Efficacy of Antimicrobial Peptoids against *Mycobacterium tuberculosis*[▽]

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Tuberculosis is a leading cause of death worldwide. Resistance of *Mycobacterium* to antibiotics can make treatments less effective in some cases. We tested selected oligopeptoids—previously reported as mimics of natural host defense peptides—for activity against *Mycobacterium tuberculosis* and assessed their cytotoxicity. A tetrameric, alkylated, cationic peptoid (1-C13$_{4mer}$) was most potent against *M. tuberculosis* and least cytotoxic, whereas an unalkylated analogue, peptoid 1$_{4mer}$, was inactive. Peptoid 1-C13$_{4mer}$ thus merits further study as a potential antituberculosis drug.

Mycobacterium tuberculosis is a Gram-positive bacterium that infects human macrophages, causing tuberculosis (TB). Approximately one-third of the world's population is infected with *M. tuberculosis*, and more than 10 new cases of TB occur every minute, accounting for 2 to 2.5 million deaths annually worldwide (9, 15, 24).

The current treatment for drug-sensitive *M. tuberculosis* infections requires long-term multiple antibiotic therapy for 6 to 12 months (10). *Mycobacterium bovis* bacille Calmette-Guérin (BCG), a vaccine against *M. tuberculosis* that is widely used for children, can fail to prevent pulmonary TB in adult populations (18). Widespread noncompliance with the full therapeutic regimen has led to the inevitable emergence of multidrug-resistant (MDR) strains of *M. tuberculosis* (17). Rising resistance to rifampin and isoniazid, which are the most commonly used anti-TB drugs (14), is cause for concern and provided the motivation for this study. In spite of constant efforts to control TB, the disease remains a grave global problem that demands the discovery of novel treatments.

Naturally occurring antimicrobial peptides (AMPs), also known as host defense peptides, serve as the first line of immune defense for most organisms and as the sole immune effector for some organisms, including insects (29). Typically, they are relatively short (<40 amino acids long) and quite cationic (+3 to +6 at pH 7) and adopt amphipathic structures that present discrete hydrophobic and hydrophilic regions when they are associated with anionic interfaces (e.g., with anionic micelles). Some natural AMPs have alkyl modifications as well (22, 27). The antimicrobial activity of these natural toxins, which are typically released from granules, is attributed to their disruptive interactions with the bacterial membrane (29) in a manner involving non-receptor-mediated, poorly understood biophysical mechanisms of action (4, 11, 29). Their apparently nonspecific modes of killing have apparently made it difficult for bacteria to acquire resistance to AMPs, in contrast to the rising resistance of bacteria to small-molecule antibiotics that bind to specific receptors, which are subject to alteration via mutation and selection. Despite the efforts of many groups, AMPs are not yet used widely as therapeutics for a number of reasons, including their high cost, expectedly low bioavailability (as a result of *in vivo* protease susceptibility), and possible immunogenicity and/or systemic toxicity (12, 25). Efforts to overcome these drawbacks have prompted the design and synthesis of various nonnatural mimics of AMPs, which offer greater bioavailability and biostability, potentially increasing pharmaceutical suitability (1, 2, 23, 25).

Oligo-N-substituted glycines (peptoids) are sequence-specific peptidomimetics that are based on a "biomimetic" peptide backbone identical to that of natural proteins but have their side chains attached to the amide nitrogen (21, 30). This structural difference makes them highly resistant to protease activity (16, 19). In this study, we investigated the activity of six different oligopeptoids as antimycobacterial compounds. Cationic and amphipathic dodecamer peptoid 1, previously shown to be active against a broad spectrum of bacteria and fungi, was used as a positive control, whereas the cationic and aliphatic dodecamer peptoid 1-*N*ssb, which is inactive against bacteria, served as a negative control (6, 21). A previously designed tetrameric, cationic, alkylated peptoid, 1-C13$_{4mer}$, also previously shown to be an active antibacterial compound (previously termed *N*tridec-1$_{4mer}$) (5) was studied alongside unalkylated peptoid 1 analogues (1-11$_{mer}$, 1-Pro$_9$) and its own unalkylated analogue, 1$_{4mer}$, to investigate the effects of N-terminal alkylation and other molecular structural features on antimycobacterial activity (Table 1). Peptoids were synthesized by the standard solid-phase submonomer method (30) and purified by reversed-phase high-performance liquid chromatography on a C$_{18}$ column with an acetonitrile-water gradient (6, 21). The structures of the four different monomers used in the six different sequence-specific peptoids are shown in Fig. 1, while the actual sequences of the compounds are listed in

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TABLE 1. Sequences and activities of six different peptoids and gentamicin against BCG and *M. tuberculosis* bacteria[a]

Name	Sequence (amino to carboxyamide)	MIC (µM)	
		BCG[b]	*M. tuberculosis* H$_{37}$Rv[c]
Peptoid 1	H-(*N*Lys-*N*spe-*N*spe)$_4$-NH$_2$	12.5–25	14.1
1-C13$_{4mer}$	H-*N*tridec-*N*Lys-*N*spe-*N*spe-*N*Lys-NH$_2$	6.3	6.6
1$_{4mer}$	H-*N*Lys-*N*spe-*N*spe-*N*Lys-NH$_2$	>100	ND[d]
1-Pro$_9$	H-(*N*Lys-*N*spe-*N*spe)$_2$-*N*Lys-*N*spe-L-Pro-*N*Lys-*N*spe-*N*spe-NH$_2$	12.5–25	14.5
1-11$_{mer}$	H-(*N*Lys-*N*spe-*N*spe)$_3$-*N*Lys-*N*spe-NH$_2$	12.5–25	14.46
1-*N*ssb	H-(*N*Lys-*N*ssb-*N*ssb)$_4$-NH$_2$	>100	>100
Gentamicin		25	ND

[a] The MIC is the concentration at which no bioluminescence or lack of color change was observed in the two different assays used. MICs are represented as the average of three replicate trials. The statistical analysis is shown in Fig. 1.
[b] Determined by luciferase assay.
[c] Determined by fluorescence assay.
[d] ND, not determined.

Table 1. Gentamicin, an antibiotic commonly used against BCG in culture, was included as a positive control (13, 28).

Antibacterial activity of peptoids against BCG. The MICs of the six different peptoids against *M. tuberculosis* were assessed using a luminescent, luciferase-expressing strain of BCG with the use of bioluminescence as an indicator of cell viability (Fig. 2) (8). BCG was grown in Middlebrook 7H9 broth in the presence of 5 µg/ml of kanamycin, shaking, for 6 weeks at 37°C. In 6-ml surgical tubes, 2:1 serially diluted peptoid solutions were incubated with BCG at 37°C for 1 h in a 2-ml total volume and with a maximum concentration of 100 µM. A 100-µl volume of the solution was transferred to a black, clear-bottom, 96-well plate, and then 2 µl of a luciferin solution was added. Bioluminescence was measured using an IVIS imaging system (a Xenogen product from Caliper LifeSciences, Hopkinton, MA). Additional peptoid was added at 3, 6, and 23 h; 1 h after each addition, changes in the bioluminescent signal intensity were measured. The MIC was defined as the concentration at which no bioluminescence was observed after 24 h and was reported as an average of three replicate trials. Error bars represent the mean ± standard deviation. Statistical differences from the control (without added antimicrobial) were determined by one-way analysis of variance (ANOVA) with

post hoc testing using the Tukey-Kramer method at the 24-h time point. Differences were considered statistically significant at a *P* value of <0.0001.

Without any added antibiotic compound, a steady increase in bioluminescence was observed, giving an indication that BCG was growing (Fig. 2). When antibiotic compounds were added, decreases in bioluminescence over the first 4 h were insignificant. For active compounds, however, a significant reduction in bioluminescence was seen after 24 h of incubation. As shown in Fig. 2, 1-C13$_{4mer}$ was the most active peptoid (MIC = 6.3 µM) and was better than gentamicin (MIC = 25 µM) at inhibiting the growth of BCG. On the other hand, the negative-control peptoids 1-*N*ssb and 1$_{4mer}$ were inactive, even at 100 µM (Fig. 2). Peptoids 1, 1-11$_{mer}$, and 1-Pro$_9$ exhibited MICs in the range of 12.5 to 25 µM.

Antimicrobial activity of oligopeptoids against *M. tuberculosis*: MABA. As another way to assess the antimycobacterial activity of these compounds, we had the peptoids tested against *M. tuberculosis* (H$_{37}$Rv) using the microplate Alamar blue assay (MABA) by an NIH/NIAID-contracted laboratory (7). The visual MIC was defined as the concentration at which the peptoids prevented the change in color of the Alamar blue solution.

The MICs of peptoids obtained by the testing lab with the MABA were consistent with the MICs obtained with the bioluminescence assay (Table 1). Again, 1-C13$_{4mer}$ demonstrated the greatest tuberculocidal activity, whereas peptoid 1-*N*ssb was ineffective. Peptoids 1, 1-11$_{mer}$, and 1-Pro$_9$ exhibited MICs in the range of 14 to 15 µM (Table 1).

Cytotoxicity of peptoids against macrophages. As a preliminary assessment of the potential biocompatibility of peptoids and their suitability for the treatment of TB infections, we measured the cytotoxicity of peptoids against the Raw 264.7 and J774 mouse macrophage cell lines by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, colorimetric viability assay (20). The reported 50% lethal dose (LD$_{50}$) represents the average value that was obtained using data from six replicate trials. Means and standard deviations are reported. Statistically significant LD$_{50}$ differences from the no-treatment control were determined by one-way ANOVA with *post hoc* testing using the Tukey-Kramer method. Differences were considered statistically significant at a *P* value of <0.0001.
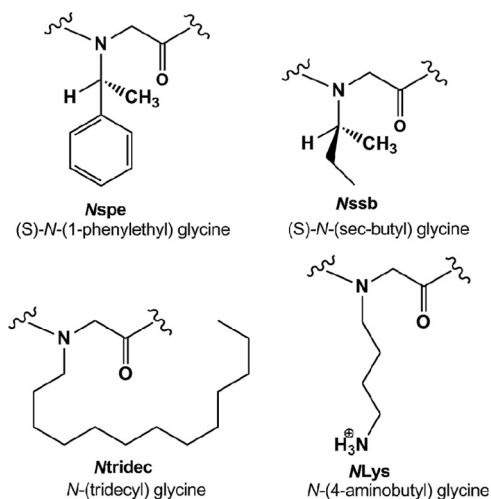

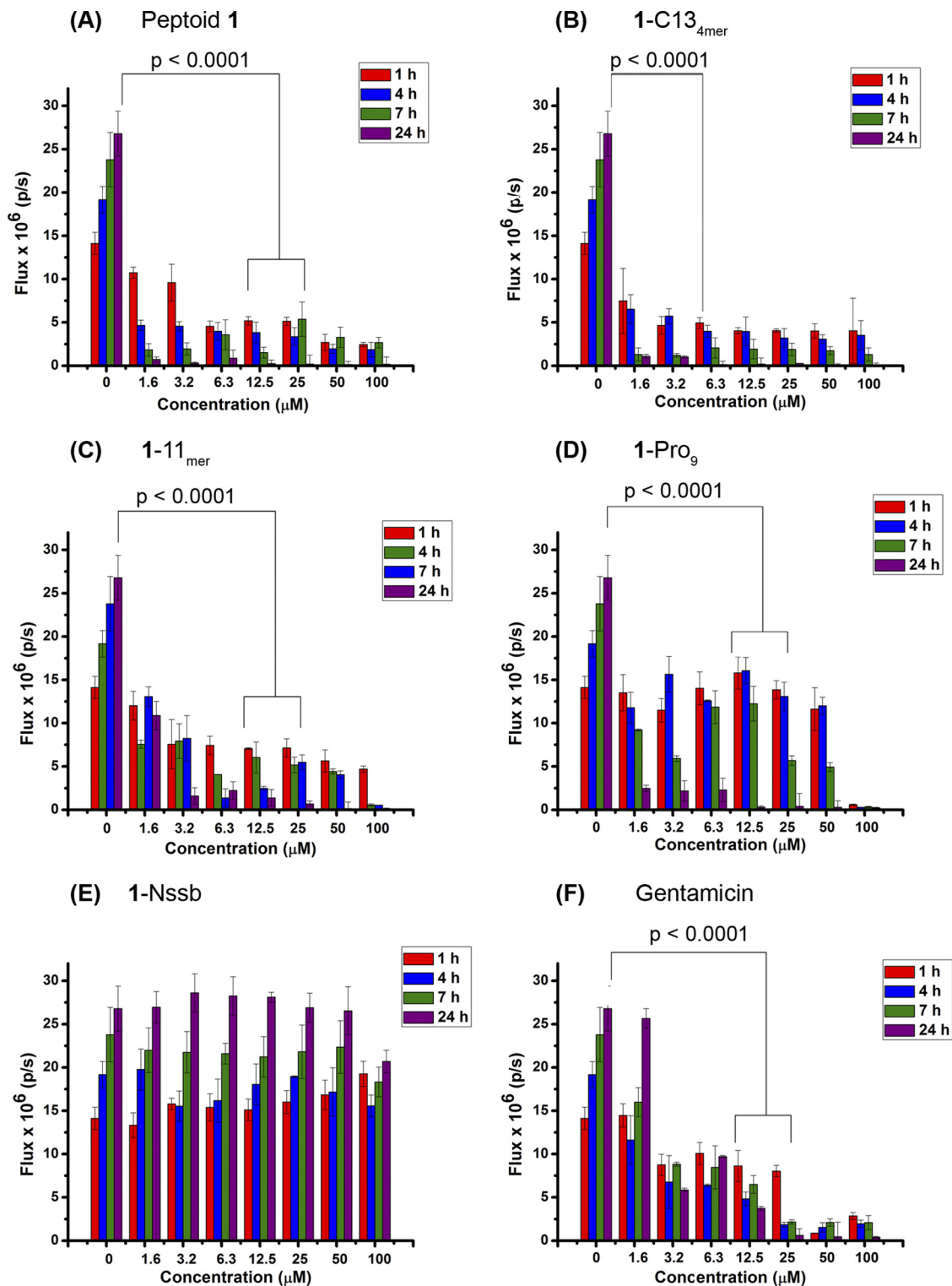FIG. 1. Peptoid submonomer chemical structures.

FIG. 2. BCG bioluminescence (in a luciferase-expressing *Mycobacterium* strain) at different concentrations of peptoid 1 (A), 1-C13$_{4mer}$ (B), 1-11$_{mer}$ (C), 1-Pro$_9$ (D), 1-*N*ssb (E), and gentamicin (F). Compounds were added at 0, 3, 6, and 23 h; bioluminescence was measured after 1 h of dosing at 37°C. Peptoid 1$_{4mer}$ was completely ineffective at reducing bioluminescence in this testing protocol (data not shown). The error bars represent standard deviations. The differences were considered statistically significant ($P < 0.0001$) with respect to a blank (no antimicrobial) at 24 h. p/s, photons/second.
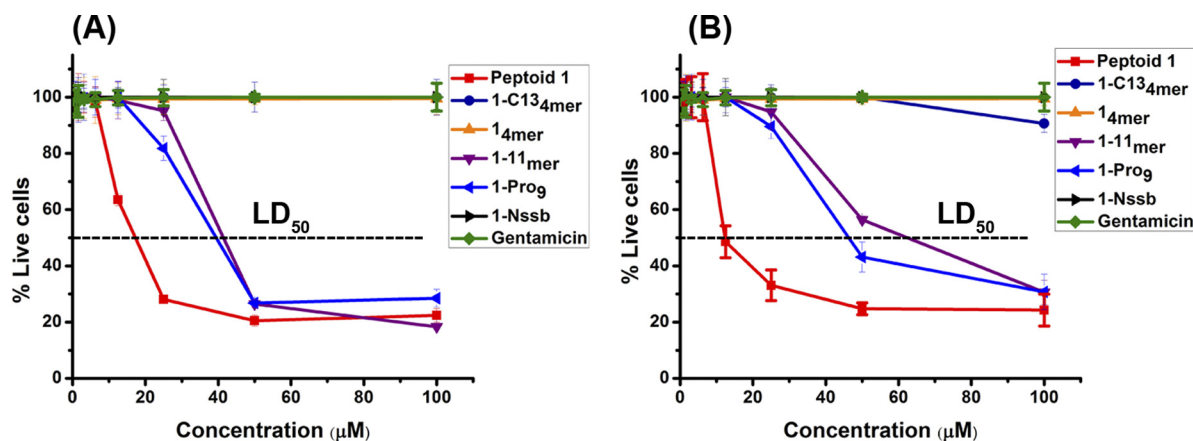
FIG. 3. Cytotoxicity of peptoids against Raw 264.7 (A) and J774 (B) mouse macrophage cell lines in culture. The LD_{50} was determined as the concentration at which 50% of the cells were killed. The error bars represent standard deviations. Differences between LD_{50} values were considered statistically significant at a *P* value of <0.0001.

The LD_{50} (the dose that killed 50% of the cells) of one of the peptoids (dodecamer 1) against macrophages was quite close to its MIC; however, other peptoids were substantially less toxic to macrophages at their MICs (Fig. 3). Peptoid $1\text{-}C13_{4mer}$ had an LD_{50} of >100 μM, hence, its LD_{50} is 15 to 20 times higher than its MIC, which is 6.3 μM. Peptoids $1\text{-}11_{mer}$ and $1\text{-}Pro_9$ had 2- to 4-fold higher LD_{50} values (~50 μM) than their MICs (~12.5 to 25 μM). As mentioned above, peptoid 1 has a very narrow biocompatibility window, with an MIC of 12.5 to 25 μM and an LD_{50} of ~20 μM. The inactive negative-control peptoids—compounds $1\text{-}N$ssb and 1_{4mer}— were also nontoxic.

Three of the six peptoids investigated in this study showed interesting tuberculocidal activity, and all three were equally active against BCG and *M. tuberculosis* (Table 1). These three different oligopeptoids ($1\text{-}11_{mer}$, $1\text{-}Pro_9$, and $1\text{-}C13_{4mer}$) may also have useful therapeutic windows, judging by their cytotoxicity in culture, with LD_{50}/MIC ratios ranging from 2 to 20 (Fig. 3). These data indicate that they should be further investigated as potential anti-TB drugs.

Peptoid $1\text{-}C13_{4mer}$ is four residues long and cationic, with a 13-carbon aliphatic tail on its N terminus. The hydrophobic tail of peptoid $1\text{-}C13_{4mer}$ gives it a substantial surfactant character—as a monomer—and would be expected to interact strongly, and disruptively, with the waxy, hydrophobic, lipid-rich outer layer of *Mycobacterium*, enabling better peptoid penetration of the bacterium, in a manner not seen in the case of unalkylated peptoid 1_{4mer}. In general, cationic surfactants are toxic to mammalian cells, as well as to bacteria, but this case, the strong self-association of this oligopeptoid enforced by its C13 tail must protect macrophages, which, unlike bacteria, are not substantially anionic (or hydrophobic, in the case of *M. tuberculosis*) on their outer membranes. Micellization of $1\text{-}C13_{4mer}$ at its MIC would be fully expected to increase its anti-*M. tuberculosis* potency, since the labile micelles, after binding to the anionic/hydrophobic bacterial membrane, would dissociate, creating a high local concentration at the cell surface (26). The cell membrane of *Mycobacterium* differs greatly from that of typical Gram-positive and Gram-negative bacteria. The outer layer of *Mycobacterium* is comprised of lipid-rich, hydrophobic layers of mycolic acid, and this aliphatic, hydrophobic wax barrier reduces the permeability of *M. tuberculosis* to the typical anti-TB drugs (3, 14). The inefficient penetration of the drugs and/or the entrapment of drugs by the waxy envelope layer are, in part, the reasons for the higher resistance of *Mycobacterium* to antibiotics (18). The very slow metabolism of *Mycobacterium* also has a tendency to render most conventional drugs less effective because the latter compounds often work by impairing particular steps in the cell's metabolic cycle.

This is the first study demonstrating the efficacy of cationic, biomimetic peptoids against the particularly troublesome class of infectious bacteria that lead to TB. We were excited to find that certain peptoids show appreciable activity against both BCG and *M. tuberculosis* while also being nontoxic or only slightly toxic to macrophages at their MICs. The cationic, amphipathic—in fact, surfactant-like—compound peptoid $1\text{-}C13_{4mer}$ was the most active and least cytotoxic. Presumably, its ability to form stable micelles is important for its selectivity. Since antimicrobial peptoids kill bacteria by a nonspecific biophysical mechanism with the potential to circumvent bacterial resistance, they may have significant pharmaceutical potential as a new class of tuberculocidal drugs against MDR strains of *M. tuberculosis*.

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