Antimalarial peptides: the long and the short of it

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Abstract

Antimicrobial peptides include a diverse array of both natural and synthetic molecules varying greatly in size, charge, hydrophobicity and secondary-structural features. Although better known as antibacterial agents, many peptides have demonstrated activity against the malarial parasite *Plasmodium* either in its vertebrate blood stages or mosquito stages or both. The antimalarial peptides reviewed here consist of (i) cationic, amphipathic 'host-defence' peptides including some (e.g. defensins and cecropins) that are naturally produced by mosquitos, (ii) other membrane-active peptide antibiotics such as gramicidins, (iii) hydrophobic peptides, most notably cyclosporins, (iv) thiopeptides, such as thiostrepton, and (v) some other naturally occurring or synthetic peptides. Many of these peptides affect membrane integrity and some are selective for parasite membranes over those of the host, while others are thought to have more specific intracellular targets. The mechanisms of action of the majority of antimalarial peptides are however either uncertain or totally unknown. Very few of these agents have been tested in rodent malaria models and none has undergone significant pre-clinical or clinical development for malaria. Issues such as metabolic lability, high cost, and a lack of information about systemic toxicity are likely to be serious obstacles to further development of peptides as antimalarial drugs. On the other hand, they offer potential advantages, including the possibility of being much less prone to resistance than the drugs in current use. An alternative to conventional chemotherapy, namely the release of malaria-refractory, transgenic mosquitos overproducing antimalarial peptides, has already passed the 'proof of concept' stage.

Key words: Malaria, *Plasmodium*, antimalarial chemotherapy, antimicrobial peptide, gramicidin, cyclosporin, thiopeptide.

INTRODUCTION

Malaria continues to be one of mankind's most intractable infectious-disease problems. The extent of malaria worldwide, the limitations of current control methods and the urgent need for new chemoprophylactic and chemotherapeutic agents have been comprehensively discussed elsewhere (1-3) and need not be restated here. It should suffice to state that, while the antimalarial drugs in use (which are for the most part artemisinins, quinoline or related compounds, or inhibitors of the folate pathway) can be effective in most situations, improvements in terms of (especially) cost and safety profile are highly desirable. Furthermore, our most useful drugs have successively fallen prey to resistance, making it essential to have a new drug 'pipeline' that will provide the replacements. The current pipeline is not flowing with new drug candidates at the rate required (4), so the search for new antimalarial agents will have to be pursued for some time to come.

Human malaria is caused by one of five species of protozoal, apicomplexan parasites of the genus *Plasmodium*, of which *Plasmodium falciparum* is the most virulent, the best characterized, and (along with *Plasmodium vivax*) the most common (5). Certain rodent malarial parasites such as *Plasmodium berghei* are used as models (as none of the human parasites normally infects rodents) and are also well characterized. Parasites of the *Apicomplexa* are important animal pathogens notable for their complex life cycles and highly specialized invasive forms. Besides the various *Plasmodium* species they include the agents of toxoplasmosis, cryptosporidiosis and several other significant diseases.

For the reader unfamiliar with malaria (those who are will want to skip this paragraph and the next), it is important to summarise briefly the parasite's life cycle, because the antimalarial actions described below relate to different stages of development. The infective form of the malarial parasite is the sporozoite, transmitted by the bite of the definitive host (female anopheline mosquito) to the intermediate host (human or other vertebrate) during a blood meal. Sporozoites eventually migrate to the liver and invade and multiply within hepatocytes. During this process, which lasts several days (depending on the species) the intermediate host is unaware of the infection. Parasites exit hepatocytes in a different form, the merozoite, which enters the blood-stream and invades erythrocytes. Erythrocytic forms can follow one of two developmental pathways. The asexual pathway involves multiplication over a defined period (~48 hours in *P. falciparum*) to form new merozoites (up to ~25–30 for *P. falciparum*). This cycle is the one primarily associated with disease pathology and generally the higher the amplification of the parasite (expressed as parasitaemia, the percentage of erythrocytes infected) the more serious the disease. The parasites of the asexual, erythrocytic cycle appear initially as 'ring' forms (whose main activity seems to be to modify the host erythrocyte surface), are later called trophozoites (which are rapidly growing and metabolically active, especially in haemoglobin digestion) and later still are defined as schizonts (dividing forms), before new merozoites appear and are released. The alternative (sexual) pathway occurs in a minority of blood-stream parasites and leads to the formation of micro- (male) and macro- (female) gametocytes over several days. It is these forms, when taken up by another mosquito, that lead to transmission of malaria. The erythrocytic

(especially asexual) forms of *P. falciparum* are readily cultured in the laboratory using donated human erythrocytes and are used for evaluating antimalarial activity *ex vivo* (often referred to in the literature as '*in vitro*'). The hepatic forms are harder to work with in the laboratory and consequently relatively little drug testing has been done with these parasite stages.

Gametocytes taken up by the vector differentiate in the mid-gut into their respective gametes, which involves an explosive exflagellation in the case of the microgametocyte. Following fertilization (which often mixes genetic material from different parasite strains), the zygote differentiates into an ookinete, which invades the midgut wall and settles on the outside, forming an oocyst. Multiplication takes place again within the oocyst, producing new sporozoites, whose migration to the salivary glands completes the cycle. The mosquito phase takes several days, with the exact duration dependent on the species and on temperature. Generally *Plasmodium* infection is believed to have little effect on the fitness of the mosquito. All of the mosquito stages can also be completed in the laboratory, most conveniently using rodent parasites.

Peptides have provided a source of antibacterial and other antimicrobial compounds for some time, although their clinical application has so far been limited (6-12). Some of them offer potential advantages over other antimicrobial drugs including wide spectrum of action, rapid cidal activity, and the existence of synthetic and biologic methods for generating a highly diverse range of analogues. Perhaps the most attractive feature is that resistance to peptides is in many cases slow to appear (13). In the case of certain peptides (see below) this is believed to be a reflection of the lack of a single, distinct molecular target, whose affinity for the peptide can readily be reduced by genetic mutations. Disadvantages include short half-lives in the body (although this can sometimes be addressed by cyclisation and/or including D- or other non-proteinogenic amino acids), neutralization by physiological salt or divalent cation concentrations or due to aggregation, immune response (though this may be mitigated by the use of host-like molecules), lack of information on systemic toxicity, and high cost of production. The purpose of this review is to discuss those peptides that have been found to have antimalarial activity and what might become of them as far as antimalarial drug development is concerned.

Along with an overview of reports of antimalarial activity of peptides from the literature, this review will concern itself with some questions that might most concern the reader with an interest in malaria chemotherapy, or antimicrobial peptides in general, or both.

1. What are the roles if any of antimalarial peptides in resistance to colonization by malarial parasites?
2. What is known about the structural requirements for antimalarial activity of peptides and how do these requirements differ (if at all) from those for other antimicrobial activities?
3. What is known about the mechanisms of antimalarial actions of the peptides and the bases of their selective toxicities for the parasite?
4. What if anything is known about malarial parasite resistance to peptides, and is there any reason to believe that peptides might be more 'resistance-proof' than other antimalarial drugs?

5. What kinds of peptides offer the most promising approaches to new antimalarial drug discovery/design and how might they be taken forward?

There have been numerous reports of peptides with antimalarial activity on various cultured stages of malarial parasites and/or in animal models of malaria. The range of amino acid compositions, secondary-structural features and lengths (from dipeptides up to molecules long enough to be called proteins) of these antimalarial peptides is impressive. Many broad-spectrum antimicrobial peptides, from various sources including anopheline mosquitos that are the vectors for malaria, are active to varying degrees on malarial parasites. These are discussed in the following sections.

In addition there have been numerous reports of antimalarial activities of substrate analogues of the various peptidases that play important roles in host haemoglobin degradation, host-cell invasion and egress, and intracellular housekeeping. These have been extensively reviewed (14-16) and (along with peptidic inhibitors of other protein-modifying enzymes such as histone deacetylases) will not be covered here due to space limitations. It is however worth mentioning an interesting approach to delivery of a peptidase inhibitor taken by Dhawan et al. (17), who used the Antennapedia homeoprotein internalization domain to enhance penetration of an ankyrin peptide into blood-stage *P. falciparum*. Also, there have been many reports of synthetic peptides or protein fragments with specific targets at parasite/host or parasite/vector interfaces, for example fragments of proteins such as adhesins that are involved in host-cell invasion. These topics have also been well covered elsewhere (18-20) and will not be discussed here. The issues related to peptides as antimalarial drugs, such as problems of oral bioavailability or metabolic stability, which have been discussed elsewhere (7), will however apply to the above groups of peptides as much as to the antimalarial peptides discussed below. Peptidic derivatives of known antimalarial drugs will also be omitted from this review.

PEPTIDES WITH ANTIMALARIAL ACTIVITY

Cationic 'host-defence' peptides

The largest and most studied category of antimicrobial peptides is the cationic peptides produced by a variety of multicellular organisms for the purpose of defence against pathogens (6). Such is the preponderance of these peptides that the term 'antimicrobial peptides' is often used to cover only this sort of peptide. They are generally ribosomally synthesised, <100 amino acids long and amphiphilic with a net positive charge in the range of +2 to +9 (6). Disulphide bridges are often present. The secondary structures are however diverse and can include predominantly α-helical, predominantly β-sheet, mixed α-helical/β-sheet or fully extended structures, and the content of loops and turns is variable. The cationic host-defence peptides have been extensively researched and have been shown to exhibit activity against a wide variety of bacteria, fungi, enveloped viruses and protozoa. As discussed below, they constitute the lion's share of reported antimalarial peptides as well. This extraordinarily

wide spectrum of activity appears to be related to their mechanisms of action, which have been shown at least in some peptides to be related to relatively non-specific interactions with membranes whose fundamental properties differ between pathogens and their hosts. This lack of specificity has set alarm bells ringing as regards potential host toxicity, and indeed close relatives of some of the peptides described here have potent haemolytic, neurotoxic or other toxic activities. Nonetheless some cationic peptides have (at least at the cellular level) clear differences between concentrations toxic to pathogen and host, sometimes of several orders of magnitude. Some are under active investigation as potential antimicrobial drugs and it remains to be seen whether the rapid and potent activities seen in cultured cells and in some cases animal models can be translated into clinical efficacy (6, 7, 12). Although this review is confined to the issue of direct parasite growth inhibition or killing, there is evidence that cationic peptides may be important effectors in such host immune functions as phagocytosis, prostaglandin release, lipopolysaccharide neutralisation and immune cell recruitment (6). For example, α-defensins are bactericidal only at high concentrations and may owe their activity *in vivo* primarily to immunomodulation. The future of antimicrobial host-defence peptides may therefore lie less in their direct potency against the pathogens and more in their ability to manipulate host immune responses to maximize pathogen killing while at the same time minimizing immunopathology.

The mosquito vector for malaria produces three classes of antimalarial peptide: cecropins, a defensin and gambicin (21). While these have activity against mosquito-stage and in some cases blood-stage parasites (Table **1**), the concentrations needed were in some cases close to those toxic to the insects, and there is doubt as to whether effective concentrations are reached in infected insects for sufficient times. Nonetheless, interference RNA-based knock-down of expression of gambicin and other secreted peptides did increase levels of *Plasmodium* infection (22). Although upregulation of peptides including gambicin can be detected in response to malaria infection, it is thought that this arm of the mosquito immune system has evolved primarily to combat bacteria and fungi and that antiparasitic activity is a 'bonus' (22). Less seems to be known about the role if any of peptides in immunity to malaria in vertebrates, but the killing of cultured *P. falciparum* by γδ T cells was suggested to be mediated by the 9-kDa peptide granulysin (23).

Dozens of cationic peptides derived from insects, vertebrates and other organisms, and derivatives thereof, have been studied for their activity on malarial parasites in the blood stages in the vertebrate host and/or the various mosquito stages (Table **1**). The structures of some of the key peptides discussed in the text are in Fig. (**1**); others are available at the Antimicrobial Peptide Database, http://aps.unmc.edu/AP/main.php (last accessed 26 July 2010). Although a wide variety of structures is represented in the table (like peptides are listed together where possible), the most striking conclusion from the data is that activity on both blood and insect stages is, with very few exceptions, in the low micromolar range at best. This contrasts with the majority of antimalarial drugs in use, which tend to have 50% inhibitory concentrations (IC_{50}) for asexual blood stages in the low nanomolar range or below, but is by-and-large consistent with the concentrations that are effective on other pathogens. Haemolysis and other host-cell toxicities are reported to be lower for many of the peptides shown, but

the ‘proof of the pudding’, efficacy and safety in rodent malaria, is wholly lacking. Another approach however is to exploit the activity of the peptides against insect-stage parasites. The idea here is to engineer mosquitos overproducing antimalarial peptides, making them refractory to *Plasmodium* infection, and to release them with the aim of replacing the wild-type, susceptible population. A proof of this concept was shown by Kim et al (24) who introduced the cecropin A (*cecA*) gene, under the control of the blood-meal-inducible, gut-specific *Aedes aegypti* carboxypeptidase gene promoter, into *Anopheles gambiae*. The gene was expressed in *A. gambiae* midguts and caused an average ~61% reduction in mean *P. berghei* oocyst numbers, 12-14 days after feeding, in two transgenic lines. This achievement has recently been surpassed by Kokoza et al (25) using *cecA* and defensin A (*defA*) genes under the control of the vitellogenin gene promoter with *A. aegypti* as a model. Mosquitos overproducing both peptides had reductions of oocyst numbers, 7 days after feeding on *Plasmodium gallinaceum*-infected chicks, that in some cases exceeded 95%. When the experiment was repeated with a second blood meal at 7 days and counting of oocysts after a further 7 days, oocysts were completely absent and no infections could be established in naïve chickens.

Some investigators have sought to improve activity and/or selectivity by modifying the naturally occurring peptides, with success in a few cases. For some peptides the structure–activity relationships (SAR) for antibacterial and antifungal activity mirrored those of antimalarial activity, and for others there were clear differences. Boman et al (26) were able to combine part of cecropin A (Fig. (**1**)), which had no measurable activity on blood stages, with part of melittin, a haemolytic bee-venom toxin, to produce a peptide with low micromolar antimalarial activity but no haemolysis at a 20-fold higher concentration. The cecropin-melittin hybrid also had improved antibacterial activity. Comparison of the two similar amphibian skin peptides dermaseptin S3 (DS3) and dermaseptin S4 (DS4; see Fig. (**1**) for structures) showed that only the latter was haemolytic at low concentrations (27). Substitutions of neutral residues by positive ones increased the activity of dermaseptin S4 derivatives on blood stages (28). Subsequent derivatisation of the K_4-S4(1-13)a dermaseptin S4 derivative at the N-terminus showed that hydrophobic groups improved activity, while negatively charged ones lessened it and positively charged ones had no effect (29). Some derivatives had greater haemolysis of infected erythrocytes than uninfected ones (29). There was strong parallelism between antimalarial and haemolytic, and to a lesser extent anti-*Escherichia coli* activities, among the dermaseptin S4 derivatives of Krugliak et al. (28); but subsequently, improved antimalarial/haemolytic activity ratios were obtained with the propionyl, butyryl and isobutyryl derivatives described by Dagan et al. (29). Antimalarial activity was eventually dissociated from infected-erythrocyte lysis, indicating that the membranes of the parasites themselves may be the target (see below). SAR among lauryl-lysine oligomers, synthetic molecules designed to mimic the first 16 amino acids of dermaseptin S3, were somewhat distinct for antimalarial, haemolytic and cytotoxic (MDCK cell) effects (30). Psalmopeotoxins (Fig. (**1**)), from the venom of a tarantula spider, were antimalarial but ineffective against the bacteria and fungi tested (31). Rearrangement of disulphide bridges in psalmopeotoxins affected activity (32). Tian et al. (33) proposed that the fruit fly-derived drosomycins possessed, in comparison with defensins, a ‘parasite-specific’ loop. Mason et al. (34) suggested that the parasite-

selectivity of histidine-rich peptides may be due to differences between normal host membranes and membranes of infected erythrocytes (decreased cholesterol, increased phosphatidylserine exposure, increased fluidity and disorder, increased content of unsaturated acyl chains) and/or parasites (very low cholesterol). Akaddar et al (35) found that the activity of the 21-amino acid mammalian peptide catestatin was completely or almost completely preserved in the central LSFR tetrapeptide only. This is of particular interest because large antimicrobial peptides usually cost more to produce and are less metabolically stable.

Wade et al (36) addressed the question of mechanism of action by comparing the activities of the physiological L-enantiomers of cecropin A, melittin, two cecropin–melittin hybrids and magainin 2 amide with the corresponding D-enantiomers. Equal activity of the two would suggest interaction with non-chiral molecules only and would be consistent with a relatively non-specific membrane effect. Activity against various bacteria and (in the case of melittin) erythrocytes showed no difference, but the D-enantiomers were ~5-fold less active on blood stage *P. falciparum,* suggesting at least some role of chiral components in the antimalarial action. The conductances of the two sets of peptides were the same in planar lipid bilayers. By contrast, the L- and D-enantiomers of the dermaseptin S4 derivatives of Krugliak et al (28) were equi-active.

A common theme in several studies is, as may be expected, the interaction of cationic peptides with membranes. In some cases infected erythrocytes were lysed at lower concentrations than uninfected ones, suggesting a basis for selectivity. In others, the membrane of the intra-erythrocytic parasite itself was affected, and Mor (11) suggests that some peptides move by "affinity-driven transfer" from host to parasite membranes. Binding of DS3 and DS4 to erythrocytes was non-saturable and appeared to be located in membranes, and fluorescent derivatives labelled the intracellular parasite as well as the host cell (27). Proteolytic cleavage experiments suggested that the N-termini were buried in the core of the hydrophobic bilayer with the hydrophobic face buried shallowly in the hydrophobic region and the positive charges directed towards the hydrophilic region, consistent with the non-pore 'carpet' mechanism (37). DS4 however exhibited higher aggregation than DS3 and unlike DS3 underwent a change in aggregation state at increasing concentrations (27). It was suggested that DS4 was more active in membrane permeabilisation because its higher aggregation state took longer to dissociate in the membrane and the absence of charge at its N-terminus favoured stronger hydrophobic interaction with the acyl chains in the (phosphatidycholine-based) outer leaflet of the erythrocyte membrane. In agreement with this idea, lauryl-lysine oligomers with lower critical aggregative concentrations were more potent on blood-stage parasites (30). Some dermaseptin S4 derivatives dissipated the parasite membrane potential and depleted intracellular K^+ (28, 29). The substantial differences in length and charge between psalmopeotoxins I (33 amino acids, +2) and II (28 amino acids, +5) conferred only a slight difference in activity (that may not be statistically significant) (31). On the other hand psalmopeotoxin I suffered a loss of activity after pre-incubation with uninfected erythrocytes but psalmopeotoxin II did not, suggesting possible selectivity of the latter for infected erythrocytes. NK-2 (Fig. (**1**)), derived from the positively charged core region of NK-lysin, was haemolytic to infected

erythrocytes above ~1 μM but not to uninfected ones at 10 μM (38). There was also evidence for parasite membrane permeabilisation (at 5 or 10 μM). Moreover, fluorescently labelled NK-2 bound to infected erythrocytes and parasites but not uninfected erythrocytes. This selectivity was suggested to be related to higher phosphatidylserine content in the outer leaflet of the infected erythrocyte membrane.

The kinetics of parasite killing or inhibition appear to be highly variable between different cationic peptides, with some showing activity within as little as 1 minute (28, 39) while others took several hours. The stage-dependence of action in blood stages (28, 40) and insect stages (39, 41-44) was highly variable between the different peptides and these data have so far offered little assistance in determining mechanisms of action.

In summary, it is likely that most or all antimalarial host defence peptides act on membranes, with chemical and physical differences between uninfected and infected erythrocyte membranes and between erythrocyte (and other host cell) and parasite membranes explaining the selective toxicity often observed. The relatively non-specific nature of such a target would offer hope that resistance would be slow to emerge (13) but this has never been studied. Moreover one would expect that D-peptides would be more refractory to proteolytic digestion as a possible resistance mechanism. At the same time we should be cautious that preconceptions from studies with other microorganisms may have disposed investigators to looking at membrane effects and ignoring other possible mechanisms. The possibility of intracellular targets for some of these peptides, as suggested for example by Akaddar et al. (35), should not be discounted.

Other membrane-active peptides

A number of ionophoric and other peptides of microbial origin, known to be active on membranes of at least some organisms (most often bacteria), have antimalarial activities (Table **2**). It is striking that the activities of some of these compounds are in the nM or ng/ml range or even lower, much more potent in general than the 'host-defence' peptides. Balanced against this is the high host toxicity of many of these agents (not shown in Table **2**): for example haemolysis and mammalian cytotoxicity were seen for tyrocidines at low-mid μM concentrations (45).

Gramicidin A (gramicidin, Fig. (**1**)) is a linear, helical, hydrophobic peptide that forms cation channels in membranes and is highly haemolytic. The haemolytic activity could be abolished (at least up to 100 nM), while preserving a degree of antimalarial activity, by replacing the indole hydrogens on all four tryptophan residues with formyl groups (tryptophan-*N*-formylated gramicidin, NFG) (46-48). It was necessary to suspend the NFG in lipid vesicles to observe full activity (46). Gramicidin A' (composed chiefly of gramicidin A) and NFG caused leakage of K^+ from infected erythrocytes to a greater extent than uninfected ones (46, 48) and this was suggested to be its mode of action on *P. falciparum* (48). The cyclic, cationic peptide gramicidin S lysed infected erythrocytes and parasites but tyrocidine A

(Fig. (**2**)) did not (45). Mature intraerythrocytic *P. falciparum* parasites (>36 h post-invasion) were the stages most susceptible to gramicidin D (49), gramicidin A, NFG and two other modified, non-haemolytic gramicidins (47), presumably reflecting their high membrane content. By contrast there was little stage-specificity of the fungal peptides evaluated by Nagaraj et al. (50). Antimalarial SAR among tyrocidines indicated that higher activity was associated with higher apparent hydrophobicity and lower side-chain surface area (45). These relationships were less marked for haemolytic and (mammalian) cytotoxic activities.

Two of the compounds listed in Table **2** have been used in models of mouse malaria but gramicidin D was toxic to the mice by parenteral routes and inactive orally (51) while intraperitoneal (i.p.) NFG had limited activity *in vivo* (52). An older report showed good activity of tyrothricin (tyrocidine + gramicidin) on *P. gallinaceum* in chickens but the doses required were close to those lethal to the birds (53).

Hydrophobic peptides

Some peptides best described as hydrophobic have been shown to have antimalarial activities (Table **3**). The best-characterised are the cyclosporins, which have a range of antimicrobial activities (54, 55). They are cyclic undecapeptides of fungal origin containing several N-methylated or other unusual amino acids.

The best known of this class, cyclosporin A (CsA; Fig. (**2**)) is a well-characterised immunosuppressant (56). Following the initial, unexpected observations of antimalarial activity of CsA in mouse models (57, 58) a number of follow-up studies showed effectiveness in *P. berghei* and *Plasmodium yoelii* rodent malaria and on cultured *P. berghei, P. falciparum* and *P. vivax* (Table **3**). One study with *P. falciparum* in owl monkeys (not shown in Table **3**) showed benefits of CsA at ≥50 mg/kg (7–12 daily doses) (59). The activities against cultured parasites, combined with effectiveness in immunosuppressed mice (58), indicated a direct antiparasitic action, though sub-antiparasitic doses were of benefit in a mouse model of cerebral malaria (60). Gametocytes seem to be susceptible to some degree but were less affected than asexual blood forms (57, 61, 62). The data on the most susceptible stage of the asexual cycle are inconsistent (61, 63) and may reflect differences in experimental design. Liver stages appear to be largely resistant (62), in agreement with the observation that CsA is more effective in rodent malaria when administration is started at the time of infection, or after the establishment of a substantial parasitaemia, than when the drug is used prophylactically (61, 62). Recrudescence of parasitaemia was seen following the end of treatment in a number of studies (57, 58, 61) and resistant *P. yoelii* and *P. berghei* parasites could be isolated (58, 61). The resistant forms were apparently not compromised for growth in mice (61) but appeared to be less virulent in the study of Nickell et al (58) and less fit than the CsA-susceptible parent for transmission through the mosquito according to Murphy et al (61). Resistant *P. falciparum* were also isolated in culture by Kumar et al (64) (see below) but their fitness was not assessed.

The classical model of immunosuppressive action of CsA in T-lymphocytes involves inhibition of the phosphoprotein phosphatase calcineurin (PP2B) by a complex of CsA and its major receptor, cyclophilin (65). Calcineurin plays a key role in signal transduction during T-cell activation. At least three cyclophilins and a calcineurin are also found in *Plasmodium* and although their functions are not known, some of the evidence (as discussed more extensively by Bell et al (66)) points towards a similar mechanism of action in malarial parasites. Foremost among this evidence is that out of 9 CsA-resistant clones isolated by Kumar et al (64), 3 had lesions in cyclophilin genes and 2 in calcineurin-subunit genes. The two cyclophilins affected had been identified as cyclosporin-binding proteins in *P. falciparum* (67). CsA has however a number of different pharmacological targets (56) including the mammalian P-glycoprotein transporter. Sequence polymorphisms in (and possibly expression levels of) a *P. falciparum* P-glycoprotein homologue affected CsA susceptibility (68). Moreover, certain non-immunosuppressive cyclosporin analogues, with reduced or abolished cyclophilin binding and/or calcineurin inhibition, were still potent antimalarial agents (69). The most potent analogue, at least against cultured parasites, was the cyclosporin D derivative, valspodar – which is well known to be non-immunosuppressive but a potent P-glycoprotein inhibitor (55, 69). Needless to say this compound would be of more interest than CsA from the point of view of developing a new antimalarial drug (see below). The mechanism of antimalarial action of cyclosporins therefore remains unproven, and it may be that CsA and valspodar have different targets. In spite of the high hydrophobicity of cyclosporins, there has been little thought given to a more non-specific action on membranes. The observation that dansylated cyclosporin bound saturably to parasites (70) may argue against this idea.

The compounds listed in Table **3** are reported to be of low host cytotoxicity but CsA has well-known nephrotoxicity in whole organisms (56). Valspodar has a good record of *in vivo* pharmacology, having reached phase-III clinical trials as a reverser of tumour multi-drug resistance (71), so would seem to be a possible candidate for 'piggy-back' antimalarial drug development.

Thiopeptides

Thiazolyl antibiotics are highly modified thiopeptides known to target the eubacterial large-subunit (LSU) ribosomal RNA (72). A few thiopeptides of this group have activity on asexual, blood-stage *P. falciparum*. Thiostrepton (Fig. (**2**)) has reported IC_{50} ranging from 1.8–10 μM (73-75a), though this is suggested to be an overestimate since solubility of the compound in culture medium is poor. IC_{50} values for thiostrepton derivatives were 1.2 μM and above (75a) and for other thiopeptides they were 35 nM for micrococcin (75), ~300 nM for GE2270A (76) and ~10 nM for amythiamicin A (76). Thiostrepton had no measurable effect on sporozoites up to 500 mg/ml (77). Thiostrepton is thought to hyperstabilize the secondary structure of the 23S rRNA, inhibiting polypeptide elongation (in bacteria) (72). Assuming the same mechanism of action, McConkey et al. (73) deduced from sequence analysis that the LSU rRNA of the plastid (or 'apicoplast', an organelle of probable cyanobacterial origin found in several apicomplexan parasites), rather than the mitochondrial protein-synthesis apparatus, was

likely to be the target in *Plasmodium*. Clough et al. (74) further demonstrated that thiostrepton inhibited protein synthesis and bound to [^{32}P]-labelled *P. falciparum* plastid transcripts. Binding was reduced if the A1067G binding-site mutation (found in *E. coli*) was introduced, and reduced still further by the A1067U change, or with the sporozoite rRNA sequence. Similar findings were recorded by Rogers et al. (78) using thermal-melt analysis. In support of this proposed mechanism of action, thiostrepton inhibited translation of the plastid elongation factor EF-Tu (78a) and affected normal plastid elongation and branching prior to replication (78b). The cyclic thiopeptide GE2270 and the related amythiamycin A had the different (but still translation-related) effect of altering the migration of recombinant, plastid EF-Tu in native electrophoretic gels (76).

Some inhibitors of apicoplast translation such as tetracycline and clindamycin display a 'delayed death' effect on asexual blood-stage *P. falciparum* whereby the drug has no apparent effect until the cycle following the one originally exposed (78b). Chaubey et al (78a) saw no effects in the first cycle using 4 μM thiostrepton and only a minor delay in development with 8 μM. By contrast, Goodman et al. (78b), using 10 μM thiostrepton, saw a clear delay in ring to trophozoite transition, and failure to progress to schizogony, in the first cycle: i.e. not a delayed-death phenotype. Nonetheless there was no marked reduction in parasitaemia until 48 h of exposure in their experiments. This first-cycle effect and the different SAR for antimalarial and antibacterial activity among thiostrepton derivatives (75a) have led to suggestions of an additional non-plastid target for thiopeptides. Schoof et al (75a) propose that this is the 20S proteasome, but their data are based on mammalian rather than parasite proteasomes and even if true, their hypothesis does not explain how these agents might be selectively toxic.

In the only study *in vivo*, four daily doses of thiostrepton (500 mg/kg i.p.) eliminated sporozoite-induced parasitaemia when started 2 days before infection, and merozoite-induced parasitaemia when started on the day of infection (77). The drug was slow to take effect on blood-stage parasites (8–10 days for clearance of parasites vs. 4 days for chloroquine). Thiostrepton also drastically reduced subsequent transmission to naïve mice, a distinct advantage not shared by many of the antimalarial drugs in use. The plastid, with its bacterial-type metabolism, is in general considered a promising source of new antimalarial drug targets (79).

Miscellaneous peptides

Table **4** shows the antimalarial activities and host cytotoxicities (where available) of a number of other peptide, depsipeptide and lipopeptide agents. Several of the peptides listed in Table **4** have activities against other protozoa and/or fungi and/or bacteria. In some cases clear targets for these compounds in malarial parasites are suggested, but in most they are not. In the study by Mizuno et al. (80) of jasplakinolide (Fig. (**2**)), a promoter of actin polymerization, the stage-dependent activity was consistent with an effect on merozoite invasion of erythrocytes (which uses an acto-myosin motor). Treated merozoites had apical protrusions and increased F-actin content (80, 81). Lazarus et al (81), in contrast, found that the trophozoite stage was most susceptible. Jasplakinolide-treated trophozoites had

an increased proportion of Triton X-100-insoluble actin (81) and a rearrangement of actin to the cell periphery (82). These changes were associated with reduced haemoglobin uptake in blood stages, and either an increased vesicle number and redistribution of vesicles to the cell periphery (82) or a reduced number and increased convolution of cytostomes, the points of uptake of erythrocyte cytosol by the parasite (81). Both studies suggested that jasplakinolide caused a defect in the endocytic pathway. In jasplakinolide-treated ookinetes, actin was relocated to a large blob at the basal end and was associated with reduced motility (83). Jasplakinolide has therefore been a useful research tool but is not parasite-selective. The known microtubule inhibitors dolastatin 10 (Fig. (**2**)) and auristatin PE were most potent on schizonts, caused growth arrest or delay at the schizont stage, and eliminated normal mitotic structures (84). Microtubules are considered to have potential as antimalarial drug targets and are already exploited by a number of antiparasitic drugs (85). SAR for the dolastatin/auristatin series showed some differences for parasites and human cells but no derivative that was clearly parasite-selective could be identified (84).

In addition to the erythrocytic-stage activities in culture shown in Table **4**, some of the cyclic β-amino acid-containing dipeptides of Sathe et al (86) had partially suppressive activity at 50–100 mg/kg p.o. in a 4-day mouse malaria test. In the only mosquito-stage study (not shown in Table **4**), angiotensin II and its related synthetic peptide VC-5 (0.5 μl of 60 μM injected into thoraces of *A. aegypti*) reduced numbers of *P. gallinaceum* sporozoites in salivary glands by 88 and 76% respectively (87). They also showed membrane damage when incubated with sporozoites, and VC-5 lowered infection rates and pre-patent periods when inoculated into chickens. They were non-haemolytic up to 100 μM but were rapidly degraded in human serum so testing against erythrocytic parasites was not done.

CONCLUSIONS

A range of peptides exhibit antimalarial activity on malarial parasites in their blood or mosquito stages or both. Some of the naturally occurring peptides of mosquitos affect the parasites found in this vector but it has been difficult to determine the magnitude of their effects in the natural setting. Engineering of transgenic mosquitos overproducing these or other peptides nonetheless appears to offer a potential route to blocking malaria transmission, albeit one fraught with practical difficulties. One drawback would be that the overproduced peptides could contain only proteinogenic L-α-amino acids. An alternative and more conventional approach would be to use peptides as drugs to kill blood-stage parasites. An ample variety of peptides have activity on cultured erythrocytic parasites but few have been shown to have potent activity *in vivo,* and those that have are not without significant effects on the host. SAR data are limited but in some cases it has been possible to distinguish antimalarial SAR from other antimicrobial and host-toxicity SAR. More systematic studies are needed to identify the structural variants that are most potent and selective on cultured parasites and to test them in mouse malaria models. Better understanding of mechanisms of action would help to guide the design of new peptides and the development of *in vitro* assays with which to compare their target-binding affinities.

In some cases antimalarial peptides are believed to act selectively on infected erythrocyte and/or intracellular parasite membranes, in which case the appropriate model membrane systems are required. In others there appears to be an intracellular target, such as a ribosomal subunit or tubulin, and this will require different kinds of target-based assays. Finally, there have been (with the single exception of cyclosporins) no studies on parasite resistance to peptides. We do not know whether peptides might be (as has been proposed for their other antimicrobial activities) more 'resistance-proof' in malaria than, for example, enzyme inhibitors. This may depend on whether they exert a relatively non-specific action on the membranes of parasites (or infected host cells) or have more specific targets. Ultimately, propensity for generating resistant parasites is something that has to be determined experimentally. Given the periods of months taken to isolate and characterise drug-resistant *Plasmodium* mutants in culture or in rodents and the limited clinical relevance of these experiments (88), this may be the most difficult aspect of future research on antimalarial peptides. Nonetheless it is research worth doing.