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REVIEW

Antimicrobial peptides for leishmaniasis

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Leishmaniasis is a parasitic disease that is endemic to American, African, Asian and southern European countries. More than 350 million individuals in 88 countries are at risk of infection from this neglected tropical disease. No effective vaccinations are available against leishmaniasis, and control of the disease relies entirely on toxic drug treatments, some of which were developed as early as the 1940s. As parasite resistance becomes more prevalent, there is increasing concern that currently used drugs will soon become ineffective treatments. Consequently, an urgent need exists to develop new classes of compounds that are active against drug-resistant strains of Leishmania. This review summarizes research aimed at investigating the potential development of antimicrobial peptide-based antileishmanial agents.

Keywords AMP, antileishmanial, antimicrobial peptide, drug resistance, *Leishmania*, leishmaniasis, therapy

Introduction

Insect vector-borne protozoan parasites of the order *Kinetoplastida* cause a range of neglected human diseases, most notably leishmaniasis, Chagas disease and African sleeping sickness. Globally, these diseases lead to greater than 100,000 deaths annually, and cause the loss of more than 4 million disability-adjusted life years (ie, healthy years of life lost as a result of premature death and disability) [1]. The mortality and morbidity caused by leishmaniasis (both visceral leishmaniasis [VL] and cutaneous leishmaniasis [CL]) is only surpassed among parasitic diseases by malaria and lymphatic filariasis [2]. The kinetoplastid parasites that cause leishmaniasis are endemic in tropical and subtropical regions and, therefore, disproportionately affect the health and economic viability of most of the developing world. *Leishmania* spp – the causative agents of leishmaniasis – infect more than 12 million individuals in five continents, and are endemic in 88 countries; thus, more than 350 million individuals are at risk of infection [1]. Instances of leishmaniasis are also not uncommon in certain regions of North America and southern Europe. In addition, the spread and severity of the disease is exacerbated by its status as a possible coinfection in patients with AIDS and the overlap in prevalence of HIV and *Leishmania* [3].

The treatment of leishmaniasis, as well as trypanosomiasis (another parasitic disease caused by kinetoplastids), is difficult [4,5]. VL, the most serious form of leishmaniasis, requires an extended, costly course of drug treatment. In addition, *Leishmania* drug resistance has recently become evident, including resistance against miltefosine, an oral alkylphospholipid that is the most recently registered drug for use against VL [6,7]. This worrying trend has led to the use of more toxic drugs in the treatment of this parasitic infection. These factors regarding currently available treatments, combined with the lack of effective prophylactic vaccines against leishmaniasis infection, make the discovery of new therapeutic agents a priority; this need has also been recognized by the WHO [8].

Leishmania spp exhibit a digenetic lifecycle, alternating between flagellated, extracellular promastigote forms in the digestive tract of the sandfly vector and, following a bite, aflagellate intra-macrophage amastigote forms in the mammalian host. The changes in environment experienced by the parasite during the course of this lifecycle are dramatic, as reflected by a radical reorganization of the cell surface (the interface with the host). Insect-stage promastigote forms possess a thick glycocalyx consisting of glycosylphosphatidylinositol (GPI)-anchored proteins and glycoconjugates, the most abundant being lipophosphoglycan (LPG) [9], which has been demonstrated to play a central role in infection [10]. In contrast to the insect stages of *Leishmania* spp, intracellular amastigotes downregulate the expression of LPG (and other surface macromolecules) and lack a conspicuous surface coat; however, similar to promastigotes, the amastigotes possess a plethora of free GPI-anchored glycolipids,

termed glycoinositolphospholipids (GIPLs). In addition, *Leishmania* amastigotes sequester host glycolipid, which is then displayed on the surface of the parasite [9]. The mode by which this relatively minimalist glycocalyx protects the amastigote from the degradative action of host macrophages remains unclear. However, when investigating potential therapies for leishmaniasis, it is important to consider the major changes that occur during differentiation of the *Leishmania* parasites with respect to surface determinants, and how these changes may be exploited in a therapeutic context.

Currently available therapies for leishmaniasis

As noted, no vaccine against *Leishmania* spp is currently available, and several issues must be addressed before such a vaccine can be developed successfully [11]. Therefore, the treatment of leishmaniasis relies entirely on chemotherapy. Pentavalent antimonials, such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), are the most commonly used first-line drugs in the treatment of both VL and CL [12,13]. These drugs have been in clinical use for more than 70 years, despite being associated with severe side effects, such as renal failure and cardiotoxicity [14], and the current requirement for intravenous administration [15]. However, the most urgent and concerning issue regarding the use of pentavalent antimonials in the treatment of leishmaniasis is the emergence of drug resistance [6]. Fortunately, such resistance has not yet been widespread, and remains isolated to the North Bihar region of India where VL is endemic [16]. This emergence of drug resistance has primarily been attributed to the misuse of these drugs, which are available freely as OTC agents in this region [17]. Furthermore, *Leishmania* spp parasite resistance to the pentavalent antimonials can be induced easily in the laboratory [18]. Combined, these observations have led to concerns that the antimonials may soon become ineffective. Second-line drugs, including amphotericin B (Fungizone) [19] and the aromatic diamidine pentamidine [20], have consequently been used increasingly in the treatment of both CL and VL. Both of these agents have been in use for more than 30 years and, similar to the antimonials, induce severe side effects [14]. A lipid formulation of amphotericin B (AmBisome) has also been developed [21], which demonstrates higher efficacy and lower toxicity compared with the original drug [13]. However, although the price of AmBisome in VL endemic, resource-limited regions has been reduced via a partnership between the WHO and the manufacturer Gilead Sciences Inc [22], the cost remains high for developing nations [23]. The aminoglycoside paromomycin (Humatin) is used as a topical, second-line therapy for CL, and is also used as a low-cost parenteral treatment for VL in India [24]. Parasite resistance towards second-line drugs for leishmaniasis has not been confirmed conclusively but there are indications, based on decreased pathogen susceptibility following patient relapse, that this may occur in the near future [25]. As a result, combination therapies are being evaluated; sodium stibogluconate plus paromomycin has been demonstrated to be effective against VL in clinical trials conducted in both Africa and India [24]. However, miltefosine, the only orally administered treatment available for VL, is also effective against CL [26,27]. Through its dual effectiveness – and being the only antileishmanial agent to have completed phase IV clinical trials – miltefosine is the most likely candidate to replace antimonials as the first-line drug treatment for leishmaniasis in the next decade [12]. Unfortunately, similar to the other antileishmanials, miltefosine is also associated with severe side effects [28] and, in addition, is teratogenic, thus precluding administration during pregnancy [29]. Furthemore, parasite resistance to miltefosine can emerge easily, as has been readily observed *in vitro* [30]. Data from controlled clinical trials revealing resistance have also suggested that miltefosine may only be effective as an antileishmanial agent for a short time period [31].

Thus, all currently used first-line and second-line drugs for the treatment of leishamaniasis have issues in terms of toxicity, cost and/or administration. Furthermore, the prospect of the emergence of widespread drug resistance indicates that there is an urgent need to develop new and effective therapies for leishmaniasis. This review summarizes research aimed at investigating the potential development of antimicrobial peptide-based antileishmanial agents.

Antileishmanial properties of antimicrobial peptides

Antimicrobial peptides (AMPs) have been identified in a wide variety of organisms, including bacteria [32], plants [33], insects [33,34] and mammals [35], and the number of new AMPs being isolated, characterized and collated in databases such as AMPer [36] continues to increase rapidly. AMPs are produced in response to infection, and represent key components of the innate immune system [37]. They can vary both in size and structure across species and, in general, are cationic, although examples of anionic AMPs have also been reported [38]. Many AMPs also exhibit broad-spectrum antibacterial activity, even against multidrug-resistant bacterial strains, and have low cytotoxicity to mammalian cells. Over the past 20 years, these attributes have precipitated considerable research efforts directed toward the development of AMP-based antibiotics [39]. In addition to displaying potent antibacterial properties, increasing numbers of AMPs have also been demonstrated to have biological activity against a range of therapeutic targets, such as cancer cells [40]. Furthermore, AMPs can possess antiviral [41], antifungal [42], and even spermicidal activity [43].

AMPs are also excellent candidates for the design of novel antiprotozoal agents; however, this possibility has not been fully investigated or exploited [44]. The reasons for the lack of research in this area can be attributed, in part, to the fact that many parasitic infections are most prevalent in developing countries, and that the development of

new antiparasitic agents is regarded as a relatively low priority by pharmaceutical companies. However, AMPs possess several attractive attributes as potential antileishmanial agents, including the lack of toxicity toward mammalian cells at concentrations required to kill *Leishmania* parasites. Furthermore, studies indicate that AMPs exert their antileishmanial activity via a disruption of biological membranes [39], a mechanism that is considerably different to those used by the currently available drugs. This novel mechanism of action may provide AMP-based antileishmanial agents with the ability to overcome the resistance observed with existing drugs, thus allowing their potential use in combination therapies.

The first AMP reported to exhibit antileishmanial activity was a dermaseptin [45]. Since this discovery, AMPs isolated from a variety of sources have demonstrated activity against a range of *Leishmania* species (Table 1) [45-61]. The largest subgroup of AMPs to be screened for antileishmanial activity were isolated from amphibian sources. This source of peptides is not surprising, as many amphibian AMPs, such as temporins A and B [51], are relatively short and, thus, are easy to prepare and modify and are good candidates for drug development. Recently, the first examples of plant-derived AMPs to exhibit antileishmanial activity were reported [60]. However, perhaps the most interesting AMP screened against *Leishmania* spp is the *Phlebotomus duboscqi* defensin [58]. This peptide was isolated from the hemolymph of *Leishmania major*-infected *P duboscqi* sandflies, the natural vector for the transmission of leishmaniasis in the Old World. *P duboscqi* defensin exhibited an IC_{50} value of 68 to 85 μM against *L major* promastigotes, thus suggesting a potential role for this AMP within *Leishmania*-infected sandflies. The investigators speculated that the defensin was involved in the control of parasite numbers within the sandfly midgut [58].

The number of AMPs that have been screened against *Leishmania* spp (Table 1) is relatively small compared with those that have been tested for activity against bacteria and fungi. In addition to the economic and social reasons for the lack of progress in this area, technical difficulties associated with cultivating *Leishmania* parasites, particularly the clinically relevant amastigote forms, may also play a role in this difference. As shown in Table 1, most of the AMPs screened thus far have yet to be tested against amastigote forms of *Leishmania*. As discussed in the *Introduction*, there are considerable differences in plasma membrane composition between the amastigote and promastigote forms of the parasite [9]. Given that the plasma membrane is the main biological target of AMPs, it is likely that the activity of these agents will vary against each of the *Leishmania* lifecycle stages. Consequently, it is difficult to ascertain whether AMPs that have been screened against *Leishmania* promastigotes will demonstrate similar effects against amastigotes, an attribute that would render them candidates for further drug development.

The mode of action of antimicrobial peptides

In general, there are two distinct mechanisms via which AMPs exert their biological activity [39]. First, AMPs can cause the disruption of the plasma membrane of the target organism. This mechanism is the most commonly used by AMPs. Second, several AMPs can traverse the plasma membrane and act against intracellular targets. Detailed studies regarding the mode of action that AMPs use against various *Leishmania* spp have not been conducted exhaustively, but both of these mechanisms have been observed to date. For example, transmission electron microcopy confirmed that membrane disruption occurred in *Leishmania* promastigotes and amastigotes treated with temporins A and B [51]. Similar studies also confirmed that other AMPs, such as bombinins, acted via this mechanism [53]. This primary mode of action may provide AMP-based drugs with one major advantage over current treatments: for drug resistance to develop against such AMP-based drugs, *Leishmania* parasites would need to alter their membrane structure and/or phospholipid composition, and such modifications would be difficult to accomplish.

Two AMPs, indolicidin [57] and histatin-5 [61], have been demonstrated to exert antileishmanial activity by acting on intracellular targets. Indolicidin had the ability not only to disrupt the parasite membrane, but also to induce autophagic cell death [57]. Histatin-5 did not cause severe disruption to the plasma membrane, but was active against both *Leishmania donovani* promastigotes and *Leishmania pifanoi* axenic amastigotes [61]. The intracellular accumulation of histatin-5 in both of these species was confirmed using confocal microscopy and labeled peptides. Further experiments indicated that histatin-5 targeted the mitochondrion, causing bioenergetic failure and leading to non-apoptotic cell death. Interestingly, the D-isomer of histatin-5 was more active against both promastigotes and amastigotes compared with the natural isomer, suggesting that the intracellular target involved in mediating antileishmanial action does not involve chiral recognition. The investigators proposed that the enhanced biological activity of the D-isomer resulted from resistance to proteolytic cleavage. This hypothesis was supported experimentally by data demonstrating accumulation of the D-isomer inside the parasite at concentrations that were higher than those of the natural histatin-5 [61].

Methods to enhance the antileishmanial activity of peptides

The inherent susceptibility of peptides toward chemical and enzymatic degradation is a major hurdle that needs to be overcome for the successful development of peptide drugs. Several different approaches, including the incorporation of non-proteinogenic amino acids, backbone cyclization and the use of encapsulating delivery strategies, have been developed in an effort to circumvent the issue of degradation [62-64]. In particular, modifications have been performed on AMPs with antileishmanial activity as part of drug development efforts.

Table 1. Selected antimicrobial peptides with activity against *Leishmania* spp.

Peptide	Source	Sequence	Activity (% growth inhibition; concentration in μM)		Reference
			Promastigotes	Axenic amastigotes	
Dermaseptin-S1	Amphibian	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	*L major* (50; 4.5) *L mexicana* (50; 1.5)	n/a	[45,46]
Dermaseptin-S4	Amphibian	ALWMTLLKKVLKAAAKALNAVLGANA	*L major* (50; 2.0)	n/a	[45,47]
Dermaseptin-01	Amphibian	GLWSTIKNVGKEAAIAAGKAALGAL-NH_2	*L amazonensis* (100; 23.4)	n/a	[48]
Dermaseptin-H3	Amphibian	GLWSTIKNVGEAAIAAGKAALGAL-NH_2	*L amazonensis* (78; 13.5)	n/a	[48]
Cecropin A	Insect	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2	*L donovani* (50; > 50.0)	n/a	[49]
Melittin	Insect	GIGAVLKVLTTGLPALISWIKRKQQ-NH_2	*L donovani* (50; 0.3)	n/a	[49]
Phylloseptin-1	Amphibian	FLSLIPHAINAVSAIAKHN-NH_2	*L amazonensis* (50; 50)	n/a	[50]
Temporin A	Amphibian	FLPLIGRVLSGIL-NH_2	*L donovani* (50; 8.4)	*L pifanoi* (50; 14.6)	[51]
Temporin B	Amphibian	LLPIVGNLLKSLL-NH_2	*L donovani* (50; 8.6)	*L pifanoi* (50; 7.1)	[51]
Temporin -1Sa	Amphibian	FLSGIVGMLGKLF-NH_2	*L infantum* (50; 18.1)	*L infantum* (50; 22.8)	[52]
Bombinin H2	Amphibian	IIGPVLGLVGSALGGLLKKI-NH_2	*L donovani* (50; 7.3)	*L pifanoi* (50; 11)	[53]
Bombinin H4	Amphibian	L*i*GPVLGLVGSALGGLLKKI-NH_2[a]	*L donovani* (50; 1.7)	*L pifanoi* (50; 5.6)	[53]
Tachyplesin-1	Crustacean	KWCFRVCYRGICYRRC	*L braziliensis* (100; 12.5)	n/a	[54]
Skin polypeptide YY	Mammal	YPPKPESPGEDASPEEMNKYLTALRHYINLVTRQRY-NH_2	*L major* (100; 5.9)	*L major*[b] (100; 6.2)	[55]
Decoralin	Insect	SLLSLIRKLIT	*L major* (50; 72.0)	n/a	[56]
Indolicidin	Bovine	ILPWKWPWWPWRR	*L donovani* (50; 35)	n/a	[57]
P duboscqi defensin	Insect	AT<u>C</u>DLLSAFGVGHAA<u>C</u>AAH<u>C</u>IGHGYRGGY<u>C</u>NSKAV<u>C</u>T<u>C</u>RR[c]	*L major* (50; 68-85)	n/a	[58]
Gomesin	Insect	Z<u>C</u>RRL<u>C</u>YKQR<u>C</u>VTY<u>C</u>RGR[c,d]	*L amazonensis* (50; 2.5)	n/a	[59]
PTH1 defensin	Plant	RNCKSLSHRFKGPCTRDSNC	*L donovani* (50; 33.4)	n/a	[60]
Histatin-5	Mammal	DSHAKRHHGYKRKFHEKHHSHRGY	*L donovani* (50; 7.3)	*L pifanoi* (50; 14.4)	[61]

[a] ***i*** D-Allo-isoleucine; [b] testing was conducted using *ex vivo* rather than axenic amastigotes; [c] underline indicates disulfide bridge between atoms; [d] **Z** pyroglutamic acid
P duboscqi *Phlebotomus duboscqi,* **PTH1** potato defensin

In the case of the wasp-venom AMP decoralin, a simple amidation of the carboxy-terminus resulted in an increase in activity against *L major* promastigotes [56]. Amidation of the carboxy-terminus is a widely applied strategy in SAR studies in peptide chemistry. This modification can enhance stability against protease degradation, or can help stabilize the α-helical secondary structure that is required for plasma membrane disruption by many AMPs. The preparation of hybrid peptides derived from two AMPs with established antileishmanial activity has also been explored. A hybrid of cecropin A and melittin [CA(1-8) M(1-18)] demonstrated a higher level of activity against *L donovani* promastigotes than cecropin A [49]. However, the activity of the hybrid peptide was reduced compared with melittin, thus rendering the significance of these data unclear. Additional studies have revealed that acylation of the hybrid peptide CA(1-7)M(2-9) with various fatty acid chains [C(2) to C(16)] could be used to enhance activity against *Leishmania* parasites [65]. Notably, activity against amastigotes with the hybrid peptide increased by 15-fold, suggesting that such a modification could be useful to apply to other antileishmanial AMPs. Interestingly, the acylated hybrid peptide Oct-CA(1-7)M(2-9) was demonstrated to be a safe and effective treatment for naturally acquired canine CL when administered by intravenous injection [66]. These results are encouraging, but given the restrictions and costs of such *in vivo* experiments similar proof of concept studies are unlikely to be conducted routinely with other promising AMP candidates.

Another factor that may be prohibitive in the development of AMP-based drugs is the high production cost involved compared with small-molecule drug development. However, this cost could be reduced by identifying the minimal peptide sequence that is capable of retaining biological activity. By screening short fragments of a known mussel defensin, Bernard and colleagues identified a cyclic peptide of only nine amino-acid residues that exhibited an ID_{50} value of 12 μM against *L major* promastigotes [67]. When adopting such an approach, it is important to investigate all potential cytotoxic properties of the peptides against mammalian cells, as a reduction in size is often accompanied by a reduction in membrane selectivity. Some SAR data have also been reported for the AMPs magainin 2 [68] and dermaseptin S4 [47,69], but these studies were performed only against promastigotes. Therefore, it is unclear whether the observations made could be used successfully to assist in the rational design of AMP analogs with enhanced activity against pathogenic *Leishmania* amastigotes.

Although some interesting observations have arisen from these modifications, there are still no specific guidelines that can be used for the optimal selection of AMP candidates. More detailed SAR data are required, particularly with respect to *Leishmania* amastigotes, so that the properties and peptide motifs that are required for biological activity can be further established.

Potential challenges to antileishmanial antimicrobial peptide drug development

Two potential issues are pertinent to the future development of AMP-based antileishmanials. First, the surface metalloprotease GP63, also known as leishmanolysin, may protect *Leishmania* from AMPs. Notably, some AMPs tested against leishmanolysin-knockout mutants of *L major* promastigotes displayed higher levels of leishmanicidal activity [70]. However, it is unlikely that leishmanolysin activity is a significant factor in the resistance of different *Leishmania* spp toward all AMPs, as certain AMPs display reduced activity toward the clinically relevant amastigote forms of the parasite, despite the fact that these forms express minimal levels of leishmanolysin [71]. A second potential hurdle relates to the intracellular targeting by *Leishmania* parasites (which generally target macrophages). As a result, any AMP drug will need to have the ability to cross several physical barriers to reach the parasite, and will need to display little or no toxicity toward the host cell. The issue of toxicity is perhaps the least problematic, as several of the AMPs that have been screened (Table 1) have demonstrated low cytotoxicity toward macrophages [61]. A more significant challenge may relate to the identification of AMPs for which promising leishmanicidal activity against axenic extracellular parasites can be transferred to *Leishmania*-infected *in vitro* macrophage models. Currently, research investigating AMPs in macrophage models has been limited [51,61], although quantitative, but technically demanding, assays are available [72]. However, if AMPs are to be developed further as antileishmanial agents, more detailed information regarding the modes of action and activity of AMPs against *Leishmania*-infected macrophages will be required.

Conclusion

The number of AMPs screened against *Leishmania* species has been steadily increasing. However, despite this increase, the total number of AMPs that have been tested against this protozoan parasite remains low compared with the number of AMPs that have been screened against bacterial species. Given this relative paucity of data, it remains difficult to derive general conclusions that would aid researchers in the rational selection and design of antileishmanial AMPs. In addition to the lack of SAR data, many of the published studies in the field have focused on the insect-stage, promastigote form of *Leishmania* rather than the clinically relevant intra-macrophage amastigote form. Given the evident difference in the surface architecture of these two lifecycle stages, it is important to screen AMPs against amastigotes as well as promastigotes, despite the inherent challenges. The mammalian stage of the *Leishmania* lifecycle that occurs within macrophages presents a hurdle to the development of any antileishmanial drug, including AMPs. In addition to concerns regarding host cytotoxicity, any compound would have to penetrate the phagolysosome within which the amastigotes persist. Few AMPs have been assessed for their ability to inhibit or clear

amastigotes from infected tissue culture macrophages or in animal models. However, those AMPs that have been tested in these systems have demonstrated encouraging results, and further research is therefore warranted.

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