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Investigating the Anti-leishmanial Effects of Linear Peptoids

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Peptoids, a class of peptide mimetics, have emerged as promising anti-infective agents against a range of bacterial infections. Herein we present the first study of the antiparasitic and specifically the anti-leishmanial properties of linear peptoids. Peptoids were identified as having promising activity against *Leishmania mexicana* axenic amastigotes, a causative agent of cutaneous leishmaniasis.

The neglected tropical disease leishmaniasis is endemic in over 80 countries worldwide; 12 million people are infected, and more than 350 million are at risk, the vast majority of whom live in resource-poor settings with very limited access to health care. Distinct insect-vector-borne *Leishmania* species can cause either cutaneous leishmaniasis (CL), which leads to significant scaring and mucosal damage (mucocutaneous leishmaniasis), or visceral leishmaniasis (VL), which causes life-threatening organ damage.[1,2] There is no vaccine for this serious widespread disease, and current treatments largely rely on pentavalent antimonials such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime).[3,4] These have been in clinical use for over 70 years, despite the associated severe side effects such as cardiotoxicity and the need for parenteral administration.

The use of pentavalent antimonials in the treatment of leishmaniasis for such a long time has led to the emergence of drug resistance.[5] Therefore, second-line drugs such as amphotericin B (Fungizone) and the aromatic diamidine pentamidine have seen increasing use. Both have been in clinical use for over 30 years and, like the antimonials, induce severe side effects and require parenteral administration. The newest and only oral drug for VL, miltefosine, is also limited by its teratogenicity.[6] Parasite resistance toward these alternative drugs has not yet been conclusively confirmed in the field, but there are indications that it may only be a matter of time.[5] Given the issues surrounding the use of the current first- and second-line drugs to treat leishmaniasis, there is clearly an urgent need to develop new and effective therapies. Recently, there has been an upsurge in interest regarding the discovery and development of safer, more effective treatments for VL. In the 2012 London Declaration on Neglected Tropical Diseases, public–private partnerships pledged to help control VL by 2020.[7] In contrast, CL, a disease that causes significant and widespread medical and social problems with 1.2 million new cases each year, remains relatively ignored.

Antimicrobial peptides (AMPs) have been proposed as one potential solution to the development of new topical anti-leishmanials to treat CL.[8] However, their inherent chemical and biological instability presents a major hurdle, and only a few AMPs are currently in clinical trials as antibacterial agents.[9] In contrast, the peptide-mimicking peptoids offer a significantly better opportunity for development from bench to market.[10] Peptoids (or poly-*N*-substituted glycines) have considerable potential for development as new topical anti-infective agents, because they are cheaper to manufacture and substantially more stable under physiological conditions.[11,12–15] Their antibacterial activity against a range of Gram-positive and Gram-negative bacteria (including MRSA), selectivity for bacterial cells, and low hemolytic activity are similar to the values reported for leading AMPs.[16,17]

Herein we report the first ever screen of a linear peptoid library against *Leishmania mexicana* parasites, a causative species of CL.[18] We identified active peptoids not only against promastigotes (insect-stage parasites), but also against the clinically more relevant axenic amastigotes (mammalian-stage parasites). Our results provide a basis for further structure–activity relationship (SAR) studies to investigate the mode of action and toxicity of these new anti-leishmanial compounds.

All the peptoids in our library (Table 1) are designed around the subunit *Nx*-*Ny*-*Ny*, which is repeated either two, three, or four times to give 6-, 9-, or 12-mers, respectively. For *Ny* we chose either the chiral aromatic building block *N*spe or the achiral *N*phe, as published work has shown that bulky aromatic building blocks are needed in close proximity to induce a helical structure within the peptoid which can enhance antimicrobial activities.[14] For the positively charged building block *Nx* we chose lysine-type amines with various side chain lengths (*N*ah, *N*Lys, and *N*ae) to study their influence on anti-leishmanial activity. It has been shown that this side chain length has an influence on antimicrobial activity.[16]

The peptoids were synthesized manually on resin with the sub-monomer method using a shaker (80 °C) and 15 min for each coupling step. All peptoids were purified by RP-HPLC to obtain a library of 18 compounds (see the Supporting Information for details regarding synthesis and characterization).

Peptoids **1**–**18** were tested against *L. mexciana* promastigotes and axenic amastigotes using a modified protocol that was established in our research group.[8a] In brief, we incubated

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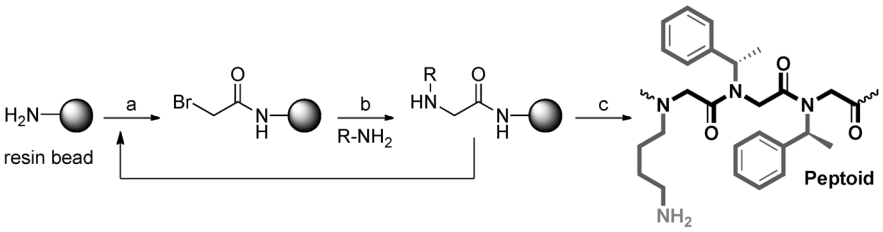
Table 1. Peptoid library synthesized on solid phase using the sub-monomer method: a) acylation step (bromoacetic acid and DIC in DMF); b) displacement step (primary amine in DMF); c) TFA cleavage.



*N*x	*N*y	Peptoid	Sequence	M_r [Da]	No. cationic side chains
*N*ah	*N*phe	**1**	(*N*ah*N*phe*N*phe)$_4$	1819.4	+4
		2	(*N*ah*N*phe*N*phe)$_3$	1368.8	+3
		3	(*N*ah*N*phe*N*phe)$_2$	918.2	+2
*N*Lys		**4**	(*N*Lys*N*phe*N*phe)$_4$	1707.1	+4
		5	(*N*Lys*N*phe*N*phe)$_3$	1284.6	+3
		6	(*N*Lys*N*phe*N*phe)$_2$	862.1	+2
*N*ae		**7**	(*N*ae*N*phe*N*phe)$_4$	1594.9	+4
		8	(*N*ae*N*phe*N*phe)$_3$	1200.4	+3
		9	(*N*ae*N*phe*N*phe)$_2$	806.0	+2
*N*ah	*N*spe	**10**	(*N*ah*N*spe*N*spe)$_4$	1930.2	+4
		11	(*N*ah*N*spe*N*spe)$_3$	1452.9	+3
		12	(*N*ah*N*spe*N*spe)$_2$	974.3	+2
*N*Lys		**13**	(*N*Lys*N*spe*N*spe)$_4$	1819.3	+4
		14	(*N*Lys*N*spe*N*spe)$_3$	1368.8	+3
		15	(*N*Lys*N*spe*N*spe)$_2$	918.2	+2
*N*ae		**16**	(*N*ae*N*spe*N*spe)$_4$	1707.1	+4
		17	(*N*ae*N*spe*N*spe)$_3$	1284.6	+3
		18	(*N*ae*N*spe*N*spe)$_2$	862.1	+2

both promastigotes and axenic amastigotes for 1 h with the appropriate concentrations of our compounds (2–100 μM). The solutions were then diluted 10-fold, followed by a further incubation for 24 h. Subsequently, alamarBlue® was added, and after 4 h the fluorescence was measured to determine the antiparasitic effect of the peptoids.

The results show that all short 6-mer peptoids **3**, **6**, **9**, **12**, **15**, and **18** were completely inactive against both forms of the

parasite, even at the highest concentration tested (Supporting Information Figure S1). The longer 9-mer peptoids **11**, **14**, and **17** with the *N*spe building block showed good activity against the *L. mexciana* promastigotes (Figure 1 a). Anti-leishmanial activity increased as the side chain length of the positively charged building block was shortened (*N*ae > *N*Lys > *N*ah), with peptoid **17** being the most active 9-mer in our library. In contrast, the 3-mer peptoids **2**, **5**, and **8** were not significantly active against the promastigotes, indicating that the less sterically hindered building block *N*phe is not favored for antiparasitic activity. The 12-mer peptoids **1**, **4**, **7**, **10**, **13**, and **16** were all active, with the *N*spe-containing peptoids again showing significantly lower ED_{50} values than the *N*phe analogues. Again, as for the analogous 9-mer peptoids, a shorter side chain (i.e., *N*ae) increased efficacy against promastigotes.

When we screened our library against the axenic amastigote form of *L. mexicana*, several peptoids showed activity at 100 μM (Figure 1 b). Peptoids **4**, **7**, **8**, **10**, and **17** were found to decrease cell viability by 40–100 %, but at lower concentrations activity was negligible. In contrast, peptoid **16** displayed a good level of activity with a calculated ED_{50} value of 17 μM. Notably, the ED_{50} value for peptoid **16** is similar to that reported for AMPs against various amastigote-stage *Leishmania* spp.[8b,19]

Because we used different amounts of serum in the promastigote (15 %) and amastigote (20 %) media, we tested the peptoids in serum-free conditions in the first 1 h of incubation to evaluate any effect of serum on activity. The results showed that the serum had only a moderate effect on the antiparasitic activity, so no inactive compounds became active, and the ED_{50} values of the active compounds were only slightly decreased (see Supporting Information Figures S2 and S3). This is contrast to previously reported work carried out on AMPs, in which a clear and measurable difference between serum and serum-free conditions was observed.[8a]

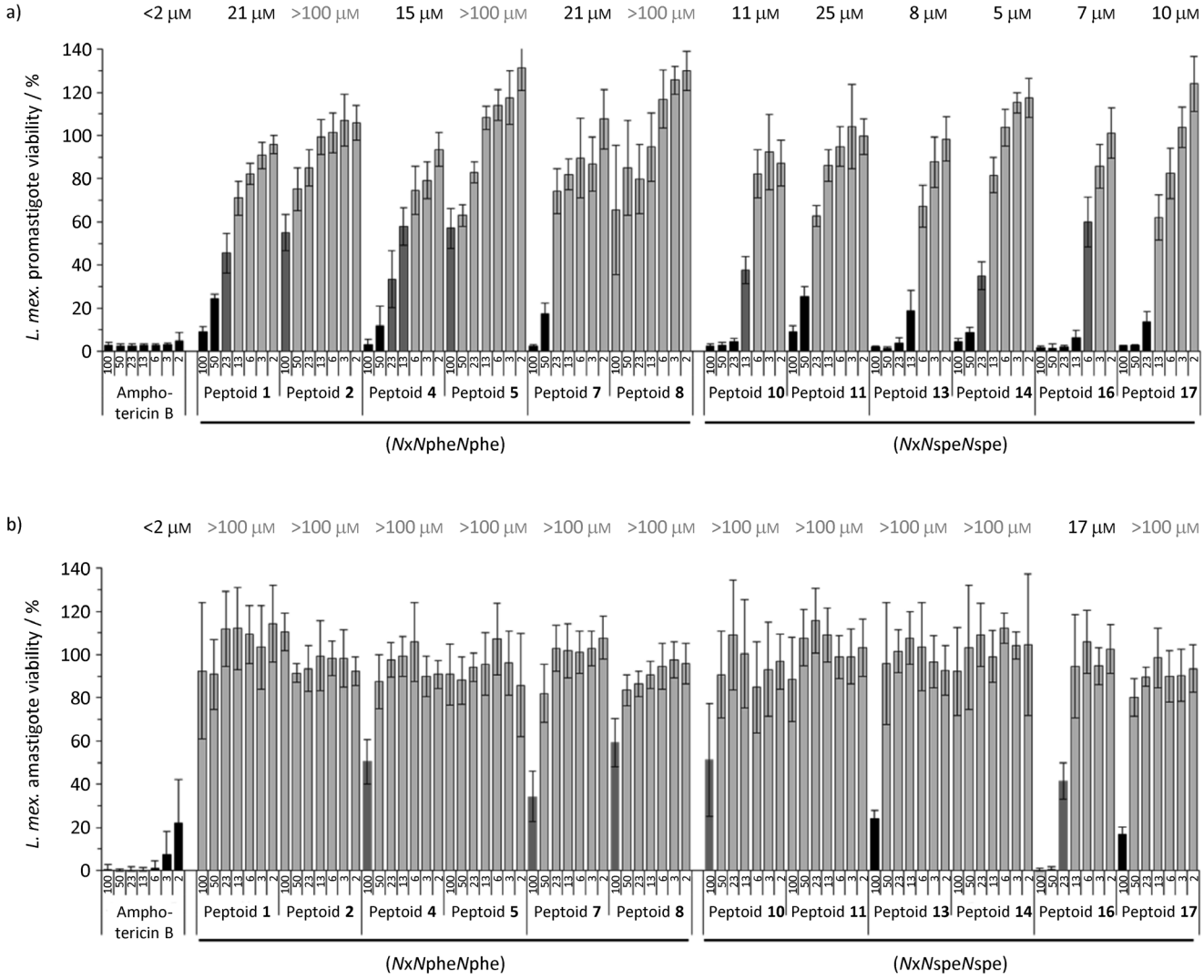


Figure 1. Using the alamarBlue® assay system, *L. mexicana* a) promastigote and b) amastigote viability in the presence of various concentrations of peptoids (2–100 μM) was determined with respect to untreated, negative DMSO controls. Amphotericin B was used as a positive control. *N*x = *N*ah, *N*Lys or *N*ae. Data points represent the mean of 2 or 3 independent experiments performed in triplicate, with standard deviation indicated; ED_{50} = 50 % effective dose. See Supporting Information Figure S1 for a complete data set including 6-mer peptoids **3**, **6**, **9**, **12**, **15**, and **18**.

This first investigation of peptoids against the parasite *L. mexicana* demonstrates that this class of peptide mimetics has anti-leishmanial activity. We identified nine peptoids with ED_{50} values in the low-micromolar range against the promastigote form of the parasite and one peptoid with a low-micromolar ED_{50} value against the axenic amastigote form of the parasite. Comparison of the sequences for the active peptoids showed that both the overall length of the peptoid as well as the nature of the individual building blocks influenced biological activity. In general, as the peptoid sequence was lengthened (i.e., moving from 6- to 12-mer), a lower ED_{50} value was obtained. The chiral aromatic building block *N*spe, which is known to stabilize the formation of a helical secondary structure, was to found increase the antiparasitic effect relative to the non-chiral *N*phe. The additional methyl groups in the *N*spe-containing peptoids is also likely to increase the overall hydrophobicity of the molecules, which may in turn help to enhance the biological activity. In the side chains that bore the positively charged amine group, we found that shorter chain lengths gave rise to more active peptoids. Overall the combination of *N*ae (short side chain) and *N*spe (chiral) building blocks gave the most potent compound in the library, peptoid **16**. This peptoid also demonstrated elevated cytotoxicity against HepG2 cells, indicating that the increase in efficacy is a generic effect. However, peptoid **16** was still significantly more potent against *L. mexicana* amastigotes than the mammalian cell line (Supporting Information Table S5).

In conclusion, a library of 18 linear peptoids were prepared and evaluated for anti-leishmanial activity. Twelve peptoids from the library prepared showed activity against the promastigote form of *L. mexicana* at ≤ 100 μM, with six (**4**, **10**, **13**, **14**, **16**, and **17**) having ED_{50} values of < 20 μM. Peptoid **16** was found to be the most active, with an ED_{50} value of 7 μM. In contrast, only seven peptoids from the library showed activity against *L. mexicana* axenic amastigotes at ≤ 100 μM. Only peptoid **16** was found to have an ED_{50} value of < 20 μM (17 μM). Our investigation also demonstrated a clear difference in the sensitivity of the two different parasite forms of *L. mexciana* to peptoids, with amastigotes being significantly more resistant. This difference is also observed for AMPs,[8,20] and it suggests that the different cellular surfaces of promastigotes and amastigotes[21] influences sensitivity to peptoids and peptides. Leading on from this, it is likely that the peptoids screened in this study target the cellular membrane of the parasites to affect their biological activity.

Experimental Section

Materials and reagents: Abbreviations for reagents are as follows: *tert*-butoxycarbonyl (Boc); 9-fluorenylmethoxylcarbonyl (Fmoc); trifluoroacetic acid (TFA); triisopropylsilyl (TIPS); *N*,*N*-dimethylformamide (DMF); *N*,*N*-diisopropylcarbodiimide (DIC); dimethylsulfoxide (DMSO). Solvents and reagents were purchased from commercial sources and used without further purification unless otherwise noted. Rink amide resin (typical loading level 0.6–0.8 mmol g^{-1}) was purchased from Merck Biosciences. Bromoacetic acid, TFA, DIC, and the amine building blocks were purchased from Sigma–Aldrich or TCI Europe. DMF was purchased from AGTC Bioproducts (National Diagnostics). Peptoid syntheses were performed manually in polypropylene syringes fitted with a polyethylene frit (20 mL, Crawford Scientific) in a heated shaker (400 rpm, 80 °C). Preparative RP-HPLC was performed with a semi-preparative PerkinElmer Series 200 LC pump fitted with a 785A UV/Vis detector using an SB-Analytical ODH-S optimal column (250 mm × 10 mm, 5 μm; flow rate 2 mL min^{-1}). Peptoids were characterized by LC–MS (TQD mass spectrometer and an Acquity UPLC from Waters) using an Acquity UPLC BEH C_8 1.7 μm (2.1 mm × 50 mm) column at a flow rate of 0.6 mL min^{-1} and a linear gradient of 5–95% solvent B over 3.8 min (A = 0.1% formic acid in H_2O, B = 0.1% formic acid in CH_3CN). Peptoid identities were also confirmed by MALDI-TOF MS analysis (Autoflex II ToF/ToF mass spectrometer, Bruker Daltonik GmBH) operating in positive ion mode using an α-cyano-4-hydroxycinnamic acid (CHCA) matrix. Data processing was done with MestReNova version 8.1.

Synthesis and characterization of linear peptoids: Fmoc-protected Rink Amide resin (normally 100–300 mg, 0.1–0.3 mmol) was swollen in DMF (1 h, room temperature) and deprotected with piperidine (20% in DMF, *v*/*v*) for 30 min. After DMF washing, the resin was treated with bromoacetic acid (8 equiv, 2 M in DMF) and DIC (8 equiv, 2 M in DMF) for 15 min at 80 °C on a heated shaker with a metal block (400 rpm, syringe was wrapped with tin foil for better heat distribution). The resin was washed three times with DMF (3 × 5 mL), before the desired amine sub-monomer was added (4 equiv, 1 M in DMF) and allowed to react for 15 min at 80 °C. The resin was again washed three times with DMF (3 × 5 mL). The bromoacetylation and amine displacement steps were repeated until the final sub-monomer had been added and the desired peptoid sequence had been obtained. Cleavage was performed with TFA (95%), TIPS (2.5%), and H_2O (2.5%) for 90 min at room temperature. The cleavage cocktail was collected in a 50 mL falcon tube and precipitated with 50 mL Et_2O. The solution was centrifuged for 15 min at 4000 rpm (RCF 2570). Afterward, the ether phase was decanted. The crude was dissolved in acidified H_2O (with 0.1% TFA) or a mixture of acidified H_2O and CH_3CN, and lyophilized. The crude products were re-dissolved in acidified H_2O (with 0.1% TFA) or a mixture of acidified H_2O and CH_3CN, and purified by RP-HPLC. A typical method used for the purification was 0–50% B over 60 min (A = 0.1% TFA in 95% H_2O and 5% CH_3CN, B = 0.1% TFA in 5% H_2O and 95% CH_3CN) at λ 220 or 250 nm. Relevant fractions were collected, lyophilized, and analyzed by LC–MS and MALDI-TOF MS.

Cell culture of *L. mexicana* (M379) promastigotes and amastigotes: *Leishmania mexicana* (MNYC/BZ/62/M379) promastigote parasites were maintained at 26 °C in Schneider's Insect medium (Sigma–Aldrich) supplemented with heat-inactivated fetal bovine serum (FBS, 15%; Biosera Ltd.). Cells were counted using a Neubauer Improved Hemocytometer. Promastigotes were transformed into axenic amastigotes by a pH and temperature shift as previously described:[21] In brief, a culture of promastigotes in the late log phase was transferred into Schneider's Insect medium supplemented with 20% heat-inactivated FBS (pH 5.5) at 5×10^5 parasites per mL. After six days, the parasites were in the metacyclic stage and used for transformation into amastigote forms by transfer in the same medium at 32 °C at 5×10^5 parasites per mL. After an additional 5–7 days, the parasites were in the amastigote stage.

Cytotoxicity assays with *L. mexicana* promastigotes and amastigotes: Cytotoxicity analyses were performed in 96-well plates (flat-bottom, Costar, Fisher Scientific) using alamarBlue® (Life Technologies) for cell viability detection as previously described.[8] *L. mexicana* promastigotes and amastigotes were pre-incubated with the

compounds in triplicate (5 mM stock solutions in DMSO; amphotericin B was used as a positive control; untreated parasites with DMSO as a negative control) in 50 μL of the corresponding media at 4×10^6 mL^{-1} for 1 h. Afterward, 40 μL were removed from each well before the addition of 90 μL of the corresponding media, followed by incubation for 24 h at appropriate temperature. Then, 10 μL alamarBlue® solution was added to each well before a 4 h incubation at the appropriate temperature prior to assessing cell viability using a fluorescent plate reader (Biotek; λ_{ex} 560 nm, λ_{em} 600 nm). To investigate the effects of serum on the efficacy of the peptoids, the assay described above was modified using serum-free medium for the pre-incubation time. For these assays, the parasites were washed three times in serum-free medium before adding them to the compound solutions. All of the experiments described above were carried out on a minimum of two separate occasions to ensure a robust data set was collected.

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Keywords: anti-leishmanial agents · antiparasitic agents · *Leishmania* · peptoids

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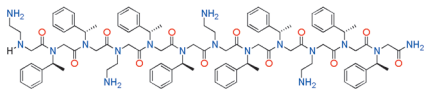

Peptoid **16**
L. mexicana promastigote ED_{50}: 7 μM
L. mexicana amastigote ED_{50}: 17 μM

***L. mex.* meets its match:** Peptoids—a class of peptide mimetics—have demonstrated their activities against a range of bacterial infections. In this work, we studied the anti-leishmanial properties of linear peptoids, which were observed to have promising activity against *Leishmania mexicana* axenic amastigotes, a causative agent of cutaneous leishmaniasis.