J Indian Inst Sci, Jan–Feb 2000, **80**, 17–23

Novel targets for antimalarial drug development*

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Abstract

Heme as well as protein biosynthetic pathways of malaria parasite *Plasmodium falciparum* have been identified by us as crucial for the survival of the parasite Intervention of either of the two pathways results in the death of the parasite, basically due to the pivotal role played by heme in these pathways

Keywords Protein synthesis chloroquine, heme, *Plasmodium falciparum*

1 Introduction

Human malaria is caused by one of the four species of *Plasmodium, P falciparum, P vivax, P malariae* or *P ovale* While vivax malaria is most common in tropical regions with typical symptoms of recurrent fever and shivering, falciparum malaria is the most fatal as it causes cerebral malaria Cerebral malaria is due to the adherance of the parasite to the endothelium of the capillaries and the venules of the brain and other organs resulting in anoxia Due to the resistance of *P falciparum* to antimalarial chloroquine and the mosquitoes to insecticides, the development of more effective newer drugs is urgently needed For developing newer drugs it is essential to understand the biology and biochemistry of the parasite with focus on metabolic pathways of the parasite which was neglected over the years as all attention was focused on vaccine development only Keeping this in mind, we have identified two biochemical pathways which can be used as targets for drug development The pathways are (i) protein synthesis and (ii) heme biosynthesis

1 1 *Life cycle of the parasite*

Infection commences with the bite of an infected mosquito to a healthy human As a result, sporozoites are released into circulation, which invade the hepatocytes of the liver Within 10–15 days, after multiple asexual schizogony, thousands of merozoites are released from the hepatocytes and invade the host RBC, where they undergo second asexual schizogony The intraerythrocytic stages of the parasite are rings, trophozoites, schizonts and merozoites Some of the parasites during their erythrocytic schizogony develop into male and female gametocytes These gametocytes are taken up by the feeding mosquito, followed by zygote formation in the midgut of this vector The subsequent formation of sporozoites in the mosquito completes the cycle

*Text of lecture delivered at the Annual Faculty meeting of the Jawaharlal Nehru Centre for Advanced Scientific Research at Bangalore on October 21, 1999

2 Food vacuole and digestion of host hemoglobin

During the intraerythrocytic cycle, the host-cell cytoplasm is consumed and an estimated 80% of the hemoglobin is degraded [1] The food vacuole of *Plasmodium* is a very unique organelle and is considered the metabolic headquarter of the parasite It is equivalent to the lysosome of higher organisms with a pH of 4 5–4 9 and contains many hydrolytic enzymes Two major activities, hemoglobin degradation and hemozoin formation, take place in the food vacuole

2 1 *Hemoglobin degradation*

Two aspartic proteases, plasmepsins I and II, and one cysteine protease, falcipain are responsible for hemoglobin degradation [2] Aspartic protease makes a single cleavage in the α chain of host hemoglobin between Phe 33 and Leu 34 residues These residues lie in the hinge region of hemoglobin and are critical for the maintenance of the integrity of the structure of hemoglobin None of the hundreds of the characterised hemoglobin variants have a homozygous defect in this region[3], thus providing evidence to the fact that the parasite has evolved a smart strategy to evade the host's immune system After initial cleavage, globin is further degraded by a cysteine protease into amino acids which are utilized by the parasite for its protein synthesis

2 2 *Hemozoin formation*

The heme generated from hemoglobin degradation gets converted into a nontoxic moiety—the malaria pigment, hemozoin The conversion of heme to hemozoin according to Slater and Cerami[4] is brought about by a specific enzyme, the heme polymerase, but others like Dorn *et al* [5] consider it a nonenzymatic process

2 3 *Mechanism of chloroquine (CQ) action*

Although the antimalarial chloroquine has been in use for almost 200 years, the mechanism of its action is still a topic of debate Some of the proposed mechanisms are (1) Interaction with DNA According to this theory, CQ intercalates with DNA and inhibits its synthesis [6] This theory is not acceptable as it considers millimolar concentrations of drug to be sufficient for the purpose, while micromolar quantities of CQ are required for therapeutic effect (2) Increase in vacuolar pH This theory proposes an accumulation of CQ in the food vacuole Due to its weak base property, CQ raises the pH of the food vacuole, leading to the death of the parasite However, it has been suggested that alkalinization of the vesicles *per se* is not adequate to explain the toxicity of chloroquine [7 8] (3) Inhibition of heme polymerase causing accumulation of free heme which is toxic to the parasite [4] The more recent theories consider, (4) CQ forming a complex with heme which caps the growing heme-polymerizing moieties, thus inhibiting hemozoin formation[9] and (5) Inhibition of protein synthesis[10] by the drug

3. Protein synthesis and mechanism of chloroquine action

We observed that chloroquine at therapeutic doses of 1–3 μM inhibits parasite protein synthesis A cell-free translation system was standardized in order to study translation initiation in detail It was also noted that 10–15 μM heme stimulates protein synthesis by three fold in the parasite lysate and chloroquine inhibits this process (Fig 1) A 50–60% inhibition of total

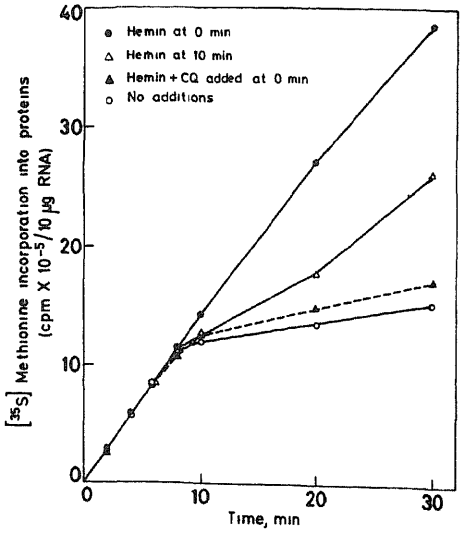

Fig 1 Inhibition of heme-dependent protein synthesis by chloroquine in rabbit reticulocyte lysate Protein synthesis was assayed as described in the text The reaction mixures (25 µl), which contained the following, were incubated at 30°C and processed 20 µM hemin added at 0 min (•), 20 µM hemin added at 10 min (Δ), 20 µM hemin plus 10 µM chlorquine added at 0 min (Δ), no additions (O) (after Surolia and Padmanaban[10])

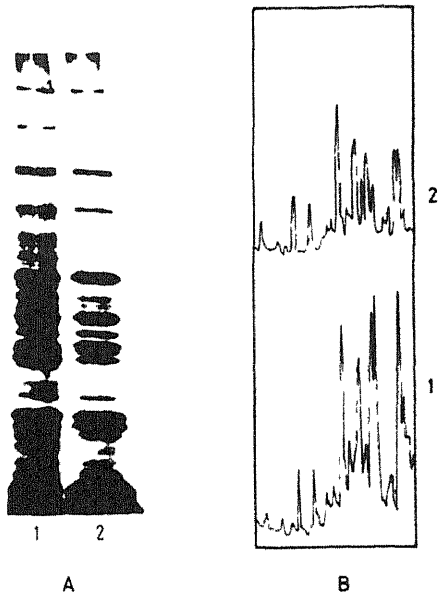

Fig 2 Effect of chloroquine *in situ* on [^{35}S] methionine incorporation into total proteins of the parasite The incorporation of [^{35}S] methionine into parasite proteins was determined by analyzing samples from control and chloroquine-treated cultures on Na DodSO$_4$/7 5% PAGE followed by fluorography (A) Autoradiogram of parasite proteins obtained from normal (lane 1) and chloroquine treated (lane 2) cultures Molecular mass is indicated in kDa (B) Densitometric scan of lanes 1 and 2 is shown in A The autoradiogram was scanned with LKB 22002 ultra scan laser densitometer (after Surolia and Padmanaban[10])

parasite protein synthesis takes place in the presence of 3μM concentrations of CQ (Fig 2) When the cultures were incubated *in situ* with CQ and the proteins of the cell-free system were ^{32}P labeled for phosphorylation, parasite initiation factor 2α (pIF-2α) gets highly phosphorylated when compared to other proteins and also the pIF-2α kinase gets autophosphorylated It was further confirmed that the parasite kinase as well as IF-2α were of parasite origin The parasite IF-2α could be quantitatively immunoprecipitated by the eukaryotic homologue (Fig 3) Based on these results we proposed that CQ sequesters heme from the system using enhanced autophosphorylation of pIF-2α kinase and pIF-2α, which in turn results in inhibition of protein synthesis culminating in the death of the parasite

We propose this as a primary and early major event Other proposed mechanisms make it imperative for CQ to reach the food vacuole, the site of hemoglobin degradation to act More importantly, for other proposed mechanisms, the effects were observed only after 24 h of incu-

bation with chloroquine, before the effect is observed In contrast, the inhibition of protein synthesis by CQ is attained within 10–15 min of incubation with the drug

4 Electron microscope autoradiography

To prove that CQ is available to the ribosomes in the parasite cytoplasm, the site of protein synthesis, electron microscope autoradiography was carried out with [^{3}H] CQ Labelled CQ was incubated with parasites for various time intervals after which the cells were prepared for electron micrography Figure 4 shows the presence of [^{3}H] CQ granules in the cytoplasm of the parasite but not in the food vacuole at that point of time proving that the protein synthesizing machinery is one of the primary targets of chloroquine

These results firmly indicate that the parasite protein synthesis is heme dependent and chloroquine inhibits the heme-dependent protein synthesis The initiation factor 2α plays an important role in regulating parasite protein synthesis The parasite lysate from cultures treated with therapeutic concentrations of chloroquine *in situ* for a very short period (30 min) results in enhanced phosphorylation of IF-2α under conditions of cell-free protein synthesis, which is inhibited by the addition of hemin to the lysate The addition of hemin, phosphorylated com-

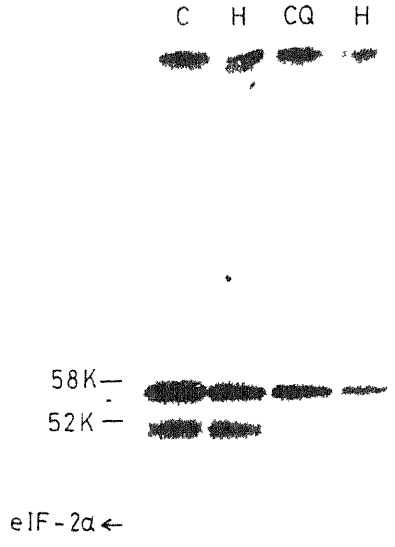

Fig 4 [^{3}H] CQ uptake by *Plasmodium* FV-food vacuole C cytoplasm

Fig 3 Effect of chloroquine treatment *in situ* on parasite eIF 2 phosphorylation in parasite lysate Parasite lysates (S-30) were prepared from control or chloroquine-treated cultures Lane 1, control lysate (no addition) lane 2 control lysate (15 μM hemin) lane 3 lysate from chloroquine-treated cultures (no addition), lane 4 lysate from chloroquine-treated cultures (15 μM hemin) Blanks run with parasite lysate from control and chloroquine-treated cultures with nonimmune serum did not reveal any phosphorylated band corresponding to eIF-2α Molecular mass is indicated in kDa (after Surolia and Padmanaban[10])

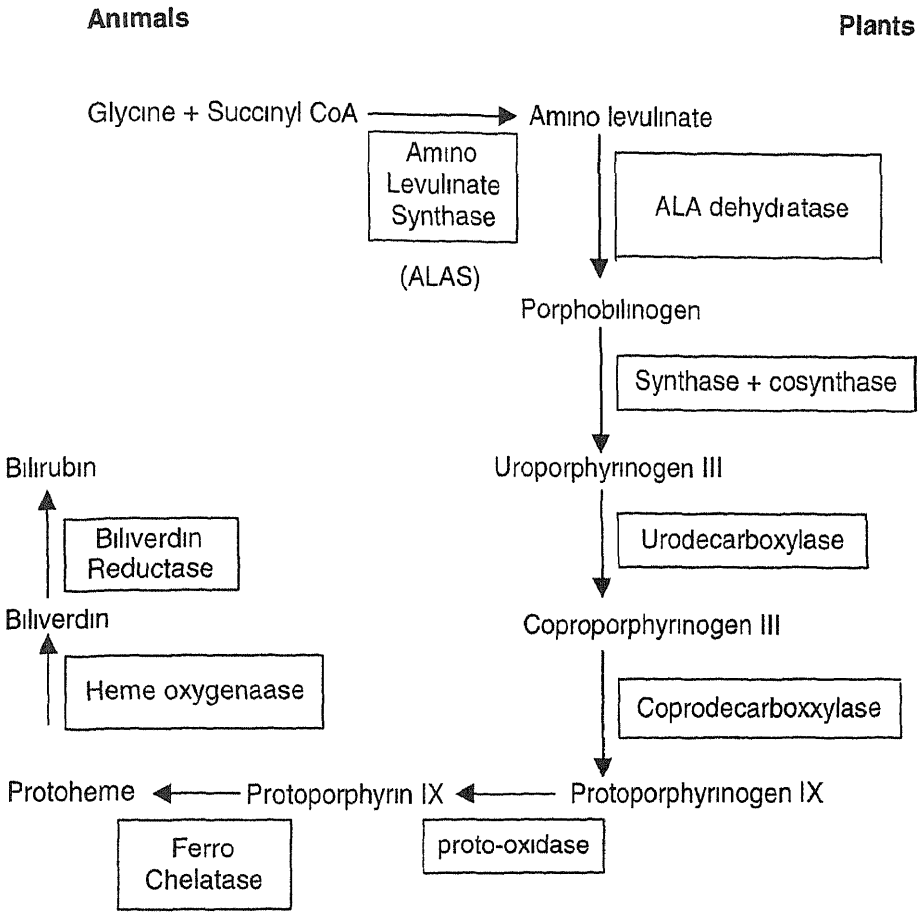

Fig 5 General scheme for heme biosynthetic pathway

pounds and IF-2 counteract the inhibited protein synthesis in the parasite lysate prepared from the *in-situ* chloroquine-treated cultures The studies also demonstrate the presence of pIF-2α kinase in parasite lysate The presence of [^{3}H] chloroquine granules in the cytoplasm of the parasite within 20 minutes of incubation of labeled chloroquine with parasite cultures proves our hypothesis that inhibition of protein synthesis by chloroquine is a major early event culminating into the death of the parasite The enhanced autophosphorylation of pIF-2α kinase and pIF-2α appears to result into this inhibition of protein synthesis

5 Heme biosynthesis by *Plasmodium*

From the protein biosynthesis data it is clear that heme is required for basal parasite protein synthesis The question is whether the parasite is capable of synthesizing its own heme Animals utilize glycine, whereas for plants, glutamate is a precursor for heme synthesis The parasite was found to utilize glycine and not glutamate as precursor for heme Figure 5 shows

Table I
de-novo* synthesis of heme by the human malarial parasite *Plasmodium falciparum

Treatment	[4 ^{14}C]ALA incorporation		[2-^{14}C] Glycine incorporation
	Unutilized ALA	Heme	ALA
			(cpm/7 7 × 10^5 uninfected or parasitized cells)
Uninfected culture	552	264	24
" + SA	1336	76	49
Parasitized culture	248	7470	2090
" + SA	2432	348	4912

Parasite cultures (trophozoites, 10% parasitemia) were treated with SA for 4 h before incubating with [4-^{14}C] ALA (10μCi/l 25 ml) or [2-^{14}C] glycine (25 μCi/l 25 ml) for 12 h Radioactivity incorporation into heme, ALA and that remaining unutilized as ALA were assessed as described in text SA-succinyl acetone (after Surolia and Padmanaban[12])

the general scheme for heme synthesis The enzymes ALA synthase and ALA dehydratase, though present in very small amounts, were detected in the parasite (Table I) Further proof of biosynthesis of heme in the parasite was obtained by using succinyl acetone, a very specific inhibitor of ALA dehydratase Parasite heme synthesis was completely inhibited when succinyl acetone was used (Table II) Succinyl acetone, when injected in infected mice, was able to prolong their life span, thus proving that parasites do have a *de-novo* heme biosynthetic pathway Subsequently, ALA synthase was cloned[11] proving further the ability of the parasite to synthesize heme

6 Inhibitors of aspartic proteases

The crystal structure of *Plasmodium* aspartic protease is known Based on the structural information available for a typical inhibitor of aspartic protease[3], small peptides were designed and synthesized with the help of molecular modeling These peptides were tested *in vitro* for their efficacy One peptide was found to have a very low IC-50

Thus, it is evident from these studies that despite the accumulation of large quantities of heme derived from red cell hemoglobin, the parasite synthesizes heme *de novo* ALA dehy-

Table II
Detection of ALA synthase and ALA dehydrase activities in *P falciparum*

Sample	ALA synthase (ALA)	ALA dehydrase (PBG)
	(nmol/l 04 × 10^7 parasitized cells)	
Red-cell supernatant	ND	6 10
Parasite pellet	0 15	0 20

ND Not detectable The activities in the red cell supernatant were determined after saponin lysis and isolation of parasite pellet (trophozoite stage, 10% parasitemia) The enzyme activities were assayed in the parasite pellet and the red cell supernatant The activity in the red cell supernatant is calculated on the basis that only 10% of the red blood cells were parasitized (after Surolia and Padmanaban[12])

dratase is an attractive chemotherapeutic target, as inhibition of this enzyme by its specific inhibitor succinyl acetone inhibits parasite growth [12] More importantly, succinyl acetone is known not to bring about significant change in overall heme content of the host The design and development of more potent hemoglobinase inhibitors may be useful for a new class of antimalarial compounds

7 Conclusions

The results obtained from our studies on the mechanism of chloroquine (CQ) action, uptake of [^{3}H] chloroquine by the parasite, *de-novo* biosynthesis of heme by the parasite and on hemoglobinase inhibitors clearly indicate that heme is a key regulatory molecule needed in vital metabolic pathways A therapeutic intervention in any of these pathways involving heme would be deleterious to the parasite These studies also indicate that various enzymes/proteins involved in these two pathways could be further explored as potential targets for the development of novel and more effective antimalarials

Acknowledgements

The research was supported in part by grants from the Department of Science and Technology (DST) and Council of Scientific and Industrial Research (CSIR), Government of India, and Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore

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