Chapter 8

Plasmepsin: Function, Characterization and Targeted Antimalarial Drug Development

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Additional information is available at the end of the chapter

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Abstract

The devastating malaria, caused by parasites of the genus *Plasmodium*, afflicts nearly half of the world's population and imposes a heavy socio-economic burden particularly to the disease-endemic Sub-Saharan Africa. Sustained efforts in malaria control have been made from the perspectives of medicine- and vaccine-based prevention and treatment of malaria and malaria transmission blockage for the past 15 years, resulting in a decreased mortality rate by 60% and a decreased malaria incidence rate by 37% globally. Nonetheless, due to the emergence and rapid spread of drug-resistant parasite strains, novel antimalarial drugs are urgently required to combat this deadly disease. Plasmepsins are deemed potential targets for novel antimalarial drug design. Plasmepsins represent an aspartic proteinase family that can be sub-categorized into seven groups based on the amino acid sequence identity. This chapter discusses our progress in understanding the biosynthesis, biological functions and enzymatic characteristics of the plasmepsin family. This led to development of various types of plasmepsin-targeted compounds and the assessment of their binding affinity and selectivity, anti-parasitic activity and cytotoxicity. The gained experience and current status in developing plasmepsin-targeted antimalarial drugs are addressed. Finally, a deeper and broader investigation on the functions and characteristics of the plasmepsin family is encouraged.

Keywords: malaria, plasmepsin, drug design, *Plasmodium*, aspartic proteinase

1. Introduction

Malaria, a life-threatening infectious disease, afflicts approximately 3.2 billion people, causes 214 million clinical cases and leads to nearly 440,000 deaths worldwide in 2015 despite the facts that malaria mortality rates decreased by 60% globally and by 66% in Africa between 2000 and 2015, and that malaria incidence rates decreased by 37% globally and by 42% in Africa for the

past 15 years [1, 2]. Nearly 90% of the malaria cases and deaths occur in Sub-Saharan Africa in 2015, loading a heavy socio-economic burden to this poorly developed region [1].

Malaria is caused by parasitic protozoa of the genus *Plasmodium*. Hundreds of *Plasmodium* species have been identified to infect reptiles, birds and mammals, including rodents and primates. Four *Plasmodium species pluralis* (spp.), *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, are known to infect man, though other malarial species of non-human primates occasionally infect human as well. Among these species, *P. falciparum* is the most deadly and *P. vivax*, the most prevalent. *P. falciparum* invades both young and mature erythrocytes and provokes malignant disease symptoms. Prevalent mainly in Africa, *P. falciparum* accounts for ~40% of the clinical cases on a global basis [1]. In contrast, *P. vivax* prefers invading young erythrocytes and causes benign symptoms; it has a wider geographical distribution than *P. falciparum* and is responsible for half of the total reported cases [1].

To complete its life cycle, the malaria parasite requires a female mosquito as the transmission vector and a vertebrate host (**Figure 1**). When a blood meal is taken, a parasite-infected mosquito inoculates sporozoites into the human host to start the exo-erythrocytic phase, in which sporozoites infect hepatocytes and mature into schizonts. Of note, in parasites such as *P. vivax* and *P. ovale*, a dormant stage, namely hypnozoites, can maintain in hypatic cells for weeks or even years before invading the bloodstream. Rupture of schizonts releases merozoites, which

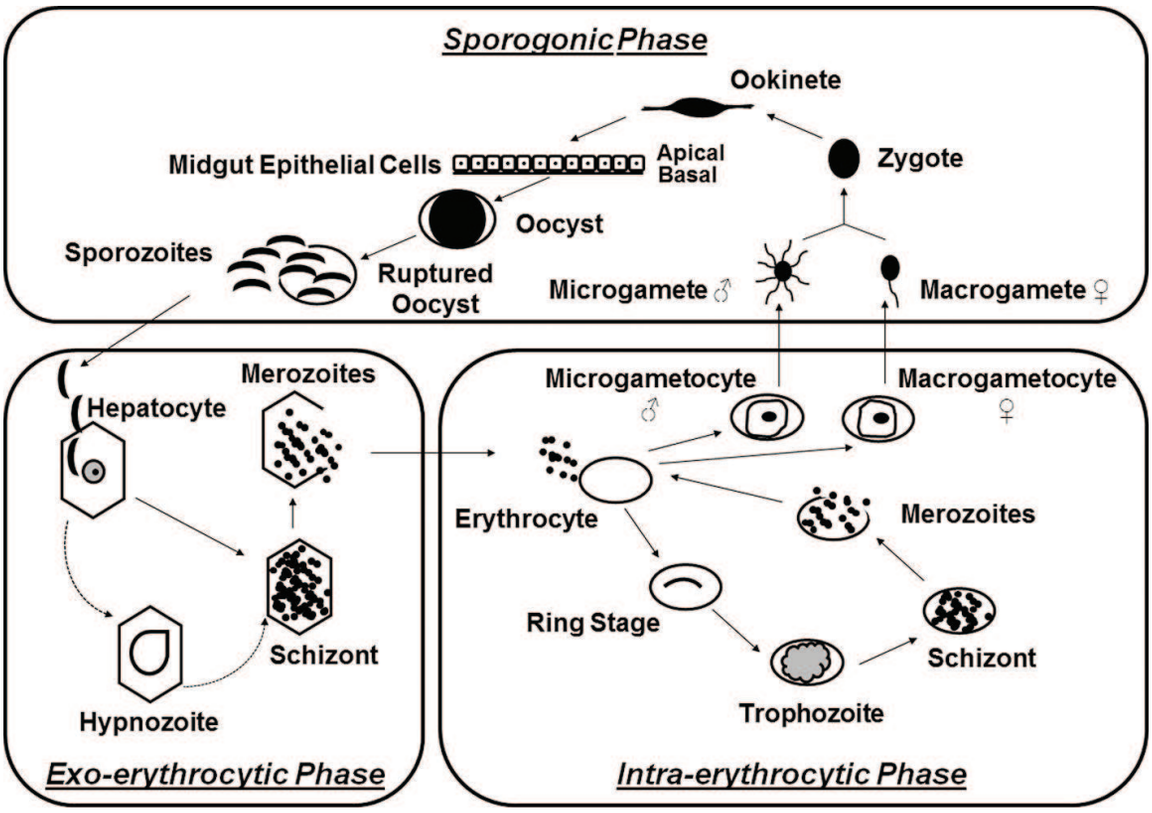

Figure 1. The life cycle of malaria parasites. Malaria parasites require a transmission vector (e.g., mosquito) and a vertebrate host (e.g., human) to complete their life cycle. The exo-erythrocytic and intra-erythrocytic phases occur in the vertebrate host, and the sporogonic phase occurs in the transmission vector.

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then infect erythrocytes to initiate the intra-erythrocytic phase. In this phase, the parasite undergoes multiple rounds of asexual replication with each cycle comprising, in sequence, the ring, the trophozoite and the schizont stage. A portion of merozoites infect erythrocyte to differentiate into gametocytes. Microgametocytes and macrogametocytes are ingested by a mosquito to start the sporogonic phase. In the mosquito's stomach, gametocytes further differentiate into gametes. Microgametes fertilize macrogametes to generate zygotes, which subsequently develop into motile and elongated ookinetes. Ookinetes penetrate the midgut of the mosquito and develop into oocysts, from which sporozoites are released and delivered to the mosquito's salivary gland, ready for the next infection.

Malaria control in the modern era arguably starts from the isolation of antimalarial quinine and quinidine from cinchona bark in early nineteenth century [3], while it was not until 1925 that pamaquine (also known as plasmoquine or plasmochin), the first synthetic antimalarial drug, was yielded. Synthesized in 1934, chloroquine (CQ), a 4-aminoquinoline compound, exhibited a strong antimalarial potency and a low toxicity and became the most extensively used drug in malaria prophylaxis and treatment between 1940s and 1960s [4–6]. The massive use of CQ, however, resulted in the emergence of CQ-resistant *P. falciparum* strains, which promoted development of novel antimalarial drugs (e.g., 8-aminoquinolines, antifolates, naphthoquinones and non-antifolate antibiotics). Of particular note among these compounds is artemisinin (AN). Extracted from the herbal plant *Artemisia annua*, AN, has been used for malaria treatment since early 1970s [7]. Though AN and its various derivatives display high antimalarial activities (e.g., [8–12]) and quick attenuation of disease symptoms [13], they have short half lives *in vivo* [14]. The combination of AN and a longer-acting drug (e.g., artemether-lumefantrine and artesunate-mefloquine) is effective for disease treatment and for deferring drug resistance development. Artemisinin-based combination therapies (ACTs) have up till now been used as a standard therapy in many countries and regions despite potentially unmatched pharmacokinetics between drugs and/or widespread resistance against the non-artemisinin components. Malaria control was also carried out by intervention of disease transmission, thanks to the discovery of insecticidal properties of dichloro-diphenyltrichloroethane (DDT) in 1939 [15]. Due to health and environmental risks, DDT was later substituted by other insecticides, such as pyrethroids, chlorfenapyr and pyriproxyfen. While both indoor residual spraying and insecticide-treated bed nets contribute to controlling epidemic outbreaks of malaria, the latter provide more effective protection for people living in temporary shelters. Nonetheless, one cannot ignore the growing emergence of insecticide-resistant vector strains and the lack of interventions targeting outdoor mosquito populations, which constitute major challenges in blocking malaria transmission. Intervention of malaria transmission has also been managed via biological control of mosquitoes at both the larval and the adult stage. Several fish species, such as *Poecilia reticulate* (guppy) and *Gambusia affinis* (mosquitofish), are able to consume mosquito larvae and reduce their population; however, these fish also pose a threat to other native aquatic predators of mosquitoes due to intraguild predation [16, 17]. In contrast, the larval dytiscid beetles *Agabus* do exhibit a selective predation on mosquitoes over alternative prey, although intraguild predation and cannibalism also occur within and between *Agabus* species [18]. In addition, the use of water-dispersible granular formulation of two *Bacillus* species in malaria control results in an efficacious elimination of the larval mosquito population with a negligible environmental impact [19]. Also of note is the

use of fungi for malaria control. Ground and aerial application of self-propagating *Lagenidium giganteum* effectively controls the larval mosquito population for at least an entire breeding season [20, 21]. Oil-based formulations of fungal entomopathogens are able to block malaria transmission by reducing adult mosquito survival and altering parasite survival/maturation in the vector [22]. Further, transgenic fungi *Metarhizium anisopliae* targeting sporozoites in mosquitoes inhibit parasite development [23]. These pieces of evidence indicate the potential of fungi as a biocontrol agent of mosquitoes. Natural products are another important source utilized to control malaria transmission. A variety of plant extracts and essential oils (e.g., the neem oil, the fenugreek oil and the extracts from Indian sandalwood) exhibit larvicidal activities and adult mosquito repellency properties (for example, see [24–30]). Moreover, natural product-synthesized silver nanoparticles show a higher potency in mosquitocidal activity than the aqueous extracts but their toxicity against other natural mosquito consumers is negligible (for example, see [31–33]). These, in addition to the time-efficiency, cost-effectiveness and eco-friendliness green-synthesis of nanoparticles, suggest the feasibility and importance of a synergistic mosquito control using botanical nano-insecticides and biological agents. Besides these antimalarial approaches, vaccines against malaria parasites have been under development since 1970s [34, 35]. Malaria vaccines are categorized into three types: exo-erythrocytic vaccines, blood-stage vaccines and transmission-blocking vaccines; sustainable prevention requires a combination of vaccines targeting multiple life stages of the parasite. RTS,S/AS01, the first and thus far the only vaccine that completes a Phase III clinical trial, targets the exo-erythrocytic phase of *P. falciparum*. Though this vaccine demonstrates a decent efficacy for prevention of clinical malaria cases in African children (age 5–17 months, efficacy 50%) and infants (age 6–12 weeks, efficacy 30%) [36], an ideal candidate aiming for global eradication would require a higher efficacy [37].

A major challenge faced by the anti-malaria campaign currently is the emergence and rapid spread of drug-resistant variants of *Plasmodium* spp. [38]. Malaria parasites have developed resistance to virtually every type of antimalarial drugs thus far used, including AN and its derivatives [39]. The lack of effective treatment of symptoms caused by drug-resistant parasites urges us to identify molecular targets, against which novel drugs can be subsequently developed to combat malaria. Plasmepsins (PMs), a family of aspartic proteinases, are considered a promising drug target.

This review focuses on the biosynthesis, biological functions and enzymatic characteristics of the plasmepsin (PM) family from human malaria parasites. The progression of PM-targeted antimalarial drug development is also discussed.

2. Plasmepsin family overview

From comparative genomic analysis of sequence information of seven *Plasmodium* spp. deposited in the *Plasmodium* genome database [40], a cohort of genes that encode PMs were identified and categorized into seven groups based on their amino acid sequence identity [41]. In *P. falciparum*, up to ten PMs have thus far been identified, namely *Pf*PMs 1, 2, 4–10 and *Pf*HAP (*H*isto-*A*spartic *P*roteinase) [42]. These PMs, encoded by genes located in five different chromosomes, are composed of the pro-segment and the mature enzyme domain. *Pf*PM5 and

*Pf*PM9 also contain extra residues at their C-termini. PMs are distinct in structural and biochemical properties, such as molecular weight and isoelectric point (**Table 1**).

PM	Chr.	Pro			Zymogen			Mature enzyme		
		# a.a.	# a.a.	% i.d.	MW (Da)	pI	# a.a.	% i.d.	MW (Da)	pI
PM1	14	123	452	62	51,461	7.23	329	70	37,050	4.82
PM2	14	124	453	61	51,481	5.29	329	69	36,915	4.62
HAP	14	123	451	52	51,694	8.23	328	59	36,979	4.97
PM4	14	121	449	—	51,047	5.19	328	—	36,955	4.38
PM5	13	83	590	25	68,481	7.66	440	25	50,844	6.50
PM6	3	84	432	29	49,434	7.75	348	29	39,352	6.44
PM7	10	76	450	28	52,329	8.44	374	28	43,317	6.09
PM8	14	45	385	26	44,255	9.38	340	29	38,976	8.85
PM9	14	212	627	27	74,184	9.63	402	25	46,970	9.28
PM10	8	232	573	30	65,115	5.22	341	29	38,604	5.38

The % i.d. data is calculated using the Basic Local Alignment Search Tool [43]. The MW and pI data of zymogens are adopted from the Plasmodium Genomics Resource [44]. The MW and pI data of mature enzymes are calculated using ProtParam [45]. Abbreviations: Pro, pro-segment; # a.a., numbers of amino acids; % i.d., percentage of identity versus PM4.

Table 1. Comparative properties of plasmepsins from the *P. falciparum* 3D7 strain.

Of note, *pfpm4, pfpm1, pfpm2* and *pfhap* cluster in a 20-kb-long region of chromosome 14, and share a high amino acid sequence identity (**Table 1**). Each non-*falciparum* parasite, however, harbors usually one gene (*pm4*) that shares with *pfpm4* the highest sequence identity, which is comparable to those shared among the four *pfpm*s. It is believed that the other three PM genes may arise from multiple gene duplication events [41]. Since these four PM paralogs were initially detected in the food vacuole (FV), an acidic organelle unique to the genus *Plasmodium* where degradation of hemoglobin of red blood cells (erythrocytes) occurs [46–48], they are named the FV *Pf*PMs. PM4s of the non-*falciparum* species are also grouped as FV PMs because they are highly homologous to the FV *Pf*PMs. *Pf*PMs 5–10 share a low amino acid sequence identity with the FV *Pf*PMs, and their sequence structures are distinct from each other and from those of the FV *Pf*PMs (**Table 1**), indicating that there exist diverse biological functions and enzymatic features among the PM family members.

3. Biosynthesis

3.1. Food vacuole plasmepsins

FV *Pf*PMs are synthesized as type II integral membrane proteins, with the putative transmembrane motif residing in the N-terminal pro-segment. Using immunoelectron microscopy (immunoEM), *Pf*PM1 and *Pf*PM2 were observed in the lumens of transport vesicles and FVs,

in the parasite plasma membrane (PPM), in small vesicular structures near PPM and in the cytostome, a morphologically variable microstructure comprising invaginated parasitophorous vacuolar membrane (PVM) and PPM [46] (**Figure 2**). Further, Klemba and colleagues probed the trafficking of *Pf*PM2 in a transgenic *P. falciparum* culture model [49]. The *Pf*M2-green fluorescence protein (GFP) fusion protein was detected by immunoEM in the membrane and lumen of FVs and in the cytostomes, consistent with the previous finding [46]. Administration of brefeldin A (BFA), an inhibitor blocking anterograde protein traffic from the endoplasmic reticulum (ER), to trophozoites retained *Pf*PM2-GFP in the ER/nuclear envelope (NE); yet this protein was detected in the cytostomes and subsequently the FVs minutes after release of the BFA inhibition. The role of Golgi apparatus in the biosynthesis of FV *Pf*PMs is not yet clear, but is doubtful, since FV *Pf*PMs are known to be unglycosylated. Taken together, these findings suggest that the biosynthesis of FV *Pf*PMs follows an "ER-to-PPM-to-FV" route (**Figure 2**). Interestingly, *Pf*PM2 has also been detected in the cytoplasm of host erythrocytes (see Section 4.2 for more discussion), leading to the hypothesis that there exists an alternative traffic route for the FV PMs.

To gain catalytic activity, FV PMs need to release their pro-segments. The cleavage site is conserved at the motif (Y/H)LG* (S/N)XXD (* represents the scissile bond) [50], which is different from the sites where *in vitro* PM auto-maturation occurs ([48, 51–54], see also discussion

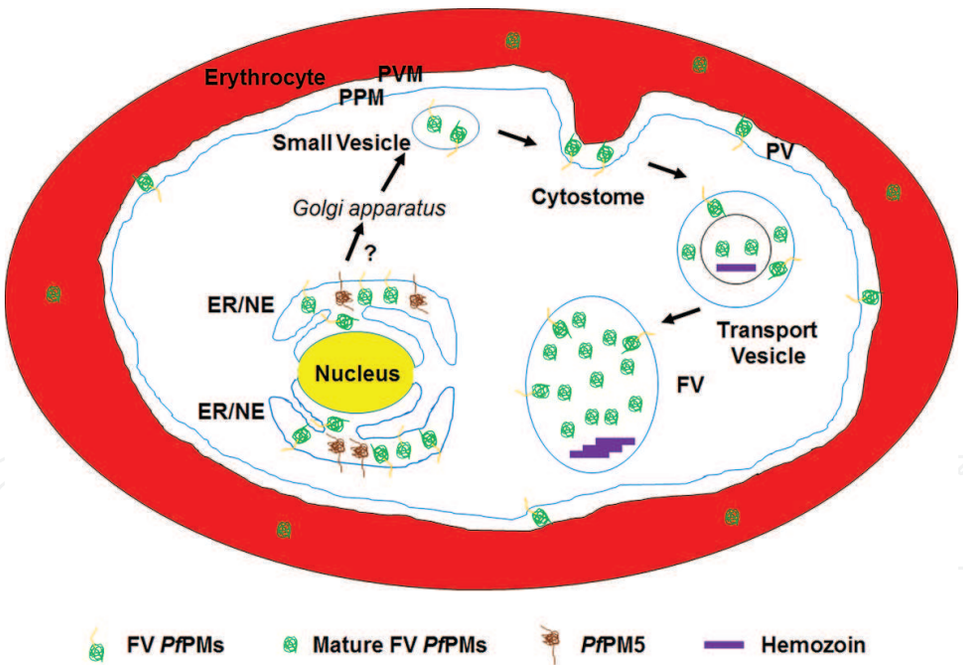

Figure 2. Biosynthesis of plasmepsins in the *P. falciparum* intra-erythrocytic phase. Food vacuole plasmepsins from *P. falciparum* (FV P*f*PMs) are expressed as type II integral membrane proteins with their N-terminal pro-segments (orange threads) spanning the endoplasmic reticulum/nuclear envelope (ER/NE) membrane. FV P*f*PMs are transported via small vesicular structures to the parasite plasma membrane (PPM), where some reside in the cytostomal vacuole. The involvement of Golgi apparatus in this secretory pathway is not clear. Endocytosis of cytostomes retains FV P*f*PMs in transport vesicles, which convey the enzymes eventually to the FV. Maturation of the FV P*f*PMs is carried out in the acidic FVs and transport vesicles. Certain FV P*f*PMs (e.g., PfPM2) are also found functionally active in the host erythrocytes, though how they are secreted outside the parasite is not yet clear. In contrast, P*f*PM5 is an ER-resident, type I integral membrane protein. PV, parasitophorous vacuole; PVM, parasitophorous vacuolar membrane.

in Section 5). This observation suggests that PM maturation in the parasite is a convertase-catalyzed *trans*-processing event. Further studies showed that the pro-segment cleavage of naturally-occurring PMs occurs in an acidic milieu, is largely completed within half an hour in cultured *P. falciparum* at the trophozoite stage, and is inhibited by tripeptide aldehyde *N*-acetyl-Leu-Leu-norleucinal (ALLN) or *N*-acetyl-Leu-Leu-methioninal [50, 55]. The identity of the convertase is believed to be the cysteine proteinases falcipain (FP) -2 and -3 in that (1) both FP-2 and FP-3 catalyze cleavage of peptide substrates at the C-terminus of the conserved glycine; (2) a membrane-permeant derivative of the cysteine proteinase inhibitor E-64 directly binds to FP-2 and FP-3 and, in turn, slows the kinetics of PM maturation in cultured parasites; and (3) both FPs are inhibited by ALLN at low micromolar magnitude *in vitro* [56]. Of note, when FPs are inhibited, the parasite can use PMs (e.g. *Pf*PM2) as alternative convertases [56], though it is not known yet whether and to what degree this alternative processing is employed.

Where does the maturation of FV PMs occur? Evidence from immunoEM shows that antibodies directed against N-terminal epitopes of mature *Pf*PM1 and *Pf*PM2 recognize the enzymes not only in the FV but also in transport vesicles [46, 49]. Of note, hemozoin crystals stemmed from hemoglobin degradation that is initiated and carried out by mature FV PMs are also observed in both the FVs and transport vesicles [57]. These findings indicate that both subcellular compartments contain catalytically active PMs. In addition, the finding that functional vacuolar proton pumps are present in the PPM [58, 59], the outer membrane of transport vesicles, suggests that the vesicular milieu is acidic. Taken together, it is conceivable that the convertase-catalyzed PM maturation also occurs in transport vesicles.

The four FV *Pf*PMs exhibit distinct temporal expression patterns in the intra-erythrocytic phase of the parasite life cycle: *Pf*PM1 and *Pf*PM2 emerge as early as the ring stage, *Pf*PM4 first appears in the early trophozoite stage, and yet *Pf*HAP is not detected until the mid-trophozoite stage; all the four continue to be expressed at the schizont stage [48]. This is expected since the FV *Pf*PMs are key enzymes to hemoglobin processing, and *Pf*PM1 and *Pf*PM2 are believed to initiate that event (for more discussion, see Section 4.1). Importantly, expression of these FV *Pf*PMs is not restricted in trophozoites and schizonts in that mass spectrometry (MS)-based analyses have identified their presence in gametocytes, merozoites, oocysts and sporozoites [60–62].

No studies, to the author's knowledge, have been reported on biosynthesis of the FV PMs from non-*falciparum* species. It is likely that they adopt a similar pattern as the *Pf*PMs due to the high sequence identity shared among these homologs.

3.2. Non-food vacuole plasmepsins

Among the non-FV PMs, PM5 is the most studied. *Pf*PM5 is synthesized as a type I integral membrane protein comprising an N-terminal pro-segment, a catalytic domain, a C-terminal transmembrane domain and a cytoplasmic tail [63]. Notably, the sequence of the pro-segment region of PM5 is highly variable among *Plasmodium* spp. [44]. *Pf*PM5 is almost exclusively detected in the ER/NE (**Figure 2**) [63]. The C-terminal transmembrane domain is essential to the ER/NE residence of *Pf*PM5 [64]. Expression of *Pf*PM5 is detected throughout the life cycle of the parasite [44, 65, 66]; in the intra-erythrocytic phase, *Pf*PM5 expression starts at the early ring stage in a scarce level and continues to increase steadily through the trophozoite and

schizont stages, which mirrors the temporal expression patterns of *Pf*PM1 and *Pf*PM2 [48, 63]. Interestingly, in contrast to the rapid maturation of the FV *Pf*PMs, no processing of the N-terminal pro-segment is observed hours after the synthesis of *Pf*PM5; also unlike the FV *Pf*PMs, *Pf*PM5 is catalytically active in the presence of the pro-segment [63].

Few studies have addressed the biosynthesis of PMs 6–10. Genes encoding *Pf*PM9 and *Pf*PM10, but not *Pf*PMs 6–8, are transcribed in parasites infecting erythrocytes [67]. In the intra-erythrocytic phase, *Pf*PM9 and *Pf*PM10 exhibit a diffuse expression pattern throughout the cytoplasm, but are excluded from the FV [48]. Of note, MS-based analysis indicates the presence of *Pf*PM9 in sporozoites and the presence of both *Pf*PM6 and *Pf*PM10 in merozoites and sporozoites [60–62]. In addition, expression of *Pf*PM7 and *Pf*PM10 is detected in zygotes and ookinetes [68, 69].

4. Biological function

4.1. Hemoglobin digestion and degradation

The primary pathological role that FV PMs play is digestion and degradation of the oxygen-carrying hemoglobin that constitutes 95% of cytosolic proteins of human red blood cells (**Figure 3**).

In the intra-erythrocytic phase, hemoglobin digestion and degradation is carried out between the ring and the early schizont stage [70, 71]. A vast majority of hemoglobin, at a millimolar concentration in erythrocytes, however, is processed within the 6–12-hour trophozoite stage [72], indicative of an enzyme-catalyzed event. The processing of hemoglobin occurs mainly

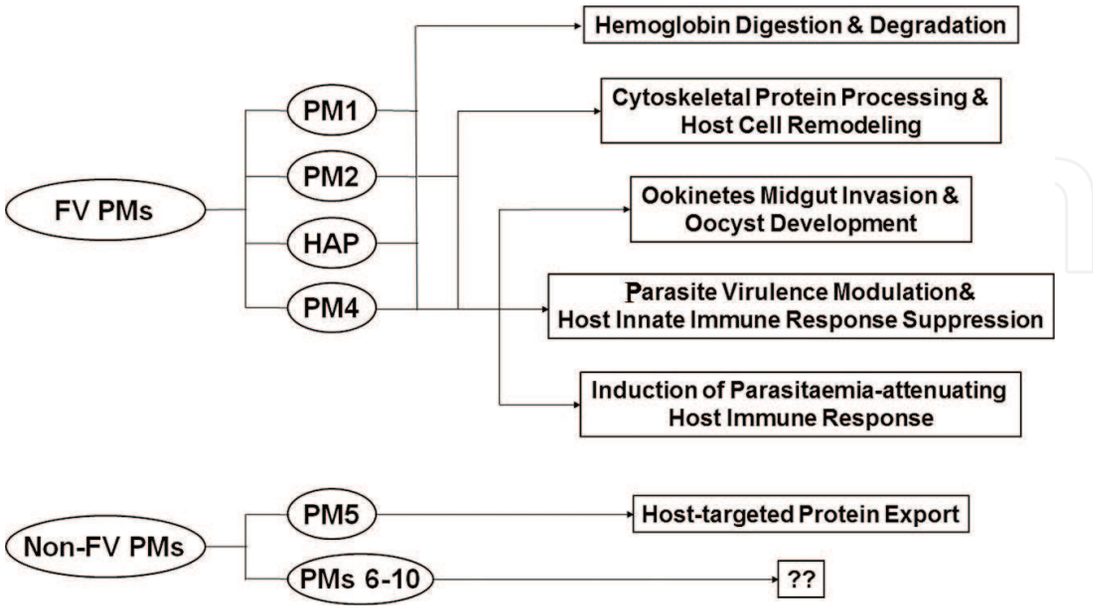

Figure 3. A diagram illustrating the connections of plasmepsins and their known biological functions.

in FVs; however, it is also carried out in vesicles arising either from micropinocytosis of cytoplasm of host cells or from endocytosis of cytostomes [57].

Early investigations establish that aspartic and cysteine proteinase activities are responsible for hemoglobin processing [73–81]. The successful isolation of FV from cultured trophozoites renders possible identification of naturally-occurring hemoglobin-processing enzymes [82]. *Pf*PM1, the first proteinase purified from isolated FVs, exhibits its cleavage specificity at the α-subunit amino acids F33-L34 (α33-34) of native hemoglobin [83]. Located in a highly conserved region among vertebrate species [84], this peptide bond is essential for maintaining the quaternary structure of the hemoglobin tetramer [85]. Breaking the α33-34 bond unravels the molecule and, in turn, leads to additional enzyme cleavages of the α- and β-subunits [47]. Sharing a 73% amino acid sequence identity with *Pf*PM1, *Pf*PM2, the second proteinase purified from FVs, also cleaves native hemoglobin at α33-34, though less efficiently than *Pf*PM1 [47]. SC-50083, a selective inhibitor of *Pf*PM1 over *Pf*PM2 by two orders of magnitude [46], blocks a majority of native hemoglobin degradation by FV protein extracts [47], indicating that *Pf*PM1 initiates the proteolysis. Of note, both *Pf*PM1 and *Pf*PM2 can further digest denatured globin into smaller peptides [47]. A third FV PM, *Pf*HAP, purified from FVs, cleaves native hemoglobin even less efficiently than *Pf*PM2 does and yet shows an efficiency in degrading denatured globin equivalent to *Pf*PM2 [48]. Similar to *Pf*HAP, *Pf*PM4 of the recombinant form prefers degrading denatured globin than native hemoglobin [48]. Other proteolytic enzymes, such as the cysteine proteinase falcipains, the metallo-proteinase falcilysin and aminopeptidases, are actively involved in further degrading hemoglobin fragments to oligopeptides and amino acids [42, 86]. These findings indicate that hemoglobin digestion and degradation in *P. falciparum* is an ordered process, in which *Pf*PM1 and *Pf*PM2 initiate the cleavage and various proteinases are involved in additional processing. Hemoglobin processing in other human malaria parasites may depend on their FV PMs that are homologous to *Pf*PM4.

The purpose of hemoglobin digestion and degradation has been under debate. Some believe that malaria parasites consume hemoglobin as a source of nutrients [87–91], which is supported by their limited capacity to *de novo* synthesis [88, 92] or exogenous amino acid uptake [93]. Nonetheless, hemoglobin degradation alone seems insufficient to maintain parasite metabolism due to its low contents of cysteine, glutamine, glutamic acid and methionine and its lack of isoleucine; in addition, hemoglobin-derived amino acids are found diffused into the host cell [89], indicating an excessive amount of hemoglobin being processed. This leads to a second hypothesis positing that the parasites degrade hemoglobin to empty space for their development and growth [94]. A third hypothesis, supported by an experimental-based modeling study, is that hemoglobin degradation is necessary to maintain the osmotic stability of infected erythrocytes such that the malaria parasite is able to grow and replicate in integrated host cells [95].

4.2. Cytoskeletal protein processing and host cell remodeling

*Pf*PM2 plays a role in remodeling host erythrocytes. In cultured schizonts, *Pf*PM2 was observed in the cytoplasm of the host cell in addition to the parasite [96], suggesting its potential interactions with cytoskeleton proteins. In support of this finding, recombinant

*Pf*PM2 exhibits hydrolysis of spectrin, actin and protein 4.1 at near neutral pH conditions [96]. In addition, schizont-expressed, naturally-occurring *Pf*PM2, but not *Pf*PM1 or falcipains, is enriched in the size exclusion chromatography (SEC) fractions that show proteolytic activity of the SH3 motif of the cytoskeletal protein spectrin [96], thus supporting its host cell remodeling role at the mature stage of the intra-erythrocytic phase. Of note, recombinant *Pf*PM4 hydrolyzes spectrin at pH 6.6 in a similar pattern as recombinant *Pf*PM2 does [97]. Further, a 37-kDa aspartic proteinase purified from the rodent malaria parasite *P. berghei* enables hydrolysis of spectrin and protein 4.1 from human erythrocytes at physiological pH [98]. Based on these pieces of evidence, it is likely that the FV PM-mediated host cell remodeling commonly occurs in the intra-erythrocytic phase of *Plasmodium* spp. (**Figure 3**).

4.3. Ookinetes midgut invasion and oocyst development

PM4 (*Pg*PM4) from the avian malaria parasite *P. gallinaceum* is involved in ookinete invasion of mosquito midguts and oocyst development during the sporogonic phase (**Figure 3**) [99]. In its mosquito host, *P. gallinaceum* expresses *Pg*PM4 in zygotes and ookinetes. In ookinetes, *Pg*PM4 is located at the apical membrane surface as well as in micronemes, an organelle of apicomplexan parasites involving in protein secretion. Monoclonal antibodies directed against *Pg*PM4 block oocyst development, but have no effects on ookinete formation. *Pg*PM4, together with chitinase and other enzymes, is speculated to hydrolyze peritrophic matrix proteins during ookinetes' midgut invasion, a critical step for parasite development. Questions remain elusive, such as how the expression of *Pg*PM4 and its orthologs is spatio-temporally regulated in the life cycle of malaria parasites, whether PM4 orthologs from other *Plasmodium* spp. play a similar role, what the natural substrates of *Pg*PM4 are, and how *Pg*PM4 recognizes and cleaves its substrates.

Of particular note, antibodies directed against the catalytic domain of either *Pf*PM7 or *Pf*PM10 decrease the prevalence of *P. falciparum* invasion of the mosquito and reduce the intensity of developed oocysts [69], indicating the involvement of mature *Pf*PM7 or *Pf*PM10 in parasite development during the sporogonic phase as well.

4.4. Host-targeted protein export

In the intra-erythrocytic phase, malaria parasites express and export hundreds of proteins, collectively named the "exportome," to infected red blood cells in order to acquire nutrients, to remodel the host cell, to avoid host immune detection, and to promote virulence [100–102]. A portion of the exportome shares at the N-terminus a pentameric sequence motif of RxLxE/Q/D (x represents any natural amino acid), known as the *Plasmodium ex*port *el*ement (PEXEL) [101] or the vacuolar transport signal [100]. A cleavage of the PEXEL motif at the C-terminus of leucine triggers the PEXEL-containing proteins to traverse the PPM and PVM, and subsequently reach the host cell [103]. PM5 catalyzes this reaction in the ER following the translation of PEXEL-containing proteins (**Figure 3**) [64, 104].

PM5-mediated PEXEL cleavage is proved to be essential to not only protein export but also parasite survival in that episomal expression of a catalytically inactive PM5 mutant decreases the level of proteins exported to host cells and slows down the parasite growth rate [64]. Interestingly, when the PEXEL motif of the *P. falciparum* erythrocyte membrane protein 3 (*Pf*EMP3) is engineered such that a signal peptidase, but not PM5, is able to conduct the cleavage, the resulting protein is transported to the parasitophorous vacuole rather than the cytoplasm of host cell, even if it has the same acetylated-xQ sequence retaining at the N-terminus as the PM5-cleaved mature *Pf*EMP3 does [104]. Meanwhile, when proteins are engineered to alter the prime side sequence of the PEXEL motif, the processed mature proteins fail to export to host erythrocytes even if PM5 performs the cleavage [105]. These findings highlight the importance of both PM5's involvement in the cleavage and the exposure of appropriate N-terminal sequence of the mature protein in host-targeted protein export. Detailed mechanisms related to how PM5-mediated PEXEL cleavage contributes to host-targeted protein export, and other potential roles of PM5 in the protein export event remain elusive.

Of particular note, the host-targeted malaria protein export is not restricted in the intra-erythrocytic phase but occurs over the course of the parasite life cycle [66, 106, 107], which coincides with the spatio-temporal expression pattern of PM5 [44, 65, 66]. It is thus conceivable that PM5 is also involved in protein export at other stages of the parasite life cycle, though no supporting evidence has been reported yet.

4.5. Other functions

Recent studies from Spaccapelo and colleagues showed the role of PM4 (*Pb*PM4) from the rodent malaria parasite *P. berghei* in maintaining virulence and suppressing innate immune responses of parasite-infected mice (**Figure 3**) [108, 109]. Supporting evidence comes from the observations that (1) the parasite with *pbpm4* genetically ablated (*Δpbpm4*) fails to elicit experimental cerebral malaria (ECM) in the ECM-susceptible mice; (2) the *Δpbpm4* is unable to kill the ECM-resistant mice as the parent strain does, but is cleared from blood after a three-week infection; and (3) after a single infection of naïve hosts by the *Δpbpm4*, these convalescent mice gain immune protection from a later parent strain infection. The mechanism by which *Pb*PM4 contributes to parasite virulence warrants further investigation.

In another study [110], recombinant *Pb*PM4 expressed and purified from *E. coli* was injected intraperitoneally (i.p.) in mice, together with the adjuvant saponin; sera obtained from the immunized mice contain antibodies that can recognize the cultured *P. berghei* strain from which the immunogen-encoding sequence originates. In addition, i.p. injecting erythorcytes infected by this *P. berghei* strain into *Pb*PM4-immunized mice boosts their production of the parasite-recognizing antibodies *in vivo*. Interestingly, three of five *Pb*PM4-immunized mice show resistance to *P. berghei* infection with the parasitaemia percentage reduced by an order of magnitude compared to naïve mice. These findings suggest that PMs are able to serve both as drug targets and as immunogens for malaria control (**Figure 3**). Though whether PM4 homologs residing in the host-infecting parasites are able to elicit a similar immune response

as purified recombinant forms is not yet clear, their potential immunogenic role in malaria prevention and treatment merits further investigation.

5. Enzymatic characterization

5.1. Food vacuole plasmepsins

5.1.1. Plasmodium falciparum plasmepsin 1

The naturally-occurring *Pf*PM1 runs as a 37-kDa monomeric protein in SEC, indicative of its mature form [47, 83]. Purified naturally-occurring *Pf*PM1 hydrolyzes native hemoglobin at α33-34 at an optimal pH 5.0 [83], within the pH range of the FV [111, 112]. This reaction is fully inhibited by pepstatin, a typical aspartic proteinase inhibitor, at nanomolar magnitude, but little by serine, cysteine or metallo-proteinase inhibitors in the millimolar range [83].

*Pf*PM1 of the recombinant form was expressed in *E. coli*. To obtain catalytically active mature enzyme, two technical obstacles were overcome: first, to avoid the potential toxicity the putative transmembrane motif exerts to *E. coli*, a truncated construct lacking the N-terminal half of the pro-segment was used [113]; second, to confer the auto-maturation capability on the truncated zymogen, this *Pf*PM1 construct was further engineered by introducing a self-cleavage site in the pro-segment [51, 52], by retaining a longer pro-segment [114], or by co-expressing with thioredoxin in one open reading frame [115]. These engineered *Pf*PM1s conduct auto-maturation at pH 4.0–5.5; however, the resulting mature enzyme retains a 7- or 12-amino-acid pro-segment [51, 52, 115]. Furthermore, the *Pf*PM1 produced by auto-maturation *in vitro* shows unanimously weaker kinetic efficiencies (k_{cat}/K_m) in cleaving hemoglobin-derived substrates than the naturally-occurring, mainly due to lower k_{cat} values [52, 115, 116]. These findings suggest that the presence of a short piece of pro-segment in the *in vitro* auto-matured *Pf*PM1 inhibits the enzyme activity and that the inhibition may occur in a different way than that it directly occupies the active site, like the case of pepsinogen and progastricsin [117–120]. In support of this, a crystal structure of the highly homologous *Pf*PM2 zymogen demonstrates that the pro-segment blocks enzyme activity by harnessing the C-terminal domain away from the N-terminal half to prevent the cooperative action of the catalytic dyad [120, 121].

The subsite specificity of *Pf*PM1 at S3 – S3' was analyzed using combinatorial chemistry-based peptide libraries [52]. In this study, the degree of accommodation of each of the 19 amino acids (i.e., norleucine and the 20 natural amino acids omitting methionine and cysteine) at each of the six subsites was quantitatively assessed. Ultimately, the peptide sequence comprising the best accommodated amino acid at each investigated position, in the order of P3–P3', is FSF*LQF (* represents the scissor bond). By comparing data to those obtained using the same method from analyzing human cathepsin D (hcatD), the most homologous human enzyme to FV PMs, a peptide sequence was deduced comprising at each position an amino acid that is well fit in *Pf*PM1, but better recognized by *Pf*PM1 than by hcatD. A peptidomimetic inhibitor (KPFSLΨLQF, where Ψ = –CH_2–NH–), converted from such peptide sequence by reducing the scissor bond to the non-cleavable methyleneamino (–CH_2–NH–),

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exhibit an inhibition of *Pf*PM1 with the dissociation constant (K_i) in nanomolar magnitude and a >5-fold selectivity for *Pf*PM1 over hcatD. In another study using a random decamer peptide library, Siripurkpong and colleagues showed that *Pf*PM1 prefers accommodating leucine and serine at S1' and S2', respectively [122]. While the two studies agreed on the S1' subsite specificity, the discrepancy at S2' may arise from difference in enzyme preparation, peptide library composition, or catalytic conditions.

5.1.2. Plasmodium falciparum plasmepsin 2

The naturally-occurring *Pf*PM2 is purified as a 36-kDa mature enzyme, separated from *Pf*PM1 by elution at a lower salt concentration [47]. As discussed in Section 3.1, the naturally-occurring *Pf*PM2 cleaves native hemoglobin at α33-34 less efficiently than *Pf*PM1 [47]; however, it digests acid-denatured globin 3-fold more efficiently than *Pf*PM1 [113]. Similar to the naturally-occurring *Pf*PM1, *Pf*PM2 is tightly inhibited by pepstatin with the K_i in sub-nanomolar magnitude [113, 116].

Unlike the case of *Pf*PM1, a recombinantly expressed truncated *Pf*PM2 zymogen lacking the putative transmembrane motif fully converts itself to mature enzyme in acidic conditions [53]. *Pf*PM2 generated from *in vitro* auto-maturation retains a 2- or 12-amino-acid pro-segment; though, the *in vitro* auto-matured enzyme and its naturally-occurring counterpart shares similar kinetic efficiencies in digesting hemoglobin-derived substrates and inhibition by peptidomimetic compounds [113, 116]. Interestingly, *Pf*PM2 can adopt the proper conformation from *in vitro* protein refolding such robustly that deleting part of (e.g. Δ112p–121p) or the entire pro-segment costs no loss of its catalytic activity [123, 124].

Beyer and colleagues studied the subsite specificity of *Pf*PM2 at S3 – S3' using the combinatorial chemistry-based peptide libraries discussed in Section 4.1.1 [125]. *Pf*PM2 prefers accommodating bulky hydrophobic residues (e.g., norleucine, leucine, isoleucine and phenylalanine) in all studied subsites except for the S2', where glutamine is the most favored. The peptide sequence comprising the most favored amino acid at each position, in the order of P3 – P3', is nLInL*LQI (nL = norleucine). A peptidomimetic inhibitor (KPnLSnLΨLQI) designed using the same approach described above exhibits an inhibition of *Pf*PM2 with the K_i at nanomolar magnitude and a >15-fold selectivity for *Pf*PM2 over hcatD. In two earlier studies, the catalytic activity of *Pf*PM2 was assessed in cleaving five sets of chromogenic octapeptides; peptide substrates within a particular set differ in amino acids substituted in one of the P4, P3, P2, P2' and P3' positions [126, 127]. The results showed that peptides with large hydrophobic amino acids (e.g. phenylalanine and leucine) residing in P3, P2 and P3' give rise to the highest k_{cat}/K_m values, consistent with the findings from the combinatorial peptide library study. In addition, Siripurkpong and colleagues reported that *Pf*PM2 digests a library of random decameric peptides most efficiently when leucine is placed in the P1' position and that the enzyme has comparable kinetic efficiencies when residues of different properties (e.g., serine, methionine, alanine and glutamine) are placed in the P2' position [122], again consistent with the previous findings. Of note, an N-terminal extension of peptide substrates to P6 enhances the kinetic efficiency of *Pf*PM2, and yet C-terminally extended peptides manifest no such effect [124]. The possible presence of a similar effect in other PM homologs is unclear yet.

5.1.3. Plasmodium falciparum histo-aspartic proteinase

HAP is a PM with the catalytic aspartic acid of the N-terminus replaced by a histidine. Naturally-occurring *Pf*HAP, purified as a monomeric mature enzyme of ~37 kDa, cleaves hemoglobin-derived substrates at an optimal pH 5.7 [48]. *Pf*HAP shows nearly no cleavage of native hemoglobin, but is able to digest acid-denatured globin and to hydrolyze α33-34 in hemoglobin-derived peptide substrates [48]. Nonetheless, *Pf*HAP cleaves α33-34 20-fold less efficiently than *Pf*PM1 and *Pf*PM2 [48, 113]. The naturally-occurring *Pf*HAP can be fully inhibited by isovaleryl-pepstatin (pepstatin A) at 1 μM and by the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) at 1 mM [48].

Catalytically active *Pf*HAP of the recombinant form was obtained using a similar strategy as the one applied to recombinant *Pf*PM1 [128, 129]. The *in vitro* auto-matured *Pf*HAP retains 4 pro-segment residues [128]. It exhibits an optimal catalytic activity at pH 5.2 and lowers kinetic efficiencies in cleaving hemoglobin-derived peptides than its naturally-occurring counterpart [128]. In addition, though pepstatin A at 1 μM completely inactivate the enzyme, PMSF at 1 mM inhibits enzyme activity by only 25% [128]. The apparent differences in enzymatic features between the naturally-occurring *Pf*HAP and the *in vitro* auto-matured may be attributable to improper folding of the recombinant protein [128] and/or the inhibition effects of the pro-segment [120].

A key question remains elusive is whether *Pf*HAP functions as an aspartic or a serine proteinase. Based on results from computational modeling, some view *Pf*HAP as a serine proteinase with a catalytic triad of H34, S37 and D214 [130], and others consider *Pf*HAP an atypical aspartic proteinase with D214 performing catalysis and H34 stabilizing the intermediate enzyme species [131]. By conducting alanine mutation of these residues related to catalysis, Parr and colleagues showed that D214A renders *Pf*HAP incapable of auto-maturation, whereas H34A and S37A do not affect auto-maturation, but lead to a lower kinetic efficiency in cleaving peptide substrates [132]. These findings support the role of D214 in enzyme catalysis, indicating that *Pf*HAP is an atypical aspartic proteinase.

5.1.4. Plasmepsin 4 orthologs

To the author's knowledge, no literatures have thus far reported the characteristics of naturally-occurring *Pf*PM4. The recombinantly expressed *Pf*PM4 zymogen lacking the putative transmembrane motif conducts auto-maturation under acidic conditions, resulting in a mature form retaining 12 pro-segment residues [48]. This mature *Pf*PM4 cleaves hemoglobin-derived peptides at an optimal pH 5.4 [48]. *Pf*PM4 digests native hemoglobin less efficiently than *Pf*PM1 and *Pf*PM2 and prefers cleaving acid-denatured globin [48]. Similar to *Pf*PM1 and *Pf*PM2, but unlike *Pf*HAP, *Pf*PM4 is fully inhibited by pepstatin A at sub-nanomolar magnitude, but not by inhibitors of other types of proteinases [48, 54].

Recombinant PM4s from the other three human malaria parasites and the rodent malarial parasite *P. berghei* were similarly produced and activated [54, 126, 133]. The subsite specificity at S3 – S3' of the five PM4 orthologs (i.e., *Pf*PM4, *Po*PM4, *Pv*PM4, *Pm*PM4 and *Pb*PM4) was investigated using combinatorial peptide libraries [125, 133]. All five PM4s unanimously prefer accommodating phenylalanine or tyrosine at S1 and S1', except that *Pb*PM4 accommodates norleucine best

at S1'. At S3, bulky hydrophobic amino acids, such as leucine, norleucine and phenylalanine, are preferred by all five enzymes. At S3', the acceptance of amino acids by the four human PM4s is broad with isoleucine accommodated best, whereas *Pb*PM4 accommodates aromatic phenylalanine and tryptophan best. For S2 and S2', all five PM4s seem to tolerate amino acids of different properties. Glutamic acid, serine and isoleucine are the most favored at S2; while for glutamine, isoleucine, glutamic acid and arginine, when accommodated in S2', each leads to a considerable peptide cleavage. The peptide sequence comprising the most favored residue by each subsite, in the order of P3 – P3', is IQF*YIL for *Pf*PM4, is FEF*YFI for *Po*PM4, is LEF*FII for *Pv*PM4, is FEF*FII for *Pm*PM4, and is FEF*nLSW for *Pb*PM4. Peptidomimetic inhibitors were designed using the same approach described in Section 4.1.1: KPVEFΨRQT for *Pf*PM4, KPLEFΨFRV for *Po*PM4, KPLEFΨYRV for *Pv*PM4, KPFELΨAWT for *Pm*PM4, and KPYEFΨRQF for *Pb*PM4. These compounds unanimously exhibit a selective inhibition of their respective PM4s over hcatD, and inhibit their respective PM4s with the K_i values at sub-nanomolar to nanomolar magnitude, except for the one designed for *Pm*PM4, which inhibits *Pm*PM4 with the K_i at micromolar magnitude. Such a poor inhibition may be due to the incorporation in the P1' position of an alanine that is poorly recognized by *Pm*PM4, indicating the key role of the P1' amino acid in determining the enzyme-ligand interaction. In another two studies, the subsite specificity of the four human PM4s was analyzed at S3, S2, S2' and S3' using chromogenic octapeptides [54, 126]. The results showed that (1) hydrophobic amino acids (e.g., phenylalanine and isoleucine) are more favored at P3 than smaller hydrophobic, polar and charged amino acids, (2) hydrophobic amino acids are favored at P2, and (3) amino acids of different properties at P2' and P3' are well tolerated. These findings are consistent with the data obtained from the combinatorial peptide library study.

5.2. Non-food vacuole plasmepsins

Thus far, enzymatic characterization of non-FV PMs has been focused on the PM5 orthologs. PM5 (*Pf*PM5) immunopurified from cultured *P. falciparum* cleaves PEXEL (RxLxQ/E/D)-containing substrates at the C-terminus of leucine at pH 5–7 [64], resembling the pH of the mammalian ER [134]. The PEXEL-cleaving activity of *Pf*PM5 is partially inhibited by pepstatin A and HIV-1 PIs (i.e., lopinavir, nelfinavir, ritonavir and saquinavir) with the IC_{50} values in the high micromolar range [64, 104]. The presence of P3 R and P1 L is key to *Pf*PM5-catalyzed PEXEL cleavage in that mutations in these two positions (e.g., P3 R-to-A or K and P1 L-to-A or I) unanimously inhibit the cleavage, and abolish the export of PEXEL-containing proteins to host erythrocytes; amino acids in the prime side positions also influence the efficiency of PEXEL cleavage and subsequent protein export [104, 105, 135]. *Pf*PM5 also digests non-canonical PEXEL motifs (e.g., RxLxxE) at the C-terminus of P1 L, which in turn, triggers host-targeted protein export [105]. Likewise, this *Pf*PM5-catalyzed non-canonical PEXEL cleavage and subsequent protein export are blocked by a P3 R-to-A mutation [105]. Of note, though deleting neither the P1' nor the P2' amino acid affects enzyme cleavage, protein export efficiency is reduced by these prime side mutations [105]. Taken together, these findings highlight the essential role of P3 R and P1 L in modulating *Pf*PM5-mediated PEXEL cleavage and the importance of the prime side peptide sequence in directing host-targeted protein export.

Two constructs of *Pf*PM5 encoding a truncated zymogen (amino acids 37–521) and a mature enzyme (amino acids 84–521) have been recombinantly expressed in *E. coli* [136, 137]. Following *in vitro* protein refolding, both the zymogen and the mature enzyme exhibit catalytic activity in cleaving PEXEL-containing peptides at an optimal pH 6.0–6.5 [136, 137]. Indeed, the pro-segment of *Pf*PM5 was shown to be non-essential for guiding the proper folding of protein [137, 138]. Subsite specificity analysis of the recombinant mature *Pf*PM5 on a peptide series of RxLxE at P2 and P1' showed that when the polar serine is placed at P1', the hydrophobic isoleucine is more favored at P2 than the charged glutamic acid and lysine; and vice versa, when isoleucine is placed at P2, serine is better accommodated at S1' than glutamic acid and the hydrophobic valine [136]. Recombinant *Pf*PM5, like the parasite-expressed, can only be partially (<50%) inhibited by pepstatin A, nelfinavir or PMSF at 100 mM; however, its catalytic activity is almost fully blocked by Cu^{2+} or Hg^{2+} at the sub-micromolar level [137]. Furthermore, the zymogen and mature form of PM5 (*Pv*PM5-Thai) from *P. vivax* Thailand

PM	Expression pattern	Subcellular location[a]	Enzymatic characteristics			
			pH[b]	Natural substrates	Subsite specificity[d]	Pepstatin A inhibition
*Pf*PM1	Intra-erythrocytic phase; merizoites; gametocytes	FV, TV	5.0	Hb	FSF*L(Q/S)F	<1 nM (K_i)
*Pf*PM2	Intra-erythrocytic phase; merizoites; gametocytes; oocysts; sporozoites	FV, TV	4.7; ~6.8	Hb; Host cytoskeletal proteins	nLInL*LQI	<1 nM (K_i)
*Pf*HAP	Intra-erythrocytic phase; merizoites; gametocytes; sporozoites	FV, TV	5.7	Hb	n.d.	1 μM (fully inhibition)
*Pf*PM4	Intra-erythrocytic phase; merizoites; gametocytes; oocysts; sporozoites	FV, TV	4.5; ~6.6	Hb[c]; Host cytoskeletal proteins[c]	IQF*YIL	<1 nM (K_i)
*Pv*PM4	Intra-erythrocytic phase	FV, TV	4.5	Hb[c]	LEF*FII	<1 nM (K_i)
*Po*PM4	n.d.	FV, TV	4.5	Hb[c]	FEF*YFI	<1 nM (K_i)
*Pm*PM4	n.d.	FV, TV	4.5	Hb[c]	FEF*FII	<1 nM (K_i)
*Pb*PM4	n.d.	FV, TV	5.0–5.5	Hb[c]; Host cytoskeletal proteins	FEF*nLSW	<1 nM (K_i)
*Pf*PM5	Intra-erythrocytic phase; merizoites; gametocytes; sporozoites	ER/NE	6.0–6.5	PEXEL-containing parasite proteins	RxL*x(Q/E/D); RxL*xxE	~20–30 μM (IC_{50})

[a]This column shows the subcellular locations of catalytically active, mature plasmepsins.
[b]This column shows the optimal catalytic pH; for *Pf*PM2 and *Pf*PM4, digestion of host cytoskeletal proteins is carried out at near neutral pH.
[c]Digestion of these natural substrates were performed *in vitro* using *recombinant* plasmepsins.
[d]This column shows the best amino acids accommodated at subsites in the order of P3 – P3′; * represents scissile bond between P1 and P1′; x represents any natural amino acid; nL = norleucine.

Table 2. Enzymatic properties of plasmepsins.

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isolates were recombinantly expressed; the purified *Pv*PM5-Thai exhibits similar enzymatic features as the recombinant *Pf*PM5 does [139].

The enzymatic properties of PMs discussed in this section are summarized in **Table 2**.

6. Plasmepsin-targeted antimalarial drug development

6.1. Evaluation of food vacuole plasmepsins as antimalarial drug targets

The establishment of the role of FV PMs in hemoglobin processing raised the question whether FV PMs can be targets of novel antimalarial drugs. Peptidomimetic compounds developed in the early stage (e.g., pepstatin A, SC-50083, Ro40-4388, and HIV-1 PIs) bind FV PMs tightly and block growth of cultured parasites [46, 51, 140, 141], suggesting that inhibition of FV PMs is a promising antimalarial strategy. Numerous types of FV PM-targeted compounds, synthetic or isolated from natural sources, have been assessed for the past two and a half decades based on criteria involving binding affinity and selectivity, inhibition potency to cultured parasite growth, and cytotoxicity to mammalian cell culture (for reviews, see for example [142, 143]). For example, certain hydroxyethylamine derivatives inhibit *Pf*PM1, *Pf*PM2 and *Pf*PM4 in nanomolar magnitude, exhibit a >30-fold binding selectivity over hcatD, and disrupt growth of cultured *P. falciparum* with IC_{50}s in the low micromolar range [144, 145]. In a series of studies, several allophenylnorstatine-based compounds were found to inhibit all four FV *Pf*PMs in nanomolar magnitude, to block parasite growth with IC_{50}s in the low micromolar range, and to have the TD_{50}s (cytotoxicity) in high micromolar magnitude to rat skeletal myoblasts [146–148]. In addition, clinically used HIV-1 PIs exhibit antimalarial activity on parasites in both the exo-erythrocytic and the intra-erythrocytic phases in the sub-micromolar to low micromolar range [149–151], inhibit *Pf*PM2 and *Pf*PM4 at low micromolar magnitude, and have a >10-fold selectivity over hcatD [141]. Interestingly, using affinity binding probes coupled to a FV PM inhibitor library, a hydroxyethyl-based inhibitor was identified that inhibits all four FV *Pf*PMs and the growth of cultured *P. falciparum* with IC_{50} at ~1 μM [152].

To assess whether FV PMs are appropriate drug targets, *pfpm*4, 1, 2 and *pfhap* were knocked out individually (i.e., Δ*pfpm4*, Δ*pfpm1*, Δ*pfpm2* and Δ*pfhap*), in combination (e.g., Δ*pfpm4*/*1* and Δ*pfpm1*/*2*/*hap*), or together as a whole (i.e., Δ*pfpm4*/*1*/*2*/*hap*). Genetic ablation of any particular gene alters neither the mRNA transcription nor the protein expression of the other three paralogs over the course of the intra-erythrocytic phase [153]. For hemoglobin metabolism, the Δ*pfpm4* strain, but not the Δ*pfpm1*, Δ*pfpm2* or Δ*pfhap*, shows a reduction in hemozoin accumulation in the FV compared to the parent line [154, 155]. Of note, genetic disruption of PM expression does affect the rate of parasite replication in that the Δ*pfpm4*, Δ*pfpm1*, Δ*pfpm2*, Δ*pfpm4*/*1* and Δ*pfpm4*/*1*/*2*/*hap* strains all exhibit a reduced growth rate in amino-acid-rich media compared to the parent line, and that when cultured in amino-acid-limited media, the Δ*pfhap* strain also demonstrates a slower growth rate [153–157]. As for cell and subcellular organelle morphology, though no morphological abnormalities are apparent in the Δ*pfpm1* and Δ*pfhap* strains, a portion of the Δ*pfpm2* shows enlarged mitochondria, and a portion of the Δ*pfpm4* exhibits a notable accumulation of electron-dense, single-membrane vesicles in the FV

[154, 156]; in addition, ceroid-like multilamellar bodies, and electron-dense, single-membrane vesicles are accumulated in the FV of the *Δpfpm4/1/2/hap* strain [155]. Taken together, genetic ablation of *pfpm*s is not lethal to the parasite in cultured conditions despite apparent metabolic and pathological abnormalities, thus it seems that FV PMs may be dispensable for parasite survival; however, one cannot overlook the potential contribution of *Pb*PM4 to the virulence of the parasite in infected mice (see discussion in Section 4.5). Understanding the pathological role of FV PMs in both cell-based and animal models may lead to a better assessment of the feasibility of PM-targeted drug development.

To better understand the relationship between enzyme inhibition and anti-parasitic activity, the effects of known FV PM inhibitors on the growth of PM-knockout parasites were investigated. When pepstatin A was administered to cultured parasite in the intra-erythrocytic phase, growth of the Δ*pfpm1*, Δ*pfpm2*, Δ*pfhap* and Δ*pfpm4/1* strains is even slightly less sensitive to the compound than that of the parent line, and yet growth of the FP-2-knockout strain is at least one order of magnitude more sensitive to pepstatin A [156, 157]. These findings indicate that the parasite may turn to other proteinases to maintain normal function when the activities of FV PMs are blocked. The effects of HIV-1 PIs on *in vitro* PM inhibition and blockage of parasite growth have been well established [141, 158]. However, the Δ*pfpm1/2/hap* and Δ*pfpm4/1/2/hap* strains share a comparable sensitivity to five HIV-1 PIs (i.e., atazanavir, lopinavir, indinavir, ritonavir and saquinavir) with the parent line [155], indicating that FV PMs may not be the target of these inhibitors in the parasite [141]. Such off-target effects are rather common among developed PM inhibitors of distinct classes (e.g., C_2-symmetric 1,2-dihydroxyethylenes [159], hydroxylethylamine transition-state isosteres [145] and amidine-containing diphenylureas [160]). The authentic targets of these inhibitors in the parasite have been under investigation [161].

Despite that FV PMs are not critical to parasite survival at the blood stage and that certain FV PM inhibitors exhibit their anti-parasitic activities with an off-target effect, it is still early to negate FV PM-targeted drug design given our limited understanding of their functions and characteristics. The continuously identified novel functions of FV PMs plus their broad spatio-temporal expression pattern over the course of the parasite life cycle are worthy of further investigation.

6.2. Developing novel antimalarial drugs targeting non-food vacuole plasmepsins

PM5 has been considered an ideal target for novel antimalarial drug design based on a series of findings: first, ablation of the gene encoding PM5 is lethal to cultured *P. berghei* [104], so is mutation of a catalytic aspartic acid of PM5 to cultured *P. falciparum* [64]; second, PM5 is evolutionarily conserved among *Plasmodium* spp. with no identified gene replication or functional redundancy [44]; third, PM5 shares a low amino acid sequence identity with human aspartic proteinases (e.g., 26% with mature hcatD, and 18% with mature human β-secretase 1 (hBACE-1)); and fourth, the expression profile of PM5 spans the entire life cycle of malaria parasites [44, 65, 66].

Two basic components were incorporated in the initial design of PM5 inhibitors: a PEXEL sequence, which provides a moderate fit of compounds to the active site of the enzyme, and

a transition-state peptidomimetic moiety, which gives rise to a tight interaction with the catalytic residues of proteinases. WEHI-916, a statine-based compound mimicking the non-prime-side RVL motif of the PEXEL, shows a strong inhibition (IC_{50} = ~20 nM) of *Pf*PM5 and *Pv*PM5, a much weaker inhibition of hcatD (IC_{50} = 25 μM), and a negligible inhibition of hBACE-1 (IC_{50}>100 μM) [162, 163]. Administration of WEHI-916 to cultured *P. falciparum* blocks the PEXEL cleavage in a dose-dependent manner, and impairs protein export to host erythrocytes [162]. Of particular interest, conditioned knockdown of *pfpm5* enhances WEHI-916-mediated inhibition of PEXEL cleavage and the sensitivity of parasite growth to this compound; whereas overexpression of *Pf*PM5 weakens the anti-parasitic potency of WEHI-916 [162]. These findings confirm that PM5 is the target of WEHI-916 in the parasite. Though, WHEI-916 has only a moderate potency (EC_{50} = 2.5 μM to the strain 3D7) in killing cultured *P. falciparum*, which may be attributed to its poor membrane permeability [162, 163]. To enhance the anti-parasitic potency of WEHI-916 while retaining its strong binding to PM5, the highly polar P3 arginine in WEHI-916 was modified to its isostere L-canavanine, and the N-terminal sulfonamide was replaced by a carbamate [164, 165]. The resulting compound WEHI-842 inhibits *Pf*PM5 and *Pv*PM5 more tightly (IC_{50} = 0.2–0.4 nM), and blocks the PEXEL cleavage and protein export more potently than WEHI-916 [165]. Importantly, WEHI-842 kills the chloroquine-sensitive 3D7 strain and multiple chloroquine-resistant *P. falciparum* strains with a potency (EC_{50} = 0.4 μM) one order of magnitude higher than that of WEHI-916, and yet it exhibits a low cytotoxicity against human cells (TD_{50}>50 μM) [165]. Taken together, WEHI-842 represents a promising lead for developing PM5-targeted antimalarial drugs.

Our limited knowledge on PMs 6–10 makes it difficult to assess the necessity and importance of developing drugs targeting these enzymes. However, the detection of these PMs in multiple stages of the parasite life cycle suggests that their role in malaria pathogenesis is non-trivial. For future PM-targeted drug development, the functions and characteristics of PMs 6-10 warrant further study.

7. Concluding remarks

Malaria, one of the deadliest infectious diseases in history, still poses a serious socio-economic problem at present. Malaria control has been effectively undertaken from multiple perspectives, including drug-based disease prevention and treatment, intervention of malaria transmission by the mosquito vector, and usage of vaccine against malaria parasites. Though, the emergence and quick spread of drug-resistant parasite strains urges us to identify new antimalarial drug targets. The subject of this review has focused on the aspartic proteinase PM family, the molecular entities deemed novel and promising targets of next-generation antimalarial drugs.

Discussed here is our understanding of the PM family members on their biosynthesis, biological functions and characteristics for the past two and a half decades. Seven groups of PMs have thus far been identified from genome comparison of a series of *Plasmodium* spp. infecting rodents, birds, humans and non-human primates. These PMs, unique in enzymatic feature and spatio-temporal expression pattern, play multifaceted roles in the pathogenicity of the malaria

parasite. Due to the seemingly dispensable role of FV PMs in parasite growth and survival, the focus of PM-targeted drug development is shifting towards non-FV PMs. Selective inhibitors of PM5 have been developed and shown strong inhibition potency to parasite growth.

On the other hand, our knowledge on PMs is still quite limited and much needs to be clarified and explored in the future studies. For example, what is the biological meaning of the presence of four FV PM paralogs in *P. falciparum*? What do the FV PM inhibitors authentically target to exert their anti-parasitic activity? What are other possible roles of PM5 than host-targeted protein export? What are the functions of PMs 6-10, and can these enzymes be antimalarial drug targets? What is the likelihood that PMs are used as immunogens in active immunization and that antibodies directed against PMs are used in passive immunization to protect hosts from malaria parasite infection? Successful PM-targeted drug development replies on a comprehensive understanding of this enzyme family.

List of abbreviations

ACTs	artemisinin-based combination therapies
ALLN	*N*-acetyl-Leu-Leu-norleucinal
AN	artemisinin
BFA	brefeldin A
Chr.	chromosome
CQ	chloroquine
(k)Da	(kilo-)dalton
DDT	dichloro-diphenyltrichloroethane
E. coli	*Escherichia coli*
E-64	L-3-carboxy-2,3-trans-epoxypropionyl-leucylamido(4-guanidino)butane
EC_{50}	half maximal effective concentration
ECM	experimental cerebral malaria
EM	electron microscopy
ER	endoplasmic reticulum
FP	falcipain
FV	food vacuole
GFP	green fluorescence protein
HAP	Histo-Aspartic Proteinase
Hb	hemoglobin
hBACE-1	human β-secretase 1
hcatD	human cathepsin D

HIV-1	human immunodeficiency virus type 1
IC_{50}	half maximal inhibitory concentration
i.p.	intraperitoneally
kb	kilo-base
k_{cat}	turnover number
k_{cat}/K_m	specificity constant
K_i	dissociation/inhibition constant
μM	micromolar
mM	millimolar
MS	mass spectrometry
MW	molecular weight
NE	nuclear envelope
nL	norleucine
nM	nanomolar
P.	*Plasmodium*
Pb	*Plasmodium berghei*
PEXEL	Plasmodium export element
Pf	*Plasmodium falciparum*
*Pf*EMP3	*P. falciparum* erythrocyte membrane protein 3
Pg	*Plasmodium gallinaceum*
pH	negative log of the hydrogen ion concentration
pI	isoelectric point
PIs	proteinase inhibitors
PM	plasmepsin
Pm	*Plasmodium malariae*
PMSF	phenylmethylsulfonyl fluoride
Po	*Plasmodium ovale*
PPM	parasite plasma membrane
PV	parasitophorous vacuole
Pv	*Plasmodium vivax*
PVM	parasitophorous vacuolar membrane
SEC	size exclusion chromatography
spp.	*species pluralis*
TD_{50}	median toxic dose
TV	transport vesicle

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