Studies on the antileishmanial properties of the antimicrobial peptides temporin A, B and 1Sa

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Given the paucity and toxicity of available drugs for leishmaniasis, coupled with the advent of drug resistance, the discovery of new therapies for this neglected tropical disease is recognised as being of the utmost urgency. As such antimicrobial peptides (AMPs) have been proposed as promising compounds against the causative *Leishmania* species, insect vector-borne protozoan parasites. Here the AMP temporins A, B and 1Sa have been synthesised and screened for activity against *Leishmania mexicana* insect stage promastigotes and mammalian stage amastigotes, a significant cause of human cutaneous disease. In contrast to previous studies with other species the activity of these AMPs against *L. mexicana* amastigotes was low. This suggests that amastigotes from different *Leishmania* species display varying susceptibility to peptides from the temporin family, perhaps indicating differences in their surface structure, the proposed target of these AMPs. In contrast, insect stage *L. mexicana* promastigotes were sensitive to two of the screened temporins which clearly demonstrates the importance of screening AMPs against both forms of the parasite. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: *Leishmania mexicana*; cutaneous leishmaniasis; drug therapy; antimicrobial peptides; temporins

Introduction

Leishmaniasis is one of the World Health Organization's 16 neglected tropical diseases and is endemic in over 80 countries worldwide. It is caused by *Leishmania* species, insect vector-borne protozoan parasites, and affects an estimated 12 million people a year with a further 350 million people living at risk of infection [1,2]. At the present time a vaccine to prevent leishmaniasis is not available despite considerable research effort in this area. Therefore, the treatment of leishmaniasis currently relies entirely on a highly limited arsenal of chemotherapeutics. The treatment of both visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) largely rely on the pentavalent antimonials such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) [3,4]. These have been in clinical use for over 70 years despite their associated problems, including severe side effects such as renal failure and cardiotoxicity [5], and the fact that they require parenteral administration [6]. However, the most pressing issue surrounding the use of pentavalent antimonials in the treatment of leishmaniasis comes from the emergence of drug resistance [7]. Fortunately this has not, so far, been widespread and remains isolated to the north Bihar region of India where VL is endemic [8]. However, *Leishmania* spp resistance to these drugs can be easily induced in the laboratory [9]. Together these observations have led to concerns that the antimonials may soon become redundant as effective antileishmanials. Second-line drugs employed in the treatment of both CL and VL include Amphotericin B (Fungizone) [10] and the aromatic diamidine Pentamidine [11]. Both of these drugs have been in clinical use for over 30 years and, like the antimonials, induce severe side effects and require parenteral

administration [5]. Parasite resistance towards these second-line drugs has not yet been conclusively confirmed in the field but there are indications that it may only be a matter of time [12]. Given the issues surrounding the use of the current first- and second-line drugs to treat leishmaniasis there is clearly a need to develop new and effective therapies to treat both CL and VL.

In recent years one class of compounds that have been investigated as antileishmanials is the antimicrobial peptides (AMPs) [13,14]. AMPs isolated from a range of organisms have been screened but to the best of our knowledge there are no reports of any of these having been tested against the clinically relevant amastigote form of *Leishmania mexicana*. *L. mexicana* infection causes CL, which although non-fatal results in ulcerated lesions that can cause severe disfigurement of skin tissue leading to social exclusion in many affected communities. In addition, CL lesions leave the patient at risk of severe secondary bacterial

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infections. Given that several AMPs have been investigated as potential treatments of other skin diseases [15] we sought to explore any application that they may have in the treatment of CL. Herein, we report the first examples of AMPs from the temporin family being screened against *L. mexicana,* a causative agent of CL.

Materials and Methods

Reagents and Solvents

Rink Amide AM resin (200–400 mesh, 0.62 mmol g^{-1} loading) and all Fmoc-protected amino acids used were purchased from Novabiochem, Merck (Nottingham, UK). PyBOP^{TM} was purchased from CEM (North Carolina, USA). HPLC-grade solvents were obtained from Fischer Scientific (Loughborough, UK) and all other reagents from Sigma-Aldrich (Gillingham, UK). Side chain protecting groups utilised for the Fmoc amino acids were *t*Bu for Ser, Pbf for Arg, Boc for Lys and Trt for Asn.

Peptide Synthesis

Peptides were prepared via Fmoc SPPS using a CEM microwave synthesiser. Peptides were synthesised on a 0.31 mmol scale (500 mg of Rink Amide AM resin). Fmoc amino acids (4.0 equiv. with respect to the resin) were coupled using PyBOP (3.9 equiv. with respect to the resin), NMM (4.0 equiv. with respect to the resin) and the CEM Microwave (10 min, 20 W, 75 °C). Fmoc deprotection was carried out using piperidine 20%, DMF and the CEM microwave (3 min, 20 W, 75 °C). Final peptide cleavage was achieved using TFA : TIPS : H$_2$O (9 ml:750 µl:750 µl) at 25 °C with stirring for a minimum of 4 h.

Peptide Purification and Characterisation

Crude peptides were purified by semi-preparative RP-HPLC (Waters, Milford, USA, Mass Directed Prep System instrument fitted with a 3100 Mass detector and diode array detector) using a 19 × 100 mm Waters XBridge RP-column; flow rate = 17 ml min^{-1}; linear gradient elution 10–90% of solvent B over 13.5 min (A = 0.1% formic acid in H$_2$0, B = 0.1% formic acid in MeOH). Peptides were characterised by analytical RP-HPLC (Waters Mass Directed Prep System instrument fitted with a 3100 Mass detector and diode array detector) using a 4.6 × 100 mm Waters XBridge RP-column; flow rate = 1 ml min^{-1}; linear gradient elution 10–90% of solvent B over 16.5 min (A = 0.1% formic acid in H$_2$0, B = 0.1% formic acid in MeOH). Peptide identities were confirmed by MALDI-TOF mass spectra analysis (Applied Biosystems^{TM}, Carlsbad, USA, Voyager-DE STR instrument operating in positive ion mode] using an α-cyano-4-hydroxycinnamic acid matrix.

Leishmania Culture

Leishmania mexicana (MNYC/BZ/62/M379) parasites were maintained at 26 °C in Schneider's Drosophila media (Sigma-Aldrich, Gillingham, UK) supplemented with heat-inactivated foetal bovine sera (15% for promastigotes and 20% for amastigotes; Biosera Ltd, East Sussex, UK). Promastigotes were transformed into axenic amastigotes by a pH and temperature shift as previously described [16]. Cells were counted using a Neubauer Improved Haemocytometer.

Table 1. Sequence, chemical and physical data for temporins

Peptide	R_t (HPLC)	M_r (Calc)[a]	M_r (obs)[a,b]
Temporin A FLPLIGRVLSGIL-NH$_2$	9.05	1395.9	1397.0
Temporin B LLPIVGNLLKSLL-NH$_2$	9.41	1390.9	1413.8
Temporin 1Sa FLSGIVGMLGKLF-NH$_2$	9.24	1379.8	1380.5

[a] The calculated (calc) and observed (obs) masses are monoisotopic.
[b] M_r (obs) are the observed protonated [M+H]$^+$ or sodiated [M+Na]$^+$ species as obtained by MALDI-TOF-MS.

Cytotoxicity Assay

Cytotoxicity analyses were performed in 96-well plates (Nunc, Langenselbold, Germany) using Alamar Blue (Invitrogen, Paisley, UK) with some modifications to the published protocol [17].

Briefly, following optimisation of the assay system, 100 µl of both promastigote and amastigote *L. mexicana* at 4 × 10^5 ml^{-1} were incubated with compounds in triplicate (amphotericin B was used as a positive control, and untreated parasites with vehicle (DMSO) as a negative control) for 24 h before incubation with Alamar Blue (Invitrogen) for 4 h prior to assessing cell viability using a fluorescent plate reader (Biotek, Leeds, UK; 560EX nm/600EM nm). To investigate the effects of serum on the efficacy of the temporins the assay described above was modified. Briefly, promastigate and amastigote *L. mexicana* were pre-incubated with the compounds in 10 µl of serum-free media at 4 × 10^6 ml^{-1} for 1 h before the addition of 90 µl of complete media [18]. All of the experiments described above were carried out on a minimum of two separate occasions to ensure a robust data set was collected.

Results and Discussion

Temporin A (**1**), temporin B (**2**) and temporin 1Sa (**3**) were chosen for this study as they had previously been shown to have activity at low micro-molar concentrations against other *Leishmania* species [18,19]. Peptides **1–3** were prepared using a CEM microwave-assisted Fmoc solid-phase procedure and their physical data are presented in Table 1.

Given the ease of cell culture, AMPs have most commonly been screened against insect stage, promastigote *Leishmania* [13]. However, in order to fully assess the efficacy of any compound, it must be assayed against pathogenic, mammalian stage amastigotes. Therefore to facilitate comparative analyses of the antileishmanial action of selected, synthesised temporins, it was chosen to utilise *Leishmania mexicana*, where axenic culture of both lifecycle stages is long established [16].

The Alamar Blue viability assay has previously been validated for microtitre plate-based analyses of promastigote *L. major* [20]; and *L. donovani, L. tropica* [21] and *L. mexicana* [17] promastigotes and amastigotes. In addition it has been utilised for screening *L. amazonensis, L. braziliensis* and *L. chagasi* promastigotes [22]. However, to facilitate comparison of the efficacy of the synthesised AMPs against both promastigote and amastigote axenic *L. mexicana*, the Alamar Blue assay was optimised to allow both lifecycle stages to be screened under equivalent conditions. To this end, and in the light of previous studies,
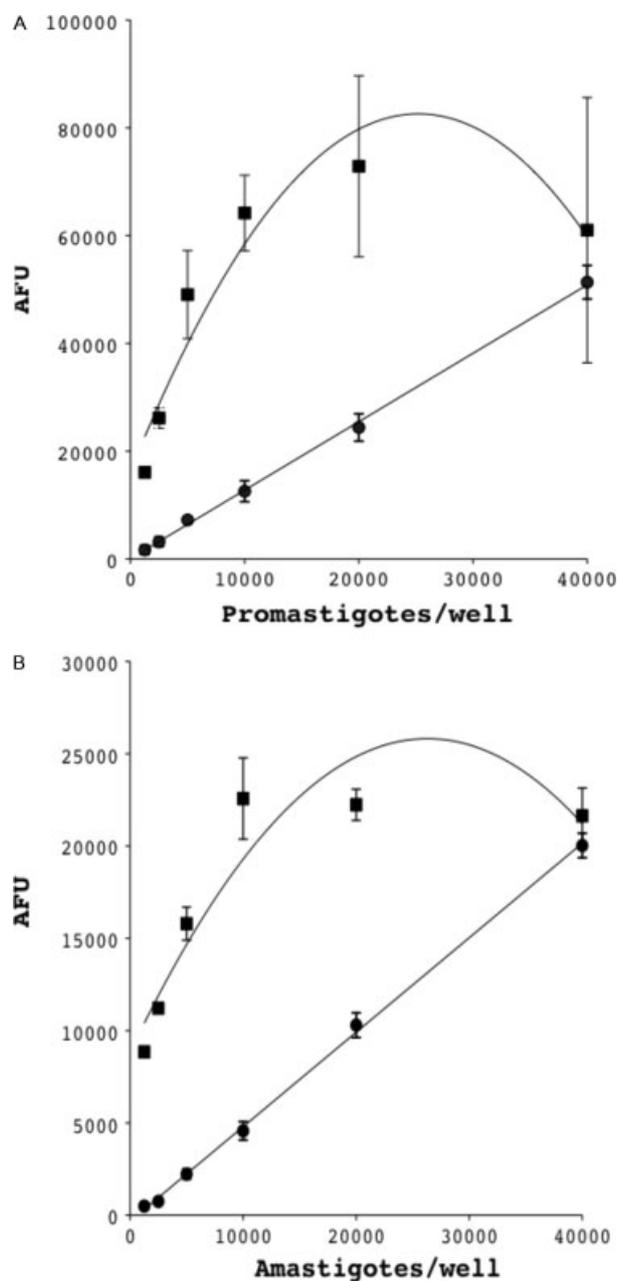
Figure 1. Twofold serial dilutions starting at 4×10^5 ml^{-1} of *Leishmania mexicana* promastigotes (A) and amastigotes (B) pre-incubated for 24 h before the addition of Alamar Blue and further incubation for 4 h (●) or 24 (■) h. Note the linear correlation between cell number and fluorescent readout with a 4-h incubation ($r^2 = 0.999$ in A and B). The lower signal seen for amastigotes (B) reflects their relatively slow rate of replication [17]. All points in triplicate with standard deviation indicated. AFU – arbitrary fluorescence units.

Figure 2. Using the Alamar Blue assay system, *L. mexicana* promastigote (A) and amastigote (B) viability in the presence of various concentrations of temporins A, B and 1Sa was determined with respect to untreated, negative controls. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 or 3 independent experiments performed in triplicate. Standard deviation indicated.

serial dilutions (starting at 4×10^4 cells well^{-1}) of both lifecycle stages were incubated for 24 h in 96-well plates at appropriate temperatures before the addition of Alamar Blue at 10% v/v for either 4 or 24 h and the subsequent assessment of cell viability by fluorescent readout (Figure 1). The data clearly show that a direct correlation of parasite numbers with readout was apparent in the case of both lifecycle stages incubated for 4 h after the addition of Alamar Blue ($r^2 = 0.999$ for both promastigotes and amastigotes). However, deviation from this
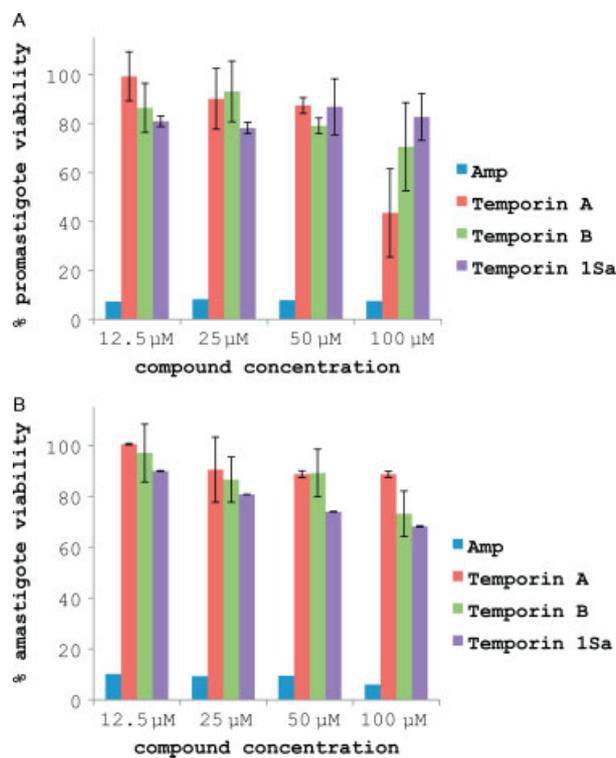
linear relationship was apparent at higher cell concentrations after 24 h incubation with the indicator, particularly with respect to the promastigotes with the correlation breaking down at more than 5×10^3 cells well^{-1} (Figure 1A). A similar pattern has previously been noted with other *Leishmania* spp. [21]. To ensure a direct correlation between readout and cell number in the AMP screen of both lifecycle stages of *L. mexicana*, a starting concentration of 4×10^5 ml^{-1} (4×10^4 cells well^{-1}), followed by incubation with Alamar Blue for 4 h, was employed in all subsequent experiments.

Prepared peptides **1–3** were screened against both promastigote and amastigote *L. mexicana* using the optimised Alamar Blue assay. In the presence of serum only temporin A demonstrated any significant antileishmanial activity against *L. mexicana* promastigotes (Figure 2A, 57% inhibition at 100 μM). None of the peptides (**1–3**) showed significant activity at the concentrations tested against *L. mexicana* amastigotes (Figure 2B). Given that the temporins investigated in this study (peptides **1–3**) have previously been shown to be active other *Leishmania* species [13], these results were unexpected. However, by pre-incubating the parasites with the temporins studied in the absence of serum the peptide activity profiles were altered. In this assay, which reflects the conditions employed in previous studies [18], temporins A and B demonstrated improved efficacy against promastigote *L. mexicana* (Figure 3A; 63% inhibition at 12.5 μM and 38% at 50 μM respectively). However, amastigote forms remained largely resistant to these compounds with only temporin A demonstrating any significant activity (Figure 3B; only 23% inhibition at 12.5 μM but 98% at 100 μM). Notably,
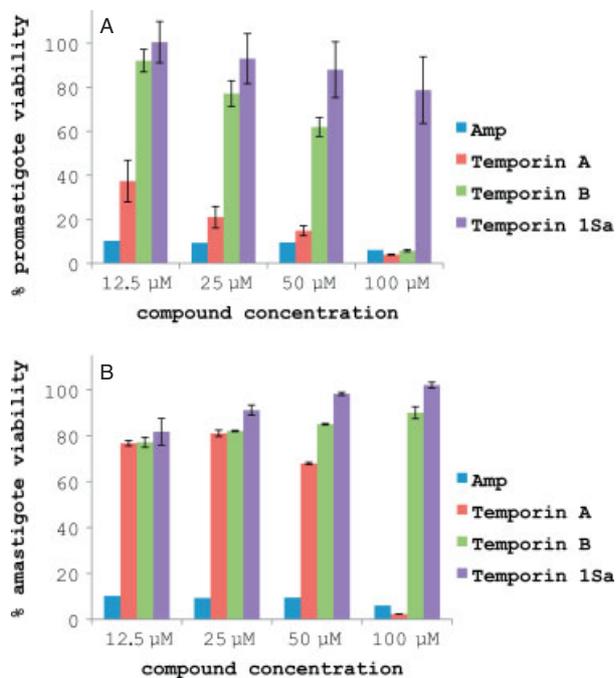
Figure 3. *L. mexicana* promastigote (A) and amastigote (B) viability after exposure to various concentrations of temporins A, B and 1Sa in the absence of serum using the Alamar Blue assay system as above. Amphotericin B (Amp) was utilised as a positive control. Data points represent the mean of 2 or 3 independent experiments performed in triplicate. Standard deviation is indicated.

temporin 1Sa remained largely inactive against both lifecycle stages.

It is notable that whilst the temporins A and B showed encouraging activity against insect stage promastigotes, a low level of activity was observed against the clinically relevant amastigote stage of *L. mexicana*. Many of the AMPs studied previously have focused on the *Leishmania* species promastigotes rather than the intra-macrophage amastigote form. Given the results obtained here it is evident that it is important to screen against amastigotes, as well as promastigotes, if lead antileishmanial AMPs are to be identified and developed.

Temporin peptides are believed to exert their antileishmanial activity through disruption of the parasite membrane [18]. Given this mode of action, it appears likely that the major differences seen in the surface structure of promastigote and amastigote *Leishmania* are responsible for the differential activity observed above [13]. However, previous studies have reported significant activity of these peptides against both promastigotes and amastigiotes of *L. pifanoi* and *L. infantum* [18,19]. This may be because of more subtle differences in the surface structure between *Leishmania* species and indicates that it may be difficult to develop an AMP that has broad-spectrum antileishmanial activity.

Conclusions

- In contrast to insect stage promastigotes, pathogenic amastigote *L. mexicana* are significantly more resistant to all the temporins tested. This demonstrates the importance of screening against both forms of the parasite.
- Results obtained suggest that amastigotes of different *Leishmania* species display varying susceptibility to peptides from

the temporin family indicating that broad-spectrum antileishmanial AMPs may be challenging to develop. In addition, the ability of AMPs to translocate the host membrane and reach intra-macrophage amastigotes has not been widely studied and remains unknown.

Supporting information

Supporting information for peptides prepared in Table 1 are provided online.

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