Chapter 7
Beyond Lysozyme: Antimicrobial Peptides Against Malaria

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1 Introduction

Antimicrobial peptides (AMPs) are components of innate immunity, the arm of the immune system in charge for the first defense against pathogens, not only in humans but also in plants, insects, and primitive multicellular organisms. AMPs are short amino acidic sequences with less than 100 residues with a secondary structure which can be used for their classification (Table 7.1) (Giuliani et al. 2007).

They have a broad spectrum of activity against many microorganisms like Gram positive and negative bacteria, fungi, and protozoa, but also viruses. Furthermore, antitumor activity for AMPs has also been reported (Hoskin and Ramamoorthy 2008).

AMPs have a rapid action (minutes to hours) but they are usually active in the micromolar range, at higher doses compared to other antibiotics.

Although the mechanisms of action of the majority of AMPs are not precisely defined, interference with membranes is recognized as the main activity. Figure 7.1 schematizes the most known hypotheses on the mode of action of AMPs on the membranes of microorganisms. The nonspecific activity on membranes gives to AMPs the advantage that they should be less prone to induce resistance in the target organisms, being their mechanism of action not connected to a specific target. However, this resistance-proof of AMPs has to be demonstrated. On the other side,

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Table 7.1 Classification of AMPs

Structure	AMPs
Linear, no Cys	Cecropin A
Cys residues	Defensins
Rich in specific amino acids (proline, glycine, histidine, tryptophan)	PR39 (proline rich), Indolicidin (tryptophan rich)

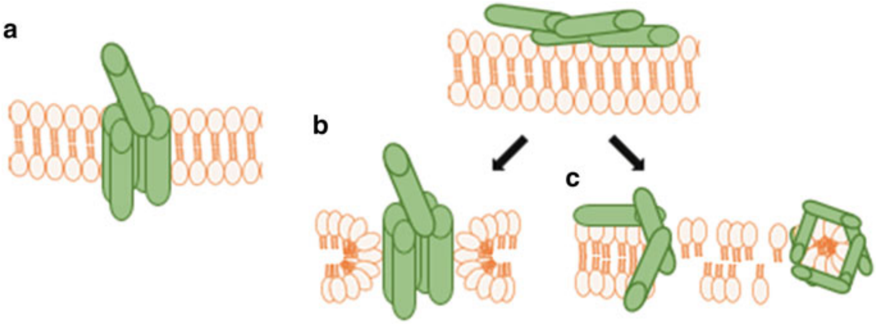

Fig. 7.1 The three model mechanisms of interaction between AMPs and biologic membranes. The image was modified from Chan et al. (2006). (**a**) Barrel/stave model. The AMPs form a pore in the membrane. (**b**) Torroidal pore. After massive AMP accumulation at the membrane surface, some AMPs acquire a transmembrane orientation and form pores, which have mixed composition (phospholipids and peptides). A curvature is induced in the membrane. (**c**) Carpet-like mechanism. The membrane surface, covered by AMPs, undergoes disruption

a disadvantage of the activity on cell membranes could be potential mammalian cell toxicity. This is true for some AMPs (e.g., gramicidin A, see paragraph 2.8), but not for others (e.g., some dermaseptin derivatives, see Sect. 2.6), which are specific for the membranes of microorganisms. In these cases, the difference in activity could be due to differences in lipid composition of membranes (cholesterol proportion or fluidity).

Beyond the activity at the membrane level, other intracellular targets such as protein or DNA synthesis have also been identified for some AMPs (Brogden 2005).

Due to their ability to penetrate cell membranes, AMPs have been proposed as vector for drug delivery (Splith and Neundorf 2011).

AMPs are difficult to be classified due to their huge diversity. The classifications can be based on different features, including amino acidic sequence (e.g., presence of cysteine residues, prevalence of particular amino acids, and presence of conserved sequences), membrane activity, secondary structure, and toxicity (Table 7.1).

2 Antimicrobial Peptides in Malaria

Some AMPs of different origin are known to affect *Plasmodium* development in different phases of the biological cycle, from asexual blood stages (cecropin, melittin, magainin, dermaseptin S4) to sexual stages in the mosquito, where AMPs can block ookinetes viability (VIDA 1-3, scorpine) or oocyst formation (VIDA 1-3) (Bell 2011). A recent work by Carter and colleagues investigated the effect of 33 AMPs on *Plasmodium* early sporogonic stages, verifying that they did not alter mosquitoes' fitness (Carter et al. 2013). Table 7.2 summarizes the antiplasmodial activity of some AMPs.

The secondary structure of AMPs has been used to predict the activity on different *Plasmodium* stages. For instance, Arrighi and colleagues designed new AMPs starting from natural or synthetic antimicrobial polypeptides and observed that peptides with no particular secondary structures (containing mainly random coils and turns) were more active on the sporogonic stages of *P. berghei* and *P. yoelii* (Arrighi et al. 2002).

Some antimalarial AMPs are hemolytic or toxic, whereas others specifically act on the membrane of infected red blood cells (RBCs) or directly on the membrane of the parasite and not on the membrane of uninfected RBCs. An example is given by dermaseptin S4, which is hemolytic and disrupts uninfected RBCs too. Development of more selective substitutes was necessary to decrease toxicity (Krugliak et al. 2000).

Table 7.2 Antimalarial activity of some representative AMPs

Activity	AMPs	Target	References
Inhibition of *Plasmodium* in vitro	Dermaseptin S4 (μM range)	Erythrocytic stages, especially trophozoites	Dagan et al. (2002), Ghosh et al. (1997), Krugliak et al. (2000)
	Vida 1-3	Ookinetes of *Pb* and *Py*	Arrighi et al. (2002)
	Scorpine	*Pb* ookinetes formation; asexual parasites	Carballar-Lejarazú et al. (2008), Conde et al. (2000)
	Cecropin, melittin, magainin e cecropin–melittin hybrids	Bloodstream forms	Boman et al. (1989), Gwadz et al. (1989), Wade et al. (1990)
Block malaria transmission in mosquitoes	Vida 1-3	Oocyst formation, *Pb* ookinetes in vitro, *Pb* and *Pf* sporogonic stages in mosquito	Arrighi et al. (2002), Carter et al. (2013)
	Defensin	Oocyst development	Shahabuddin et al. (1998)
	Melittin	*Pb* ookinetes in vitro, *Pb* and *Pf* sporogonic stages in mosquito	Carter et al. (2013)

2.1 Antimalarial AMPs Source

Antimalarial AMPs can be produced by mammalian hosts and mosquito vectors, as well as other organisms, which are not related to malaria (Table 7.3).

AMPs are part of the immune defense of mosquitoes, and *Plasmodium* infection can modulate AMPs expression in the *Anopheles* mosquito (Fig. 7.2). Vizioli and

Table 7.3 Sources of representative antimalarial AMPs

	AMPs	Origin
Human	Defensin	
Mosquito	Defensin	*A. gambiae*
	Gambicin	*A. gambiae*
	Cecropins	*A. gambiae*
Other organisms	Metalnikowin	*Palomena prasina*
	Scorpine	venom of *Pandinus imperator*
	Cecropin A	*Hyalophora cecropia*—Cecropia moth
	Magainin 2	Skin and stomach of *Xenopus laevis*

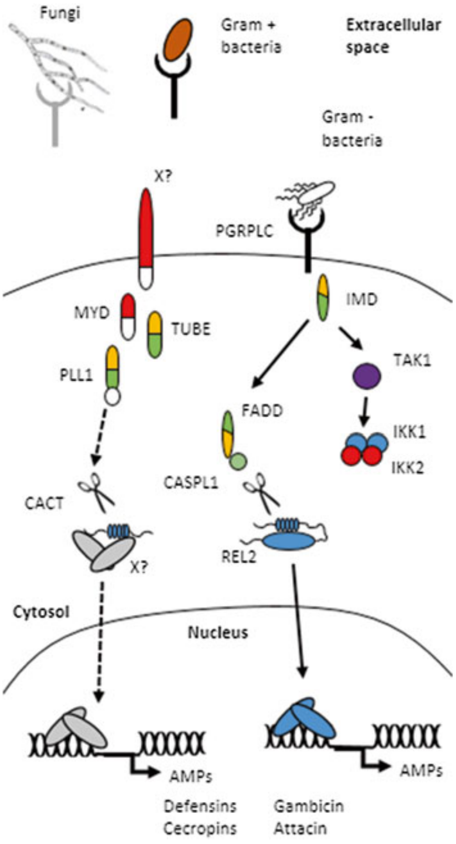

Fig. 7.2 The insect immune response to microorganisms. Common immune pathways in *Drosophila* and *Anopheles*. Proteins known in *Drosophila* with unknown ortholog in *Anopheles* are defined as "X?"

colleagues demonstrated that *Anopheles* mosquitoes fed upon mice infected with *P. berghei* expressed higher mRNA levels of cecropin A compared to mosquitoes fed with parasites unable to develop in the insect (Vizioli et al. 2000). Another example is described by Herrera-Ortiz and colleagues, who demonstrated that the mRNAs of attacin, cecropin, and gambacin were overexpressed in the midgut and abdominal tissue of mosquitoes fed with *P. berghei*-infected mouse blood (Herrera-Ortiz et al. 2011).

The majority of AMPs with antimalarial activity described by Carter and colleagues were derived from bee/wasp venoms (Carter et al. 2013).

Other examples of organisms producing AMPs with antimalarial activity are the scorpio *Pandinus imperator*, from which scorpine was isolated; the Cecropia moth which produces Cecropins; and *Xenopus laevis*, from which Magainin was extracted.

2.2 Defensins

Defensins represent the most important human AMPs as they are present at high concentrations (up to millimolar ranges) in epithelial and phagocytic cells. Their structure is characterized by a fold rich in beta-sheets and disulfide bonds between pairs of cysteines. The direct role of human defensins in malaria is not clear. Overexpression of a rat defensin (NP-1) was observed in a rat malaria model. Such enhancement was associated to protection of the young rats from lethal infection. That work supported a role for defensin in the immunity reaction to malaria infection (Pierrot et al. 2007). However, no direct studies on human defensins and malaria have been published.

Defensins are also part of the immune system of mosquitoes: their structure differs from that of human defensins, since it contains an alpha-helix linked to a beta-sheet. The role of mosquito defensins in malaria infection is better described compared to human defensins (Dixit et al. 2008; Hoffmann 1997; Meredith et al. 2008). Defensin expression, constitutive in mosquitoes midgut, is further induced by malaria infection (Richman et al. 1997; Vizioli et al. 2001b). The injection of defensin in *Aedes egypti* inhibited the development of *Plasmodium* sexual stages, resulting in oocyst abnormal development (Shahabuddin et al. 1998). The treatment of sporozoites with defensin decreased their viability.

However, a reverse genetic approach demonstrated that defensin is not necessary in *A. gambiae* (Blandin et al. 2002). The gene of defensin was disrupted in *A. gambiae* by treatment with dsRNA. This knockdown approach decreased the mosquito resistance to bacterial infections but did not alter the ookinete/oocyst formation or oocyst number after infection with *P. berghei*.

Table 7.4 Amino acid sequence of the major antimalarial AMPs discussed in this chapter

Name	Amino acid sequence
Scorpine	GWINEEKIQKKIDERMGNTVLGGMAKAIVHKMAKNEFQCMANMDMLGNCEKHCQTSGEKGYCHGTKCKCGTPLSY
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ
CA(1-13) M(1-13)	KWKLFKKIEKVGQGIGAVLKVLTTGL
CA(1-8) M(1-18)	KWKLFKKIGIGAVLKVLTTGLPALIS
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
Dermaseptin S4	ALWMTLLKKVLKAAAKAALNAVLVGANA
Gambicin	MVFAYAPTCARCKSIGARYCGYGYLNRKGVSCDGQTTINSCEDCKRKFGRCSDGFITECFL

CA cecropin A, *M* melittin

2.3 Scorpine

Scorpion venom is a rich source of peptides with different pharmacological activities. Interestingly, AMPs have been found in scorpion venom, and they may have different functions: the defense of scorpions from bacterial infection, the immobilization of their prey, or the synergistic activity with other venom toxins (Simard and Watt 1990).

In particular, scorpine (amino acid sequence in Table 7.4) is extracted from the venom of the scorpion *Pandinus imperator*. It was tested for the first time against *Plasmodium* due to its similarity, in the peptide sequence, to cecropins and defensins, already known for their antimalarial activity (Conde et al. 2000).

Scorpine decreased in a dose-dependent manner the fecundation of *P. berghei* parasites (measured as number of rosettes) and the formation of ookinetes (Conde et al. 2000). The inhibition of ookinetes formation in *P. berghei* was confirmed by Carballar-Lejarazù and colleagues, who also demonstrated the inhibition of asexual *P. falciparum* parasites in vitro (Carballar-Lejarazú et al. 2008). The authors used recombinant scorpine produced by transfected *A. gambiae* cells (cell line Sua 5.1). The plasmid for transfection was designed in order to make scorpine expressed under the control of the *A. gambiae* serpin promoter. They also created transgenic *Drosophila*, demonstrating that the expression of scorpine is not toxic to the insect. Such a paper was proposed as a proof of concept for the development of recombinant mosquitoes, an approach already proposed by Possani et al. (2002), as described below.

2.4 Cecropins, Melittin, and Cecropin–Melittin Hybrids

Cecropins are a group of insect-derived inducible antibiotic peptides from the giant silk moth *Hyalophora cecropia*. Cecropins A and B AMPs were fully characterized by Boman and colleagues, a work published by *Nature* and reproduced on

Fig. 7.3 Structure of cecropin. Image from the PFAM protein database (Punta et al. 2012) of the Wellcome Trust Sanger, Hinxton, UK (http://pfam.sanger.ac.uk/family/Cecropin)

The Journal of Immunology representing a pillar article in immunology (Steiner et al. 2009) (see Fig. 7.3 for cecropin structure). Cecropin B affected oocyst development in the *A. gambiae - P. cynomolgi* model (Gwadz et al. 1989). Some derivatives, namely Shiva-1, Shiva-2, and Shiva-3, were designed starting from the cecropin amino acidic sequence (Rodriguez et al. 1995; Yoshida et al. 2001). They inhibited the sexual stages of *P. berghei* as well as ookinete and sporozoite development in the mosquito model.

The structural conformation of melittin was described by Wade and colleagues as percentages of alpha-helixes, beta-sheet, and random coils (Wade et al. 1990).

Few years later the possibility of improving the antibacterial and antimalarial activities by creating hybrids between cecropin and melittin was explored (Boman et al. 1989). The properties of cecropins along with melittin and megainin to form ion channels in biologic membranes were studied in the 1990s (Wade et al. 1990). The amino acidic sequence of cecropin A, melittin, and two hybrids is reported in Table 7.4.

2.5 *Magainin*

Magainins were originally isolated from the skin of the African clawed frog *Xenopus laevis* (Zasloff 1987). Magainin (amino acidic sequence in Table 7.4) is active against different bacteria, such as *Escherichia coli*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa*, by forming pores in the membranes. Magainin affects the viability of others microorganisms, including *Saccharomyces cerevisiae* and *Plasmodium* spp (Gwadz et al. 1989). Some derivatives were developed. However, none of them were approved by FDA after clinical trials since they did not display increased activity compared to existing antibacterials or because they implicated toxicity issues. The structural conformation as percentages of alpha-helixes, beta-sheet, and random coils (see Fig. 7.4) was described by Wade and colleagues (Wade et al. 1990).

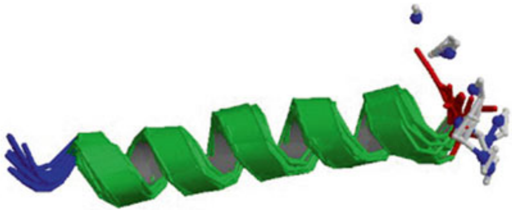

Fig. 7.4 NMR structure of magainin-2 in DPC micelles, ten structures. Picture from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (Berman et al. 2000). Protein chains are colored from the N-terminal to the C-terminal using a rainbow (spectral) color gradient (http://www.rcsb.org/pdb/explore/explore.do?pdbId=2MAG)

2.6 Dermaseptins

Dermaseptins are a family of AMPs isolated from frogs of the *Phyllomedusa* genus with cytolytic activity against bacteria, protozoa, yeast, and filamentous fungi. Ghosh and colleagues compared hemolytic dermaseptin S4 (amino acidic sequence in Table 7.4) with nonhemolytic dermaseptin S3 for their physical properties (aggregation in solution and dissociation in membranes, binding to and interaction with RBCs) and for the effect on *P. falciparum* growth in vitro (Ghosh et al. 1997). Several derivatives were prepared starting from dermaseptin S4, with many showing a selective activity on the membrane of infected RBCs compared to the activity on the membranes of normal RBCs (Krugliak et al. 2000). The effects of dermaseptin S4 and its derivatives on malaria parasites were further investigated with respect to stage specificity (Dagan et al. 2002; Efron et al. 2002).

2.7 Gambicin

Gambicin (amino acidic sequence in Table 7.4) was first isolated from the conditioned medium of the *Anopheles gambiae* cell lines 4a-3A and 4a-3B (Vizioli et al. 2001a). The activity on different microorganism was tested and gambicin inhibited the growth of *Micrococcus luteus*, *E. coli* SBS363, and *Neurospora crassa*. Gambicin was also effective against *P. berghei* ookinetes. Moreover, as other AMPs, the expression of gambicin was enhanced by *Plasmodium* infection. In 2006, Dong and colleagues studied the immune response of *Anopheles gambiae* to the human *P. falciparum* or the murine *P. berghei* malaria parasites (ookinete stage) by DNA microarray analyses and RNAi gene silencing assays (Dong et al. 2006). The two species induced the expression of different genes and the authors confirmed the different ability to modulate the mosquito immune response to malaria.

2.8 Other Antimalarial AMPs

A possible classification of antimalarial AMPs is described by Bell (2011). Cationic, amphipathic “host-defense” peptides such as defensins and cecropins were treated in this chapter. Other membrane-active peptide antibiotics, such as gramicidin, have high activity on *Plasmodium* in the nanomolar range but they are also toxic for mammalian cells. Cyclosporine A, representative of the hydrophobic peptides class, was studied in all the *Plasmodium* stages and is active especially in the murine models. Thiopeptides, such as thiostrepton, have antimalarial activity but quite high IC_{50}. Some other naturally occurring or synthetic peptides have been shown to have antimalarial activity. The antiprotozoal activity of AMPs from amphibian origin was reviewed by Rivas and colleagues (Rivas et al. 2009).

3 Potential Application of AMPs in Malaria Research and Control

AMPs have been investigated as potential drugs against different *Plasmodium* stages and in particular against the erythrocytic phase, which is largely associated with the symptoms of the disease (Khadjavi et al. 2010). Recently, the interest of the research community and health authorities has moved toward elimination/eradication programs. To reach this ambitious goal, blocking transmission becomes an important step and AMPs could be reevaluated for their activities against the sexual stages, occurring throughout the mosquito vector.

The most described application for AMPs in malaria is mosquito and parasite engineering to reduce or interrupt malaria parasite transmission (Carter and Hurd 2010). Possani and colleagues proposed to insert the genetic code for bioactive peptides extracted from scorpion venom (scorpine mainly) into *Anopheles* mosquitoes to make them resistant to malaria infection (Possani et al. 2002). The authors started from evidence from the literature that *P. gallinaceum* ookinetes injected in *Drosophila melanogaster* were able to develop into sporozoites identical to those obtained in mosquitoes and, as expected, able to infect chickens. They designed a strategy involving *Drosophila* as an investigation tool to study AMPs toxicity against insects and *Plasmodium* development within the insect. However, the authors did not go beyond the design of this strategy and did not show results of the transgenic work, only referring to preliminary, encouraging results.

A big issue with these transgenesis approaches is represented by the ethical concern in releasing transgenic insects in the environment.

A different approach is to engineer those microorganisms living in mosquitos’ midgut. In this case, the aim is to make the vector resistant to malaria parasites. *Metarhizium anisopliae* fungi were transfected with salivary gland and midgut peptide 1 (SM1), scorpine, or an antibody that agglutinates sporozoites. Mosquitoes were infected with this microorganism, leading to a reduction of sporozoites production by more than 50 %, with the best result, 98 % reduction, obtained with scorpine (Fang et al. 2011).

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