Anti-infective Agents

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Amphiphilic Tobramycins with Immunomodulatory Properties**

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Abstract: *Amphiphilic aminoglycosides (AAGs) are an emerging source of antibacterials to combat infections caused by antibiotic-resistant bacteria. Mode-of-action studies indicate that AAGs predominately target bacterial membranes, thereby leading to depolarization and increased permeability. To assess whether AAGs also induce host-directed immunomodulatory responses, we determined the AAG-dependent induction of cytokines in macrophages in the absence or presence of lipopolysaccharide (LPS). Our results show for the first time that AAGs can boost the innate immune response, specifically the recruitment of immune cells such as neutrophils required for the resolution of infections. Moreover, AAGs can selectively control inflammatory responses induced in the presence of endotoxins to prevent septic shock. In conclusion, our study demonstrates that AAGs possess multifunctional properties that combine direct antibacterial activity with host-directed clearance effects reminiscent of those of host-defense peptides.*

The world is facing an enormous threat from the emergence and dissemination of bacteria that are resistant to almost all currently available antibiotics.[1,2] Two strategies, multiple-component antibiotic adjuvants[3] and single-component-based antibacterial polypharmacology[4] are currently under investigation to combat bacterial resistance. Both strategies seek to exploit multiple modes of action. Recently, amphiphilic aminoglycosides (AAGs) have emerged as a source of antibacterial agents to combat bacterial resistance.[5–14] Mode-of-action studies have shown that AAGs can show different modes of action[9,11–13] to AGs, which bind to the 30S ribosomal subunit, thereby leading to the disruption of protein synthesis.[15] For instance, it was shown that the antibacterial effect of a neamine-based AAG against *P. aeruginosa* was caused by changes in membrane depolarization and permeability and not by inhibition of protein synthesis.[9,11] Strong evidence for membrane-targeting interactions of AAGs were also reported for amphiphilic neomycin and tobramycin analogues.[12,13]

Encouraged by the multimodal activity of cationic amphiphilic host-defense peptides (HDPs) in the host-directed clearance of an infection,[16–18] we developed an interest in exploring whether AAGs can show HDP-like properties. AAGs that combine direct antibacterial effects with the induction of immunomodulatory responses in host immune cells may display superior efficacy against multiple-drug-resistant (MDR) bacteria. It is noteworthy that for cationic amphiphilic HDPs like LL-37, the direct antibacterial activity is antagonized by physiological concentrations of divalent cations and polyanions, and other host factors.[16,17] However, HDP-mediated protection has been observed in several in vivo infection models, thus suggesting that the broad range of immunomodulatory activities exhibited by these peptides is the predominant function of HDPs for the resolution of microbial infections.[17,19–21] With this in mind, we set out to explore the potential immunomodulatory properties of AAGs. We were initially interested in developing multitargeting AAGs that combine the direct antibacterial effect of AGs with the membrane-targeting effects of AAGs. We selected tobramycin (**1**; Scheme 1) as the parent aminoglycoside since it is indispensable in intravenous or inhaled therapy to treat *P. aeruginosa* lung infections in cystic fibrosis patients.[22] Previous studies have shown that amphiphilic tobramycin analogues bearing a lipophilic group at C-6“ or C-5 retain potent antibacterial activity.[12,23] Furthermore, it was shown that C-6”-modified amphiphilic tobramycin targets bacterial membranes as its major mode of antibacterial action,[12] while C-5-modified tobramycin analogues containing positively charged small hydrophobic chains retain their capacity to interfere with protein synthesis.[23] Moreover, there is crystallographic evidence that the C-5 hydroxy group in tobramycin is not involved in direct contacts to model RNA, thus suggesting that structural modifications at this position may not interfere with RNA binding.[24]

We report herein our investigations into the antimicrobial properties of the C-5-substituted amphiphilic tobramycin analogues **4a–f** (Scheme 1), which were prepared from tobramycin (**1**) by using phase-transfer catalysis for the alkylation (Scheme 1). Compounds **4a–f** were tested for antibacterial activity by determining the minimal inhibitory concentration (MIC) against a panel of bacterial strains, including tobramycin-resistant clinical isolates (Table S1 in the Supporting Information). Our results show that the amphiphilic tobramycin analogues **4d–f**, which bear lipophilic tetradecyl, hexadecyl, and octadecyl ether appendages, respectively, show good activity against gram-positive bacteria (GPBs; MIC = 2–16 $\mu g\,mL^{-1}$) and reduced activity

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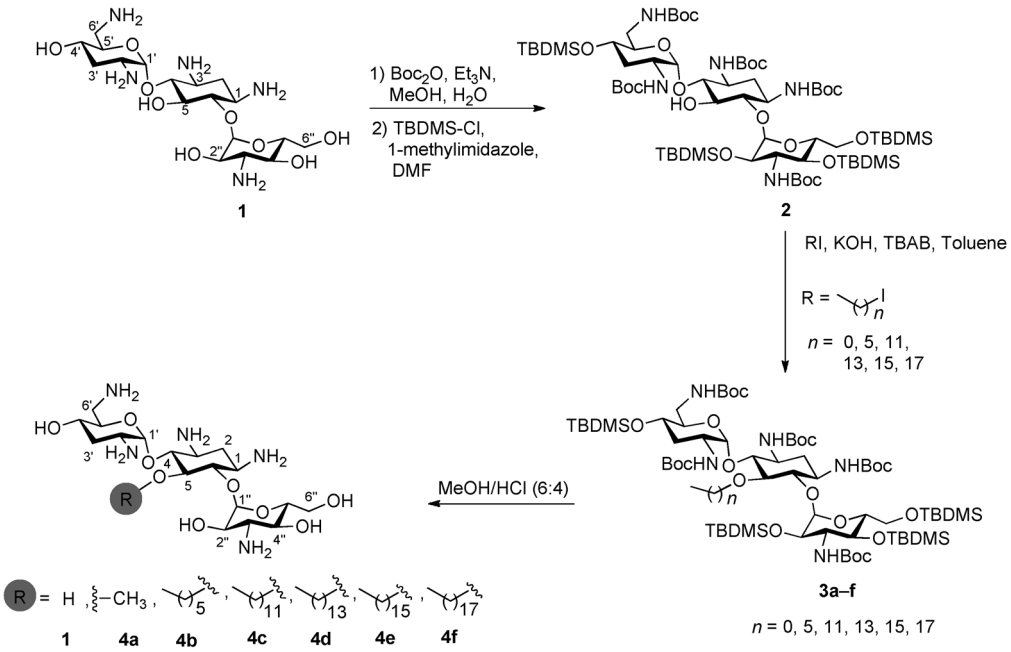

Scheme 1. Synthesis of amphiphilic tobramycins. Boc = *tert*-butoxycarbonyl, TBDMS = *tert*-butyldimethylsilyl, DMF = *N*,*N*-dimethylformamide, TBAB = tetra-*n*-butylammonium bromide.

(MIC = 16–256 μg mL^{-1}) against gram-negative bacteria (GNBs; Table S1). The most active AAG **4f** contains an octadecyl ether chain and consistently displayed the highest activity against both GPBs (MIC = 2–4 μg mL^{-1}) and GNBs (MIC = 16–128 μg mL^{-1}). Notably, when compared to tobramycin (**1**), an 8-fold reduction in MIC was observed for **4f** against resistant GNB strains including a tobramycin-resistant *E. coli* strain and a tobramycin-resistant *P. aeruginosa* strain, while a 4-fold or higher reduction was observed against tobramycin-resistant *S. maltophilia.* By contrast, the poorly amphiphilic tobramycin analogue **4b**, which contains a weakly lipophilic hexyl ether chain, showed poor antibacterial activity (MIC ≥ 128 μg mL^{-1}) against most bacterial strains tested (Table S1). Overall, the activity of the AAGs **4d–f** is comparable to the antibacterial activity often observed for antimicrobial peptides and HDPs like LL-37.

Next, we explored the immunomodulatory properties of the most potent amphiphilic tobramycin ether analogues **4d–f**, while the nonamphiphilic tobramycin methyl ether **4a** served as a negative control. There is little data on the immunomodulatory effects of tobramycin, although it has been suggested that a tobramycin–copper complex may display anti-inflammatory properties.[25] We monitored the cytotoxic effects of amphiphilic tobramycin analogues in human monocytic THP-1 cells (ATCC TIB-202). The release of lactate dehydrogenase was monitored in the tissue culture (TC) supernatants after 24 h stimulation to assess cytotoxicity. The amphiphilic tobramycin ether analogues **4d–f** showed negligible or less than 10% cytotoxicity at 5–10 μM, with a dose-dependent cytotoxicity response between 20 and 80 μM. By contrast, the nonamphiphilic control analogue **4a** showed 20% cytotoxicity at all concentrations tested (Figure S1 in the Supporting Information). It should be noted that the immunomodulatory properties of the HDP cathelicidin LL-37 and its analogues are typically studied at concentrations of 2.5 to 10 μM.[26,27] Therefore, we selected a dose of 10 μM for further assessment of the immunomodulatory activity of these analogues.

Plastic-adherent macrophage-like THP-1 cells were stimulated with **4a** and **4d–f** (10 μM), and the TC supernatants were monitored for production of the pro-inflammatory cytokines TNF-α and IL-1β as well as the chemokines Gro-α and IL-8 after 24 h, and production of the anti-inflammatory cytokine IL-1RA after 48 h of stimulation. None of the compounds induced the production of either TNF-α, IL-1β, or Gro-α (Figure 1). Likewise no production of the IL-1-antagonist IL-1RA was observed (data not shown). By contrast, AAGs **4d–f**, but not **4a**, significantly induced the production of the chemokine IL-8 in macrophages (Figure 1). Previous studies have shown that HDPs such as LL-37 and indolicidin show similar activity: they do not induce the production of TNF-α but are potent inducers of the neutrophil chemokine IL-8.[27,28] However, LL-37 can also induce the production of other chemokines such as MCP-1 and Gro-α, which act as chemoattractants for other leukocytes such as macrophages.[27,29] The fact that **4d–f** selectively induced IL-8 but not Gro-α suggests that these analogues may be selectively chemoattractant to neutrophils. Since the chemokine IL-8 is a potent neutrophil chemotactic factor required for the resolution of infections,[30,31] our results suggest that

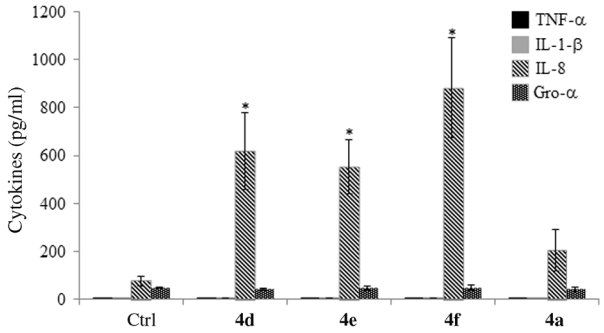

Figure 1. Plastic-adherent human macrophage-like THP-1 cells were stimulated with the tobramycin analogues (10 μM). TC supernatants were monitored after 24 h by ELISA for the production of the cytokines TNF-α and IL-1β, as well as the chemokines IL-8 and Gro-α. The values shown are an average of at least three independent experiments ± standard error (* $p < 0.05$).

the three AAGs **4d–f** may, in addition to their antibacterial activity, be able to mediate the recruitment of immune cells, in particular neutrophils, to the site of infection.

Previous studies have demonstrated that amphiphilic cationic HDPs, such as LL-37, can neutralize bacterial products such as lipopolysaccharide (LPS) and switch the signaling of Toll-like receptors to the NF-κB pathway induced by bacterial ligands to control bacterial infections and pathogen-induced inflammation.[27,32] Since the primary target cell type involved in the immunomodulatory activity of HDPs and their analogues has been shown to be macrophages,[33] we monitored LPS-induced cytokine production in the presence and absence of **4d–f** and **4a** in THP-1 macrophages after 24 h stimulation as previously described.[26,27] The analogues **4d–f** abrogated the production of LPS-induced pro-inflammatory TNF-α when the compounds were added either at the same time as LPS stimulation (Figure 2A) or 30 min prior to LPS stimulation (Figure 2B). Furthermore, **4d** and **4e** significantly suppressed LPS-induced IL-1β production when added either at the same time or 30 min prior to LPS stimulation (Figure 2). The analogue **4f** significantly suppressed LPS-induced IL-1β production when added simultaneously with LPS stimulation (Figure 2A) but not when added 30 min prior to stimulation (Figure 2B). The analogues **4d–f** also significantly suppressed the LPS-induced production of chemokines IL-8 and Gro-α by between 50 and 70% when added either simultaneously or 30 min prior to LPS stimulation, but they did not completely neutralize chemokine production (Figure 3). The nonamphiphilic analogue **4a** did not suppress the LPS-induced production of either pro-inflammatory cytokines or chemokines under any conditions (Figure 3 and Figure 4). Next, to determine whether the ability of the analogues to suppress LPS-induced cytokine production was due to binding to LPS, we tested the activity of the analogues in LPS-primed macrophages. The cells were stimulated for 30 min with LPS, followed by removal of the TC media and washing of the cells to ensure the removal of residual LPS in the TC media. Subsequently, the cells were stimulated with the tobramycin analogues 30 min after LPS stimulation. The analogues **4d** and **4e** significantly suppressed LPS-induced TNF-α production, even when added 30 min after LPS stimulation (Figure 4A), which suggests that the effect of **4d** and **4e** in controlling LPS-induced TNF-α may be due to the alteration of intracellular signaling mechanisms rather than direct LPS binding. However, none of the compounds significantly altered the LPS-induced production of either IL-1β, IL-8, or Gro-α when added 30 min after LPS stimulation (Figure 4). This is consistent with previous studies demonstrating that HDPs such as cathelicidins LL-37 and BMAP-27 inhibit TNF-α production,[34] whereas defensin HNP-1 promotes IL-1β production[35] in LPS-primed macrophages. Previous studies have also demonstrated that HDPs, for example, LL-37 and BMAP-27, induce the expression of several chemokines and do not neutralize LPS-induced chemokines.[27,29,36] Taken together, these results suggest that that the selective modulation of endotoxin-induced inflammatory responses by certain HDPs, as with **4d** and **4e**, maybe in part mediated through alteration of the intracellular signaling downstream of pattern-recognition receptors in macrophages.

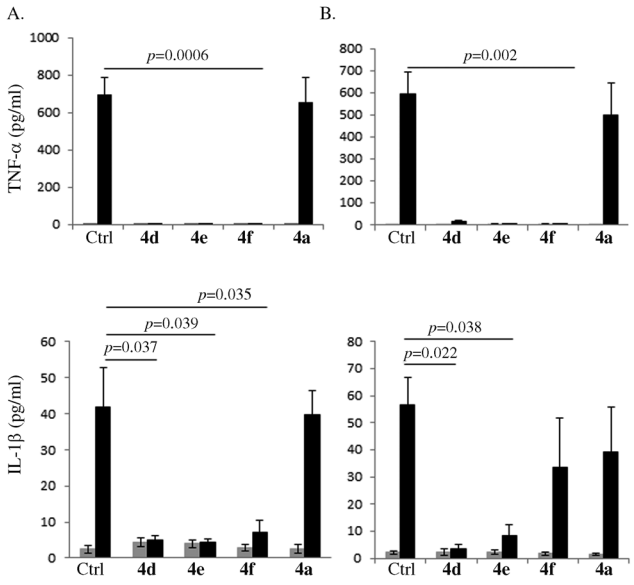

Figure 2. Plastic-adherent human macrophage-like THP-1 cells were stimulated with 10 ng mL^{-1} of bacterial LPS (gray = without LPS, black = with LPS), in the presence and absence of the tobramycin analogues (10 μM). The tobramycin analogues were added either at the same time as LPS stimulation (A) or 30 min prior to LPS stimulation (B). TC supernatants were monitored after 24 h by ELISA for the production of the pro-inflammatory cytokines TNF-α and IL-1β. The values shown are an average of at least three independent experiments ± standard error.

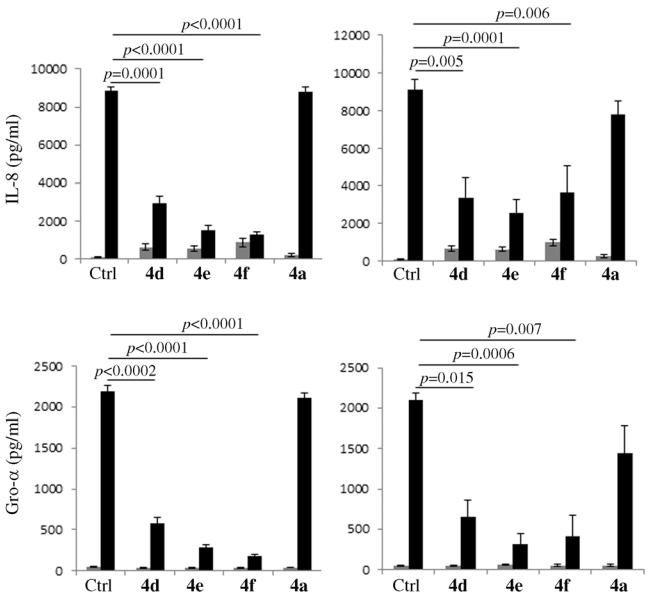

Figure 3. Plastic-adherent human macrophage-like THP-1 cells were stimulated with 10 ng mL^{-1} of bacterial LPS (gray = without LPS, black = with LPS) in the presence and absence of the tobramycin analogues (10 μM). The tobramycin analogues were added either at the same time as LPS stimulation (A) or 30 min prior to LPS stimulation (B). TC supernatants were monitored after 24 h by ELISA for the production of the chemokines IL-8 and Gro-α. The values shown are an average of at least three independent experiments ± standard error.

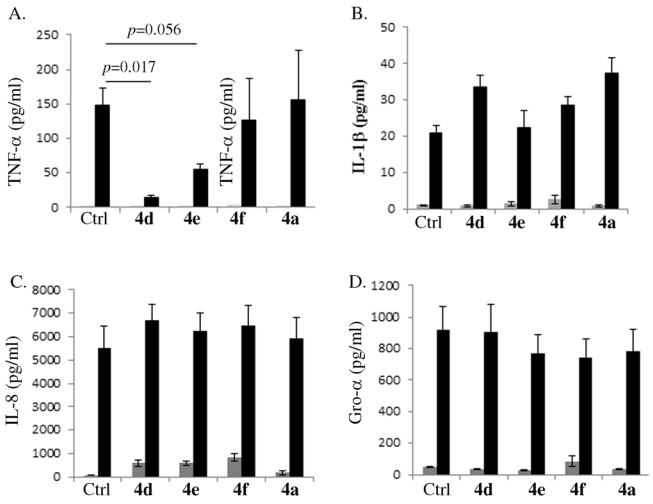

Figure 4. Plastic-adherent human macrophage-like THP-1 cells were stimulated with 10 ng mL^{-1} of bacterial LPS (gray = without LPS, black = with LPS) for 30 min, the TC medium was removed and the cells washed with fresh medium, followed by stimulation with tobramycin analogues (10 μM) for 24 h. TC supernatants were monitored by ELISA for production of the pro-inflammatory cytokines TNF-α (A) and IL-1β (B), as well as the chemokines IL-8 (C) and Gro-α (D). The values shown are an average of at least three independent experiments ± standard error.

In summary, our results demonstrate for the first time that AAGs, besides their direct antibacterial activity, can also induce immunomodulatory responses at concentrations that are nontoxic to host cells. The multimodal activity of AAGs, whereby direct antibacterial activity is combined with an immunomodulatory response, is encouraging since immunomodulatory compounds are becoming increasingly important in anti-infective therapy. Although AAGs were originally designed to overcome bacterial resistance by targeting both ribosmal RNA and the bacterial membrane, our study shows that AAGs can also influence host immune responses. We have shown that AAGs can boost the innate immune response, specifically the recruitment of immune cells such as neutrophils required for resolution of infections. Furthermore, AAGs can selectively control inflammatory responses induced in the presence of endotoxin to prevent septic shock. As with certain amphiphilic HDPs, for example, LL-37, AAGs exhibit modest direct antibacterial activity and immunomodulatory properties for the control of both infection and pathogen-induced hyperinflammation. AAGs thus represent a promising avenue for the development of multifunctional molecules for the prevention or treatment of bacterial infections.

Keywords: aminoglycosides · amphiphiles · antibiotics · drug design · host-defense peptides

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