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ORIGINAL ARTICLE

Antibacterial activity of amphiphilic tobramycin

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Amphiphilic aminoglycoside antimicrobials are an emerging class of new antibacterial agents with novel modes of action. Previous studies have shown that amphiphilic neomycin-B and kanamycin-A analogs restore potent antibacterial activity against Gram-positive neomycin-B- and kanamycin-A-resistant organisms. In this paper, we investigated the antibacterial properties of a series of amphiphilic tobramycin analogs. We prepared tobramycin–lipid conjugates, as well as tobramycin–peptide triazole conjugates, and studied their antibacterial activities against a panel of Gram-positive and Gram-negative bacterial strains, including isolates obtained from Canadian hospitals. Our results demonstrate that the antibacterial activity of amphiphilic tobramycin is greatly affected by the length and nature of the hydrophobic lipid tail, whereas the nature of the polycationic headgroup or the number of cationic charges appear to be less important. Replacement of the hydrophobic tail by a fluorinated lipid confers good activity against two *Pseudomonas* strains and reduces hemolytic activity. However, susceptibility studies in the presence of bovine serum albumin indicate that all amphiphilic tobramycin analogs are strongly protein-bound, leading to a typical four- to eight-fold increase in MIC.
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INTRODUCTION

Polycationic antibacterials (PAs) containing multiple positively charged amino and/or guanidino functions define a structurally diverse class of antibacterials with broad-spectrum activity and different modes of action.[1] PAs can be divided into amphiphilic and non-amphiphilic PAs. Amphiphilic PAs are comprised of the naturally occurring cationic antimicrobial peptides, synthetic mimics of antimicrobial peptides, polycationic lipopeptides, lipids and surfactants, whereas non-amphiphilic PAs are represented by aminoglycoside antibiotics. The polycationic charges in PAs ensure accumulation at polyanionic microbial cell surfaces in Gram-negative and Gram-positive bacteria, respectively.[2] Several PAs including the aminoglycoside gentamicin, certain antimicrobial peptides including defensins, gramicidin S variants and others, and lipopeptides such as polymyxins transit the outer membrane by interacting at sites at which divalent cations crossbridge adjacent polyanionic polymers. This causes a destabilization of the outer membrane that is proposed to lead to self-promoted uptake of PAs and/or other extracellular molecules.[2] After transit through the outer membrane, PAs contact the anionic surface of the cytoplasmic membrane. Here, depending on the structure of the Pas, several scenarios can be envisaged. Amphiphilic PAs may insert themselves into the cytoplasmic membrane, thereby either disrupting the physical integrity of the bilayer, via membrane thinning, transient poration and/or disruption of the barrier function, or translocation across the membrane and acting on internal targets.[2] Non-amphiphilic PAs like aminoglycosides must cross the membrane to bind to ribosomal RNA.

Our previous work has shown that amphiphilic aminoglycosides such as neomycin, kanamycin and neamine analogs restore potent antibacterial activities against certain Gram-positive and Gram-negative organisms, including multidrug-resistant isolates obtained from Canadian hospitals.[3–7] For instance, when compared with their parent aminoglycosides, both neomycin-B-based hexacationic C_{16}-lipid **7** or kanamycin-A-based tetracationic lipid C_{16}-lipid **6** (Figure 1) displayed 64- to 32-fold enhanced antibacterial activity against methicillin-resistant *Staphylococcus aureus*, whereas a 4- to 8-fold decrease in MIC was observed against two *Pseudomonas aeruginosa* strains.[4] Similarly, conjugation of neomycin-B and kanamycin-A to an ultrashort hydrophobic dipeptide led to a 16-fold lower MIC against methicillin-resistant *Staphylococcus aureus* and a 4- to 32-fold lower MIC against *Pseudomonas aeruginosa* strains. The physicochemical similarities between amphiphilic aminoglycosides and amphiphilic PAs suggest a membranolytic mode of action.[8,9] This hypothesis is supported by (a) the observed concentration-dependent hemolytic activity of **6** and **7**;[5] and (b) the requirement for a strongly hydrophobic lipid segment to induce potent antibacterial activity[4] and a recent study of C5"-modified neomycin-B-based polycationic lipids that have demonstrated that polycationic neomycin–lipid conjugates synergistically enhance the antibacterial activity of antibiotics interacting with intracellular targets such as amikacin and neomycin.[9] To further explore the structural activity relationships in this class of polycationic amphiphiles, we now became interested in studying the antibacterial effects of amphiphilic tobramycin analogs. To the best of our knowledge, this is the first study on amphiphilic

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tobramycin analogs. We were particularly interested in exploring how the nature of the hydrophobic lipid tail affects antibacterial activity in amphiphilic tobramycin analogs, and how the activity of amphiphilic tobramycin compares with amphiphilic kanamycin-A and neomycin-B.

MATERIALS AND METHODS

Antibacterial agents

Two classes of amphiphilic tobramycin analogs were prepared. The first class are tobramycin-based polycationic lipids **2**, **3**, **4** and **5** (Figure 1), whereas the second class are tobramycin-based triazole conjugates **9** and **10** (Figure 2). We also studied the corresponding kanamycin-A- and neomycin-B-based polycationic lipids **6**, **7** and **8,** together with their triazole conjugates **11** and **12**. Tobramycin, kanamycin, neomycin and 6'-azidodeoxy-tobramycin **1** served as reference compounds. All synthetic compounds were prepared following our previously established methodology.[3,4,6] The identity of all amphiphilic tobramycin, neomycin-B and kanamycin-A analogs were assessed by EI-MS, ^{1}H NMR, and ^{13}C NMR (see Supplementary Information). Tobramycin, kanamycin-A and neomycin-B were purchased from Sigma Aldrich Canada Ltd (Oakville, Ontario, Canada).

Bacterial strains

American Type Culture Collection (ATCC) strains as well as clinical isolates from the Canadian Intensive Care Unit (CAN-ICU) study were used, including *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 33592, *Staphylococcus epidermidis* ATCC 14990, methicillin-resistant *Staphylococcus epidermidis* (Cefazolin-CZ MIC $>32\,\mu g\,ml^{-1}$) CAN-ICU 61589, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 27270, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *E. coli* ATCC (Gentamicin resistant) CAN-ICU 61714, *E. coli* ATCC (Amikacin MIC $32\,\mu g\,ml^{-1}$) CAN-ICU 63074, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* (Gentamicin resistant) CAN-ICU 62308, *Stenotrophomonas maltophilia* CAN-ICU 62584, *Acinetobacter baumannii* CAN-ICU 63169 and *Klebsiella pneumoniae* ATCC 13883.[10]

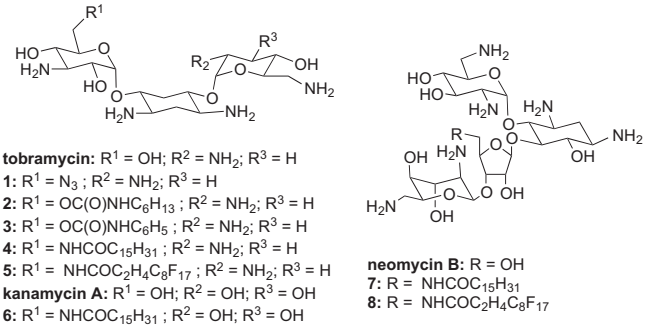

Figure 1 Structures of amphiphilic tobramycin-, kanamycin-A- and neomycin-B-based polycationic lipids.

MIC determination

Antibacterial activity against Gram-positive and Gram-negative organisms was assessed via broth macrodilution using Clinical and Laboratory Standards Institute (CLSI) methodology.[10] Stock solutions of amphiphilic aminoglycosides were prepared to a concentration of $512\,\mu g\,ml^{-1}$ in water. Organisms were subcultured and isolated on blood agar, suspended in 3 ml of Mueller-Hinton broth at the turbidity of a 0.5 M McFarland standard, and diluted to approximately 10^{5} CFU ml^{-1} before introduction into tubes containing serially diluted lipopeptide antibiotic in Mueller-Hinton broth. The turbidity resulting from lipopeptide solution in broth required the creation of control tubes lacking microbes serving as turbidity controls. All tubes were incubated overnight for 16–20 h at 37 °C. In some cases, the MIC study was performed in the presence of 4% bovine serum albumin to study protein binding.

Hemolytic activity

In-vitro toxicity was determined using a sheep red blood cell hemolytic assay as previously described.[11] Pilot experiments have shown that both human and sheep erythrocytes respond similarly in this hemolytic assay (data not shown). In brief, the erythrocytes were washed and resuspended in Tris-buffered saline. The cell suspension will be combined with varying concentrations (low to very high) of kanamycin, neomycin, tobramycin and amphiphilic aminoglycosides. The samples were centrifuged and the absorbance of the supernatants was measured at 540 nm. Tris-buffered saline and Triton X were used as negative and positive controls, respectively. The toxicity was assessed by percent hemolysis.[11]

RESULTS AND DISCUSSION

In this study, a total of seven amphiphilic and non-amphiphilic tobramycin-based analogs as well as certain corresponding amphiphilic kanamycin and neomycin analogs were synthesized. Our curiosity was driven by previous observations from our laboratory[3–5]

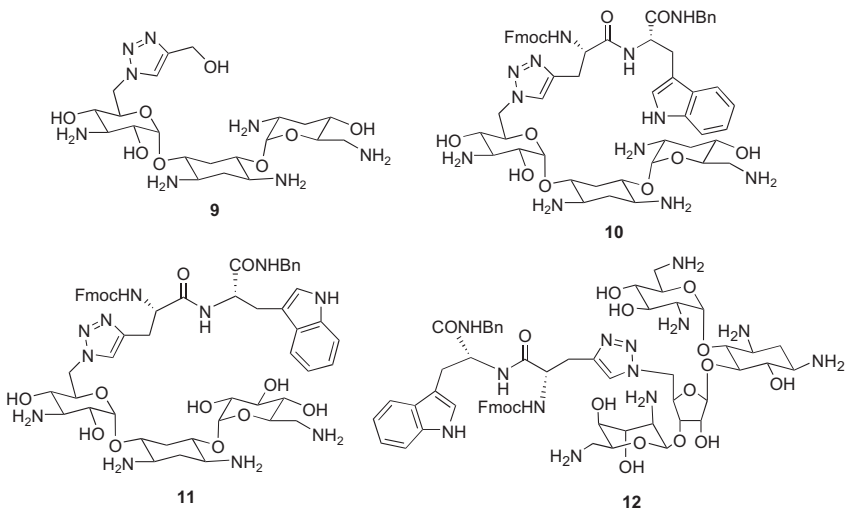

Figure 2 Structures of amphiphilic tobramycin-, kanamycin-A- and neomycin-B-based triazole peptide conjugates.

and others,[7,8] which have shown that conversion of the non-amphiphilic aminoglycosides neomycin-B and kanamycin-A into amphiphilic aminoglycosides enhances antibacterial activity against aminoglycoside-resistant and multidrug-resistant Gram-positive bacteria.[3–5] The compounds were selected to explore how the nature of the cationic aminoglycoside-based headgroup, the number of cationic charges and the nature of the hydrophobic lipid tail affects antibacterial activity in this class of compounds. At first, we explored how the nature of the hydrophobic tail affects the antibacterial activity in tobramycin-based amphiphiles. The single primary hydroxy group of tobramycin was converted into an amino function and conjugated to various fatty acid-based lipid tails. We selected a C_{16}- and fluorinated C_{12}-lipid tails. We also explored aromatic tails and shorter C_6 lipid tails in the form of carbamates. Our results clearly demonstrate that amphiphilicity is critical for antibacterial activity as tobramycin-C_6 lipid **2** and tobramycin-phenylcarbamate **3** are nearly devoid of antibacterial activity (Table 1). In contrast, tobramycin-C_{16}-lipid **4** displays good antibacterial activity against most Gram-positive (MIC $\leqslant$4–8 $\mu g\,ml^{-1}$), and some Gram-negative organisms including *E. coli* and one *Pseudomonas aeruginosa* strain. The potent activity of **4** (MIC <0.25) against methicillin-resistant *Staphylococcus epidermidis* is especially noteworthy. Replacement of the C_{16}-lipid in **4** by a partially fluorinated lipid tail with comparable hydrophobicity[12] as in compound **5** does not improve the antibacterial activity and generally leads to an expected two-fold reduction in MIC when the increase in MW is considered. It is noteworthy that compound **5** displays good antibacterial activity (MIC = 16) against two *Pseudomonas aeruginosa* strains.

Next, we studied how the nature of the polycationic headgroup and the number of polycationic charges affects the antibacterial activity. Besides the pentacationic tobramycin-based headgroup, we selected the tetracationic kanamycin-A and the hexacationic neomycin-B headgroups. With the exception of methicillin-resistant *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, conjugation of the three differently charged headgroups to a C_{16} hydrophobic tail results in almost identical antibacterial activity for compounds **4**, **6** and **7**, indicating that the nature of the polycationic headgroup is not critical for the antibacterial activity. Interestingly, a four- to eight-fold reduction in MIC is observed against *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus epidermidis* when the polycationic headgroup of neomycin and kanamycin is replaced by tobramycin, indicating that the pentacationic tobramycin-based headgroup appears to be optimal for both organisms. In addition, as observed for tobramycin analog

Table 1 Antibacteral activities (MIC $\mu g\,ml^{-1}$) of amphiphilic aminoglycoside antimicrobals

Control organism	*Tobramycin*	*1*	*2*	*3*	*4*	*5*	*9*	*10*	*Kanamycin*	*6*	*11*	*Neomycin*	*7*	*8*	*12*
Staphylococcus aureus ATCC29213	0.5 (0.5)	8 (4)	256	256	8 (32)	16 (64)	2 (2)	8 (16)	4	8	16	1	4	16	8
MRSA ATCC33592	0.5 (1)	8 (4)	512	512	8 (64)	32 (256)	64 (128)	16 (32)	>512	16	32	256	8	64	16
Staphylococcus epidermidis ATCC14990	$\leqslant$0.25 ($\leqslant$0.25)	8 (4)	128	64	4 (32)	8 (64)	1 (2)	8 (16)	2	2	8	0.25	2	8	4
MRSE CAN-ICU 61589	2 (2)	4 (16)	128	256	<0.25 (32)	$\leqslant$0.25 (64)	16 (64)	16 (64)	128	2	16	0.5	2	4	8
Enterococcus faecalis ATCC29212	8 (16)	16 (16)	256	256	8 (64)	16 (512)	64 (64)	32 (64)	n.d.	8	n.d.	n.d.	n.d.	64	n.d.
Enterococcus faecium ATCC27270	16 (8)	128 (128)	256	512	4 (32)	0.5 (64)	128 (128)	16 (64)	n.d.	8	n.d.	n.d.	n.d.	32	n.d.
Streptococcus pneumoniae ATCC49619	2 (2)	2 (1)	512	256	128 (512)	128 (512)	16 (16)	64 (128)	8	64	64	32	64	64	64
Escherichia coli ATCC25922	0.5 (0.5)	8 (4)	64 (128)	8 (8)	32 (128)	64 (256)	4 (8)	16 (8)	8	32	32	4	32	32	16
Escherichia coli CAN-ICU 61714	8 (8)	32 (32)	>512	512	32 (128)	64 (512)	256 (256)	32 (256)	16	32	32	8	64	32	32
Escherichia coli CAN-ICU 63074	8 (8)	8 (8)	128 (256)	32 (64)	8 (64)	64 (512)	64 (64)	32 (128)	32	32	32	n.d.	n.d.	64	n.d.
Pseudomonas aeruginosa ATCC27853	0.5 (0.5)	4 (4)	>512	512	128 (256)	16 (64)	8 (16)	32 (128)	>512	64	128	512	128	128	128
Pseudomonas aeruginosa CAN-ICU 62308	16 (32)	128 (256)	>512	512	16 (64)	16 (64)	256 (512)	16 (64)	>512	64	16	512	128	32	64
Stenotrophomonas maltophilia CAN-ICU 62584	>512 (>512)	>512 >512	>512	>512	256 (512)	>512 (>512)	>512 (>512)	256 (>512)	>512	<128	n.d.	>512	n.d.	>512	n.d.
Acinetobacter baumannii CAN-ICU 63169	16 (8)	256 (128)	>512	>512	256 (512)	512 (>512)	512 (>512)	256 (512)	16	<128	n.d.	32	n.d.	512	n.d.
Streptococcus pneumoniae ATCC13883	>0.25 (>0.25)	16 (8)	128	4 (4)	32 (128)	128 (256)	2 (2)	256 (512)	0.5	16	n.d.	$\leqslant$0.25	n.d.	512	n.d.
% Hemolytic activity at (100 or 500 $\mu g\,ml^{-1}$)	<0.5 (<0.5)	1.2 (1.7)	0.8 (1.0)	1.5 (1.6)	37% (100)	1.3 (27.1)	0.8 (0.7)	12.4 (56.5)	<0.5 (<0.5)	20 (n.d.)	3.9 (n.d.)	<0.5 (<0.5)	56 (n.d.)	1.1 (9.4)	9.9 (n.d.)

Abbreviations: ATCC, American Type Culture Collection; BSA, bovine serum albumin; CAN-ICU, Canadian Intensive Care Unit; MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis*; n.d., not defined.
Values in brackets were determined in the presence of 4% BSA.

5, replacement of the hydrophobic tail by a fluorinated lipid tail does not enhance antibacterial activity.

We also studied how substitution of the fatty acid-based lipid tail by a hydrophobic dipeptide sequence affects antibacterial activity in amphiphilic tobramycin analog **10** (Figure 2). For comparison with previously synthesized amphiphilic peptides **11** and **12**, we selected the hydrophobic Fmoc-protected dipeptide sequence consisting of triazol-modified glycine and tryptophan.[6] A previous study has shown that the hydrophobic Fmoc-protecting group in this dipeptide sequence improves antibacterial activity.[6] We also prepared the less amphiphilic tobramycin-based triazole analog **9** as a reference compound. Our results demonstrate that replacement of the lipid tail in **4** by a hydrophobic peptide sequence in compound **10** results in a two- to four-fold increase in MIC against most Gram-positive strains, whereas the same or a two-fold increased MIC was observed against most Gram-negative organisms. The same trend is observed with amphiphilic kanamycin-A peptide triazole conjugate **11** and neomycin-B triazole conjugate **12**.

To further explore the potential toxicity of amphiphilic tobramycin analogs, we studied their hemolytic activities at two different concentrations (Table 1). Our results demonstrate that amphiphilic tobramycin analogs with highly hydrophobic tails such as compounds **4** and **10** show concentration-dependent hemolytic activity, as it is observed for other amphiphilic aminoglycoside analogs such as compounds **6** and **7**. Generally, it appears that a hydrophobic peptide tail results in reduced hemolytic activity when compared with a C_{16} lipid tail for all three types of aminoglycosides. Moreover, replacement of the hydrophobic lipid tail in both compounds **4** and **7** by a fluorinated lipid tail as in compounds **5** and **8** significantly reduces the hemolytic activity, suggesting that fluorinated lipid tails may reduce cytotoxicity. However, both amphiphilic tobramycin–lipid conjugates experience a typical four- to eight-fold reduction in antibacterial activity against most organisms in the presence of 4% bovine serum albumin, indicating that these compounds bind strongly to proteins. A similar antibacterial deactivation has previously been observed with other classical cationic amphiphiles such as benzethonium chloride.[13]

In summary, we have established that the antibacterial activity of amphiphilic tobramycin analogs is greatly affected by the length and nature of the lipid tail. In contrast, the nature of the polycationic headgroup or the number of the cationic charges appear to be less important for induction of antibacterial activity. The most potent antibacterial is tobramycin-C_{16} lipid **4**, which displays good Gram-positive activity, but reduced Gram-negative activity. Replacement of the C_{16} lipid tail by a fluorinated lipid tail confers good activity against two *Pseudomonas aeruginosa* strains and reduces hemolytic activity. However, susceptibility studies in the presence of bovine serum albumin indicate that all amphiphilic tobramycin analogs are strongly protein-bound, leading to a typical four- to eight-fold increase in MIC.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)