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Haemoglobin Degradation and Haem Polymerization as Antimalarial Drug Targets

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To assist in the discovery of new classes of antimalarial compounds, and to thus ensure the long-term future of antimalarial drug development, it is essential to better characterize parasite cell biology at the molecular level. The best antimicrobial drug targets involve cellular processes that are both unique and essential for the organism. The obligate requirement of malarial parasites to reside inside the host's erythrocytes, and their dependence, during this stage of their life cycle, on the ingestion and degradation of haemoglobin represents such a critical process (Olliaro & Goldberg 1995). Proteases involved in haemoglobin degradation, and the sequestration of the toxic haem generated as a result, are the subject of the chemotherapeutic approaches outlined in this article.

The Biology of Haemoglobin Degradation

The degradation of haemoglobin takes place in an acidic lysosomal organelle called the digestive vacuole (Olliaro & Goldberg 1995). Estimates of the amount of haemoglobin degraded during the parasite's occupancy of the host erythrocyte has varied from 25 to 80% (Ball et al 1948; Groman 1951; Roth et al 1986). The reasons for this high level of metabolic activity are probably two-fold. Firstly, the amino acids released from haemoglobin are a potential source of nutrients. This is supported by evidence that parasite growth in culture requires supplementation with amino acids (methionine, cysteine, isoleucine, glutamine and glutamate) that are absent or of low abundance in haemoglobin (Divo et al 1985; Francis et al 1994). Secondly, the parasite requires space to grow and develop in the erythrocyte and may need to degrade haemoglobin to achieve this. This is supported by evidence that not all the products of haemoglobin degradation are required by the parasite (Vander Jagt et al 1992). Reconstitution of erythrocytes containing as little as 6% of the original haemoglobin can support parasite growth (Rangachari et al 1987) and a large proportion of the amino acids generated by haemoglobin degradation are secreted from the parasite (Zarchin et al 1986).

Several types of protease are involved in haemoglobin degradation. Using specific inhibitors to study haemoglobin degradation in-vitro, aspartic protease, cysteine protease, serine protease and metalloprotease activities have all been detected in digestive vacuole extracts (Goldberg et al 1990). However, only two aspartic proteinases (plasmepsins I and II) and one cysteine proteinase (falcipain) have been characterized at the molecular level. These are discussed in detail later.

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Degradation of haemoglobin releases ferrous (iron II) haem. This powerful reducing agent is a generator of free radicals, which are thought to be inactivated in the food vacuole by peroxidases and superoxide dismutases (Olliaro & Goldberg 1995). In the presence of peroxide antimalarial drugs, such as artemisinin, free radical derivatives and other reactive derivatives of the drugs are generated (Haynes & Vonwiller 1996; Posner 1997). These cannot be inactivated and are lethal for the malarial parasite, probably due to alkylation of proteins, lipids and other cellular components (Meshnick et al 1996).

The ferric (iron III) haematin resulting from the oxidation of ferrous (iron II) haem remains toxic for the parasite, as it is membrane interactive and potentially lytic (Fitch et al 1982, 1983). In many organisms, haem moieties are degraded by a haem oxygenase system (Schacter 1988), but such a process operating in the food vacuole would probably result in greater oxidative stress for the parasite. Instead, the monomeric iron III haematin is detoxified by polymerization into a form of polymeric β-haematin called haemozoin, or malaria pigment (Slater et al 1991; Bohle et al 1994). It is believed that inhibition of haem polymerization is lethal for the parasite.

A schematic representation of the process of haemoglobin degradation operating in the digestive vacuole is shown in Fig. 1. The potential of haem polymerization, and the proteinases involved in haemoglobin degradation, as targets for chemotherapy are explored in more detail in the following sections.

Inhibition of Haem Polymerization

Haem polymerization was originally thought to be an enzyme-mediated process (Slater & Cerami 1992). However, it was later demonstrated that the original activity observed was protein independent and that the polymerization process was essentially physicochemical in nature (Dorn et al 1995). More recently it has been postulated that histidine-rich proteins may form a structural focus for binding haem moieties and initiating haem polymerization in the parasite (Sullivan et al 1996). For a more detailed discussion see Ridley (1996).

Haem polymerization is inhibited by chloroquine in-vitro and probably represents the target for this drug in the parasite (Slater & Cerami 1992; Dorn et al 1995; Ridley 1996). As the process is protein independent, it was proposed that chloroquine inhibition is mediated by binding to monomeric haematin, preventing its incorporation into the growing β-haematin chain (Egan et al 1994; Dorn et al 1995). This reconciles chloroquine inhibition of haem polymerization with earlier work suggesting that haematin, or ferriprotoporphyrin IX, is the chloroquine receptor in malarial parasites (Chou et al 1980). Using isothermal titration microcalorimetry measurements it has also been demonstrated that the strength of

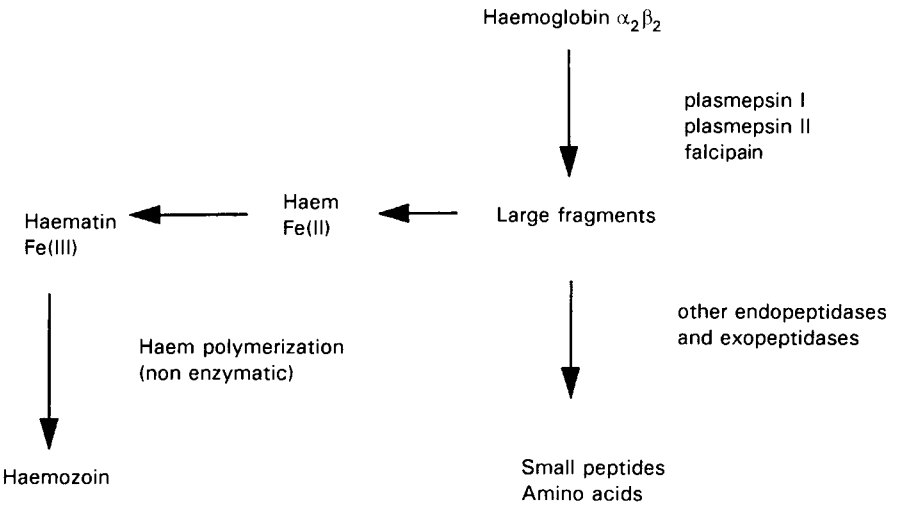


FIG. 1. Overview of haemoglobin degradation in the acidic digestive vacuole of malaria parasites.

binding of quinoline antimalarials to haematin correlates with their ability to inhibit haem polymerization in-vitro (Dorn et al unpublished). Another consequence of the finding that haem polymerization is a physicochemical process, and that its inhibition by chloroquine does not involve interaction with parasite proteins, is that chloroquine resistance develops as a result of altered drug transport and altered drug accumulation within the parasite (Ward et al 1995). The ultimate goal of work on haem polymerization is to discover new classes of molecules that both inhibit the process of haem polymerization, and are not affected by the mutations affecting intraparasitic drug accumulation. A high throughput haem polymerization assay has been developed to screen chemical libraries in order to discover molecules that meet these criteria.

In addition to the screening approach, it was recently discovered that 4-aminoquinolines with shortened side chains, though similar in structure to chloroquine, can nevertheless overcome chloroquine resistance (Ridley et al 1996). Some examples of such compounds are shown in Table 1. It is surprising that compounds so closely related in structure to chloroquine manage to overcome chloroquine resistance to such a large extent. It suggests that whatever mechanisms are contributing to chloroquine resistance they are extremely structure-specific. Although promising, several aspects of these compounds require further optimization. Firstly, they retain some cross-resistance with chloroquine, i.e. they are less active against highly resistant chloroquine strains. Secondly, the desalkyl metabolites, which in the case of chloroquine contribute significantly to overall activity, are totally inactive against chloroquine resistant strains. Thirdly, the compounds retain similar toxic liabilities to chloroquine and this needs to be rigorously assessed.

A second class of quinoline compounds, the bisquinolines, was also shown to be active against chloroquine resistant strains (Vennerstrom et al 1992). These compounds have been investigated in some detail and the (*S*,*S*)-enantiomer of *trans*-N^1,N^2-bis(7-chloroquinolin-4-yl)cyclohexane-1,2-diamine, Ro 47-7737, was found to be particularly active against both chloroquine sensitive and chloroquine resistant strains (Fig. 2, Ridley et al 1997). The long half-life of this compound resulted in exceptionally good curative and prophylactic activities in animal models, with a low propensity for recrudescence. Unfortunately, some toxic liabilities, especially phototoxicity, with an attendant danger of photocarcinogenicity, prevented its consideration for clinical trials.

In summary, quinoline-containing compounds still offer hope for the development of new antimalarials. However, minimalisation of toxic side-effects is essential. It should be remembered that chloroquine itself has a very low therapeutic ratio (Good & Shader 1982; Kelly et al 1990) and might have proven difficult to register in today's regulatory environment.

Inhibition of proteinases involved in haemoglobin degradation

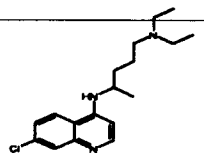
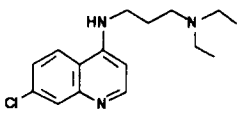
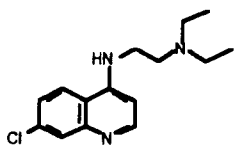
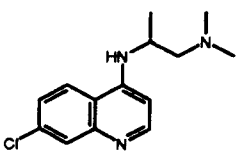
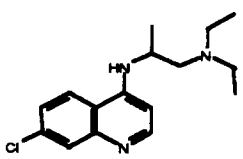
The three proteinases studied in most detail are a cysteine proteinase, falcipain, and two aspartic proteinases, plasmepsins I and II. Because of the difficulty of working with the small quantities of enzyme available from parasite culture, the genes encoding the proteinases have been cloned and attempts made to prepare recombinant enzyme. In addition, proteinase inhibitors from a variety of sources have been tested for their ability to inhibit parasite growth.

The cysteine proteinase, falcipain

A cysteine proteinase activity has been identified in food vacuoles (Goldberg et al 1990; Gluzman et al 1994) and generic inhibitors of cysteine proteinases have antiparasitic effects (Rosenthal et al 1988, 1989, 1991, 1993). It is postulated that these inhibitors act at an early step in the haemoglobin degradation pathway (de Gamboa & Rosenthal 1996). A cysteine proteinase gene, encoding the enzyme falcipain, has been cloned from most of the human malarial species (Rosenthal 1996) and it is assumed that this enzyme is responsible for the activity seen in the food vacuole extracts. However, this is not yet proven, as the food vacuole proteinase has not been purified to homogeneity for detailed molecular characterization.

There is a report that active recombinant enzyme has been successfully expressed (Salas et al 1995), but most enzyme

Table 1. Inhibition of *P. falciparum* growth in culture by selected 4-aminoquinolines with shortened side chains.

Compound	Chain length		IC50 (nM)	
			NF54	K1
Chloroquine	n = 4		16 ± 4	315 ± 82
Ro 47-0543	n = 3		18 ± 5	59 ± 15
Ro 41-3118	n = 2		24 ± 6	49 ± 14
Ro 47-9396	n = 2		22 ± 5	50 ± 15
Ro 48-0346	n = 2		25 ± 6	61 ± 20

IC50 values are the means of a minimum of 22 independent experiments and are presented with s.d.s. The K1 strain is resistant to chloroquine and the NF54 strain is sensitive to chloroquine. Adapted from Ridley et al

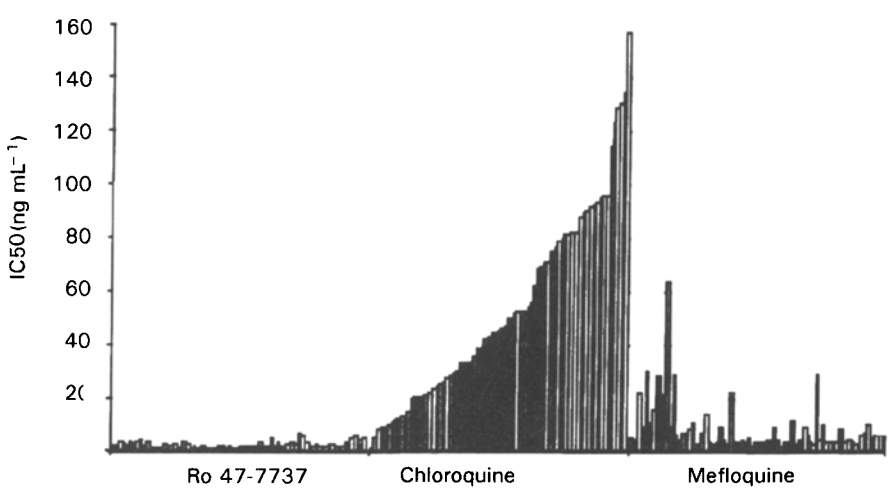


FIG. 2. Activity of the bisquinoline Ro 47-7737, compared with chloroquine and mefloquine, against over 70 strains of *P. falciparum* malaria grown in culture. Growth was monitored over a 72-h period.

inhibitor studies rely so far on an assay involving hydrolysis of a fluorogenic substrate using malarial cell extracts (Rosenthal et al 1989). The homology of the enzyme's primary amino acid sequence to cysteine proteinases of known structure has allowed molecular models to be developed, and these models have been used to design inhibitors (Ring et al 1993). Based on modelling, it was also claimed that some chalcones with antimalarial activity worked through inhibition of cysteine

proteinases, but no enzyme inhibitory data was given to confirm this (Li et al 1995). Peptidic vinylsulphones are a novel class of cysteine proteinase inhibitor shown to inhibit falcipain activity and to inhibit *P. falciparum* in culture (Rosenthal et al 1996). It was claimed that these compounds were non-toxic following oral application daily for four weeks at 30 mg kg^{-1}, but no in-vivo efficacy data was shown to demonstrate how this value compares with therapeutic doses and no pharmacokinetic evidence was given that the peptidic compounds were absorbed. The relevance of this claim is therefore unclear.

In summary, classical cysteine proteinase inhibitors inhibit parasite growth in culture, confirming falcipain as a target. Several new classes of inhibitors show activity in culture, but no specificity against human enzymes has yet been demonstrated for these compounds and no in-vivo activity has been reported.

The aspartic proteinases, plasmepsins I and II

The first aspartic proteinase to be definitively localised to the digestive vacuole of *P. falciparum* was plasmepsin I (Goldberg et al 1991). It was postulated that this enzyme initiated the process of haemoglobin degradation by cleaving the Phe_{33}-Leu_{34} bond of the α-chain of haemoglobin. The enzyme was purified, the N-terminal sequence obtained and the gene cloned (Francis et al 1994). It initially proved difficult to express functional recombinant enzyme (Luker et al 1996) and this has only recently been achieved (Moon et al 1997).

More rapid progress at the molecular level was made with a second aspartic proteinase, plasmepsin II. The gene encoding this enzyme was first identified as part of a genomic sequencing project (Dame et al 1994) and was later successfully expressed in an active form (Hill et al 1994). In the meantime it was confirmed that this proteinase was also present in food vacuoles and that it had a substrate preference for denatured haemoglobin over native haemoglobin (Gluzman et al 1994). The recombinant enzyme has been crystallised and an X-ray structure determined (Silva et al 1996).

It is unclear why there should be two aspartic proteinases in the food vacuole. However, the availability of recombinant plasmepsin I (Moon et al 1997) now allows the specificity of the two enzymes to be assessed in detail. The distinct kinetic parameters obtained for plasmepsin I and plasmepsin II using peptide substrates, and their different susceptibility to some inhibitors, suggests that the enzymes may play different roles and have different specificities. This is reinforced by the fact that the gene encoding plasmepsin I is expressed in the ring stages, whereas the gene encoding plasmepsin II is expressed in the trophozoite stages (Moon et al unpublished).

Aspartic proteinases are a proven class of targets for cardiovascular diseases (renin) and for HIV infections (HIV protease) and there is a lot of information available on enzyme structure, mechanism and inhibition (Fusek & Vetvicka 1995). It was, therefore, possible to build up a structural model of *P. falciparum* plasmepsin I, based on gene sequence information, in the absence of crystals (Fig. 3). This advance information, coupled with the libraries of aspartic protease inhibitors already available, has assisted in an early search for inhibitors of plasmepsins.

A limited number of studies using plasmepsin inhibitors have been reported. An inhibitor of plasmepsin I inhibited *P. falciparum* growth with an IC50 of around 2 to 5 μM (Francis et al 1994) and parasite growth was inhibited at the trophozoite stage, as expected. Compounds of proven specificity for plasmepsin I have also been identified that inhibit *P. falciparum* growth at sub-micromolar levels (Moon et al 1997). A highly specific inhibitor of plasmepsin I with a K_i of 0·01 nM (compared to 1500 nM for plasmepsin II) has been reported (Luker et al 1996) but no data on the ability of this compound to inhibit parasite growth in culture was given.

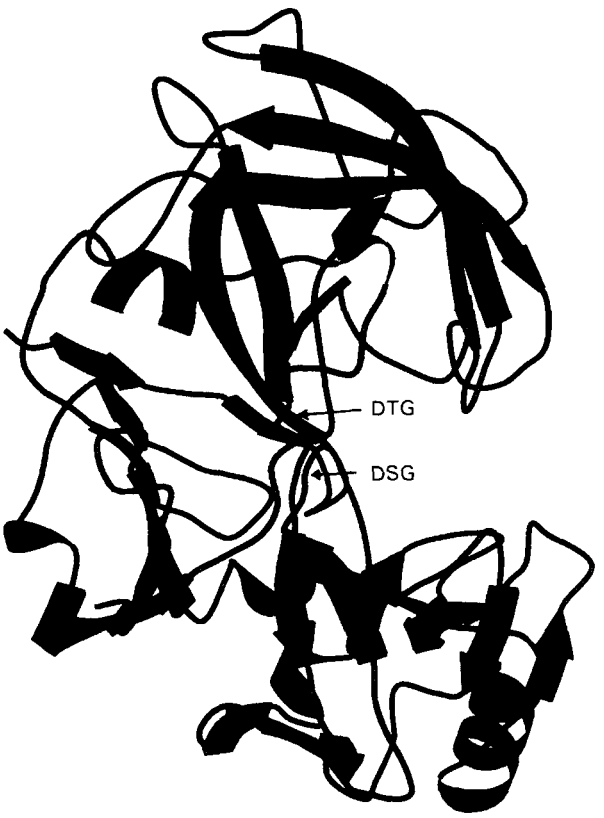


FIG. 3. Computer model of plasmepsin I, based on crystal structures of homologous aspartic proteinases. The two active-site aspartic acid (D) residues are indicated as part of their characteristic triplet (DTG and DSG) amino acid residue motifs. (This model was kindly provided by D. Bur).

With respect to plasmepsin II, an initial investigation of a limited number of inhibitors showed no striking specificities (Hill et al 1994). A compound with a 40-fold specificity of inhibition over cathepsin D ($K_i = 0{\cdot}55$ nM) has been reported, but only minimal inhibition was observed against malarial parasites in culture at a concentration of 20 μM (Silva et al 1996).

In summary, the data available indicate that both plasmepsin I and plasmepsin II are valid drug targets, as inhibitors of these enzymes inhibit parasite growth in culture. Specificity against some human enzymes has been demonstrated but, like the falcipain studies, no compounds have yet been reported with in-vivo activity.

Conclusions

Inhibition of haem polymerization and the proteinases involved in haemoglobin degradation are valid approaches for the discovery of new antimalarial drugs. The challenge in the

field of haem polymerization is to discover new classes of inhibitor that lack a quinoline moiety in the hope that these will both better overcome chloroquine resistance and that they will not suffer the toxic liabilities of quinolines. The challenge in the proteinase field is to convert the proteinase inhibitor activity observed in culture into an in-vivo activity in animal models and to demonstrate specificity against human enzymes. It may be that this is easier to control for the aspartic proteinase inhibitors, as the number of human aspartic proteinases is limited (Fusek & Vetvicka 1995). The availability of recombinant enzyme for high-throughput assays and crystallization studies will greatly assist these efforts.

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