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New Targets for Antimalarial Drug Discovery

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The emergence and spread of parasite resistance to in-use antimalarials urges for novel compounds to be discovered and developed. Current research focusses on targets of the growing and dividing parasite (ring, trophozoite, schizont) in the red blood cell (RBC). Those processes are metabolically intense and contain essential targets for parasite survival. Mature intra-erythrocytic stages are also responsible for the severe manifestation of falciparum malaria. *Plasmodium falciparum* is the main focus because it is the most common of the four *Plasmodium* species which commonly infect humans, causes a potentially life-threatening infection, and is also the species which shows the highest levels of resistance to various antimalarials.

Research Priorities for Antimalarial Drug Discovery

The Steering Committee on Drugs for Malaria (CHEMAL) of the World Health Organization's (WHO) Special Programme for Research and Training in Tropical Diseases (TDR) is committed to a concerted, systematic effort for antimalarial drug development involving: identification of chemotherapeutic targets; development of methods to expedite experimentation (gene cloning, protein expression, high throughput assay development, determination of specificity); identification of leads, optimization of inhibitors; and the subsequent preclinical and clinical development work. This is being approached by combining efforts of university and government researchers in the discovery area and then developing potential drugs through contracts and industrial partners. Special emphasis is being put on establishing Research & Development (R & D) capability in the developing world. Target generation and validation is one of CHEMAL's top priorities. Emphasis is being put on validating selected targets, and on obtaining and testing compounds for specific inhibition of the target and for activity against the whole parasite. There is a recognized need for more information on the basic metabolic and biochemical processes of the malaria parasite in order to identify the targets for the future. CHEMAL has focussed on targets generation and validation and is now concentrating on those that have been validated, but has also come to recognize that not many of those investigated will ever become validated targets and will generate effective and safe compounds. Hence the need for the discovery and characterization of unique drug targets with specific consideration for the targets which differentiate host and parasite enzymes, as well as for the molecular modelling of host and parasite enzymes. The putative target must be an essential feature of the parasite life-cycle, be parasite-specific (e.g., targets in the digestive vacuole: proteinases, haem polymerization; mitochondrion/plastid) and/or show differential sensitivity from any analogous process in the host (e.g., dihydrofolate reductase, signal transduction). It is also important that some known specific inhibitors exist as a starting point for synthesis (e.g., dihydrofolate reductase, proteinase inhibitors).

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Status of New Targets for Antimalarial Drug Development

Through a combination of research in academic centres, governmental research organizations and drug discovery efforts in pharmaceutical companies, a number of new potential target pathways have been identified and these are summarized in the Table 1 and below. Efforts to develop lead compounds for these putative targets are a priority of CHEMAL.

The Plasmodium digestive vacuole

The digestive vacuole (an acidic lysosome-like organelle) contains key processes including haemoglobin metabolism and detoxification and also drug accumulation, which represent potential chemotherapeutic targets (Olliaro & Goldberg 1995). Haemoglobin is ingested by the malaria parasite and processed in the vacuole, where it is digested, and haem, the eventual by-product, is detoxified (Goldberg et al 1990).

Haemoglobin digestion: proteinases inhibitors. To date, three enzymes have been shown to account for the majority of haemoglobin degradation: two aspartic proteases (aspartic haemoglobinase I and II, or plasmodium aspartic proteinase I and II, systematically renamed Plasmepsin I and II by the IUB Nomenclature Committee) have been isolated (Gluzman et al 1994) and the respective genes cloned and sequenced (Dame et al 1994; Francis at al 1994); and one cystein protease (falcipain) (Rosenthal et al 1988, 1991; Rosenthal & Nelson 1992). The aspartic and cystein proteinases are analogous to human cathepsin D and L, respectively. Proteinase inhibition is identified as a priority project for multiple reasons, although the demonstration of the ultimate therapeutic potential of drugs targeted against malaria proteinases remains to be determined. The genes are cloned and sequenced and recombinant proteins expressed in active form and automated assays for proteinase inhibition are available also for homologous human enzymes. Significant differences in amino acid sequence from the corresponding host enzymes and preliminary data with inhibitors substantiate the validity of proteinases as targets. There is potential for piggy-backing on compound libraries from other indications: proteinases are key enzymes in a variety of infectious and non-infectious diseases ranging from HIV to hypertension and arthritis, and inhibitors are being actively sought and developed (Olliaro et al 1996).

Table 1. Potential target pathways for antimalarial therapy.

Target	Validated	Expressed active form	Assay for automation	Mammal target
Haemozoin polymerization	?	NA	Y	?
Plasmepsin I	by inhibition	Y	Y	Y
Plasmepsin II	?	Y	Y	Y
Falcipain	by inhibition	Y	Y	Y
pmfmdr-1	?	Y	Y	Y
DHFR-TS	Y	Y	Y	Y
PPPK-DHPS	Y	Y	Y	Y
HGPRT	Y	Y	N	Y
Ca-dependent protein kinase C	by inhibition	?	N	Y
Ribonucleotide reductase	by inhibition	Y	N	Y
DNA polymerase alpha	?	N	N	Y
Organelle RNA polymerase	N	N	N	Y
Tubulin	N	N	N	Y
Superoxide dismutase	N	?	N	Y
Phosphocholine cytidylyl transferase	?	N	N	?

DHFR-TS, dihydrofolate reductase and thymidylate synthase; PPPK-DHPS, dihydropterate synthase and 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinease; HGPRT, hypoxanthine-guanine phosphoribosyl transferase.
? = uncertain; NA = not availabe; Y = yes; N = no.

Haem detoxification. The parasite has developed means to polymerize the otherwise toxic free haem resulting from haemoglobin proteolysis. Haem polymerization is targeted by antimalarial drugs of the 4-aminoquinoline and arylamino-alcohol families, although the process has not been entirely elucidated yet. A haem polymerase activity was initially described (Chou & Fitch 1992; Slater & Cerami 1992), but subsequent studies have revealed that haem can polymerize non-enzymatically under conditions physico-chemically similar to those of the digestive vacuole (Egan et al 1994; Dorn et al 1995) and this may be initiated by histidine-rich proteins (HRP) (Sullivan et al 1996a). Recent experimental data support the hypothesis that quinoline-haem complexes terminate haem chain extension (Sullivan et al 1996b). The continued interest in haem polymerization, although it is not yet completely validated as a target, stems from various factors: it is an essential feature of the parasite life-cycle and there are no alternative biochemical pathways which circumvent the target; it is unique to the parasite; it is a stable target as resistance to quinoline drugs occurs via different mechanisms; and an assay is now available for screening compounds (Ridley 1997).

Oxidative stress, free radicals and artemisinin-type compounds. *P. falciparum* is eminently susceptible to oxidative stress (Clark et al 1989) and needs to rely on the host to combat it (Fairfield et al 1983). Free radicals are commonly generated in the RBC by the oxidation of haemoglobin to methaemoglobin and presumably also in the digestive vacuole when Fe^{2+} is oxidized to Fe^{3+} in the presence of molecular oxygen. Superoxide radicals (whether they diffuse from the RBC through anionic channels or are produced locally) are converted to hydrogen peroxide by superoxide dismutase (SOD).

Artemisinin-derived intermediates were shown to act as alkylating agents, forming covalent adducts with human serum albumin, haemin and the RBC membrane (Yang et al 1993; Asawamahasakda et al 1994a; Hong et al 1994). The endoperoxide bridge appears essential for antimalarial activity, the bridge-lacking deoxyarteether being found inactive as such adducts can not be formed (Brossi et al 1988; Asawamahasakda et al 1994b). It has been suggested that haem catalyses the reductive decomposition of the endoperoxide group into a free radical (Meshnick et al 1993; Meshnick 1994) and other electrophilic intermediates (Posner & Oh 1992). This hypothesis is supported by the findings that free radical scavengers antagonize activity (Krungkrai & Yuthavong 1987) but the specific reactions involving free radicals have not been defined so far.

More work is underway to generate both more stable derivatives of artemisinin itself, and totally synthetic trioxanes and tetroxanes. For the former, attention is generally focussing on derivatives which will have slower metabolic breakdown and bypass generation of the dihydro metabolite of artemisinin (the major metabolite of the currently used artemisinin derivatives). Evidence that these derivatives may be less neurotoxic (a prime worry about all standard artemisinin derivatives) is emerging. Other derivatives have enhanced activities in-vitro against both chloroquine-resistant and -sensitive strains of *P. falciparum*. Developments in the synthetic trioxane and tetroxane area are exciting (Posner et al 1994, 1996). Synthetic trioxanes have been prepared which have in-vitro activities ranging from equal to that of artemisinin to several thousand times greater than artemisinin. Also important from drug production and regulatory viewpoints, candidate compounds can be prepared easily, in either an enantiomerically pure or achiral form. These criteria also apply to tetroxanes, which are very easily prepared in a minimum number of steps from cheap, accessible starting compounds.

Drug transport. Another potential target in the food vacuole are transport mechanisms. There is mounting evidence that drug transport plays a major role in mediating drug resistance. One such transporter has been identified in *P. falciparum*, the pfmdr1 gene (Volkman et al 1993), and this gene has been implicated in mefloquine resistance and cross-resistance to halofantrin (Wilson et al 1993). Reversal of resistance has been observed by compounds which are known to inhibit mdr

function in other systems such as penfluridol, verapamil and desipramine (Bitonti et al 1988; Kyle et al 1993; Oduola et al 1993). This has lead to the proposal that such reversers could be used in combination with antimalarial drugs to treat drug-resistant infections. This strategy is complicated by the potential interaction of these reversal drugs with the human homologue, the P-glycoprotein, and therefore reversal drugs must be tested on both the parasite and human homologue in order to identify compounds with differential specificity.

Nucleic acids

Nucleic acid metabolism of *Plasmodium* (Hassan & Coombes 1988) differs significantly from the corresponding human pathways. The malaria parasite is unable to synthesize purine de novo and must rely on the host erythrocyte for its main source of purine precursors during its intra-erythrocytic growth (Scheibel & Sherman 1988). Conversely, whilst humans are able both to synthesize and to salvage pyrimidine nucleotides, the intra-erythrocytic malaria parasites can only synthesize pyrimidine nucleotides de-novo, being unable to salvage either pyrimidine bases or nucleosides. The de novo synthesis of pyrimidines also involves para-aminobenzoic acid (PABA) and folate cofactors. In contrast to mammalian cells, malaria parasites are unable to salvage exogenous folates and synthesize these cofactors de novo.

Purine metabolism; hypoxanthine. Although adenosine triphosphate (ATP) is the predominant purine present in human erythrocytes, there is considerable evidence that hypoxanthine, formed during the ATP catabolism, is the immediate purine precursor utilized by the parasite and that the parasite-specific enzymes for hypoxanthine salvage exist. Hypoxanthine salvage rather than adenosine salvage is therefore a potential target for new drug development. Among the various enzymes involved in the hypoxanthine salvage, hypoxanthine-guanine phosphoribosyl transferase (HGPRT), is probably the most promising as a potential chemotherapeutic target since the relevant gene has been cloned and expressed (Vasanthakumar et al 1990) and a mammalian enzyme is available for testing for specificity. However, the yield of both the native and the recombinant enzyme is low and an assay for automation is not yet available.

Pyrimidine metabolism and electron transport. At least four enzymes needed for the conversion of carbamyl phosphate to thymidylate (i.e., dihydroorotate dehydrogenase, orotate phosphoribosyltransferase and orotidine 5′-phosphate decarboxylase) differ from the host enzymes by comprising bifunctional complexes (Gero et al 1981; Rathod & Reyes 1983; Krungkraiet al 1990). A novel naphthoquinone antimalarial, atovaquone, is now available in fixed combination with proguanil. Atovaquone has no cross-resistance with known antimalarials. Although it inhibits parasite dihydroorotate dihydrogenase, the primary target appears to be blockade of pyrimidine synthesis by the inhibition of the respiratory chain of malarial mitochondria at complex III (Hudson 1993). Selectivity and specificity of the target is attributed to different lipophilicity and possibly to different amino acid sequence at the binding site of the protozoan ubiquinone compared to that of mammalian cells.

Other inhibitors of this pathway are being sought. For example, dihydroorotase (DHOase), which catalyses the reaction carbamyl phosphate to dihydroorotate, has been purified and various inhibitors have been synthesized and tested (Krungkrai et al 1992; Seymour et al 1994).

Folate metabolism. Some of the most widely used antimalarials inhibit folate metabolism: sulphonamides and sulphones, which prevent the formation of dihydropterate, are usually combined with pyrimethamine, a dihydrofolate reductase inhibitor. Unlike in mammalian cells, PPPK-DHPS (dihydropterate synthase; and 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase) and DHFR-TS (dihydrofolate reductase and thymidylate synthase) exist in malaria parasites as bifunctional enzymes (Ivanetich & Santi 1990). The genes for DHFR and DHPS are now sequenced and the enzymes expressed in their active form. DHFR mutants are also available. The test can be formatted for high-throughput screening against the human enzymes and there are massive libraries of compounds to test. It is not clear, though, if this can lead to new effective antimalarials. Resistance occurs due to one gene mutation (Hyde 1989; Foote et al 1990; Peterson et al 1990), hence compounds are prone to resistance and are also likely to have a short lifespan. Other enzymes that constitute possible chemotherapeutic targets are serine hydroxymethyltransferase (SHMT) (Rueenwongsa et al 1989), methylene tetrahydrofolate reductase (MTHFR) and methionine synthase (MS) (Asawamahasakda & Yuthavong 1993).

Phospholipid metabolism

Drug discovery and development often ensue from long-term strategic research projects even in the absence of precisely identified targets. One such example is a new family of inhibitors of *Plasmodium* phospholipid metabolism. Intra-erythrocytic stage parasites require large amounts of phospholipid (PL), which is synthesized from plasmatic free fatty acids and polar heads. An accessible target in this pathway is the choline transporter, which provides the intracellular parasite with choline, a precursor required for synthesis of phosphatidylcholine (PC), the major parasite PL. A synthesis effort has produced several hundred molecules with significant structure-activity work. First-generation compounds contained a quaternary ammonium for high antimalarial activity but had availability problems. Newer bioisosters of quaternary ammonium revealed improved absorption and less toxicity (H. Vial, personal communication). In-vitro, the compounds possess high antimalarial activity against chloroquine-sensitive and -resistant *P. falciparum* in the lower nanomolar range, with no cross-resistance with known antimalarials. Selected compounds are also highly potent in-vivo in the *Aotus* monkey model. Predevelopment work is underway on selected leads.

Other pathways

Ongoing research is pointing at a variety of putative chemotherapeutic targets which still await confirmation or have not yet produced significant leads. Some seem more promising than others, namely protein kinase C (Zhao et al 1993), organelle RNA polymerase (Wilson al 1991; Geary & Jensen 1983; Strath et al 1993), ribonucleotide reductase and DNA polymerase alpha. These are targets of known drugs both in humans and in other microbial agents and thus have the potential as targets in the parasite system. Emphasis on target validation is critical at this point.

Perspective

While there are several potential targets for chemotherapeutic intervention which have been identified and are in various stages of target validation or lead identification and optimization, experience in other systems has demonstrated that only a limited number of such leads ever reach even the early stages of drug development. Thus, there is a continuing need for target identification in *Plasmodium*, as there is in other microbial systems. A novel approach to target identification has been initiated through the Malaria Genome Sequencing Project in which the goal will be to determine the complete sequence of *P. falciparum* genome. Advances in sequencing technology have now made such an approach feasible. The complete sequence of several bacterial pathogens has now been completed as has the complete sequence of *Sacchromyces cervisae*. Through a comparison of the sequences of microbial genomes, common essential target enzymes will be identified, as will elements unique to each organism. It is too early to evaluate this approach, however pharmaceutical companies are making major investments in the sequencing of several bacterial pathogens and thus such an investment by the public and philanthropic sectors in *P. falciparum* is timely and appropriate.

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