

Review

The proteasome of malaria parasites: A multi-stage drug target for chemotherapeutic intervention?

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ABSTRACT

The ubiquitin/proteasome system serves as a regulated protein degradation pathway in eukaryotes, and is involved in many cellular processes featuring high protein turnover rates, such as cell cycle control, stress response and signal transduction. In malaria parasites, protein quality control is potentially important because of the high replication rate and the rapid transformations of the parasite during life cycle progression. The proteasome is the core of the degradation pathway, and is a major proteolytic complex responsible for the degradation and recycling of non-functional ubiquitinated proteins. Annotation of the genome for *Plasmodium falciparum*, the causative agent of malaria tropica, revealed proteins with similarity to human 26S proteasome subunits. In addition, a bacterial ClpQ/hslV threonine peptidase-like protein was identified. In recent years several independent studies indicated an essential function of the parasite proteasome for the liver, blood and transmission stages. In this review, we compile evidence for protein recycling in *Plasmodium* parasites and discuss the role of the 26S proteasome as a prospective multi-stage target for antimalarial drug discovery programs.

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1. Introduction

The proteasome is a major proteolytic complex responsible for the degradation and recycling of proteins and therefore plays an important role in intracellular protein quality control. The proteasome mediates the degradation of many short-lived proteins that are involved in cell cycle regulation, signal transduction and apoptosis, and is also responsible for the recycling of abnormal or dam-

aged proteins, which would otherwise accumulate and become harmful to the cell (reviewed in Pickart and Cohen, 2004). The proteasome is part of the ubiquitin-proteasome system (UPS), which manages proteostasis in the cell. Via an UPS-specific enzymatic cascade, proteins become labelled with a small ubiquitin (Ub) tag. The type of ubiquitination then determines whether a protein is designated for further roles in cellular processes like DNA repair, trafficking or signal transduction, or whether it will be degraded by the proteasome (reviewed in Hendil and Hartmann-Petersen, 2004; Pickart and Cohen, 2004; Clague and Urbe, 2010). Because eukaryotic proteostasis is central to cell development, deficiencies

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can lead to metabolic, oncogenic, neurodegenerative and cardiovascular disorders (reviewed in Balch et al., 2008).

Protein regulation appears to be important for the rapid transformations of the malaria parasite during life cycle progression in target organs of the human host and the mosquito vector, including stages having high replication rates. Shifts in temperature, to which the parasite is exposed when rapidly adapting from human to mosquito, and vice versa, might additionally induce a stress response requiring management by the UPS. *In silico* predictions indicate that over half of the parasite proteins represent targets for ubiquitination (Ponts et al., 2011). The human malaria parasite, *Plasmodium falciparum*, appears to possess a functional eukaryotic proteasome and a bacterial ClpQ/hslV threonine peptidase-like protein complex (reviewed in Chung and Le Roch, 2010; Tschan et al., 2011), but their specific roles within the parasite are not known.

The proteasome has long been explored as an anti-cancer drug target (reviewed in Kisselev and Goldberg, 2001), based upon the observation that proteasome inhibition can induce apoptosis preferentially in cancer cells. In 2003 the proteasome inhibitor bortezomib (Velcade®, PS-341) was approved by the US Food and Drug Administration for the treatment of multiple myeloma (Kane et al., 2003). Since then, a stream of new proteasome inhibitors have been pursued in clinical trials (reviewed in Orłowski and Kuhn, 2008; de Bettignies and Coux, 2010).

In *Plasmodium*, inhibitor studies reveal an essential role of the proteasome for the liver, blood and transmission stages (Gantt et al., 1998; Lindenthal et al., 2005; Reynolds et al., 2007; Kreidenweiss et al., 2008; Prudhomme et al., 2008; Czesny et al., 2009; Schoof et al., 2010; Aminake et al., 2011), thus suggesting the proteasome as a promising multi-stage target in malaria therapy. The World Health Organization (WHO) recommends artemisinin-based combination therapies for the treatment of malaria, with component drugs having independent targets in order to gain control of drug-resistant parasites (WHO Guidelines for the Treatment of Malaria 2010). Ideally, such antimalarials would further exhibit activities against the liver and transmission stages of the pathogen. Inhibitors targeting the plasmodial proteasome might well fulfill these requirements, and antimalarial drug development programs might benefit from developments in anti-cancer proteasome inhibitors having improved specificity, tolerance and bioavailability.

While the *Plasmodium* proteasome begins to draw attention as an antimalarial drug target, our understanding of protein regulation in malaria parasites remains rudimentary. It is inferred that the structure and function of the plasmodial UPS is similar to other eukaryotes, based on a generally high level of conservation. However, primary data on these assumptions are fragmented, and it is not clear to what degree the UPS has adapted to the requirements of the highly specialized malaria parasite. To reveal detailed function of the parasite UPS, it will be necessary to study UPS-mediated protein regulation and proteasomal degradation of target proteins. This review highlights current knowledge of the plasmodial proteasome; investigates the range of UPS proteins in the parasite; and discusses the potential role of the proteasome as a target for antimalarial drugs.

2. The composition of the proteasome

The 26S proteasome is a 2.5 MDa complex involved in the regulated degradation of ubiquitinated proteins. It is composed of more than 33 subunits (SUs), which form a proteolytic barrel-like 20S core particle (CP), capped by two 19S regulatory particles (RPs) (Fig. 1). The RP is involved in ATP-dependent recognition, binding and unfolding of ubiquitinated proteins, while the CP is important for proteolysis (reviewed in Marques et al., 2009; Bedford et al., 2010; Xie, 2010). The CP is formed by four staged heptameric rings: two outer rings, consisting of seven α -SUs per

ring, and two inner rings composed of seven β -SUs each. Substrate peptide bonds are hydrolyzed by N-terminal active site threonine residues, which are embedded in the core of the CP's β -SUs. Three of the seven different β -SUs are proteolytically active; namely, β 1, β 2 and β 5, displaying caspase-, trypsin-, and chymotrypsin-like activities, respectively (Arendt and Hochstrasser, 1997; Heinemeyer et al., 1997). More recent data suggest that the performance of the different active sites are interdependent and may have specific functional relevance (Kisselev et al., 2006; Britton et al., 2009).

Because the proteolytic sites are sequestered in the closed barrel, activators are required to facilitate access to the CP, thus ensuring that protein degradation occurs only if the substrate is unfolded (reviewed in Gallastegui and Groll, 2010). In addition to the 19S RP, it was shown for the human and yeast proteasomes that the CP is able to associate with one of two known ATP-independent activators, the 11S/PA28 heteroheptamer complex and the large heat-repeat containing protein PA200/Blm10 (reviewed in Stadtmüller and Hill, 2011). While 11S/PA28 and PA200/Blm10 are reported to preferentially support hydrolysis of peptides, the RP is involved in the degradation of proteins with higher complexity (reviewed in O'Donoghue and Gordon, 2006).

The CP can associate with one or two of the 19S RPs. The RP recognizes the ubiquitinated protein, assists in deubiquitination and unfolds the substrate, which is subsequently translocated into the CP cavity. The RP is divided into two sub-complexes, the base and the lid (Fig. 1; reviewed in Bedford et al., 2010; Gallastegui and Groll, 2010). The RP base comprises six different AAA-type ATPase SUs, the regulatory particle triple A proteins (Rpt1-6), as well as four non-ATPase SUs, the regulatory particle non-ATPase proteins Rpn1, Rpn2, Rpn10 and Rpn13. Rpn10 and Rpn13 are ubiquitin receptors, while Rpn1 and Rpn2 function as scaffold. Rpn10 acts as a linker protein between the base and the lid. The lid consists of nine Rpn-type SUs; namely, Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15.

In *P. falciparum*, 14 putative proteins homologous to the yeast CP were identified (Mordmüller et al., 2006). CP-SUs α 5 and β 5 were recently shown to be present in the cytoplasm and nucleus of blood stage parasites, particularly in trophozoites and schizonts (Fig. 2A, B; Aminake et al., 2011; Aminake and Pradel, unpublished observations). This is in accordance with recent findings reporting a peak of ubiquitinated proteins in these stages (Ponts et al., 2011). The proteasome is further expressed in gametocytes of both genders during their differentiation from stage I to stage V (Fig. 2A, B; Aminake et al., 2011; Aminake and Pradel, unpublished observations), pointing to high protein turnover activities in these stages.

In order to identify parasite homologs to proteasomal SUs, we performed a BLAST search using human proteasome SU proteins (<http://www.uniprot.org>) as queries against the *P. falciparum* 3D7 genome (<http://plasmodb.org>; Aurecochea et al., 2009). Predicted homologs of proteasome precursor or SU sequences were ranked as having high score segment pairs and low *P*-values. The computational analysis revealed plasmodial homologs for all of the human 26S proteasome SUs (Table 1). These *in silico* results provide a first indication for the presence of a 26S proteasome in malaria parasites. Biochemical studies will be required to provide direct evidence for the physical presence, interaction and proteolytic activities of the predicted SUs.

We further identified a homolog to the human ATP-independent activator 11S/PA28, PF10370c, showing an identity of 33% (*P*-value of 2.1×10^{-24}), indicating that the plasmodial CP might also be regulated independently from the RP. No homolog of PA200/Blm10 was identified.

3. HslV: ancestral relict or essential enzyme?

In addition to homologs of the eukaryotic proteasome SUs, a predicted bacteria-like proteasomal predecessor was identified

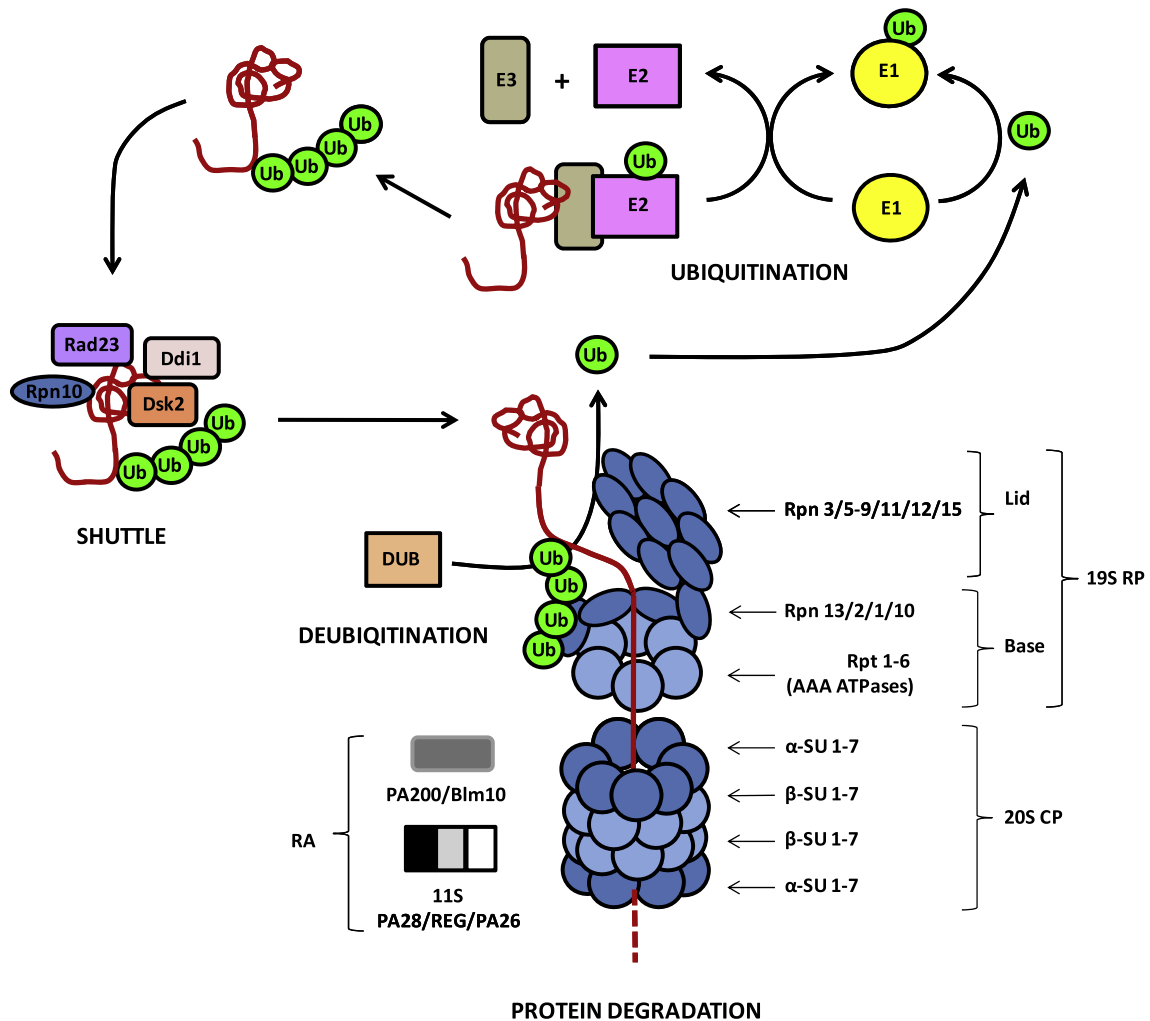


Fig. 1. The UPS of eukaryotes. The schematic depicts the structure of the 26S proteasome as well as protein ubiquitination, shuttle and deubiquitination, as experimentally demonstrated in human and yeast. UPS proteins identified in *P. falciparum* are framed in black, proteins without homologs in *P. falciparum* are framed in grey. CP, core particle; DUB, deubiquitinating enzyme; E, ubiquitinating enzyme; RA, regulatory activator; RP, regulatory particle; SU, subunit; Ub, ubiquitin.

with similarity to ClpQ/hslV threonine peptidase of *Escherichia coli*, which was termed PfhsIV (PFL1465c; Mordmüller et al., 2006). Bacterial HslV exhibits moderate sequence similarities with eukaryotic β -SUs (Rohrwild et al., 1996). The threonine peptidase typically associates with the ATPase HslU, and the HslV/U complex consists of two inner rings of six HslV-SUs per ring and two outer rings of six HslU-SUs each. In *E. coli*, HslV is involved in the degradation of regulatory or misfolded proteins, but it appears unessential for the bacterium (Missiakas et al., 1996; Kanemori et al., 1997; Seong et al., 1999). In *P. falciparum*, an HslU homolog, PfhsIU (PFI0355c), was recently identified and *in silico* analyses predicted an interaction between PfhsIU and PfhsIV (Subramaniam et al., 2009).

The HslV threonine-peptidase has been identified in a variety of protists (reviewed in Tschan et al., 2011), including localization within the kinetoplast of *Trypanosoma brucei*, where it is involved in replication of mitochondrial minicircles (Li et al., 2008). In *P. falciparum*, PfhsIV was originally described to localize to the cytoplasm of blood stage parasites, where it is proteolytically active and exhibiting threonine protease, chymotrypsin-like and peptidyl glutamyl peptide hydrolase activities (Ramasamy et al., 2007). A follow-up study assigned the nucleus-encoded PfhsIV to the mitochondrion, to which it is transported by an N-terminal targeting sequence (Tschan et al., 2010).

While PfhsIV is abundantly expressed in the asexual blood stages, new data from our laboratory indicate that PfhsIV expression decreases in gametocytes (Fig. 2C; Aminake and Pradel, unpublished observations). Considering that in gametocytes the mitochondrion becomes highly branched (Okamoto et al., 2009) while the parasite shifts from glycolysis towards mitochondrial respiration (van Dooren et al., 2006), the down-regulation of the mitochondrion-assigned enzyme in these stages warrants future study.

The attractiveness of PfhsIV as a drug target is based on the absence of a homolog in the human host (Ramasamy et al., 2007). However, it is not clear whether PfhsIV is essential for the blood stages or other life cycle stages of *Plasmodium* parasites. It might be investigated if protease/proteasome inhibitors, discussed below in Section 7, exert their effect on PfhsIV instead of the 26S proteasome. Reverse genetics approaches to disrupt the PfhsIV gene locus should shed light on these questions, and within the next years we can expect to better understand the prokaryotic proteasome.

4. Ubiquitin-tagging of proteins

Protein degradation is triggered by the carboxy-terminal tagging of the 76 amino acid long protein Ub to surface-exposed lysine

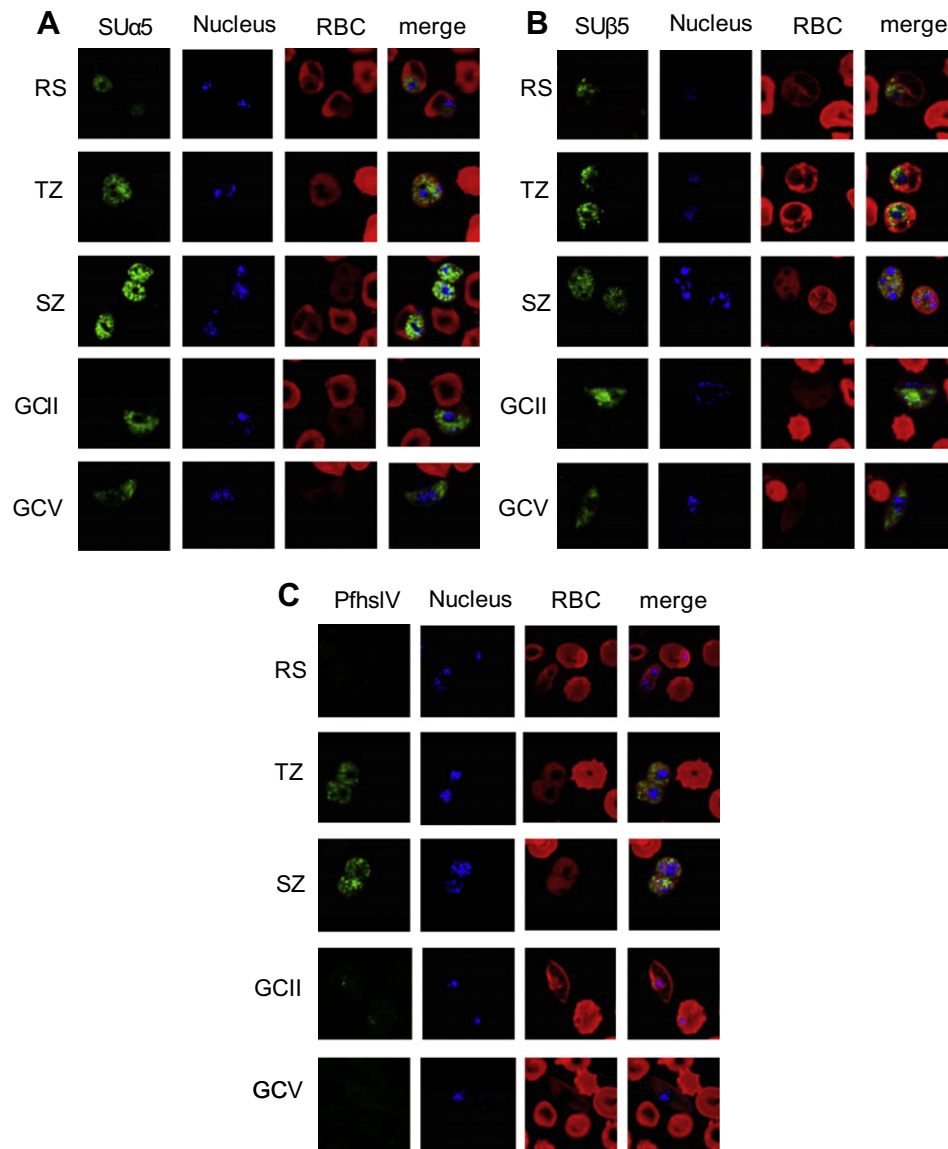


Fig. 2. (A) Subcellular localization of proteasome SUs and of PfhsIV in the blood and gametocyte stages of *P. falciparum*. Indirect immunofluorescence assays, using mouse polyclonal antisera against α -SU type 5 (A) and β -SU type 5 (B) revealed localization of the two proteins in the asexual blood stages as well as in the gametocyte stages (stages IIb and V depicted). Antibodies antisera against PfhsIV (C) labeled the protein in the trophozoite and schizont stages, but not in gametocytes (green). Nuclei were highlighted by Hoechst nuclear staining (blue); and erythrocytes were counterstained by Evans Blue (red). GC, gametocyte; RBC, red blood cell; RS; ring stage, SZ, schizont; TZ, trophozoite. Bar, 5 μ m. For detailed methods, see Supplementary data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

residues of redundant or misfolded proteins, thereby marking them for degradation by the proteasome. Once the initial Ub is attached, poly-ubiquitination of the substrate can occur through the sequential transfer of additional Ubs. Substrates targeted for degradation require an Ub chain of at least four residues. Proteins might also be mono-ubiquitinated at single or multiple sites without being targeted for degradation, and then function in cellular signaling processes like DNA repair, endocytosis or trafficking (reviewed in Hicke, 2001; Hofmann, 2009; Clague and Urbe, 2010; Grabbe et al., 2011).

Ub can be isolated as an 8.5 kDa protein from the cell either by degradation of poly-Ub proteins or by proteolytic cleavage of Ub-fusion proteins. In *P. falciparum*, a poly-ubiquitin encoding gene, *pfpUb*, comprises five tandem repeats of the Ub open reading frame (Table 2; Horrocks and Newbold, 2000). *PfpUb* is expressed in all life cycle stages with a predominant expression in intraerythrocytic parasites (Bozdech et al., 2003; Le Roch et al., 2003). Genes

encoding two additional Ub-fusion proteins are present in *P. falciparum*, the uba52 homolog L40/UBI and S31/UBI (Table 2; Ponts et al., 2008). The Ubs are fused to ribosomal proteins L40 and S27a. The *P. falciparum* genome also encodes for an 8.5 kDa Nedd8 ortholog, an ubiquitin-like protein (ULP) which becomes covalently conjugated to a limited number of cellular proteins in a manner analogous to ubiquitination. Lastly, the genome encodes for a number of additional ULPs, including homologs to SUMO, Hub1, Urm1 and Atg8 (Table 2; Ponts et al., 2008). Proteins conjugated by ULPs are generally not targeted for degradation by the proteasome, but rather function in diverse regulatory activities.

Ub conjugation involves three groups of serially-connected enzymes, the Ub-activating enzyme E1, the Ub-conjugating enzyme E2 and the Ub ligase E3 (Fig. 1). In an initial step, E1 binds Ub to form an Ub-E1 thioester and transfers the residue to E2. Subsequently, E3 interacts with both E2 and the target protein, resulting in the transfer of Ub from E2 to the substrate lysine residue. Unlike

Table 1
Homologs of human proteasome subunits α and β identified in *P. falciparum*.

Proteasome subunits	<i>Homo sapiens</i> (accession number)	<i>P. falciparum</i> (Plasmodb code)	Identities (%)	P-value
<i>CP subunits</i>				
Proteasome subunit α type 1	P25786	PF14_0716	44	8.7e–52
Proteasome subunit α type 2	P25787	PFF0420c	57	3.2e–70
Proteasome subunit α type 3	P25788	PFC0745c	34	3.7e–44
Proteasome subunit α type 4	P25789	PF13_0282	53	3.4e–66
Proteasome subunit α type 5	P28066	PF07_0112	54	5.5e–66
Proteasome subunit α type 6	P60900	MAL8P1.128	45	3.1e–56
Proteasome subunit α type 7	O14818	MAL13P1.270	53	2.6e–59
Proteasome subunit β type 1	P20618	PFE0915c	43	4.0e–47
Proteasome subunit β type 2	P49721	PF14_0676	37	6.3e–33
Proteasome subunit β type 3	P49720	PFA0400c	43	3.7e–44
Proteasome subunit β type 4	P28070	MAL8P1.142	42	6.0e–41
Proteasome subunit β type 5	P28074	PF10_0111	53	1.2e–59
Proteasome subunit β type 6	P28072	PF11545c	29	8.3e–27
Proteasome subunit β type 7	Q99436	PF13_0156	53	1.6e–66
<i>RP base subunits</i>				
26S protease regulatory subunit 7 or RPT1	P35998	PF13_0063	70	1.2e–159
26S protease regulatory subunit 4 or RPT2	P62191	PF10_0081	77	1.5e–168
26S protease regulatory subunit 6B or RPT3	P43686	PFD0665c	64	4.6e–133
26S protease regulatory subunit 10B or RPT4	P62333	PF13_0033	67	2.8e–142
26S protease regulatory subunit 6A or RPT5	P17980	PF11_0314	71	7.3e–158
26S protease regulatory subunit 8 or RPT6	P62195	PFL2345c	74	2.0e–155
26S proteasome non-ATPase regulatory subunit 2 or RPN1	Q13200	PFB0260w	37	1.5e–143
26S proteasome non-ATPase regulatory subunit 1 or RPN2	Q99460	PF14_0632	43	1.3e–161
26S proteasome non-ATPase regulatory subunit 4 or RPN10	P55036	PF08_0109	41	4.6e–37
26S proteasome non-ATPase regulatory subunit RPN13	Q16186	PF14_0138	35	6.6e–17
<i>RP lid subunits</i>				
26S proteasome non-ATPase regulatory subunit 3 or RPN3	O43242	MAL13P1.190	35	3.4e–73
26S proteasome non-ATPase regulatory subunit 12 or RPN5	O00232	PF10_0174	39	1.6e–82
26S proteasome non-ATPase regulatory subunit 4 or RPN6	O00231	PF14_0025	35	5.3e–51
26S proteasome non-ATPase regulatory subunit 6 or RPN 7	Q15008	PF11_0303	37	1.1e–76
26S proteasome non-ATPase regulatory subunit 7 or RPN8	P51665	PFI0630w	39	9.3e–55
26S proteasome non-ATPase regulatory subunit 13 or RPN9	Q9UNM6	PF10_0298	26	1.2e–38
26S proteasome non-ATPase regulatory subunit 14 or RPN 11	O00487	MAL13P1.343	63	1.2e–100
26S proteasome non-ATPase regulatory subunit 8 or RPN 12	P48556	PFC0520w	32	1.6e–25
26S proteasome non-ATPase regulatory subunit RPN 15 or DSS1	P60896	MAL7P1.117	61	0.53

E1 and E2, the E3 enzymes are specific to the protein substrate. The E3 ligases provide substrate selectivity through a specific substrate recognition domain or via other cofactors; and can accordingly be divided into four major classes, the HECT, the RING, the PHD and the U-box E3 enzymes (reviewed in Shi and Grossman, 2010).

In *P. falciparum*, eight putative E1-like enzymes, 14 putative E2-like enzymes and 54 putative E3 ligases were identified (Table 2; Ponts et al., 2008, reviewed in Chung and Le Roch, 2010). Only limited information is available on the functionality of these enzymes in malaria parasites. A homolog for the Ub-conjugating enzyme 13 was identified in *P. falciparum* (PFUBC13, Table 2), and it was shown that the PfUBC13 activity is regulated by the protein kinase PfPK9 (Philip and Haystead, 2007). Le Roch and co-workers recently reported ubiquitination activity and an essential function of three of the parasite-specific E3 ligases (Chung and Le Roch, 2010). The authors pointed out that these enzymes might represent a prospective novel type of drug target due to their high divergence and essentiality for the malaria parasite. In accordance with this statement, recent studies on the human E3 ligase MDM2 and the small molecule inhibitor MI-63 indicated that the targeted inhibition of E3 enzymes can be achieved (Canner et al., 2009).

The transport of ubiquitinated substrate to the proteasome is mediated by shuttle proteins (Fig. 1). Rpn10, described above as a 19S RP base protein, is also present in the cell without any proteasome association and appears to function as a substrate shuttle. Other extra-proteasomal Ub-binding proteins functioning as shuttles are Rad23, Dsk2 or Ddi1 (reviewed in Hartmann-Petersen and Gordon, 2004; Madura, 2004; Welchman et al., 2005). These proteins contain an Ub-like domain UbL, which can bind to the Ub receptors of the proteasome, as well as the Ub-binding domain, UbA (reviewed in Bedford

et al., 2010). Orthologs of Rad23, Dsk2 and Ddi1 (Table 2) can be identified in *P. falciparum*; namely, PF10_0114, PF11_0142 and PF14_0090, exhibiting 29%, 27% and 34% identities, respectively (P-values of 1.0e–24, 5.2e–34 and 3.5e–32).

As the delivery of Ub-tagged proteins to the proteasome is essential for proteolysis, drug targeting of the shuttle proteins would in theory be an attractive approach to interfere with the UPS. However, little is known on their suitability as drug targets. One compound, termed girolin, is linked with blocking the delivery of Ub-tagged proteins to the proteasome (Tsukamoto et al., 2004). However, its exact mode of action is not well known, and in malaria parasites could also involve translation termination (Benoit-Vical et al., 2008).

5. Protein deubiquitination

Prior to degradation by the proteasome, the Ubs are removed from the substrate to make them available for subsequent ubiquitination cycles. Ub removal is mediated by de-ubiquitinating enzymes (DUBs; Fig. 1). DUBs are proteases involved in ULP maturation, Ub removal and pUb chain editing (proof-reading; reviewed in Bedford et al., 2011). While some DUBs disassemble Ub chains non-specifically, the majority of them exhibit substrate-specificity. In humans, DUBs are classified into five conserved families, JAMM, UCH, USP, OTU and MJD. All DUBs are cysteine proteases, with the exception of the JAMM zinc metalloproteases (reviewed in Shi and Grossman, 2010).

The 19S RP lid component Rpn11 exhibits de-ubiquitinating activity. Other DUBs are not part of the proteasome, but interact

Table 2
UPS components identified in *P. falciparum*.

UPS component	Plasmodb code	UPS component	Plasmodb code
<i>Ub and ULPs^a</i>		<i>E3 Ub-ligases^a</i>	
polyUb	PFL0585w	HECT	MAL8P1.23
L40/UBI	PF13_0346	HECT	PF11_0201
S31/UBI	PF14_0027	HECT	MAL7P1.19
Nedd8	MAL13P1.64	HECT	PFF1365c
SUMO	PFE0285c	Cullin	PF08_0094
Hub1	PFL1830w	Cullin	PFF1445c
Atg8	PF10_0193	F-box	PFF0960c
Urm1	PF11_0393	F-box	PFF0710w
<i>E1 Ub-activating enzymes^a</i>		F-box	PFL1565c
UBA1	PFL1245w	U-box	PF08_0020
UBA2	PFL1790w	U-box	PFC0365w
UBA3	MAL8P1.75	U-box	PF07_0026
UBA4	PF13_0344	RING finger	PFL2440w
UBA1-like	PF13_0182	RING finger	PF11_0330
Atg7	PF11_0271	RING finger	PF10470w
Other	PF13_0264	RING finger	MAL13P1.405
Other	PF11_0457	RING finger	PFE1490c
<i>E2 Ub-conjugating enzymes^a</i>		RING finger	PF14_0215
UBC9	PFI0740c	RING finger	PFF1325c
UBC12	PFL2175w	RING finger	PFC0610c
UBC13	PFE1350c	RING finger	PFL0275w
UEV	PFC0255c	RING finger	PFF0165c
Other	PFL0190w	RING finger	PF10_0117
Other	PF13_0301	RING finger	PF11_0244
Other	PFF0305c	RING finger	PFF0355c
Other	PF10_0330	RING finger	PF10_0046
Other	PF08_0085	RING finger	PF14_0054
Other	PFC0855w	RING finger	PFC0740c
Other	PFI1030c	RING finger	PF10_0276
Other	PFL2100w	RING finger	PFD0765w
Other	PF14_0128	RING finger	PF10_0072
Other	MAL13P1.227	RING finger	PFE0610c
<i>DUBs^a</i>		RING finger	PFB0440c
UCH	PF13_0096	RING finger	PFC0510w
UCH	PFE0835w	RING finger	MAL13P1.216
UCH	MAL7P1.147	RING finger	PFL0440c
UCH	PFD0655c	RING finger	PF13_0188
UCH	PFA0220w	RING finger	PFE0100w
UCH	PF14_0145	RING finger	PF14_0416
UCH	PFD0165w	RING finger	PFF1180w
UCH	PFI0225w	RING finger	PFF0755c
PfUCHL3	PF14_0576	RING finger	MAL13P1.302
PfUCH54	PF11_0177	RING finger	MAL13P1.345
USP14	PFE1355c	RING finger	MAL7P1.155
Josephin	PFL1295w	RING finger	PFC0690c
Josephin	PF11_0125	RING finger	PFL1705w
Mov34	MAL13P1.343	RING finger	PFC0845c
Mov34	PFI0630w	RING finger	PFI0805w
Mov34	PFI0895c	RING finger	PFL1620w
Mov34	PFD0265w	RING finger	PFC0175w
Mov34	PF10_0233	RING finger	PF14_0139
Mov34	PF11_0409	RING finger	PFC0425w
DUF862	PFL0865w	RING finger	MAL13P1.224
DUF862	PF10_0069	RING finger	MAL13P1.122
DUF862	PFI0940c	<i>Shuttle proteins</i>	
OTU	PF10_0308	Rad23	PF10_0114
OTU	PF11135c	Dsk2	PF11_0142
OTU	PF11_0428	Ddi1	PF14_0090
WLM	PF10_0092		
Peptidase_C48	PFL1635w		
Peptidase_C48	MAL8P1.157		
Peptidase_C54	PF14_0171		

^a Modified from Pons et al. (2008).

In *P. falciparum*, two independent studies identified 18 and 29 DUBs, respectively (Table 2; Ponder and Bogyo, 2007; Pons et al., 2008). Homologs of human UCH37, PfUCH54; and of UCHL3, PfUCHL3 (Table 2), were identified in *P. falciparum* and known to exhibit deubiquitinating and deNeddylating activities (Artavanis-Tsakonas et al., 2006, 2010; Frickel et al., 2007). A putative homolog of human USP14 (PFE1355c) is also present in *P. falciparum* (33% identities, P -value = $6.9e-43$).

Due to their intrinsic protease activity, plasmodial DUBs might represent excellent targets for antimalarial drug discovery. Parasite cysteine proteases have been of interest due to their involvement in hemoglobin degradation (reviewed in Rosenthal, 2011). Comprehensive studies in malaria parasites would be feasible to determine the effect on Ub-protein accumulation of antiplasmodial cysteine protease inhibitors and currently available DUB inhibitors (such as Ubal, UbVS or cyclopentenon; reviewed in Daviet and Coland, 2008). The deNeddylating activities of PfUCH54 and PfUCHL3 might be of particular interest for drug design, since such activity is not known for the mammalian homologs of the two DUBs.

6. Inhibitors of the 20S proteasome

Several distinct kinds of proteasome inhibitors can be discerned (reviewed in Verdoes et al., 2009). First, chemically reactive β -lactone natural products such as lactacystin or salinosporamide A have been found (Fig. 3). These mechanism-based “suicide” inhibitors undergo covalent attachment to the threonine in the active centers of the β -SUs by nucleophilic opening of the β -lactone ring. For lactacystin, the β -lactone containing intermediate ormulide is formed slowly *in situ* from its cystein thioester. These compounds are of high appeal in basic science, but their reactive promiscuity and high toxicity render potential applications remote.

Secondly, a large variety of substrate mimetics has been reported which emulate the endogenous peptides processed by the proteasome and thereby achieve higher selectivity amongst other cellular proteases. Both natural products (epoxomicin, fellutamide B) and designed compounds (MG-132, bortezomib) have been developed and intensively characterized (Fig. 3), in some cases leading to success in the clinic (bortezomib). Most of these substrate-competitive molecules carry a chemically reactive “warhead” at the C-terminus to deactivate the proteasome (e.g. epoxyketone, aldehyde, boronic acid) intermittently or even irreversibly. However, recently reversible high-affinity inhibitors of this kind have been reported as well (Blackburn et al., 2010), which do not undergo a covalent attachment. Furthermore, by appropriate tailoring of the chemical structures, subsite-specificity for the CP-SUs β 1, β 2, or β 5 was achieved (Britton et al., 2009).

Thirdly, several complex natural products of the cyclic peptide (argyrins) or peptide alkaloid type (TMC compounds, thiostrepton) inhibit proteasome function (Fig. 3). Apparently, none of these inhibitors undergoes covalent attachment to the proteasome, but rather high activity was found for these compounds or their derivatives. In the case of the argyrins, efficacy in cancer-focused animal models was demonstrated (Nickeleit et al., 2008). While the binding site for TMC-95A was structurally characterized and found to be located at the active centers of the β -SUs (Groll et al., 2006), the site of action is much less clear for argyirin and thiostrepton. Both compounds are quite large and might not easily pass the entry rim formed by the α -SUs. Hence, although potential binding of argyirin at the active centers was recently suggested by molecular modeling (Stauch et al., 2010), other less direct or allosteric modes of action could also be involved, especially for thiostrepton derivatives.

Overall, the intense search for drug candidates in the anti-cancer area has generated a constant stream of non-covalent and covalent

with the complex (proteasome-interacting proteins, PIPs; reviewed in Xie, 2010); among them Ubp6/USP14 and Uch2/Uch37 of yeast and human. Uch2/Uch37 interacts with the proteasomal Ub receptor Rpn13, and both Ubp6/USP14 and Uch2/Uch37 may serve as “proof-reading” enzymes to remove Ub chains from proteins that are mistakenly encountered and ubiquitinated by the UPS (reviewed in Kraut et al., 2007).

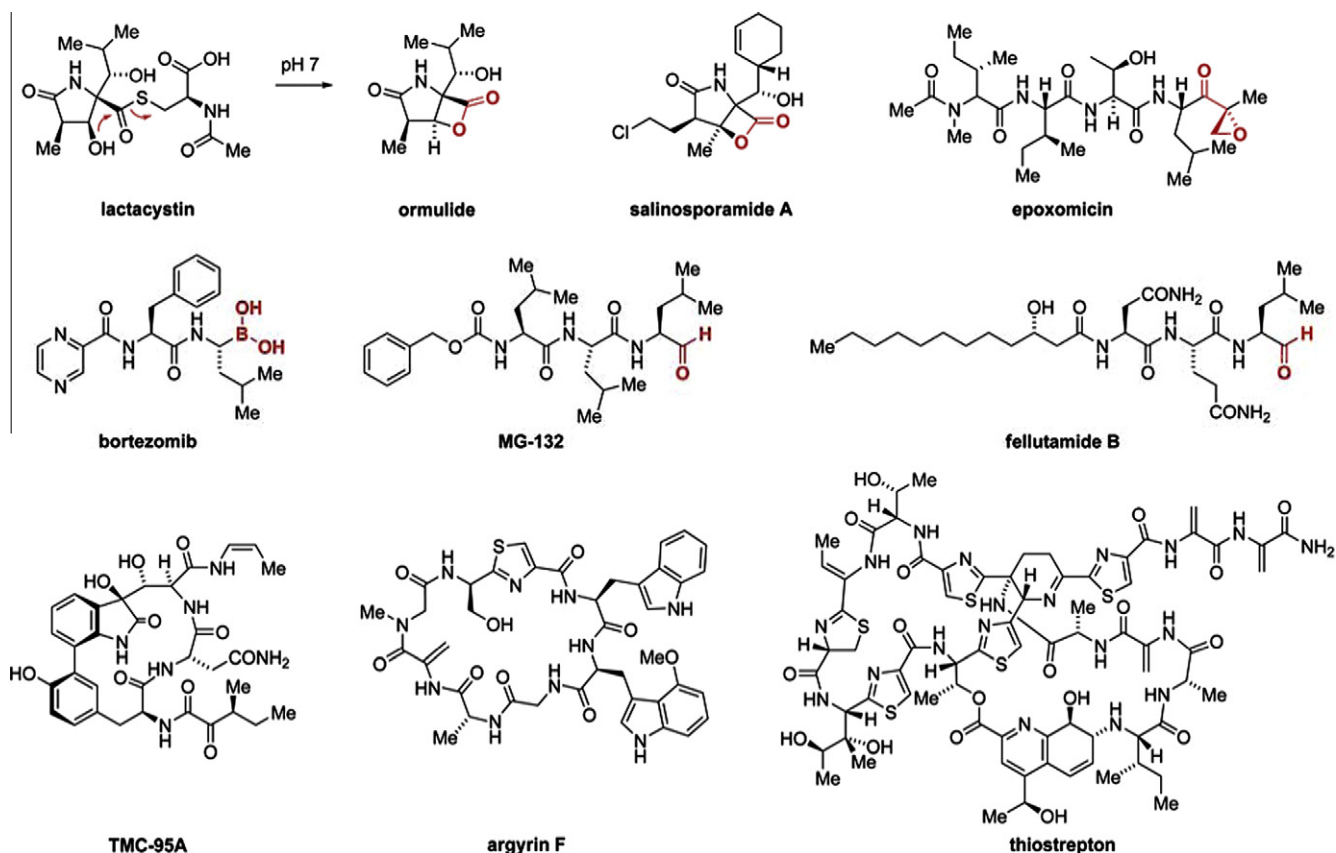


Fig. 3. Chemical structures of proteasome inhibitors. Functional groups undergoing covalent attachment to the proteasome active centers are highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lent inhibitors of the 20S proteasome, accompanied by constantly improving structural data. While it remains to be clarified to which degree functional or molecular inter-species selectivity between humans and *Plasmodium* parasites can be achieved, this broad basis of bioactive lead compounds with broadly tested toxicity profiles will be highly useful in the future to inspire new compound designs and support screening campaigns in *Plasmodium* infection models.

7. The plasmodial proteasome: a multi-stage drug target?

Due to the essential roles of the UPS in all eukaryotic cells, one might expect that proteasome inhibitors show activities against malaria parasites as well. While it was shown in yeast and human

that the CP-SUs $\beta 1$, $\beta 2$ and $\beta 5$ display caspase-like, trypsin-like and chymotrypsin-like activities, respectively, such distinct activities have not been confirmed for *Plasmodium*, and interaction of active site-targeting inhibitors with CP-SUs have yet to be demonstrated. However, epoxomicin was reported to bind parasite CP-SUs $\beta 2$ and $\beta 5$ (Mordmüller et al., 2006) and bortezomib, which exhibits anti-plasmodial activities, has a high specificity for $\beta 5$ (Oerlemans et al., 2008), providing indirect evidence for the proteolytically active sites of the plasmodial proteasome.

Among active-site targeting proteasome inhibitors, lactacystin, salinosporamide A, MG 132, epoxomicin, and bortezomib were tested for efficacy against *P. falciparum* and reported to inhibit parasite growth *in vitro* at low nanomolar concentrations (Table 3; Gantt et al., 1998; Reynolds et al., 2007; Kreidenweiss et al.,

Table 3

The antimalarial effect of selected proteasome inhibitors.

	Liver stages (100% inhibition) [μM]	Blood stages (IC_{50}) [μM]	Gametocytes (100% elimination) [μM]	Transmission (100% reduction) [μM]
Bortezomib	n.d.	0.03–0.56 ^{c,d}	n.d.	n.d.
Epoxomicin	n.d.	0.002–0.03 ^{d,g}	0.03 ^{f,g}	0.1 ^f
Lactacystin	9.0 ^a	1.2–1.5 ^a	n.d.	n.d.
MG-132	n.d.	0.04–0.05 ^{e,g}	0.5 ^g	n.d.
MLN273	1.0 ^b	0.04 ^b	n.d.	n.d.
Salinosporamide A	n.d.	0.01 ^e	n.d.	n.d.
Thiostrepton	n.d.	8.9–16.7 ^g	8.9 ^g	n.d.

n.d., not determined.

^a Gantt et al. (1998).

^b Lindenthal et al. (2005).

^c Reynolds et al. (2007).

^d Kreidenweiss et al. (2008).

^e Prudhomme et al. (2008).

^f Czesny et al. (2009).

^g Aminake et al. (2011).

2008; Prudhomme et al., 2008; Aminake et al., 2011). Proteasome inhibitors are fast acting, and drug-treated parasites were reported to be arrested prior to DNA replication (Reynolds et al., 2007; Kredenweiss et al., 2008; Aminake et al., 2011), thus at a time point when protein ubiquitination is at a peak (Ponts et al., 2011). Ubiquitinated protein accumulation was observed in the parasite lysate after treatment of the parasite with proteasome inhibitors, providing indirect evidence that the proteasome is affected (Lindenthal et al., 2005; Aminake et al., 2011).

In addition to their effect on blood stage parasites, the proteasome inhibitors lactacystin and MLN273 (a compound related to bortezomib) were reported to have an effect on the liver stages *in vitro*, when applied in the micromolar range (Table 3; Gantt et al., 1998; Lindenthal et al., 2005). Both inhibitors blocked the development of exoerythrocytic forms in liver hepatoma cells *in vitro*, and lactacystin was further shown to inhibit infectivity in the *P. berghei* mouse model *in vivo* (Gantt et al., 1998). The effect of other proteasome inhibitors on the liver stages of *Plasmodium* has not been tested. Few antimalarials known to target liver stage are available; for example, the 8-aminoquinolines primaquine and tafenoquine. The fact that proteasome inhibitors exhibit activities against the liver and blood stages, makes them particularly interesting for malaria therapy. MG-132 and epoxomicin were recently reported to exhibit gametocytocidal activities *in vitro* in their IC₅₀₋₉₀ concentrations, thereby eliminating the gametocytes from the cultures within two days of drug incubation, and in consequence reducing parasite transmission from the human to the mosquito (Table 3; Czesny et al., 2009; Aminake et al., 2011). Gametocytocidal activities have been demonstrated for few antimalarials, including primaquine and artemisinin (Pukrittayakamee et al., 2004). The development of novel antimalarials with gametocytocidal activities is therefore urgently needed and is currently promoted by the WHO and the Bill and Melinda Gates Foundation.

Gametocytes are the sexual precursor cells of the malaria parasite, which form in the human blood in response to stress, and likely other unknown cues (reviewed in Talman et al., 2004). Gametocytes develop from stage I to stage V during a period of approximately 10 days, before continuing their life cycle in the mosquito vector (reviewed in Pradel, 2007; Kuehn and Pradel, 2010). When investigating the gametocytocidal effect of epoxomicin in detail, we recently observed that the inhibitor exerted its effect on gametocytes during maturation from the early to the mature stages (Aminake et al., unpublished observations). The maturation of gametocytes is accompanied by high protein turnover rates, which is particularly reflected by the sequential expression of a high number of surface-associated proteins, like Pfs16, Pfs230, Pfs48/45 and the LCCL-domain proteins (reviewed in Pradel, 2007; Kuehn and Pradel, 2010), and these changes in the composition of surface proteins might be regulated by the plasmodial UPS.

Thiostrepton was recently identified as an inhibitor of the proteasome, although the compound was described in the 1950s as a strong antibiotic with high activity against Gram-positive strains (reviewed in Bagley et al., 2005) and is used as a topical antibiotic in veterinary medicine. Its activity is mediated by tight binding to the GTPase-associated region of the bacterial 70S ribosome, which then stalls during protein translocation (Harms et al., 2008; Jonker et al., 2011). Due to genetic and functional similarity of the ribosomes in bacteria and eukaryotic organelles, thiostrepton impairs protein biosynthesis in the mitochondrion and in the apicoplast of malaria parasites (McConkey et al., 1997; Tarr et al., 2011). However, the rapid killing induced by thiostrepton and its derivatives is contrary to the delayed death effect of other apicoplast-targeting antibiotics (reviewed in Pradel and Schlitzer, 2010), and this might be explained by invoking an additional target. The target was recently identified as the proteasome by cell microscopy, enzymatic

activity studies and phenotype profiling (Schoof et al., 2010; Aminake et al., 2011). While thiostrepton exhibits only moderate antiplasmodial activities *in vitro*, derivatives are active in the low micromolar range (Table 2; Schoof et al., 2010; Aminake et al., 2011). Taken together, thiostrepton derivatives combine in one molecular structure two modes of action against *P. falciparum*, and this unique duality might reduce the likelihood of resistance.

8. Future perspectives

The plasmodial proteasome can be viewed as a candidate multi-stage drug target, and progression of our understanding might benefit from the experience and reagents gained during the development of anti-cancer proteasome inhibitors. It will nonetheless remain challenging to identify inhibitors which are sufficiently specific to the microorganism; that is, with a high therapeutic index allowing treatment of human malaria. In the case of aggressively dividing myeloma a sufficient therapeutic window was found for bortezomib, which suggests that proteasome inhibitors could become an effective treatment or at least component for an acute therapy against the rapidly developing blood stages of *Plasmodium*. To reach a sufficient therapeutic index and to target also the more hidden and diluted liver and gametocyte stages, species-selective agents would be highly desirable. Along this line, encouraging data was recently reported for *Mycobacterium tuberculosis*, for which a species-selective proteasome inhibitor was found (Lin et al., 2009). Therefore, further characterization of the biochemistry and cell biology of the UPS in *Plasmodium* will not only help unravel its unique biology in a multi-stage parasitic organism, but also potentially enable the discovery of new antimalarial drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpddr.2011.12.001.

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