Heme polymerization by malarial parasite: A potential target for antimalarial drug development

Amit Vikram Pandey and Virander Singh Chauhan*

Malaria and Structural Biology Group, International Centre for Genetic Engineering and Biotechnology, P.O. Box 10504, Aruna Asaf Ali Road, New Delhi 110 067, India

Fast spreading drug resistance to commonly used antimalarial drugs like chloroquine and pyrimethamine has posed urgent requirement for newer drugs. Identification of critical biochemical processes necessary for parasite survival and their characterization is necessary for targeted drug design. Despite prolonged research, biochemistry of malaria parasite is poorly understood. Many common biochemical pathways, well characterized in other organisms, are yet to be looked at in malaria parasite. Polymerization of heme to form hemozoin pigment is a process unique to *Plasmodia*. This has attracted much attention in the recent years as a potential target for antimalarial chemotherapy, as specific inhibitors designed against such a process have the chance of high selectivity in interaction with metabolism of the host harbouring the parasite. Although the last word has yet to be said on mechanism of heme polymerization by malaria parasite, a consensus is evolving between various research groups working in this area, about its potential as a target for antimalarial design. In this review we have discussed the problem of heme polymerization and its possible use as a target for drug design as well as provide an account of the work being done in this direction by us as well as other research groups.

MALARIA has long been a major killer of humankind, especially in the tropical and subtropical regions of the world. More than half of the global population is at the risk of being infected by malaria. Earlier hopes of malaria eradication by removal of its mosquito vector had only limited success due to the development of insecticide resistance by mosquitoes. However, the major jolt to human fight against malaria has come from the emergence of drug resistant strains of *Plasmodia*. Chloroquine, the most widely used drug for clinical treatment of malaria has lost its efficacy mainly due to its indiscriminate use as an over the counter drug in many countries[1].

Though more than hundred years have elapsed since the discovery of malaria parasite and its transmission, detailed biochemistry of *Plasmodia* remains poorly understood. Surprisingly, despite being used for centuries for treatment of malaria, mechanism of action of quinine and its related synthetic analog chloroquine, is still unclear[2]. Rational design of inhibitors against specific biochemical targets has been hampered by poor understanding of the molecular mechanisms involved in antimalarial action of these drugs. Only folate antagonists like pyrimethamine, proguanil and sulfadoxine have well characterized mechanism of action[3].

Hemozoin formation by malaria parasite

Malaria parasite resides inside the erythrocytes of the infected host during asexual blood stage of its life cycle. Hemoglobin, the major protein present inside the erythrocytes is used for nutrient supply by the malaria parasite[4]. *Plasmodia* degrade hemoglobin and use the amino acids derived from proteolytic digestion for their biosynthetic requirements. This hemoglobin catabolism in malaria parasite is carried out in a specialized acid compartment digestive vacuole[5]. Hemoglobin degradation is a highly ordered process involving several proteases[6,7]. An aspartic protease, plasmepsin I, makes the first cleavage to denature the hemoglobin tetramer, followed by action of another aspartic protease plasmepsin II[8,9]. Denatured globin formed by the action of plasmepsins is further degraded into small peptides by other proteases. A cysteine protease, falcipain, has been characterized from *P. falciparum*, which degrades denatured globin[10,11]. Small peptides have been shown to be transported from digestive vacuole to cytoplasm where these are broken down into amino acids by a serine protease[12] (Figure 1).

Large amounts of toxic-free heme is released as a byproduct of hemoglobin degradation[13]. Heme, when present in hemoglobin is in ferrous form, and is nontoxic. When released free from hemoglobin, it is converted into ferric form that is highly toxic, inhibiting vacuolar proteases and damaging parasite membranes[14,15]. Detoxification of heme is therefore necessary for the survival and growth of malaria parasite[16–18]. In the host, detoxification of heme is achieved by an enzyme heme oxygenase, which breaks heme to form biliverdin. Another enzyme, biliverdin reductase, converts biliverdin into

*For correspondence. (e-mail: virendra@icgebnd.ernet.in)

bilirubin, which is converted into a water soluble conjugate and excreted through urine. Malaria parasite does not seem to use this pathway for the heme catabolism. Instead, it has evolved a unique pathway of heme polymerization to avoid heme toxicity[19]. Inside the food vacuole of malaria parasite, heme is converted into hemozoin, popularly known as 'malaria pigment'. This hemozoin pigment is a polymer of heme units linked through an iron-carboxylate bond[20,21] (Figure 2). This bond is formed by the linking of central ferric iron of one heme unit with the propionate side chain of another heme. Number of heme units in a hemozoin molecule is yet to be established. Recent crystallographic data suggests cross linking between hemozoin chains through hydrogen bonding between propionate side chains[21]. This pigment is inert in parasite and released into the host blood supply after infected erythrocytes burst open at the end of parasite life cycle. Hemozoin gets deposited inside the tissues of the infected host[22,23]. Level of hemozoin pigment deposited in the tissues increases with the increase in parasitemia[23].

The plasmodial enzymes involved in digestion of hemoglobin have attracted much attention as possible targets for antimalarial design. Recently, crystal structure of plasmepsin II complexed with pepstatin A, has been solved and, currently, it is being used for inhibitor design[9]. Attempts to obtain the *P. falciparum* cysteine protease in large quantities for functional as well as structural studies have so far been unsuccessful[10] as enzyme expressed in bacterial system could not be made in active form. However, recent experiments in our laboratory, directed towards making enzymatically-active *P. falciparum* cysteine protease, falcipain, expressed in *E. coli* with a fusion protein attachment, have met with considerable success, and detailed studies to characterize this enzyme as well as to get the active enzyme without any fusion partner are under progress (unpublished data).

Hemozoin and antimalarial action of quinolines

Chloroquine was first used in 1940s for the treatment of malaria. Numerous conflicting theories have been put forward over the past five decades to describe its antimalarial mode of action[1]. One of the earliest observed morphological effects of chloroquine treatment on malarial parasite, is pigment clumping and digestive vacuole enlargement[24]. Quinoline antimalarial drugs have been shown to accumulate in food vacuole of malaria parasite[25–30], and these drugs form complexes with heme[31,32] (Table 1). Most of the blood schizontocidal antimalarial drugs are active against only those stages of the parasite which actively degrade hemoglobin and

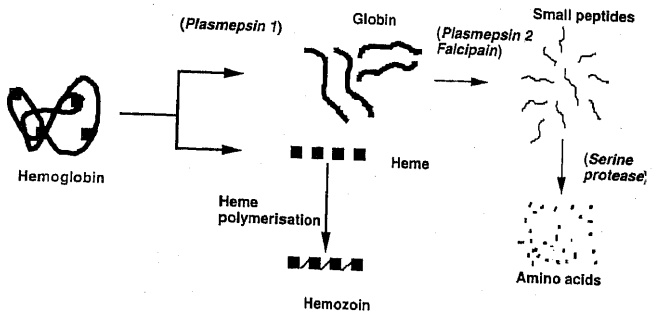

Figure 1. Schematic representation of hemoglobin catabolism in the malaria parasite.

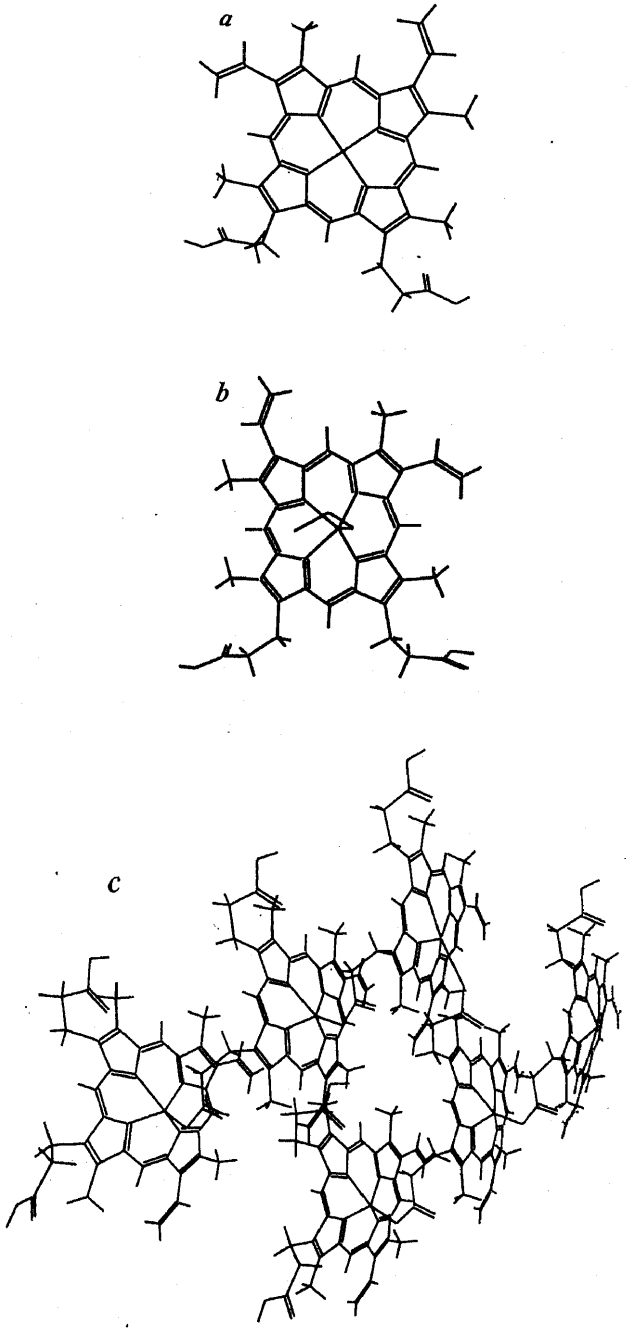

Figure 2. *a*, Structures of heme; *b*, heme–acetate adduct and *c*, hemozoin.

produce hemozoin[33,34]. The process of heme polymerization is unique to malaria parasite and therefore has emerged as an attractive target inside the parasite for the design of specific inhibitors. However, the efforts in this direction are hampered due to poor understanding of the mechanism of heme polymerization. Several factors responsible for catalysing heme polymerization have been reported by various research groups[35]. Initially an enzyme 'heme polymerase' was identified in *P. falciparum* trophozoites, which converted heme into hemozoin in acidic environment[36]. Similar activity has been identified in *P. berghei* and *P. yoelii*[37,38]. In a later report, polymerization of heme has been described as a spontaneous chemical process, which does not require any parasitic material[39,40]. Autocatalytic conversion of heme, mediated by preformed hemozoin and involvement of phospholipids has also been reported[41,42]. However, subsequent to these reports, we have shown that hemozoin formation under the conditions of parasite food vacuole required presence of parasitic material and could not proceed spontaneously[43]. It seems that initially, ferric iron of heme interacts with the carboxylic acids in the buffer used in the heme polymerization assay, forming a bond with similar infra-red spectral characteristics as the iron-carboxylate bond between ferric iron and the propionate side chains of heme in hemozoin. However, we found that these heme–carboxylic acid adducts have different solubility properties than hemozoin, and could be easily separated. Hemozoin is insoluble in organic solvents (methanol, ethanol, and acetone) and mildly alkaline bicarbonate buffer (100 mM, pH 9.0), whereas free heme and other small molecular weight heme–carboxylic acid adducts are soluble in these solvent conditions. This difference in the solubility has enabled us to differentiate between hemozoin and heme–carboxylic acid interaction adducts[43] (Table 2). These adducts prevent chain elongation, since further addition of heme units is blocked (Figure 2 *b*). This prevents the formation of a crystalline structure, so these products are short-length molecules, and thus have solubility characteristics like monomeric heme. Studies from other research groups[44,45] have subsequently confirmed these findings.

Table 1. Properties of blood schizontocidal antimalarials

Type of drug	Binding with heme	Inhibition of heme polymerization	Inhibition of proteases
4-Amino quinolines	+	+	+
Quinine	+	++	+
Quinoline methanols Mefloquine, Halofantrine	+	++	+
Endoperoxides artemisinin	+	+++	++

Number of + signs is indicative of the increasing order of the property[1,2,5,32]. Endoperoxide antimalarials are better inhibitors of heme polymerization and proteases than quinoline drugs (Pandey, A. V., Tekwani, B. L. and Chauhan, V. S., unpublished data).

Histidine-rich proteins and heme polymerization

There are several reports of histidine-rich proteins (HRPs) in the literature from different malaria species[46–50]. The first report of the presence of an HRP was from avian malaria parasite, *P. lophurae*, which shares considerable homologies with *P. falciparum*[46]. Three major HRPs (PfHRP I, II and III) have been described in human malaria parasite *P. falciparum*[51]. Recently HRP from *P. falciparum* have been reported to mediate hemozoin

Table 2. Comparison of solubility properties and IR spectral characteristics of heme, hemozoin, β-hematin and heme–acetate incubation products

Sample	Solvent			IR peaks at 1660 and 1207 cm^{-1}
	Bicarbonate	SDS	Methanol	
Hemin	++++	++	++++	Absent
Hematin	++++	+++	++++	Absent
Malarial hemozoin	±	–	–	Present
β-hematin	±	–	–	Present
Heme–acetate incubation products				
0.1 M 37°C 6 h	++++	+++	++++	ND
0.5 M 37°C 6 h	++++	+++	++++	ND
1.0 M 37°C 6 h	++++	+++	++++	Present
4.5 M 37°C 6 h	++++	+++	++++	Present
4.5 M 60°C 30 min	++++	+++	++++	Present
4.5 M 80°C 12 h	++	++	++	Present

++++, > 90% solubility; ++, 40–60% soluble; ±, sparingly soluble; –, insoluble, ND, not determined. Hemin is hematin chloride, hemozoin was purified from *Plasmodium yoelii*, β-hematin was synthesized as described previously[19]. Table adapted from our results published in *FEBS Lett.*, 1996, **393**, 189–192.

formation and a PfHRP II has been localized in the digestive vacuole of malaria parasite[52]. PfHRP I is a knob-associated HRP reported to be involved in cyto adherance[53]. The other two HRPs (II and III) consist of repetitive hexapeptide units containing histidine and alanine pairs[54]. PfHRP II has a Mr of 35,138. The major repeat sequence in PfHRP II is a hexapeptide, which is present 33 times in its sequence. Out of the 33 repetitive hexapeptides, 30 have the sequence Ala–His–His–Ala–Ala–Asp, while in the rest three repeats, aspartate in the sixth position is replaced by tyrosine. Apart from this there are 18 repeats of tripeptide Ala–His–His in the sequence of PfHRP II. PfHRP III on the other hand has a Mr of 26,739 and unlike PfHRP II it has two distinct regions of tandem repeats. The first coding region that starts at nucleotide 122, has 3 repeats of Ala–His–His, 2 repeats of Ala–His–His–Val–Ala–Asp, 13 repeats of Ala–His–His–Ala–Ala–Asn and a Ala–His–His–Ala–Ala–Asp. This is followed by a 26 amino acid gap with no apparent repeats. The second repeat unit in PfHRP III is different from the HRP II with a sequence of Asp–(Asp/Gly)–Ala–His–His. PfHRP II as well as PfHRP III have a coding region for a hydrophobic leader peptide separated by an intron, from the exon-encoding tandem repeats rich in alanine and histidine (Figure 3). The leader peptide in the two proteins shows 93% homology. Similarities between PfHRP II and III, especially in hexapeptide repeats, indicate towards a common evolutionary origin.

A *P. falciparum* strain, HB3, which lacks the gene for PfHRP III, survives and completes its life cycle[55]. However, the growth of this parasite strain, lacking PfHRP III, is much slower than those containing this protein. A significant role of PfHRP III is indicated from the experiments of parasite cross with parents with and without PfHRP III. All the 15 daughter strains obtained after the cross contained PfHRP III, pointing towards a strong bias during their selection[55]. This finding suggests a prominent role for this protein in parasite metabolism. These proteins highlight the redundancy of metabolic pathways in malarial parasite, both having similar function in heme polymerization process. However, *P. falciparum* could survive and complete the malaria life cycle even in the absence of PfHRP II and III. The *P. falciparum* strain, which lacked both of these HRPs but still formed hemozoin, was found to have a 10.6 kDa protein rich in histidine (PfHRP IV), recognized by peptide antibodies against PfHRPs[52].

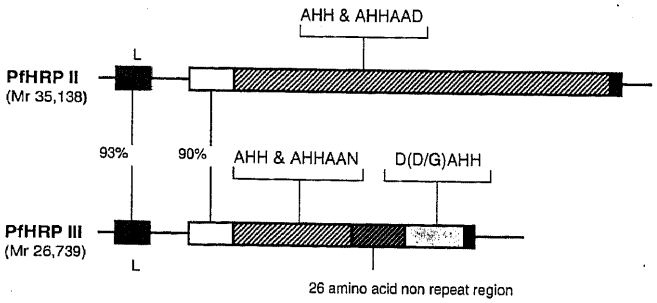

Figure 3. A schematic diagram representing the PfHRP II and PfHRP III gene sequences. In both of these genes, exons coding for hydrophobic leader peptide L and tandem repeats are separated by an intron of 0.15 kb. Major repeats in PfHRP II are AHH and AHHAAD. PfHRP III has two distinct repeat regions, first region with repeats of AHH and AHHAAN is separated by a 26 amino acid nonrepeat region from the second repeat region containing the pentapeptide sequence DD/GAHH. High level of homology is observed between the two genes. Diagram adapted from Wellems *et al.*, *Cell*, 1987, 49, 633–642.

Since almost the entire structure of PfHRP II consists of the repeat units with histidine pairs, we wondered if these structures were the heme-binding sites in this protein. We have recently found that hexapeptide repeats Ala–His–His–Ala–Ala–Asp in PfHRP II are indeed the heme-binding sites in this protein[38]. Heme-binding with synthetic peptides based on this repetitive sequence was proportional to the number of repeat units in the synthetic peptide. However, these peptides as such were unable to initiate heme polymerization, indicating that these sequences alone are not sufficient to polymerize heme. It may be possible that a larger number of repeats (> 3) are necessary to provide the structures that are required for heme polymerization, or it may be that these repeats alone are not sufficient and other structural feature of HRPs may have a role in hemozoin formation. On the other hand, the native as well as recombinant PfHRPs II and III have been shown to promote hemozoin formation (ref. 52, A. V. Pandey and V. S. Chauhan, Manuscript under preparation).

We also found that heme binding with synthetic peptides containing repetitive hexapeptide sequence of PfHRP II could be replaced by chloroquine. In our experiments, chloroquine inhibited the heme–peptide complex formation, and instead a heme–chloroquine complex was formed[38]. These results indicate that the greater affinity of heme for chloroquine, compared to the hexapeptide repeats (heme-binding sites) may lead to inhibition of heme binding to PfHRPs by chloroquine. Inhibition of heme binding to the hexapeptide repeats, which are possibly the nucleating sites for heme polymerization, would disrupt the heme detoxification machinery of malaria parasite. Preincubation of chloroquine with trophozoite extract does not have any effect on polymerization activity, thus direct action of drug on catalytic moiety may be ruled out[1]. Our peptide heme interaction experiments also suggest similar mechanism. We also found that chloroquine does not interact with the repeat peptide or the recombinant histidine-rich protein itself (A. V. Pandey and V. S. Chauhan, Unpublished data).

Inhibition of heme polymerization by quinoline antimalarial drugs occurs at much higher drug concentrations

than present in plasma[1,36–38]. Direct interaction of these drugs with heme, the substrate for hemozoin formation, may be the factor responsible for requirement of high drug concentration for significant inhibition of the process. However, since quinoline drugs accumulate to very high concentration levels inside the parasite digestive vacuole, appropriate doses of drugs required for inhibition of heme polymerization are available at the site of action[26–30]. Recent reports on chloroquine accumulation in malaria parasite suggest that drug-resistant strains of malarial parasite accumulate less chloroquine[56,57] compared to the chloroquine susceptible strains of the parasite. Lowering of the effective chloroquine concentration at the site of action would adversely effect the inhibition of hemozoin formation and might be responsible for parasite tolerance to drug. It would be interesting to characterize the factors responsible for the transport of these drugs to the digestive vacuole. Structural modification of these drug molecules could lead to the differences in the levels of drug accumulation between resistant and susceptible strains and provide new leads for the design of novel drugs. Isolation and characterization of drug transporters from the resistant as well as susceptible strains of malaria parasite may also throw some light over the probable causes of drug resistance.

Mechanism of heme polymerization

One of the most attractive features of this process is that it is found only in malaria parasite. An analysis of the studies on heme polymerization by different research groups suggests that there may be more than one factor responsible for a favourable environment for hemozoin formation in the digestive vacuole of plasmodia. One such factor is the pH of the vacuole which is in the range of 4.7–5.2. At this pH half the propionate side chains of heme are deprotonated. This makes the formation of iron carboxylate bond between propionate side chain of one heme with central ferric ion of another heme. Another factor is the presence of a catalyst that could bind heme, though not very strongly, so as to release the product after polymerization is complete. Increasing number of heme units in the growing polymer, as well as cross linking between chains would lead to the loss of solubility of the polymer in the food vacuole environment. After a certain number of heme units are linked together, the polymer may lose its ability to bind with the catalyst, resulting in chain termination. PfHRP II may be one such catalyst involved in heme polymerization inside the food vacuole. Multiple heme binding sites present in this protein make it a good candidate for catalysing heme polymerization. Several heme molecules attached to different binding sites of this protein may come in close proximity to enable the formation of an iron–carboxylate bond (Figure 4).

In the parasite, hemozoin formation starts at the ring stage and goes on till the trophozoite stage. The process of heme polymerization is linked with the degradation of hemoglobin, as the release of heme after hemoglobin digestion poses immediate demand for its detoxification. In the absence of any catalyst, this reaction is extremely slow and would require more than a week to complete[57]. Since the time period between ring to trophozoite stage is around 24 h for *P. falciparum* and detoxification of heme has to be achieved quickly to avoid membrane damage, requirement of one or more catalyst to speed up the polymerization of heme is absolutely necessary for the parasite. Attempts to stop this process by antimalarial drugs may be either by reaction with the substrate (heme) or interaction with the catalytic site. All the known inhibitors of heme polymerization studied so far tend to inhibit hemozoin formation by direct interaction with heme[58]. All these compounds bind to heme, although, any significant correlation between heme-binding capacity and ability to inhibit hemozoin formation has not been achieved[59]. However, since a very small quantity of heme is sufficient to disrupt the biochemical processes of the parasite, even a slight shift in the heme polymerization process towards free heme would be enough to cause damage. Moreover, complex of heme with antimalarial drugs, though may not be able to fit in the growing hemozoin molecule, could still retain the damaging properties of either heme or the antimalarial drug and would be able to disrupt the biochemical processes of the parasite.

Transport of the heme polymerization catalyst inside the digestive vacuole could be another target for drug design. For example, although PfHRP II has been localized in the digestive vacuole of *P. falciparum*, it

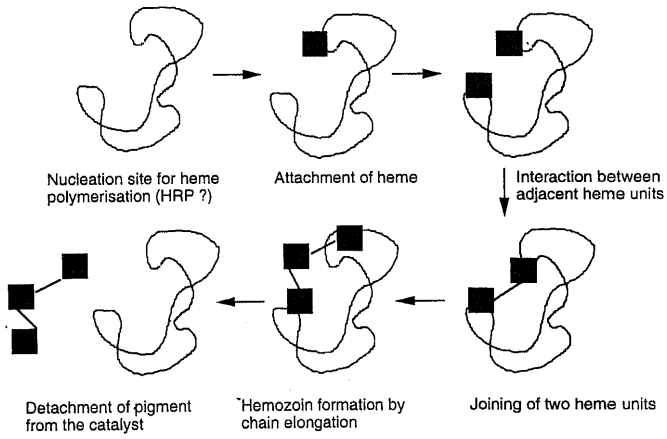

Figure 4. Proposed mechanism of heme polymerization by a catalyst. Several heme molecules attach to different binding sites available on the catalyst. Some of these heme units are in favourable orientation to form dimers through formation of iron–carboxylate bond. These small units are further elongated until the hemozoin molecule detaches from the catalyst due to changes in solubility properties.

is not known how the protein reaches there, as it is synthesized in the parasite cytoplasm. A possible carrier of PfHRP II to the site of heme polymerization may be hemoglobin itself. PfHRP II is thrown out by the malaria parasite to the erythrocyte cytoplasm and plasma, and large amounts of this protein are generally present in the infected erythrocyte. It may be taken inside along with hemoglobin to the digestive vacuole. Another HRP, PfHRP III, that could catalyse the formation of hemozoin *in vitro* is present inside the parasite cytoplasm and is ingested along with hemoglobin during pinocytosis. All these proteins obviously would be able to withstand the action of proteolytic enzymes present inside the digestive vacuole. In the initial report of heme polymerase by Slater and Cerami[36], protease treatment of the trophozoite lysate was found not to have any effect on the heme polymerization.

Another interesting aspect of this pathway is the recent discovery of peptide transport from digestive vacuole to parasite cytoplasm[12]. An attempt to block the movement of the peptides generated after globin digestion might be a useful addition to the host armor in fight against the parasite. Transport of other enzymes involved in hemoglobin digestion in the food vacuole after their biosynthesis in the parasite cytoplasm may also follow similar route. Due to difference in pH of the parasite cytoplasm (~ 7.2) and food vacuole (~ 5.0), these enzymes would be activated only after their entry into the digestive vacuole[26].

Concluding remarks

Design of inhibitors against heme polymerization must take into account the transport of the drug molecules to the site of action. An effective drug will have to cross several membranes to reach the digestive vacuole. Reports on structure–activity relationship of chloroquine analogs suggest that variation in side chain of chloroquine

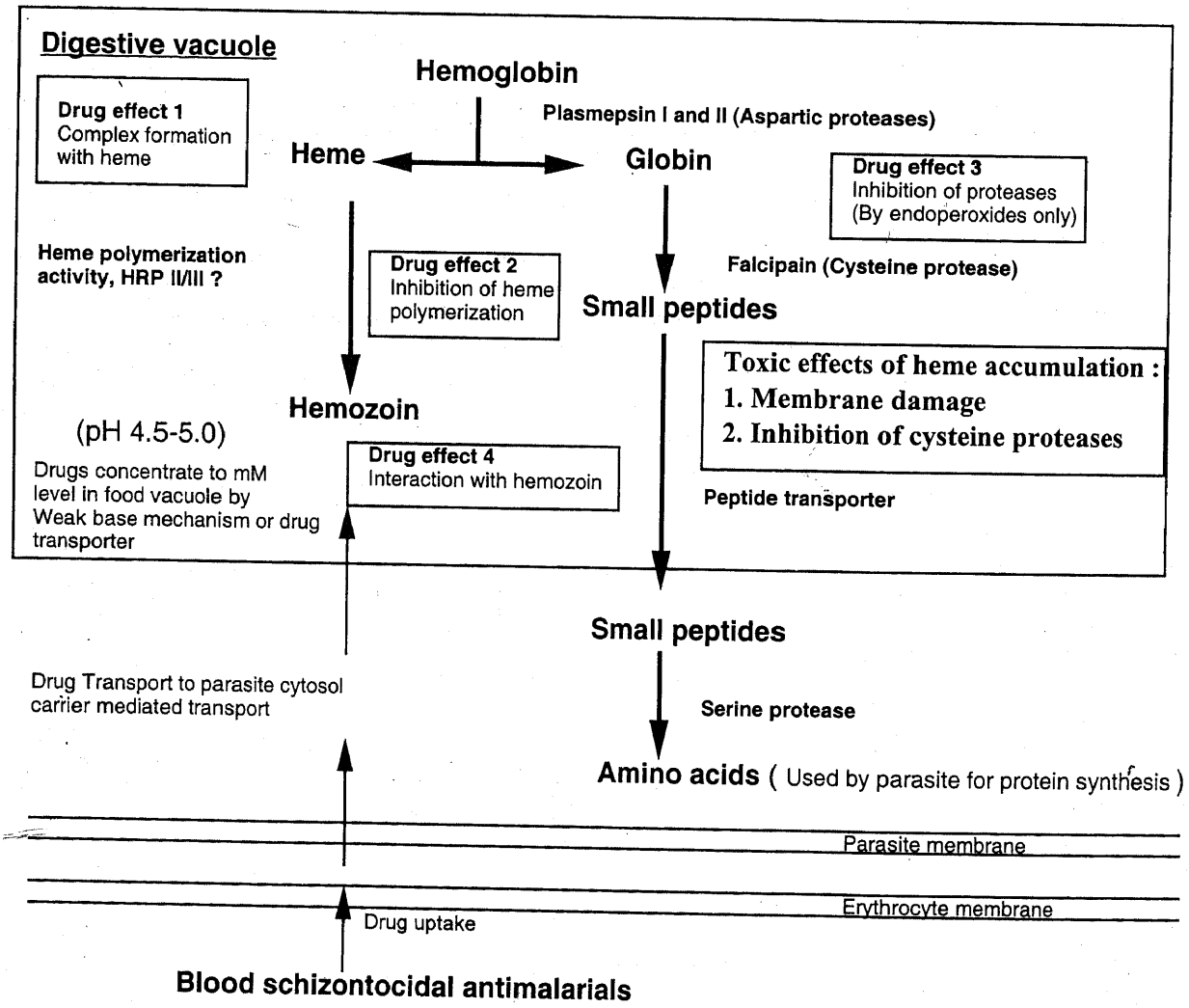

Figure 5. A schematic diagram describing the hemoglobin degradation and heme polymerization processes inside the digestive vacuole of malarial parasite, and possible site of action of antimalarial drugs.

is able to revert the resistance[60,61], suggesting either selective recognition of structures by the drug transporter in malaria parasite or different mechanism of action of some of these analogs. Hence, compounds with structures different from chloroquine, which could inhibit heme polymerization, should be able to overcome the drug resistance mechanism of the malaria parasite. Artemisinin, an endoperoxide antimalarial, with a different structure from chloroquine also binds to heme and accumulates in parasite food vacuole[62]. We have recently found that this drug could inhibit heme polymerization as well as hemoglobin degradation by malaria parasite (A. V. Pandey *et al.*, submitted for publication). Activity of artemisinin against chloroquine resistant strains of malaria parasite seems to supports this hypothesis.

A schematic diagram of hemoglobin degradation and related pathways is given in Figure 5. Detailed structure–activity relationship study of heme polymerization and related metabolic pathways of malaria parasite may allow the development of compounds that could inhibit these targets and bypass the resistance mechanism. A proper understanding of events inside the parasite digestive vacuole would be essential for antimalarial design. Inhibiting the hemoglobin catabolic pathway and related events at multiple sites would be a more promising approach towards chemotherapy of malaria. Currently we are investigating the heme polymerization mediated by HRP, and the proteases involved in the hemoglobin degradation, in order to evaluate their potential as targets for antimalarial drug development. An ideal approach for new antimalarial development would be to inhibit both aspartic and cysteine proteases as well as heme polymerization. This multiple step attack against parasite although may not result in a single wonder drug but a combination of drugs, directed against more than one target, would have less chance of falling quick prey to now well-known drug resistance in malaria[63].

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