Antibacterial Compounds

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Di-*N*-Methylation of Anti-Gram-Positive Aminoglycoside-Derived Membrane Disruptors Improves Antimicrobial Potency and Broadens Spectrum to Gram-Negative Bacteria

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Abstract: *The effect of di-N-methylation of bacterial membrane disruptors derived from aminoglycosides (AGs) on antimicrobial activity is reported. Di-N-methylation of cationic amphiphiles derived from several diversely structured AGs resulted in a significant increase in hydrophobicity compared to the parent compounds that improved their interactions with membrane lipids. The modification led to an enhancement in antibacterial activity and a broader antimicrobial spectrum. While the parent compounds were either modestly active or inactive against Gram-negative pathogens, the corresponding di-N-methylated compounds were potent against the tested Gram-negative as well as Gram-positive bacterial strains. The reported modification offers a robust strategy for the development of broad-spectrum membrane-disrupting antibiotics for topical use.*

Disruption of bacterial membranes is increasingly drawing attention as a strategy for antibiotic development. Membrane-disrupting antibiotics are effective against dormant and slow-growing bacteria that are frequently not susceptible to antibiotics that inhibit bacterial intracellular targets.[1] Very few membrane-disrupting antibiotics are currently in clinical use, however.[2]

The cationic lipopeptide antibiotic colistin, comprised of a mixture of colistin A and B (Figure 1 A), is an example of an antibiotic for treatment of Gram-negative bacterial infections. Colistin effectively disrupts the Gram-negative bacteria outer membrane by binding to lipopolysaccharide (LPS), a major component of the outer leaflet of the outer membrane.[3] Cationic lipopeptide antibiotics bind to the lipid A core of LPS (Figure 1 A) orders of magnitude more tightly than do calcium and magnesium cations that hold the highly negatively charged LPS units together.[4] By displacing the natural bivalent cations, lipopeptide antibiotics disrupt the outer membrane leading to uncontrolled cell permeability and cell death. Molecular modeling suggests that the phosphate groups of lipid A, which are negatively charged under physiological conditions, interact with the positively charged diaminobutyric acid residues of lipopeptides.[5] The recent increase in the percentage of Gram-negative strains that are resistant to cationic lipopeptides highlights the need for the development of new and broad spectrum Gram-negative outer membrane disruptors.[6]

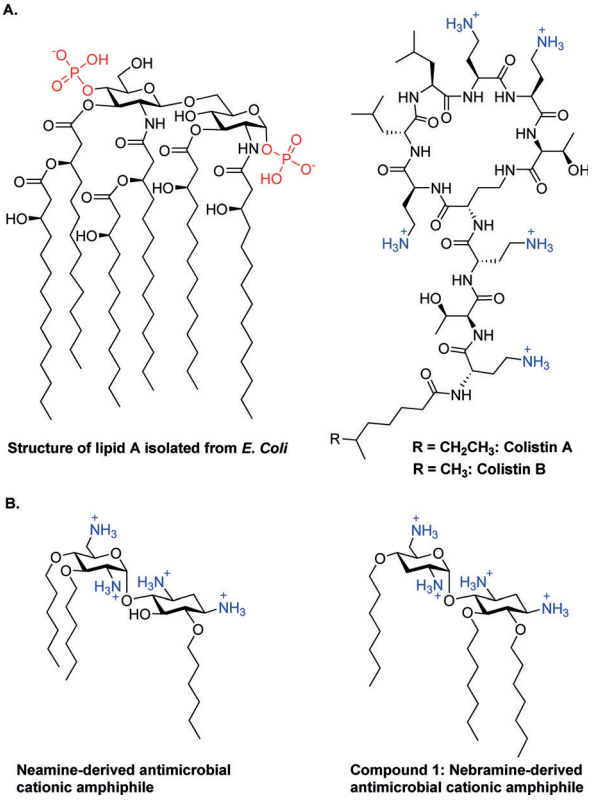

Figure 1. A) Structure of lipid A, the target of colistins. B) Neamine- and nebramine-derived anti-Gram-negative cationic amphiphiles.

In recent years, we as well as others have investigated the utilization of aminoglycoside (AG) antibiotics, natural pseudo-oligosaccharides that act primarily as inhibitors of functional protein synthesis,[7] as scaffolds for development of amphiphilic cationic bacterial membrane disruptors.[8–13]

Antimicrobial AG-derived cationic amphiphiles also have anti-fungal activity[14] and immunomodulatory properties.[15] Furthermore, some AG-derived cationic amphiphiles have affinity for LPS, suggesting that like other cationic amphiphiles these compounds may be used for the development of endotoxin-neutralizing agents.[16] Most of the reported AG-derived antimicrobial cationic amphiphiles are effective primarily against Gram-positive bacteria; however, cationic amphiphiles derived from neamine, developed by Décout and co-workers, and nebramine, developed by our group (com-

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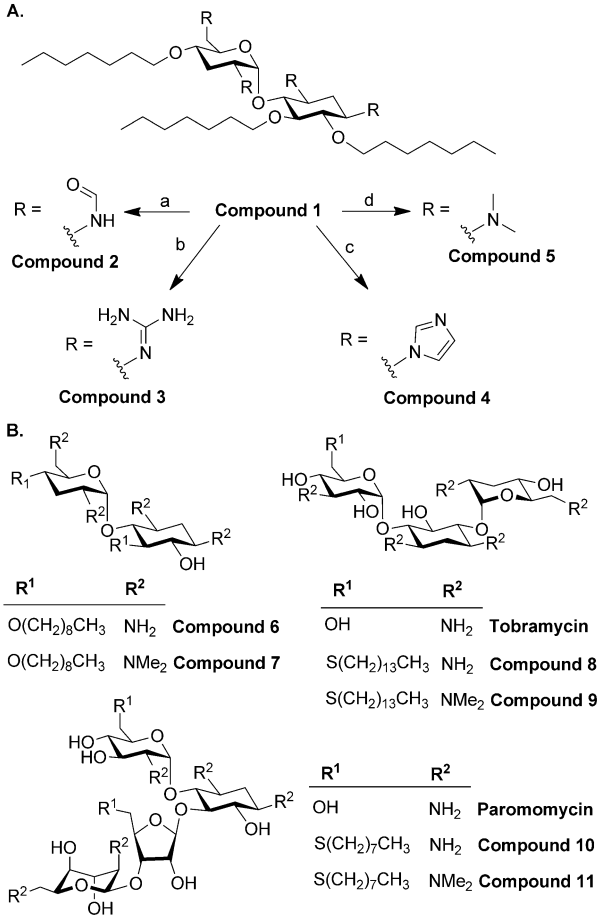
pound **1**, Figure 1B), are effective against several Gram-negative pathogens.[13,17] The anti-Gram-negative activity of these cationic amphiphiles was associated with their affinity for LPS.[18]

In the search of a strategy to broaden the efficacy spectrum of anti-Gram-positive AG-derived cationic amphiphiles to Gram-negative bacteria, we investigated the role of the positively charged primary amines of these membrane-disrupting agents on antimicrobial properties.

We chose the nebramine-derived cationic amphiphile **1** (Figure 1B) as a model compound since it exhibited antimicrobial efficacy against both Gram-positive and Gram-negative pathogens. The four primary amines of **1** were converted to the corresponding formamides, which are not charged under physiological conditions, or to the corresponding guanidine, imidazole, or dimethylated groups to afford the corresponding tertiary amines (compounds **2**–**5** respectively, Scheme 1A). The groups that are positively charged under physiological conditions in compounds **1** and **3**–**5** vary by several orders of magnitude in their basicity with pK_a values ranging from approximately 6 for the imidazole group to around 12.5 for the guanidine group.



Scheme 1. Synthesis of aminoglycoside-derived cationic amphiphiles. Reagents and conditions: a) formamide, reflux (41%); b) *N*,*N*′-di-Boc-*N*″-triflylguanidine, Et_3N, 1,4-dioxane/H_2O (5:1) (80%); c) glyoxal 40% in aqueous solution, formaldehyde, ammonium acetate, MeOH (67%); d) formaldehyde, $NaBH_3CN$, acetic acid, MeCN/H_2O (3:1) (58–98%).

The antimicrobial activity of compounds **1**–**5** was evaluated against five Gram-positive and six Gram-negative strains representing common opportunistic bacterial pathogens responsible for a wide range of infections. Minimum inhibitory concentration (MIC) values were determined using the broth double dilution method (Table 1).[12,19] Elimination of the positive charge of **1** by conversion of primary amines to the corresponding formamides (compound **2**) led to total loss of antimicrobial activity, thereby demonstrating the significance of the positive charge in facilitating the antimicrobial activity of these membrane disruptors. Modification of the primary amines of **1** to the corresponding guanidines (compound **3**) or to imidazole groups (compound **4**) led to loss of anti-Gram-negative activity; however, the anti-Gram-positive activity of these compounds was retained. Surprisingly, di-*N*-methylation of the four primary amines of **1** to afford compound **5** led to a significant and general enhancement in antimicrobial activity (Table 1).

Intrigued by the improvement in antimicrobial activity caused by di-*N*-methylation of compound **1**, we similarly modified three structurally different AG-derived antimicrobial cationic amphiphiles that were previously developed in our group: the pseudo-disaccharide nebramine-derived cationic amphiphile with two *n*-nonyl ethers (compound **6**, Scheme 1B),[13] the pseudo-trisaccharide tobramycin-derived cationic amphiphile (compound **8**) with a *n*-tetradecyl thioether unit,[11] and the pseudo-tetrasaccharide paromomycin-derived cationic amphiphile with two *n*-octyl thioethers (compound **10**).[12] These three AG-derived antimicrobial cationic amphiphiles demonstrated marked anti-Gram-positive activity, and modest to no activity against several Gram-negative strains.

Compounds **6**, **8**, and **10** were converted in a single step to the corresponding di-*N*-methylated analogues **7**, **9**, and **11** respectively (Scheme 1B). Compounds **7**, **9**, and **11** exhibited identical or up to eight-fold improvement in anti-Gram-positive activity relative to the parent compounds (Table 1). A dramatic effect was observed on efficacy against the six Gram-negative strains tested: Whereas the parent compounds were inactive to modestly active against the tested *E. coli* and *K. pneumoniae* strains and the clinical isolate of *S. sonnei*, the corresponding di-*N*-methylated analogues had significant anti-Gram-negative activity. For example, tobramycin-derived compound **8** did not exhibit potent antimicrobial activity against all six strains of Gram-negative bacteria tested (Strains F–K, MICs $\geq 64\ \mu g\,mL^{-1}$). In contrast, its di-*N*-methylated analogue **9** had MICs in the range of 2-8 $\mu g\,mL^{-1}$ against all six tested Gram-negative strains.

Compared to the antibiotics colistin, tobramycin, and gramicidin D that are in clinical use, all of the di-*N*-methylated compounds in this study had either a broader antimicrobial spectrum or improved antimicrobial activity, or both. The parent compounds **6**, **8**, and **10** with primary amines were significantly less potent than the quaternary ammonium salt cetrimonium bromide against the tested bacteria (Table 1). Cetrimonium bromide is a clinically used topical antiseptic. On the other hand, while 48% of the overall MIC values measured for the di-*N*-methylated analogues **7**, **9**, and **11** were two- to eight-fold higher than those measured for

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Table 1: Antibacterial activity: MIC values ($\mu g\,mL^{-1}$) against Gram-positive (+) and Gram-negative (−) strains.[a]

Compound	A (+)	B (+)	C (+)	D (+)	E (+)	F (−)	G (−)	H (−)	I (−)	J(−)	K(−)
Colistin	>64	>64	>64	>64	>64	4	2	16	8	8	2
Tobramycin	>64	32	4	4	2	2	4	2	4	2	16
Gramicidin D	2	0.25	8	32	1	>64	>64	>64	>64	>64	>64
Cetrimonium bromide	0.5	0.5	8	1	0.5	4	8	8	8	8	4
1	2	2	4	4	8	8	16	16	16	16	32
2	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
3	4	2	4	8	8	64	64	>64	>64	>64	>64
4	4	2	4	8	8	>64	>64	>64	>64	>64	>64
5	1	1	0.5	2	2	2	2	8	8	16	2
6	2	1	2	2	4	64	32	64	64	64	32
7	1	1	1	2	2	2	2	8	8	16	2
8	8	2	8	4	16	64	64	64	>64	>64	64
9	4	0.5	4	2	2	2	4	4	8	8	4
10	4	2	16	4	8	>64	64	>64	>64	>64	>64
11	2	1	2	2	4	8	8	16	16	16	4

[a] (A) Methicillin-resistant *S. aureus* ATCC 33592, (B) *S. pyogenes* Rosenbach ATCC 14289, (C) *S. aureus* Cowan, (D) *L. monocytogenes* ATCC 19115, (E) *E. faecalis* ATCC 29212, (F) *E. coli* ATCC 25922, (G) *E. coli* ATCC 9637, (H) *K. pneumoniae* K21, (I) *K. pneumoniae* K36, (J) *K. pneumoniae* K55, (K) *S. sonnei* clinical isolate 6831.

cetrimonium bromide, 28% of the values were identical and 24% were lower (Table 1).

In an attempt to explain the di-*N*-methylation effect, we investigated the possibility that this modification enhanced LPS affinity and, hence, facilitated the disruption of the outer membrane of Gram-negative bacteria. LPS affinity was evaluated by measuring the increase in fluorescence as a result of competitive displacement of the LPS-binding fluorescent dye BODIPY-cadaverine by the tested molecules.[20] We used LPS isolated from *E. coli* (serotype O111:B4) that was purified by ion-exchange chromatography and tested for efficacy as a Toll-like receptor ligand. The anti-Gram-negative and LPS targeting antibiotic colistin, the membrane-disrupting non-cationic antimicrobial peptide mixture gramicidin D, the ribosome-targeting AG tobramycin and the quaternary ammonium antiseptic agent cetrimonium bromide were used as controls.

The results of the LPS-BODIPY-cadaverine displacement experiments indicated that there was no correlation between the ability to displace the fluorescent dye in the complex and the anti-Gram-negative activity of the di-*N*-methylated amphiphilic AGs (Table 2). The parent compounds **6**, **8**, and **10** that were effective against Gram-positive strains were also more potent in displacing BODIPY-cadaverine from its complex with LPS than were the corresponding di-*N*-methylated analogues **7**, **9**, and **11** that were effective against the Gram-negative strains tested (Table 2). A similar effect was observed for the tetra-guanidine compound **3**, which effectively displaced BODIPY-cadaverine from its complex with LPS and yet was ineffective against the tested Gram-negative strains.

As the anti-Gram-negative activity of the di-*N*-methylated AG-derived cationic amphiphiles did not appear to be associated with improvement in LPS affinity, we reasoned that, compared with the parent AG-derived antimicrobial cationic amphiphiles, di-*N*-methylation significantly increased hydrophobicity of the molecules, and thereby generally improved the interactions with membrane lipids, resulting in non-specific membrane disruption. To quantitatively assess the increase in hydrophobicity, we calculated the partitioning coefficient (logP) values for each of the AG-derived cationic amphiphiles (Supporting Information, Table S1).

Table 2: LPS-BODIPY-cadaverine displacement assay.[a,b]

Compound	EC_{50} [μM]	Hill coefficient	Y_{max} [%]
Colistin	7.5	1.1	52
Tobramycin	ND	ND	ND
Gramicidin D	ND	ND	ND
Cetrimonium bromide	137.1	1.2	71
1	29.9	1.7	74
2	ND	ND	ND
3	46.6	1.3	95
4	181.5	2.4	97
5	101.1	0.9	61
6	9.8	2.8	77
7	20.3	1.16	66
8	13.9	1.1	100
9	8.1	14.1	50
10	15.4	1.0	97
11	41.2	0.3	84

[a] LPS of *E. coli* (serotype O111:B4) was incubated with BODIPY-cadaverine and the tested compound. Fluorescence was measured at 580 nm, maximal probe displacement (Y_{max}) of compound **8** was defined as 100%.[b]ND not determined for $Y_{max} < 20\%$.

The calculated logP values indicated that, in comparison to the parent compounds, the corresponding di-*N*-methylated analogues were significantly more hydrophobic and, interestingly, always by approximately 3 orders of magnitude compared with the parent compounds.

Further support for the hypothesis that di-*N*-methylation improves antimicrobial activity by increasing non-specific van der Waals interactions with membrane lipids was obtained through evaluation of erythrocyte membrane disruption (hemolysis) induced by the cationic amphiphiles in this study. The hemolytic effect was evaluated in an assay using

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laboratory rat red blood cells (erythrocytes).[12] Compared to compounds **6**, **8**, and **10**, the corresponding di-*N*-methylated analogues **7**, **9**, and **11** were considerably more hemolytic (Figure 2).

Interestingly, the antiseptic agent cetrimonium bromide and the antibiotic gramicidin D, which are both in topical clinical use, were significantly more hemolytic than all of the AG-derived antimicrobial cationic amphiphiles synthesized for this study. At a low concentration of 4 $\mu g\,mL^{-1}$ cetrimonium bromide and gramicidin D caused approximately 18% and 48% hemolysis, respectively (Figure 2D). Notably, compounds **3** and **4**, the guanidine and imidazole analogues of the nebramine-derived antimicrobial cationic amphiphile **1**, had dramatically improved bacterial membrane selectivity compared to that of the parent compound **1** (Figure 2D). Of the AG-derived membrane disruptors evaluated in this study, the imidazole analogue **4** displayed the greatest specificity: at a concentration of 256 $\mu g\,mL^{-1}$, compound **4** caused approximately 18% hemolysis, whereas at the same concentration the parent **1** caused 100% laboratory-rat erythrocyte hemolysis.

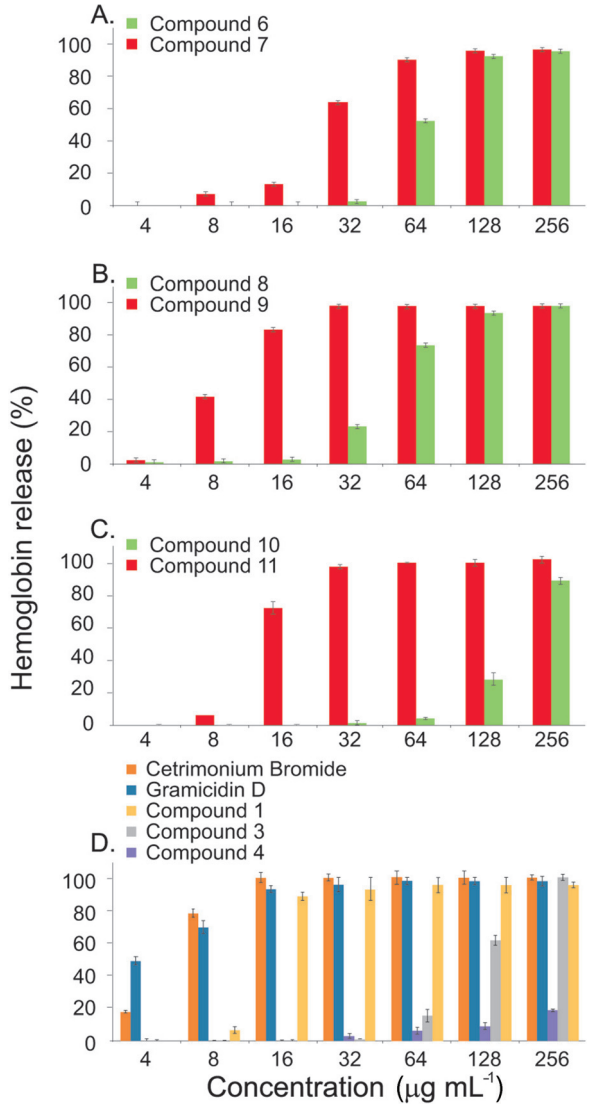

Figure 2. Hemolytic activity tests: Rat erythrocytes were incubated with increasing concentrations of the tested compounds for 1 hour at 37°C. Released hemoglobin absorbance was measured at 550 nm. The results are expressed as percentage of hemoglobin released relative to the positive control (Triton X100) for A) nebramine-derived compound **6** and di-*N*-methylated analogue **7**, B) tobramycin-derived **8** and di-*N*-methylated analogue **9**, C) paromomycin-derived **10** and di-*N*-methylated analogue **11**, D) cetrimonium bromide, gramicidin D, nebramine-derived cationic amphiphile **1** and its guanidine and imidazole analogues **3** and **4**.

To conclude, we report a chemical modification of AG-derived bacterial membrane disruptors that improved key aspects of their biological activity. We showed that di-*N*-methylation of the primary amines of AG-derived cationic amphiphiles resulted in novel membrane disruptors that demonstrated a general improvement in antimicrobial activity and, most notably, broadened their antimicrobial spectrum from Gram-positive strains to several Gram-negative strains as well. The marked improvement in antimicrobial properties that resulted from the di-*N*-methylation suggests a general direction for the development of new families of potent and broad spectrum AG-derived bacterial-membrane-disrupting antibiotics with high potential for the treatment of persistent topical infections.

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