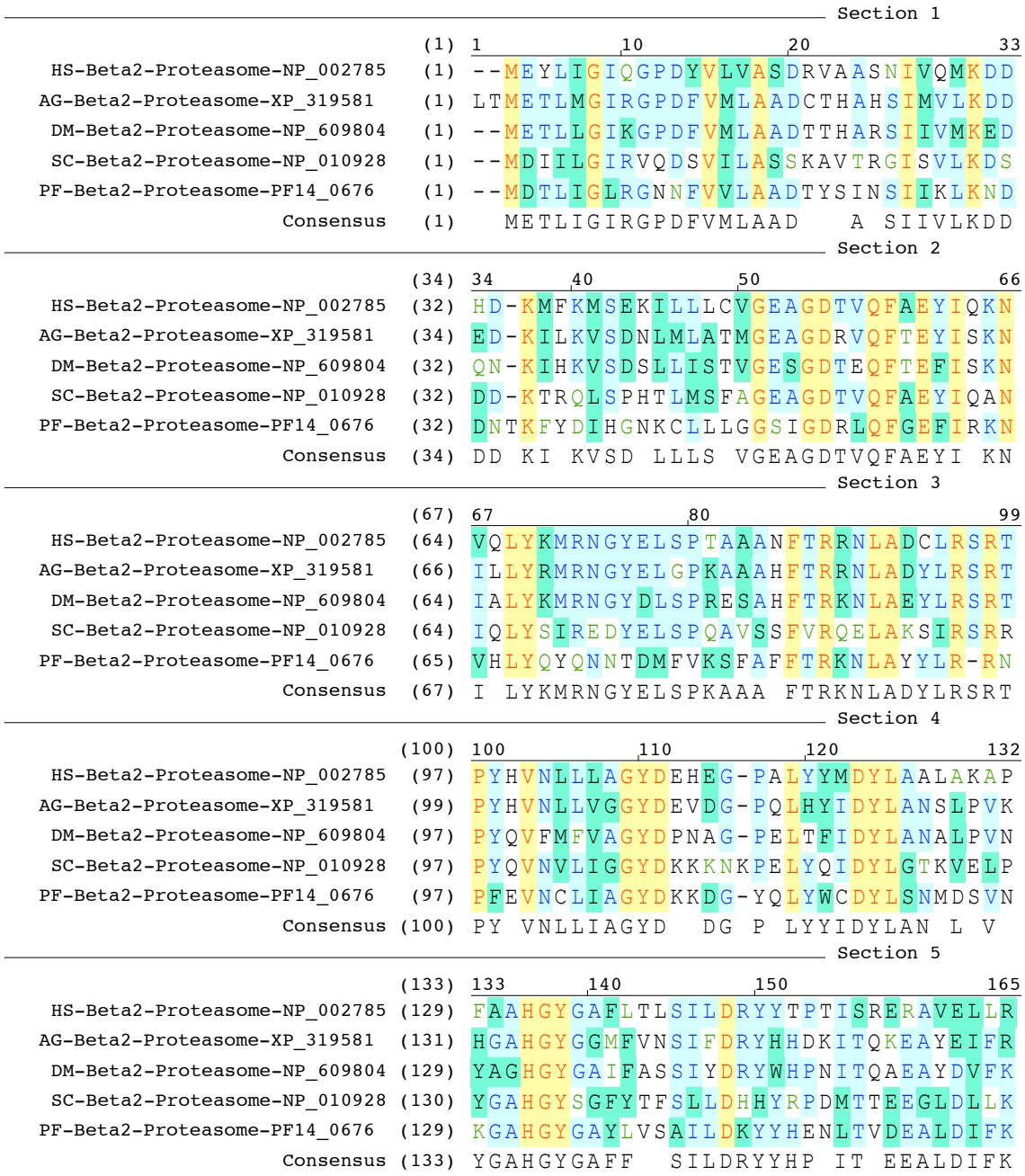

Section 6
(161) 161 170 180 192
AG-Alpha7-XP_315431 (159) KANATGRSAKTVREFLEEHYS-PAAVSTEEGT
DM-Alpha7-Proteasome-NP_525092 (159) KANATGRSAKVVREFFEKSYR-EEEVANEHGA
HS-Alpha7-Proteasome-NP_002783 (157) KANAIGRGAKSVREFLEKNYT-DEAIETDDLT
SC-Alpha7-Proteasome-NP_014604 (160) SAQTIGRNSKTVREFLEKNYDRKEPPATVEEC
PF-Alpha7-Proteasome-MAL13P1.270 (157) KAQAIGKNAKIVQEFLEKNYQ---ENMEQKDC
Consensus (161) KANAIGR AKTVREFLEKNY E VATEE
Section 7
(193) 193 200 210 224
AG-Alpha7-XP_315431 (190) ITLAIRALLEVVQSGQKSLEVAVMRRDEPMKM
DM-Alpha7-Proteasome-NP_525092 (190) VKLAIRALLEVAQSGQNNLEVAIMENGKPLKM
HS-Alpha7-Proteasome-NP_002783 (188) IKLVIKALLEVVQSGGKNIELAVMRRDQSLKI
SC-Alpha7-Proteasome-NP_014604 (192) VKLTVRSLLEVVQTGAKNIEITVVKPDSDIVA
PF-Alpha7-Proteasome-MAL13P1.270 (186) IFLALKAIFEVVELSSKNVEVALLT-EKDLTF
Consensus (193) IKLAIRALLEVVQSG KNIEVAVMR D LKM
Section 8
(225) 225 230 240 255
AG-Alpha7-XP_315431 (222) LDAQTIEEYVKKIELAKEEEAEKKKAKK---
DM-Alpha7-Proteasome-NP_525092 (222) LDTDVITDYVKIIEKEKEEELEKKKQKK---
HS-Alpha7-Proteasome-NP_002783 (220) LNPEEIEKYVAEIEKEKEE-NEKKKQKKAS-
SC-Alpha7-Proteasome-NP_014604 (224) LSSEEINQYVTQIEQEKQEQQEQDKKKSNH
PF-Alpha7-Proteasome-MAL13P1.270 (217) IEEQEINSMVELIDQERTKNNEQNE------
Consensus (225) LD EEI YV IE EKEE NEKKK KK

Multiple Sequence Alignment: Proteasome β-1 Subunit




Section 6
(166) 166 180 198
HS-Beta1-Proteasome-NP_002784 (165) AGGSASAMLQPLLDNQVGFK--------NMQNV
AG-Beta1-Proteasome-XP_315096 (155) AGGSAGPLLQPVLDNQIGQK--------NMLNA
DM-Beta1-Proteasome-NP_524115 (157) AGGTAGTLLQPVLDNQIGHKNM------NLEDA
SC-Beta1-Proteasome-NP_009512 (157) AGGAAASLIMPFLDNQVNFKNQYEPGTNGKVKK
PF-Beta1-Proteasome-MAL13P1.270 (165) CVGSGSQLILPILDNRVEQKN---------QLI
Consensus (166) AGGSAS LLQPVLDNQVG KN NM
Section 7
(199) 199 210 220 231
HS-Beta1-Proteasome-NP_002784 (190) EHVPLSLDRAMRLVKDVFISAAERDVYTGDALR
AG-Beta1-Proteasome-XP_315096 (180) DPEPVKMEKAISIIKDTFISATERDIYTGDSVI
DM-Beta1-Proteasome-NP_524115 (184) DKIKLTKERAVSVASDTFISAAERDIYTGDSVL
SC-Beta1-Proteasome-NP_009512 (190) PLKYLSVEEVIKLVRDSFTSATERHIQVGDGLE
PF-Beta1-Proteasome-MAL13P1.270 (189) KNTNFNLGDDINFVKDAITSATERDIYTGDKTL
Consensus (199) D LSLERAI LVKDTFISATERDIYTGDSLL
Section 8
(232) 232 240 250
HS-Beta1-Proteasome-NP_002784 (223) ICIVTKEGIREETVSLRKD
AG-Beta1-Proteasome-XP_315096 (213) INIITKDGIKEETLHLRKD
DM-Beta1-Proteasome-NP_524115 (217) INIITKDGIEVRTLTLRQD
SC-Beta1-Proteasome-NP_009512 (223) ILIVTKDGVRKEFYELKRD
PF-Beta1-Proteasome-MAL13P1.270 (222) IYVIDKMGINVNTLDLKQD
Consensus (232) I IITKDGIR ETL LRKD

Multiple Sequence Alignment: Proteasome β-2 Subunit



Section 6

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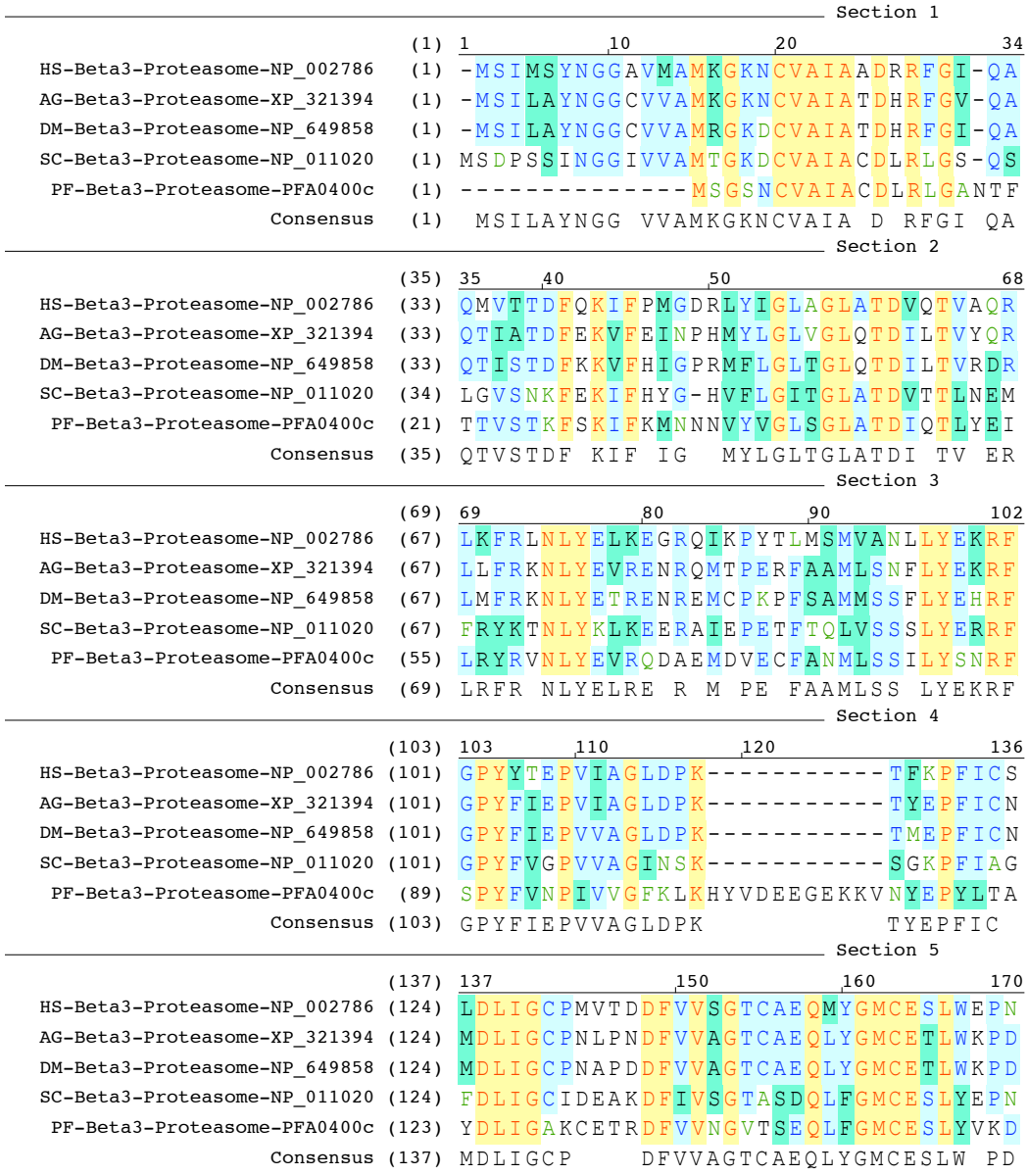
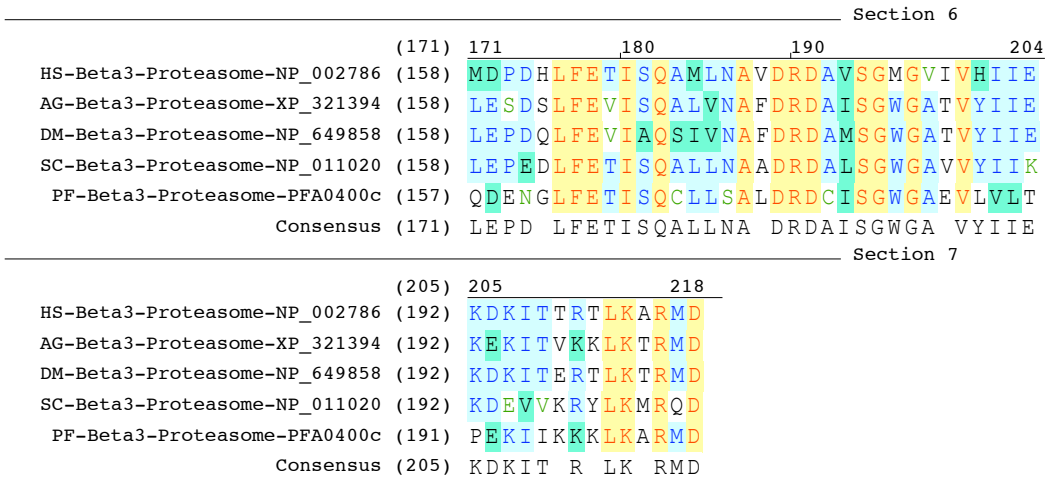
HS-Beta2-Proteasome-NP_002785     (162) KCLEELQKRFILNLPTFSVRIIDKNGIHDLDNI
AG-Beta2-Proteasome-XP_319581     (164) KGVTEIHKRLILNLPNFKVAVIDKDGVKYLDDI
DM-Beta2-Proteasome-NP_609804     (162) KCIAEIQKRLVVNLKNFTVAVVDKDGVRDLEPI
SC-Beta2-Proteasome-NP_010928     (163) LCVQELEKRMPMDFKGVIVKIVDKDGIRQVDDF
PF-Beta2-Proteasome-PF14_0676     (162) LCFEELKKRFLLTQINYELRIMYDNKVETQYVT
                        Consensus (166) KCV EL KRLILNL NF VRIIDKDGVR LD I
```

Section 7

```
                                  (199) 199         209
HS-Beta2-Proteasome-NP_002785     (195) SFPKQGS----
AG-Beta2-Proteasome-XP_319581     (197) TPDSLKQASAA
DM-Beta2-Proteasome-NP_609804     (195) SAASLAA----
SC-Beta2-Proteasome-NP_010928     (196) QAQ--------
PF-Beta2-Proteasome-PF14_0676     (195) V----------
                        Consensus (199) S
```

Multiple Sequence Alignment: Proteasome β-3 Subunit




Section 6
(171) 171 180 190 204
HS-Beta3-Proteasome-NP_002786 (158) MDPDHLFETISQAMLNAVDRDAVSGMGVIVHIIE
AG-Beta3-Proteasome-XP_321394 (158) LESDSLFEVISQALVNAFDRDAISGWGATVYIIE
DM-Beta3-Proteasome-NP_649858 (158) LEPDQLFEVIAQSIVNAFDRDAMSGWGATVYIIE
SC-Beta3-Proteasome-NP_011020 (158) LEPEDLFETISQALLNAADRDALSGWGAVVYIIK
PF-Beta3-Proteasome-PFA0400c (157) QDENGLFETISQCLLSALDRDCISGWGAEVLVLT
Consensus (171) LEPD LFETISQALLNA DRDAISGWGA VYIIE
Section 7
(205) 205 218
HS-Beta3-Proteasome-NP_002786 (192) KDKITTRTLKARMD
AG-Beta3-Proteasome-XP_321394 (192) KEKITVKKLKTRMD
DM-Beta3-Proteasome-NP_649858 (192) KDKITERTLKTRMD
SC-Beta3-Proteasome-NP_011020 (192) KDEVVKRYLKMRQD
PF-Beta3-Proteasome-PFA0400c (191) PEKIIKKKLKARMD
Consensus (205) KDKIT R LK RMD

Multiple Sequence Alignment: Proteasome β-4 Subunit

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DM-Beta4-Proteasome-NP_649529       MLNN-YNSLAQPMWQNGPAPGEFYNFTGGQTPVQQLPRELTTMGPYGTKH 49
HS-Beta4-Proteasome-XP_317860       MYPMGGNSMAGPFWSNGPAPGAFYNFPGSTVAGGAMQARSDTPGEFGTQR 50
HS-Beta4-Proteasome-NP_002787       -MEAFLGSRSG-LWAGGPAPGQFYRIP-STPDSFMDPASALYRGP--ITR 45
SC-Beta4-Proteasome-NP_116708       -------MNHDPFSWGRPADSTYGAYN----------TQIANAGASPMVN 33
PF-Beta4-Proteasome-MAL8P1.142      -------------------------------------------------M 1


DM-Beta4-Proteasome-NP_649529       STASSTTGTSVLGIRYDSGVMLAADTLVSYGSMARYQNIERVFKVNKNIL 99
HS-Beta4-Proteasome-XP_317860       SYYPVTTGTSVVGLMFKDGVIIAADKLISYGSLARFHDVDRVYRINDKTV 100
HS-Beta4-Proteasome-NP_002787       TQNPMVTGTSVLGVKFEGGVVIAADMLGSYGSLARFRNISRIMRVNNSTM 95
SC-Beta4-Proteasome-NP_116708       TQQPIVTGTSVISMKYDNGVIIAADNLGSYGSLLRFNGVERLIPVGDNTV 83
PF-Beta4-Proteasome-MAL8P1.142      TLGPVVTGTSVIAIKYKHGIMIAADRKASYGSYAKFQNVERIFKINNKTV 51
                                    :  . .*****:.: :. *:::***   ****  ::..:.*:  :... :

DM-Beta4-Proteasome-NP_649529       LGGSGDFADIQSIKRNIDQKMIE----DQCCDDNIEMKPKSLASWMTRVL 145
HS-Beta4-Proteasome-XP_317860       LGIGGDFADFQYIKRHIDQKVID----DQCLDDKNEMKPRSFYNWLTRVM 146
HS-Beta4-Proteasome-NP_002787       LGASGDYADFQYLKQVLGQMVID----EELLGDGHSYSPRAIHSWLTRAM 141
SC-Beta4-Proteasome-NP_116708       VGISGDISDMQHIERLLKDLVTENAYDNPLADAEEALEPSYIFEYLATVM 133
PF-Beta4-Proteasome-MAL8P1.142      MGFSGELADAQYLHELLTRKNINN--LSEKKRKEDMYTPQHYHSYVSRVF 99
                                    :* .*: :* * :.. :     :    .          *    .::: .:

DM-Beta4-Proteasome-NP_649529       YNRRSRMNPLYIDVVVGGVDN------------------------------ 166
HS-Beta4-Proteasome-XP_317860       YNRRSEFQPLYLDLVIGGMQ------------------------------ 166
HS-Beta4-Proteasome-NP_002787       YSRRSKMNPLWNTMVIGGYA------------------------------ 161
SC-Beta4-Proteasome-NP_116708       YQRRSKMNPLWNAIIVAGVQS------------------------------ 154
PF-Beta4-Proteasome-MAL8P1.142      YVRKNRIDPLFNNIIIAGINSQKYDNNDDNVLLYTNKNNDDEQNEYKNNE 149
                                    * *:..::**:  :::.*

DM-Beta4-Proteasome-NP_649529       ------EGTPYLANVDLRGRSYEDYVVATGFARHLAVPLVREKKPKDRDF 210
HS-Beta4-Proteasome-XP_317860       ------DGEPFLGHVNLRGRSYTSNVVATGYGTHLALPLLREWSENPTAY 210
HS-Beta4-Proteasome-NP_002787       ------DGESFLGYVDMLGVAYEAPSLATGYGAYLAQPLLREVLEKQPVL 205
SC-Beta4-Proteasome-NP_116708       ------NGDQFLRYVNLLGVTYSSPTLATGFGAHMANPLLRKVVDRESDI 198
PF-Beta4-Proteasome-MAL8P1.142      EYKEIHKDDLYIGFVDMHGTNFCDDYITTGYARYFALTLLRDHYKDN--- 196
                                          ..  ::  *:: *  :    ::**:. ::* .*:*.

DM-Beta4-Proteasome-NP_649529       TAV---EASELIRTCMEVLYYRDTRNISQYTVGVCSVN-GCGVEG----P 252
HS-Beta4-Proteasome-XP_317860       QTLGQPEANDLMKRVMEVLWYRDCRSDPKYSQAVCTAD-GVKVDA----D 255
HS-Beta4-Proteasome-NP_002787       SQT---EARDLVERCMRVLYYRDARSYNRFQIATVTEK-GVEIEG----P 247
SC-Beta4-Proteasome-NP_116708       PKTTVQVAEEAIVNAMRVLYYRDARSSRNFSLAIIDKNTGLTFKK----N 244
PF-Beta4-Proteasome-MAL8P1.142      --MTEEEARILINECLRILYFRDATSSNFIQIVKVTSK-GVEYEEPYILP 243
                                           *   :   :.:*::**  .           . *   .

DM-Beta4-Proteasome-NP_649529       FQVN-ENWTFAETIKGY----- 268
HS-Beta4-Proteasome-XP_317860       CFVA-QNWELAHTIKGY----- 271
HS-Beta4-Proteasome-NP_002787       LSTE-TNWDIAHMISGFE---- 264
SC-Beta4-Proteasome-NP_116708       LQVENMKWDFAKDIKGYGTQKI 266
PF-Beta4-Proteasome-MAL8P1.142      CVLNSADYVYPSTLLPPAGCMW 265
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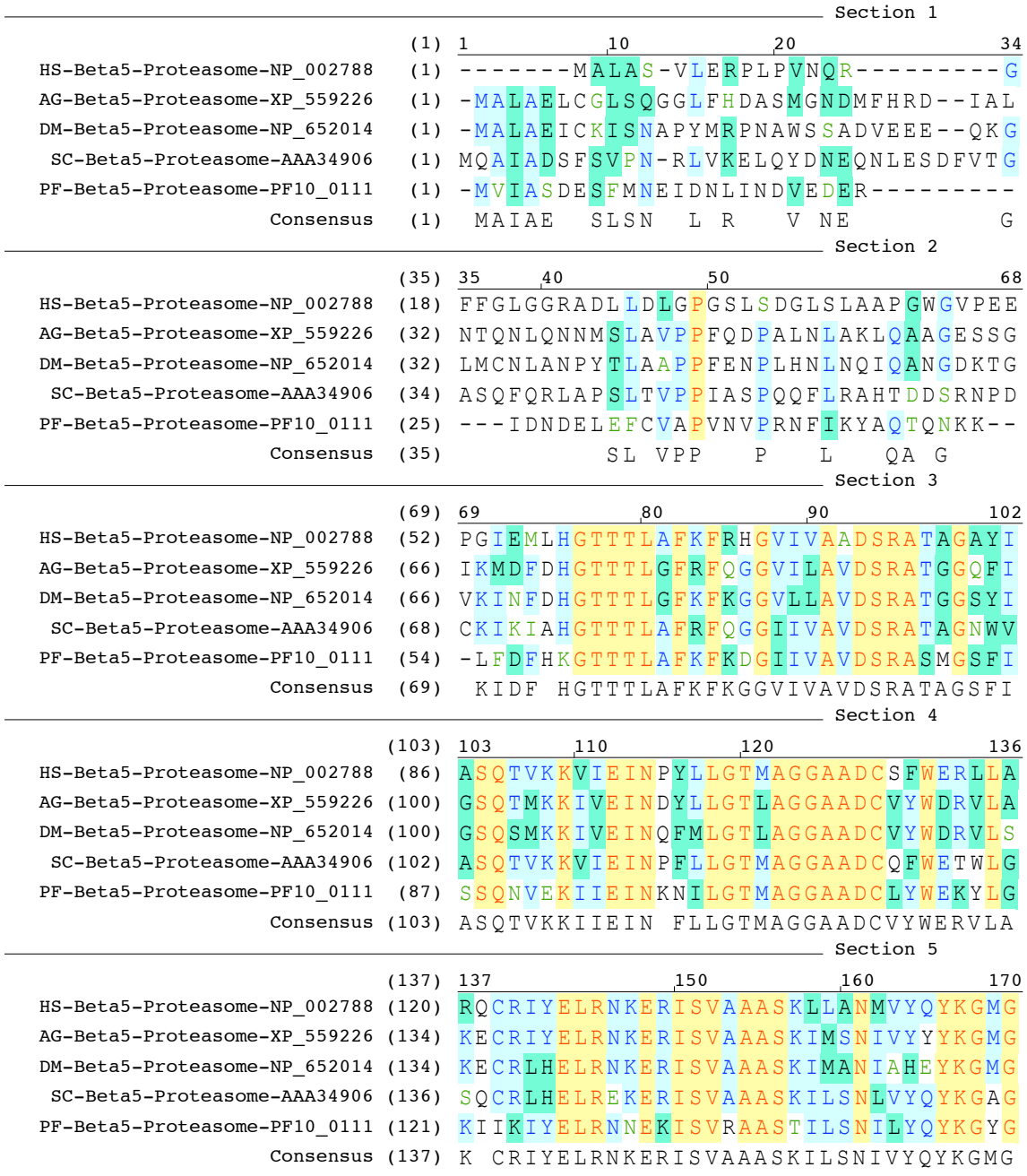
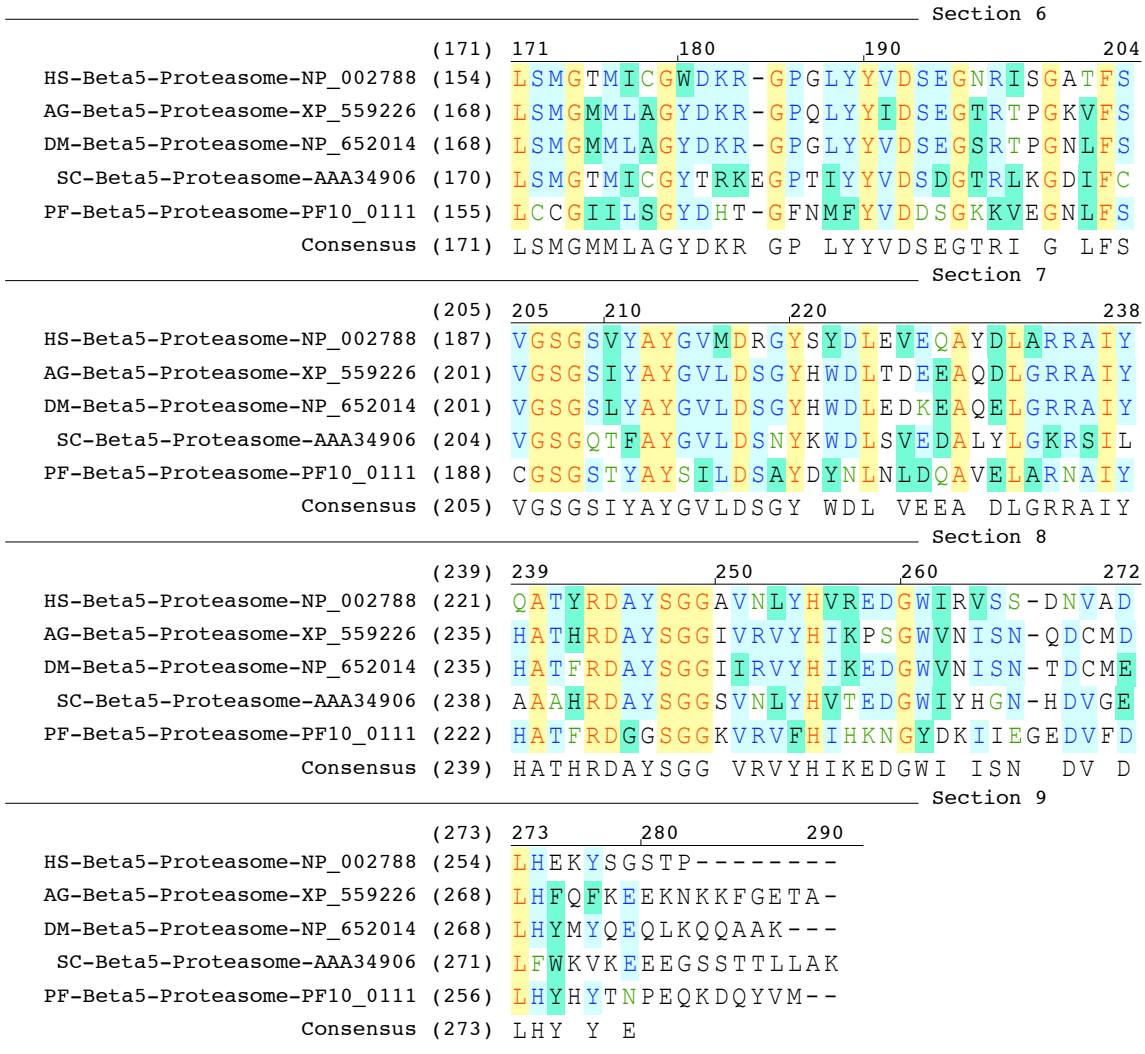
Multiple Sequence Alignment: Proteasome β-5 Subunit

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AG-Beta5-Proteasome-XP_559226 (168) LSMGMMLAGYDKR-GPQLYYIDSEGTRTPGKVFS
DM-Beta5-Proteasome-NP_652014 (168) LSMGMMLAGYDKR-GPGLYYVDSEGSRTPGNLFS
SC-Beta5-Proteasome-AAA34906 (170) LSMGTMICGYTRKEGPTIYYVDSDGTRLKGDIFC
PF-Beta5-Proteasome-PF10_0111 (155) LCCGIILSGYDHT-GFNMFYVDDSGKKVEGNLFS
Consensus (171) LSMGMMLAGYDKR GP LYYVDSEGTRI G LFS
Section 7
(205) 205 210 220 238
HS-Beta5-Proteasome-NP_002788 (187) VGSGSVYAYGVMDRGYSYDLEVEQAYDLARRAIY
AG-Beta5-Proteasome-XP_559226 (201) VGSGSIYAYGVLDSGYHWDLTDEEAQDLGRRAIY
DM-Beta5-Proteasome-NP_652014 (201) VGSGSLYAYGVLDSGYHWDLEDKEAQELGRRAIY
SC-Beta5-Proteasome-AAA34906 (204) VGSGQTFAYGVLDSNYKWDLSVEDALYLGKRSIL
PF-Beta5-Proteasome-PF10_0111 (188) CGSGSTYAYSILDSAYDYNLNLDQAVELARNAIY
Consensus (205) VGSGSIYAYGVLDSGY WDL VEEA DLGRRAIY
Section 8
(239) 239 250 260 272
HS-Beta5-Proteasome-NP_002788 (221) QATYRDAYSGGAVNLYHVREDGWIRVSS-DNVAD
AG-Beta5-Proteasome-XP_559226 (235) HATHRDAYSGGIVRVYHIKPSGWVNISN-QDCMD
DM-Beta5-Proteasome-NP_652014 (235) HATFRDAYSGGIIRVYHIKEDGWVNISN-TDCME
SC-Beta5-Proteasome-AAA34906 (238) AAAHRDAYSGGSVNLYHVTEDGWIYHGN-HDVGE
PF-Beta5-Proteasome-PF10_0111 (222) HATFRDGGSGGKVRVFHIHKNGYDKIIEGEDVFD
Consensus (239) HATHRDAYSGG VRVYHIKEDGWI ISN DV D
Section 9
(273) 273 280 290
HS-Beta5-Proteasome-NP_002788 (254) LHEKYSGSTP--------
AG-Beta5-Proteasome-XP_559226 (268) LHFQFKEEKNKKFGETA-
DM-Beta5-Proteasome-NP_652014 (268) LHYMYQEQLKQQAAK---
SC-Beta5-Proteasome-AAA34906 (271) LFWKVKEEEGSSTTLLAK
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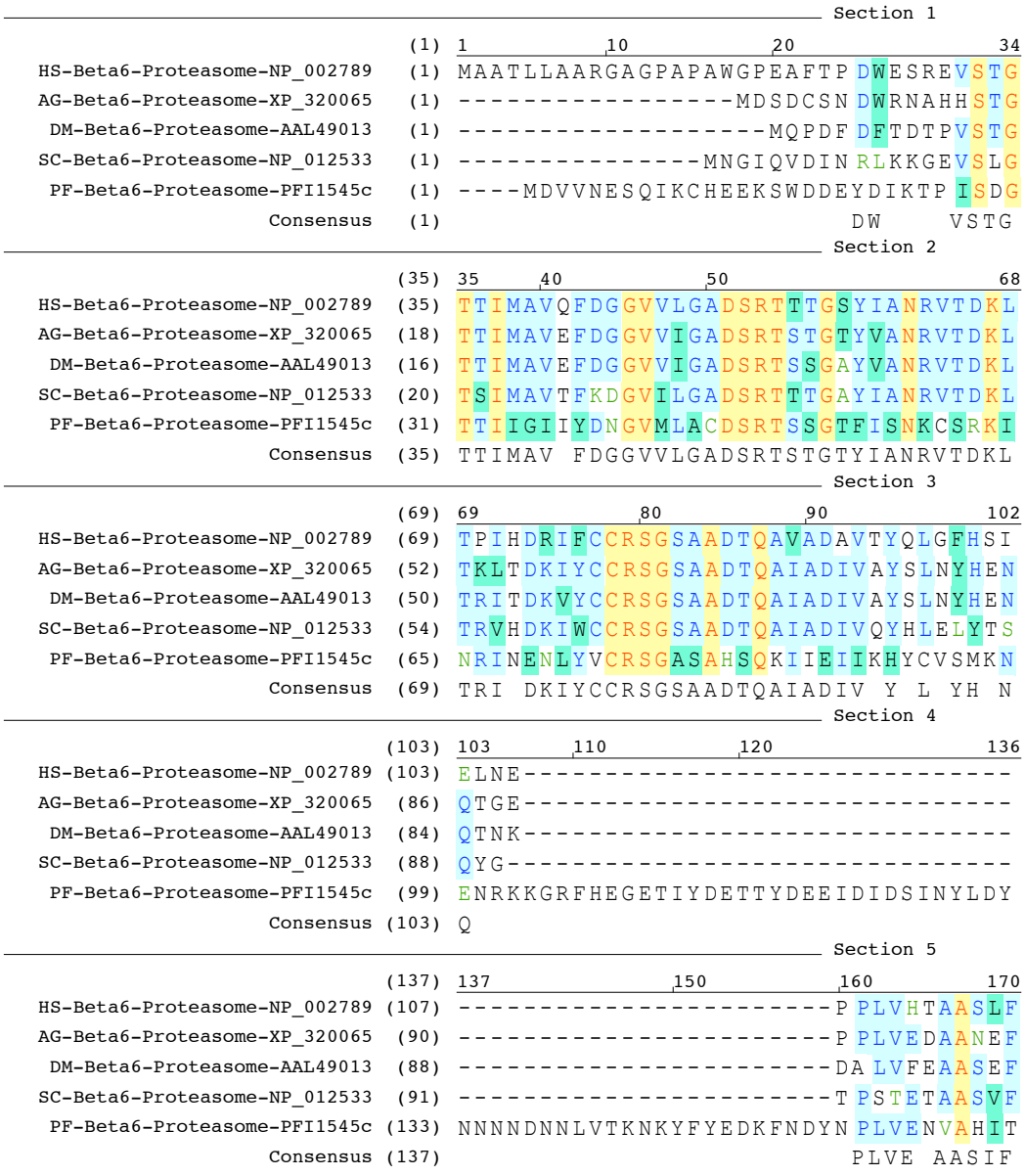
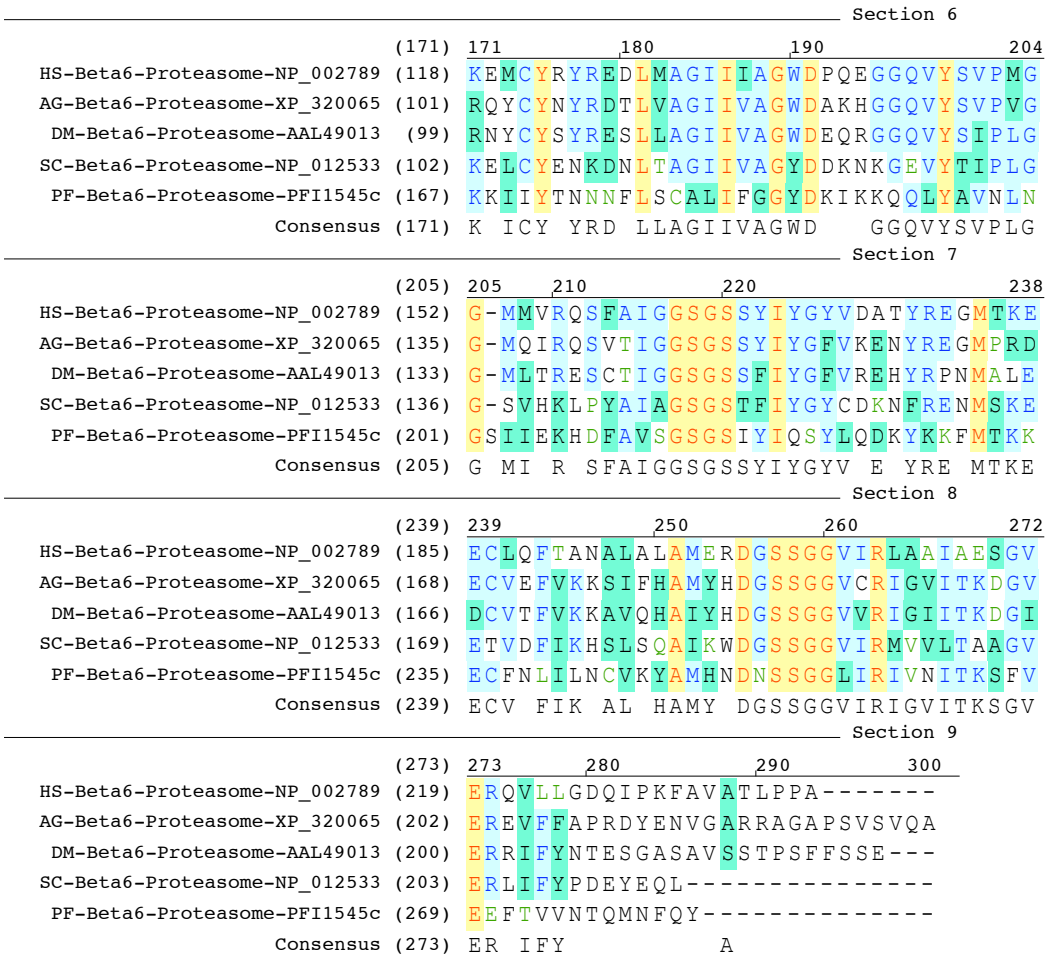
Consensus (273) LHY Y E

Multiple Sequence Alignment: Proteasome β-6 Subunit

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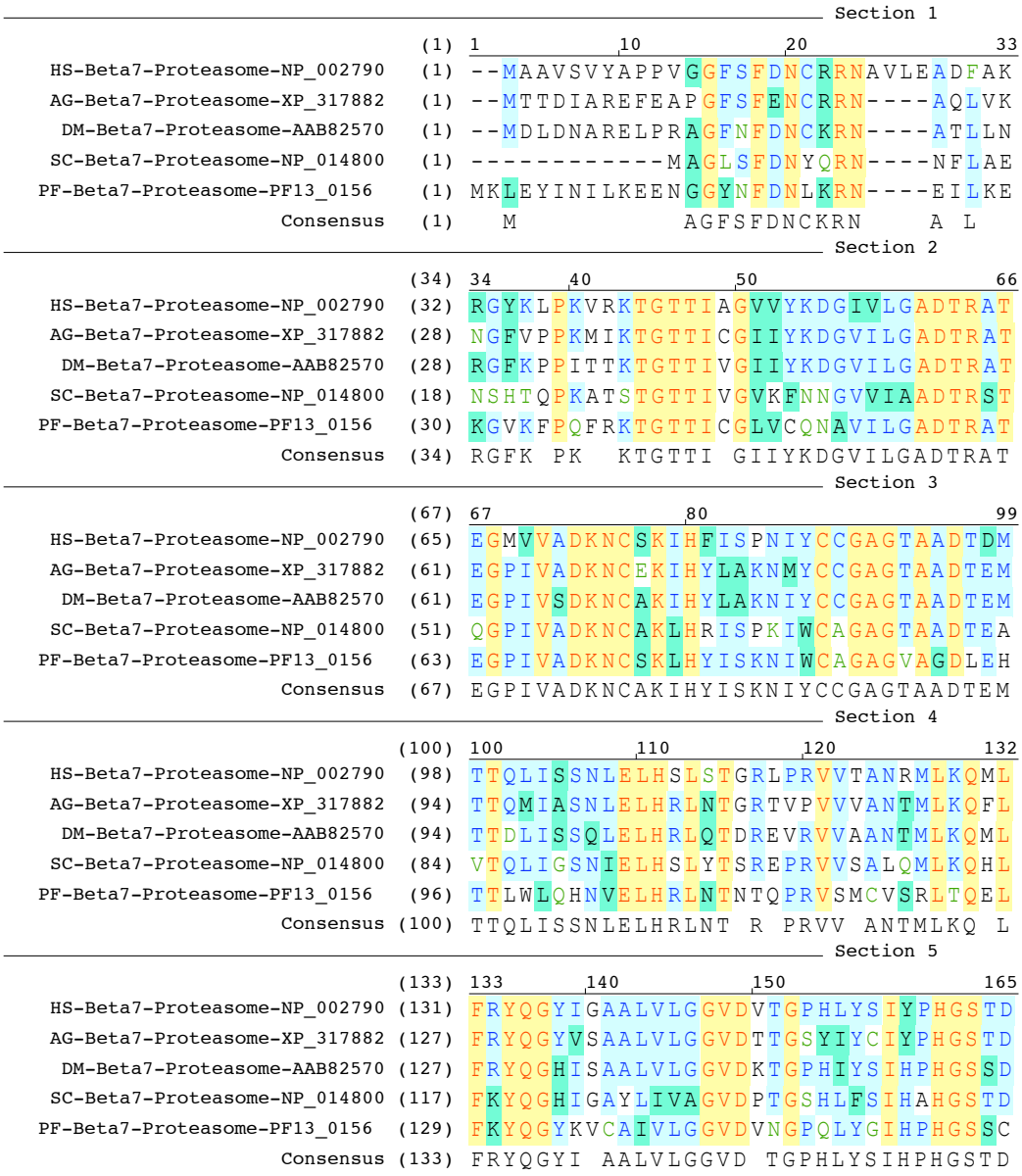
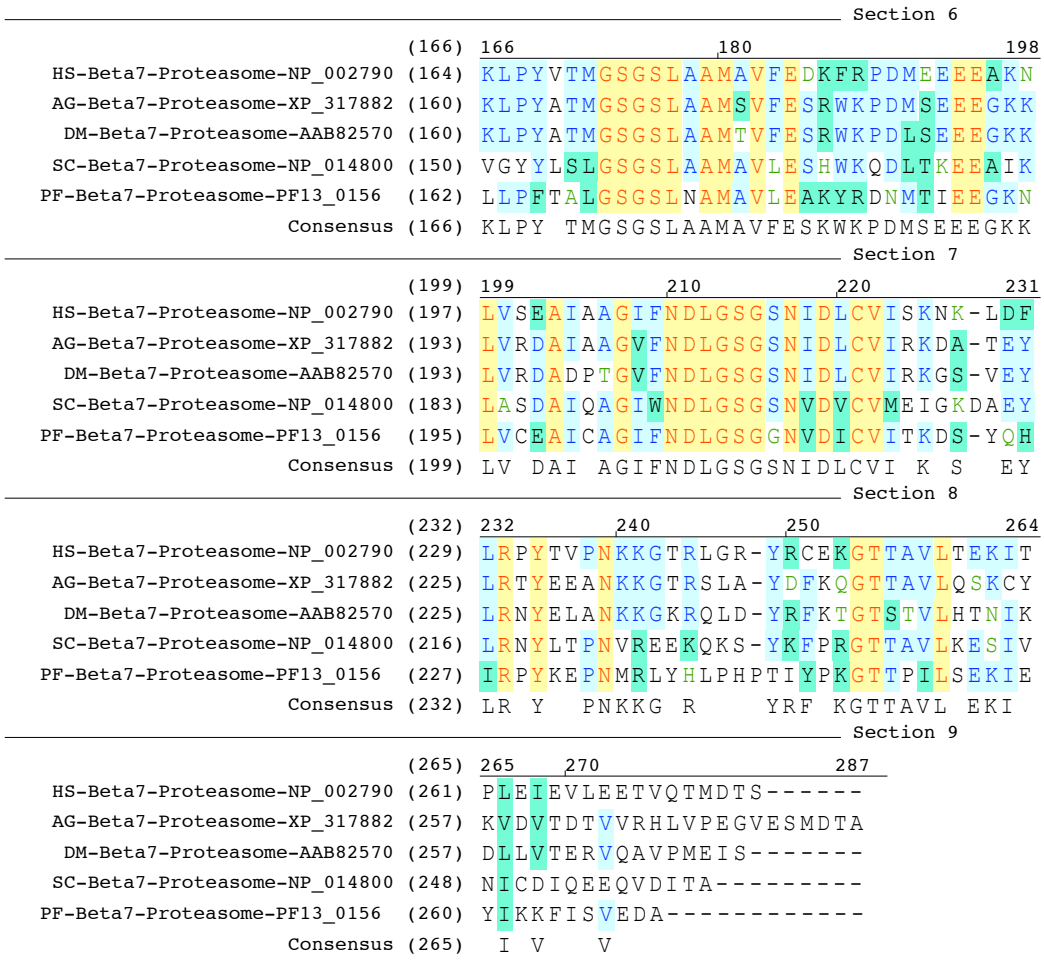
DM-Beta6-Proteasome-AAL49013 (99) RNYCYSYRESLLAGIIVAGWDEQRGGQVYSIPLG
SC-Beta6-Proteasome-NP_012533 (102) KELCYENKDNLTAGIIVAGYDDKNKGEVYTIPLG
PF-Beta6-Proteasome-PFI1545c (167) KKIIYTNNNFLSCALIFGGYDKIKKQQLYAVNLN
Consensus (171) K ICY YRD LLAGIIVAGWD GGQVYSVPLG
Section 7
(205) 205 210 220 238
HS-Beta6-Proteasome-NP_002789 (152) G-MMVRQSFAIGGSGSSYIYGYVDATYREGMTKE
AG-Beta6-Proteasome-XP_320065 (135) G-MQIRQSVTIGGSGSSYIYGFVKENYREGMPRD
DM-Beta6-Proteasome-AAL49013 (133) G-MLTRESCTIGGSGSSFIYGFVREHYRPNMALE
SC-Beta6-Proteasome-NP_012533 (136) G-SVHKLPYAIAGSGSTFIYGYCDKNFRENMSKE
PF-Beta6-Proteasome-PFI1545c (201) GSIIEKHDFAVSGSGSIYIQSYLQDKYKKFMTKK
Consensus (205) G MI R SFAIGGSGSSYIYGYV E YRE MTKE
Section 8
(239) 239 250 260 272
HS-Beta6-Proteasome-NP_002789 (185) ECLQFTANALALAMERDGSSGGVIRLAAIAESGV
AG-Beta6-Proteasome-XP_320065 (168) ECVEFVKKSIFHAMYHDGSSGGVCRIGVITKDGV
DM-Beta6-Proteasome-AAL49013 (166) DCVTFVKKAVQHAIYHDGSSGGVVRIGIITKDGI
SC-Beta6-Proteasome-NP_012533 (169) ETVDFIKHSLSQAIKWDGSSGGVIRMVVLTAAGV
PF-Beta6-Proteasome-PFI1545c (235) ECFNLILNCVKYAMHNDNSSGGLIRIVNITKSFV
Consensus (239) ECV FIK AL HAMY DGSSGGVIRIGVITKSGV
Section 9
(273) 273 280 290 300
HS-Beta6-Proteasome-NP_002789 (219) ERQVLLGDQIPKFAVATLPPA-------
AG-Beta6-Proteasome-XP_320065 (202) EREVFFAPRDYENVGARRAGAPSVSVQA
DM-Beta6-Proteasome-AAL49013 (200) ERRIFYNTESGASAVSSTPSFFSSE---
SC-Beta6-Proteasome-NP_012533 (203) ERLIFYPDEYEQL---------------
PF-Beta6-Proteasome-PFI1545c (269) EEFTVVNTQMNFQY--------------
Consensus (273) ER IFY A

Multiple Sequence Alignment: Proteasome β-7 Subunit




Section 6
(166) 166 180 198
HS-Beta7-Proteasome-NP_002790 (164) KLPYVTMGSGSLAAMAVFEDKFRPDMEEEEAKN
AG-Beta7-Proteasome-XP_317882 (160) KLPYATMGSGSLAAMSVFESRWKPDMSEEEGKK
DM-Beta7-Proteasome-AAB82570 (160) KLPYATMGSGSLAAMTVFESRWKPDLSEEEGKK
SC-Beta7-Proteasome-NP_014800 (150) VGYYLSLGSGSLAAMAVLESHWKQDLTKEEAIK
PF-Beta7-Proteasome-PF13_0156 (162) LLPFTALGSGSLNAMAVLEAKYRDNMTIEEGKN
Consensus (166) KLPY TMGSGSLAAMAVFESKWKPDMSEEEGKK
Section 7
(199) 199 210 220 231
HS-Beta7-Proteasome-NP_002790 (197) LVSEAIAAGIFNDLGSGSNIDLCVISKNK-LDF
AG-Beta7-Proteasome-XP_317882 (193) LVRDAIAAGVFNDLGSGSNIDLCVIRKDA-TEY
DM-Beta7-Proteasome-AAB82570 (193) LVRDADPTGVFNDLGSGSNIDLCVIRKGS-VEY
SC-Beta7-Proteasome-NP_014800 (183) LASDAIQAGIWNDLGSGSNVDVCVMEIGKDAEY
PF-Beta7-Proteasome-PF13_0156 (195) LVCEAICAGIFNDLGSGGNVDICVITKDS-YQH
Consensus (199) LV DAI AGIFNDLGSGSNIDLCVI K S EY
Section 8
(232) 232 240 250 264
HS-Beta7-Proteasome-NP_002790 (229) LRPYTVPNKKGTRLGR-YRCEKGTTAVLTEKIT
AG-Beta7-Proteasome-XP_317882 (225) LRTYEEANKKGTRSLA-YDFKQGTTAVLQSKCY
DM-Beta7-Proteasome-AAB82570 (225) LRNYELANKKGKRQLD-YRFKTGTSTVLHTNIK
SC-Beta7-Proteasome-NP_014800 (216) LRNYLTPNVREEKQKS-YKFPRGTTAVLKESIV
PF-Beta7-Proteasome-PF13_0156 (227) IRPYKEPNMRLYHLPHPTIYPKGTTPILSEKIE
Consensus (232) LR Y PNKKG R YRF KGTTAVL EKI
Section 9
(265) 265 270 287
HS-Beta7-Proteasome-NP_002790 (261) PLEIEVLEETVQTMDTS------
AG-Beta7-Proteasome-XP_317882 (257) KVDVTDTVVRHLVPEGVESMDTA
DM-Beta7-Proteasome-AAB82570 (257) DLLVTERVQAVPMEIS-------
SC-Beta7-Proteasome-NP_014800 (248) NICDIQEEQVDITA---------
PF-Beta7-Proteasome-PF13_0156 (260) YIKKFISVEDA----------
Consensus (265) I V V

Appendix IV:
Materials and Methods for Appendices

Materials and Methods for Appendices

Figure S1: Activity Assay Linear Range

Jurkat cells used for this experiment were obtained from the laboratory of W. Johnson (Biology Department, Boston College, Chestnut Hill, MA) and were originally Clone E6-1, TIB-152® from ATCC (Manassas, Virginia). Jurkat cells were grown in RPMI supplemented with 10% human serum, and lysed by agitation with 0.1 mm glass disruption beads in assay lysis buffer (see protocol). Lysate was clarified by centrifugation at 5,000 rpm in a microcentrifuge, and supernatant was saved for "enzyme" sample. Lysate was diluted 1:2 in assay buffer by serial dilution to obtain 12 "enzyme" samples of different concentrations. Each enzyme sample was added to 2 μL assay substrate and incubated for 90 minutes at 37^{o}C and measured for fluorescence at excitation/emission 380 nm/460 nm, respectively. Data were analyzed using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA).

Figure S2: Parasite growth in minimal media

Cultures volumes of 180 μL with 1% hematocrit and 1% parasitemia were grown in minimal medium (see protocol above) supplemented with different amounts of Albumaxx II (Life Technologies, Inc., Grand Island, NY), alongside cells from the same culture grown in standard RPMI complete medium (see Methods, Chapter II) supplemented with differing amounts of Albumaxx II. Cultures were grown under standard conditions and processed in the same manner as SYBR Green® growth assays (see methods, Chapter II and Chapter III). Data were analyzed using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA), using a linear regression algorithm.

Figure S3: HRPII secretion

3D7 cultures were incubated in parasite minimal medium (export medium, see protocol above) in a 10 mL volume each, inoculated with 2% hematocrit and 1% parasitemia at ring stage, supplemented with 0.5% Albumaxx II, then were exposed to MG132, mefloquine, or DMSO at concentrations indicated. One culture was harvested for t = 0 samples, and remaining cultures were supplemented with 12.5 nM or 50 nM MG132, 100 nM mefloquine, or DMSO vehicle, and incubated for 12 hr or 24 hr under standard conditions (See Chapter 2, methods). After incubation, cultures were separated by centrifugation at 500xg. A volume of 5 mL supernatant medium was concentrated down to 200 µL using 3,000 NMWL Amicon filters (EMD Millipore, Inc., Billerica Massachusetts). Western and Coomassie stain were obtained in the same manner as described in Chapter II, Methods. Samples were normalized by volume, and 12 µL concentrated medium was added per well. Primary antibody was anti-HRPII (*Plasmodium falciparum*, Santa Cruz Biotechnology, Dallas, TX).

Figure S4: HRPII Accumulation

Cultures were set up in the same manner described above, except in a 25 mL volume, and incubated with 12.5-100 nM MG132, 7.5-60 nM mefloquine, or DMSO vehicle. Parasites were harvested by saponin lysis, as described in Chapter II, Methods. Protein was extracted from parasites by agitation with 2 mm disruption beads in T-NET lysis buffer (50 nM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100). Samples were normalized by keeping lysate volume proportional to original culture volume, with

the goal of all samples having the same number of cells per unit volume. Westerns and Coomassie-stained gels were set up as described above and in Chapter II, Methods. Primary antibody was anti-HRPII, same as above.

Multiple Sequence Alignments

All *P. falciparum* gene sequences were obtained from the PlasmoDB website, Release 6.0: http://plasmodb.org/plasmo/ (EuPathDB Project Team, Athens, GA). Sequences from other organisms were obtained from NCBI/BLAST website: http://blast.ncbi.nlm.nih.gov/Blast.cgi (The National Library of Medicine, Bethesda, MD). Multiple sequence alignments were performed using ClustalW software [European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI), Hinxton, Cambridge, UK]. For β4 alignment, the following chart is a guide for colors:

AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G
Others	Grey	Unusual amino/imino acids, etc.

For all other alignments, colors are for visualization only, with yellow columns showing conserved residues, blue showing partial conservation, and green showing amino acid property conservation (e.g., conservation in terms of polarity, charge, etc.).