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Evaluation of amphiphilic aminoglycoside–peptide triazole conjugates as antibacterial agents

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ABSTRACT

The solid- and solution-phase synthesis of amphiphilic aminoglycoside–peptide triazole conjugates (APTCs) accessed by copper(I)-catalyzed 1,3-dipolar cycloaddition reaction between a hydrophobic and ultrashort peptide-based alkyne and a neomycin B- or kanamycin A-derived azide is presented. Antibacterial evaluation demonstrates that the antibacterial potency is affected by the nature of the peptide component. Several APTCs exhibit superior activity against neomycin B- and kanamycin A-resistant strains when compared to their parent aminoglycoside while displaying reduced activity against neomycin B- and kanamycin A-susceptible strains.

Aminoglycoside antibiotics (AAs) constitute a large family of clinically important drugs used in the treatment of Gram-positive and Gram-negative bacterial infections. AAs bind to specific sites in prokaryotic ribosomal RNA affecting the fidelity of protein synthesis.[1] Although AAs exhibit potent bactericidal activity, their widespread use has been compromised by toxicity and the worldwide emergence of resistant strains.[2] Cationic antimicrobial peptides (AMPs) form another large class of antibacterials that are currently studied for clinical use. Naturally occurring AMPs typically contain 10–50 amino acids are amphiphilic and are rich in lysine or arginine and hydrophobic amino acids.[3] However, shorter, amphiphilic peptide sequences as short as di- or tri- or hexapeptides with antibacterial activity are known.[4–6] Numerous studies with linear, cyclic and diastereomeric AMPs have strongly supported the hypothesis that their physicochemical properties, rather than any precise sequence, are responsible for their activities.[3] It is generally believed that the amphiphilic topology is essential for insertion into and disruption of the cytoplasmic membrane.[3] In particular, the ability to rapidly kill bacteria and the relative difficulty with which bacteria develop resistance in vitro, make AMPs attractive targets for drug development.[3a]

Herein, we describe the synthesis and antibacterial properties of a new class of amphiphilic aminoglycoconjugates resembling ultrashort cationic peptide antibacterials termed aminoglycoside–peptide triazole conjugates (APTCs). The motivation behind this approach is based on the observation that cationic amphiphiles such as cationic lipids, cationic peptides, and lipopeptides, chlorhexidine, and quaternary ammonium salts have been used for several decades with little or no occurrence of resistance.[7,8] Our working hypothesis is that conversion of aminoglycoside antibiotics into cationic amphiphiles may enhance antibacterial activity against resistant strains in this old class of antibiotics. As method of ligation we selected the click-based copper-catalyzed azide–alkyne cycloaddition reaction[9–12] between a terminal peptide-based alkyne introduced in the form of propargylglycine (Pra) and the azides **1**[13] and **2**[14] easily prepared from neomycin B and kanamycin A (Fig. 1). Click-based cycloaddition reaction of azides **1** and **2** to nonpeptide-based alkynes have previously been reported in solution and on the solid phase[13,14] but no reports on the synthesis and antibacterial activity of amphiphilic APTCs exist. Neomycin B and kanamycin A were selected due to their commercial availability in multi-gram quantities, low price and low susceptibility against multi-drug resistant bacteria. The C5″–(C6″)-position of the azide group in **1** and **2** was based on easy synthetic accessibility and previous reports that have indicated that modified C5″-analogs of neomycin B retain high binding affinity to RNA[15] and antimicrobial activities.[16] To be compatible with solution- and solid-phase peptide chemistry using the Fmoc-strategy the remaining amino groups in **1** and **2** were protected as *tert*-butylcarbamate (Boc). CuI-catalyzed cycloaddition of azides **1** and **2** to peptides **3–8** (Fig. 1) afforded APTCs **9–11**, **13**, **14**, **16–20** (Fig. 2).

Peptides **3–8** were selected as representative examples of soluble or immobilized ultrashort and hydrophobic peptide sequences.

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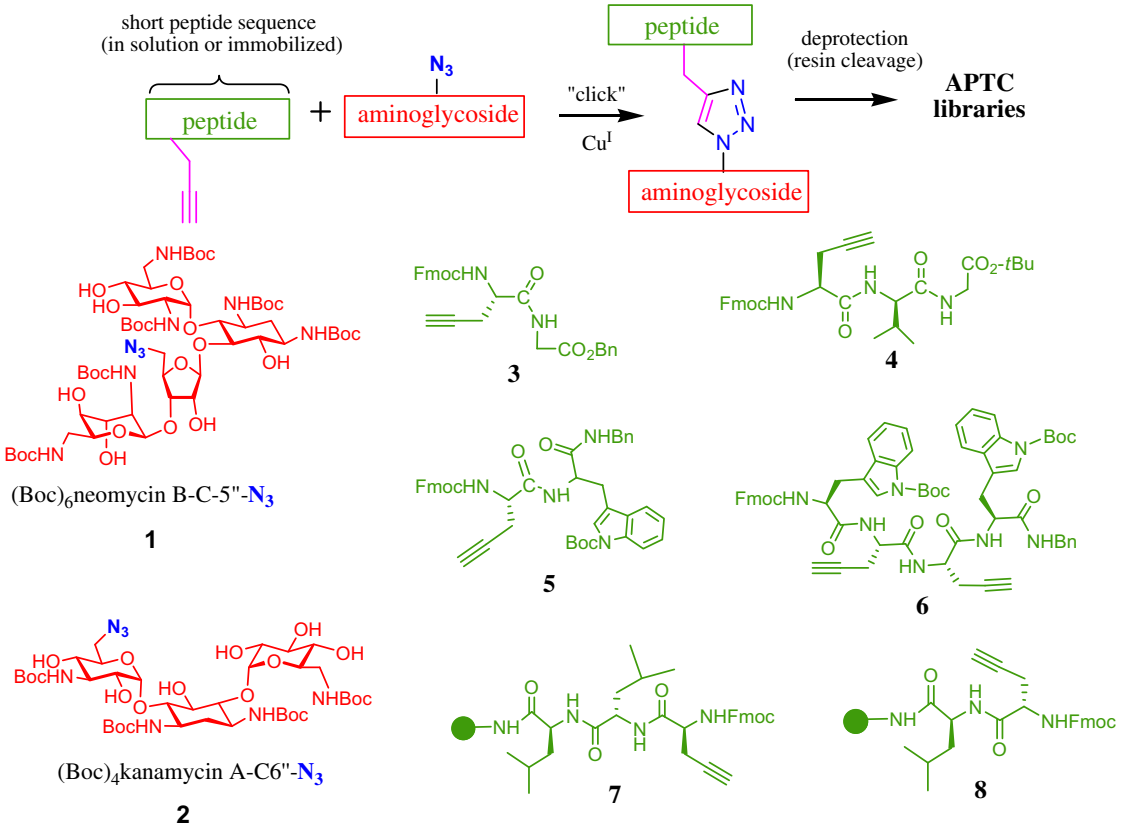

Figure 1. Click-based glycoconjugation of neomycin B- and kanamycin A-based azides **1** and **2** to hydrophobic alkyne-based peptides **3–8** that form a novel class of ultrashort antibacterial peptides termed aminoglycoside–peptide triazole conjugates (APTCs).

Short peptide sequences were selected to reduce the risk of metabolic proteolytic cleavage while at the same time reducing the production costs. Soluble peptides **3–6** are hydrophobic peptides in which hydrophobicity is gradually increased. Dipeptide **5** and tetrapeptide **6** containing tryptophan units were selected based on the preference for this hydrophobic amino acid in short and cationic peptide antibiotics.[17,18] The Fmoc-protecting group was retained in these peptides because previous reports have shown that Fmoc-protection in cationic peptide antibacterials enhances antibacterial activity.[5] Tetrapeptide **6** containing two alkyne moieties was selected to explore divalency effects while immobilized peptides **7** and **8** were chosen to study solid-phase ligation with azide **1**. Guanidinylation of the amino groups in conjugates **11** and **14** using *N*,*N*′-diBoc-*N*′-triflylguanidine[19] produced guanidine conjugates **12** and **15**. These analogs were selected to explore basicity affects in APTCs. In addition, it is known that replacement of lysine by arginine in ultrashort cationic peptide antibacterials enhances antibacterial activity.[4] The antibacterial potency of all APTCs were determined by measuring the minimal inhibitory concentration (MIC) against a broad selection of Gram-positive and Gram-negative pathogens including three AA-resistant strains obtained from a national surveillance study assessing antimicrobial resistance in Canadian intensive care units (CAN-ICU)[20] (Table 1). Our data demonstrate that the antibacterial activity of APTCs is affected by the nature of the peptide chain. For instance, APTCs **13** and **16** containing a carboxylate display weak antibacterial activity against most bacterial strains while APTCs **14** and **17** exhibit relatively potent antimicrobial activity against Gram-positive and most Gram-negative strains. More importantly, APTCs **11**, **12**, **14**, **15** and **17**, **19** and **20** can overcome the acquired resistance of MRSA against neomycin B and kanamycin A. Similarly, kanamycin A–dipeptide conjugate **15** shows a 16-fold lower minimal inhibitory concentration against MRSE when compared to kanamycin A. In addition, kanamycin A–dipeptide conjugate **14** exhibits a $\geqslant$32-fold lower MIC against kanamycin A-resistant *Pseudomonas aeruginosa* and a eightfold lower MIC (compared to gentamicin) against Gentamicin-resistant *P. aeruginosa*. Furthermore, enhancement of the amphiphilicity in APTC via increase in hydrophobicity enhances antibacterial activity. For instance, Fmoc-protected APTC **11** displays a eightfold lower MIC against MRSA when compared to unprotected peptide **10** suggesting that the amphiphilic nature of APTCs is crucial for their antibacterial activity. It is noteworthy that the guanidinylated APTCs **12** and **15** lead to a 2–4-fold enhancement in Gram-positive activity when compared to their nonguanidinylated analogs **11** and **14**, respectively. This is in contrast to observations with nonamphiphilic guanidinylated AAs that exhibit reduced antibacterial activity.[1b,21] Moreover, our results show that amphiphilic peptides **17** and **18** containing two aminoglycoside units do not improve antibacterial activity. In vitro toxicity studies show that most APTCs exhibit little to no toxicity against mammalian erythrocytes at a concentration of 100 μg/mL (Table 1). However, substantial hemolytic activities (>20%) are seen at higher concentrations (500–1000 μg/mL).

In summary, we have prepared a new class of amphiphilic aminoglycoconjugates via click-based glycoconjugation of neomycin B- and kanamycin A-derived azides **1** and **2** to short hydrophobic peptides in solution and on the solid phase. Our results demonstrate that APTCs display enhanced antibacterial activity against neomycin B-, kanamycin A-resistant MRSA, kanamycin A-resistant MRSE and gentamicin-resistant *P. aeruginosa*. However, most APTCs display reduced antibacterial activity against neomycin B- and kanamycin A-susceptible strains. In vitro toxicity measurements demonstrate that most APTCs are selective for

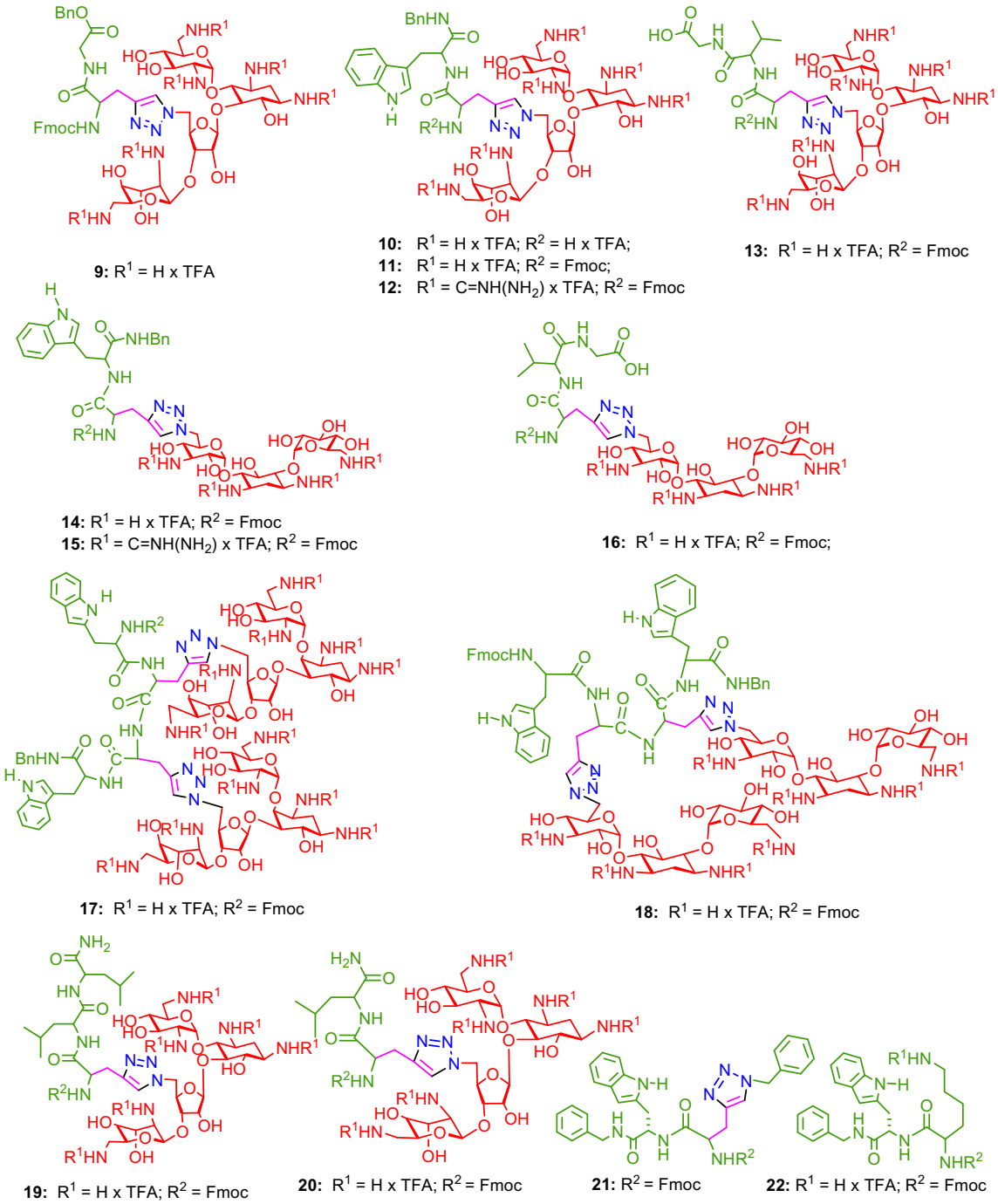

Figure 2. Amphiphilic neomycin B- and kanamycin A-based peptide triazole conjugates (APTCs) prepared by click-based glycoconjugation of azides **1** and **2** to hydrophobic alkyne-modified peptides **3**–**8**. Peptides **21** and **22** are negative controls.

bacterial cells and show little hemolytic activity against mammalian erythrocytes at MIC concentration although significant hemolytic activity occurs at higher concentrations.

The physicochemical similarities between APTCs and AMPs suggest a membranolytic mode of action. Further evidence for a membranolytic mode of action of APTCs is supported by (a) the observed concentration-dependent hemolytic activity of all APTCs; (b) the requirement for a strongly hydrophobic peptide segment to induce potent antibacterial activity; (c) the precedence of a plethora of cationic antibacterial amphiphiles with membranolytic mode of action and (d) a recent study of C5″-modified neomycin–lipid amphiphiles that have demonstrated that polycationic neomycin–lipid conjugates are able to synergistically enhance the antibacterial activity of antibiotics interacting with intracellular targets such as amikacin and neomycin.[22] In addition, we and others have recently shown that the antibacterial activity of C5″-modified neomycin–lipid conjugates against MRSA is strongly dependent on the length of the lipid moiety.[23,16c] For instance, C5″-modified neomycin–C_6 lipid does not inhibit the growth of MRSA (MIC >512 μg/mL) while C5″-modified neomycin–C_{16} lipid displays potent anti-MRSA activity (MIC = 4 μg/mL). This is consistent with observations obtained with other cationic amphiphiles with membranolytic mode of action that have clearly indicated that the presence of a hydrophobic moiety above a certain thresh-

Table 1
Minimal inhibitory concentration (MIC) in μg/ml of APTCs **9**–**20**, gentamicin, neomycin B, kanamycin A (positive controls) and **21** and **22** (negative controls) against Gram-positive and Gram-negative organisms

Organism	Compound																
	Genta-micin	Neo-mycin B	Kana-mycin A	**21**[m]	**22**	**9** *N*[k]	**10** *N*[k]	**11** *N*[k]	**12** *N*[k]	**13** *N*[k]	**14** *K*[l]	**15** *K*[l]	**16** *K*[l]	**17** *N*[k]	**18** *K*[l]	**19** *N*[k]	**20** *N*[l]
S. aureus[a]	1	1	4	128	512	16	32	8	4	16	16	4	16	8	32	32	32
MRSA[b]	2	256	>512	128	512	64	128	16	8	256	32	8	>256	16	32	32	32
S. epidermidis[c]	0.25	0.25	2	128	256	4	8	4	1	8	8	2	4	2	8	16	8
MRSE[d]	32	0.25	128	64	512	8	8	8	2	8	16	8	64	4	16	16	16
S. pneumoniae[e]	4	8	8	128	>512	>512	>512	64	64	128	64	64	8	64	64	>128	>256
E. coli[f]	1	2	8	128	512	32	32	16	32	32	32	64	128	32	64	64	64
E. coli[g] (Gent-R)	128	4	16	128	512	32	32	32	64	32	32	64	32	64	64	128	64
E. coli[h] (Amik-R)	8	32	32	128	n.d.	64	n.d.	n.d.	64	256	32	64	>256	16	32	128	64
P. aeruginosa[i]	8	512	>512	128	>512	512	512	128	128	256	128	128	>256	128	128	128	>256
P. aeruginosa[j] (Gent-R)	128	512	>512	128	>512	512	256	64	128	256	16	128	>256	32	64	64	>256
(%) Hemolysis[n] at 100 μg/ml	n.d.	<0.5	<0.5	n.d	n.d	1.0	1.0	9.9	n.d	0.8	3.9	13.7	n.d	1.5	10.2	1.0	2.2

n.d. = not determined.
[a] ATCC 29213.
[b] Methicillin-resistant *S. aureus* ATCC 33592.
[c] *S. epidermidis* ATCC 14990.
[d] Methicillin-resistant *S. epidermidis* Canadian Intensive Care Unit (CAN-ICU) 61589.
[e] ATCC 49619.
[f] ATCC 25922.
[g] CAN-ICU 61714.
[h] CAN-ICU 63074.
[i] ATCC 27853.
[j] CAN-ICU 62308.
[k] Neomycin B-based conjugate.
[l] Kanamycin A-based conjugate.
[m] Dissolved in DMSO and diluted with H_2O.
[n] Human erythrocytes.

old is critical for antibacterial activity.[3c,24–26] However, RNA-mediated interactions leading to additive or synergistic effects when combined with cell permeation cannot be ruled out.[27] In addition, it is noteworthy that the hemolytic activity of the highly potent neomycin–C_{16} lipid conjugate is significantly elevated (56% hemolysis at 100 μg/mL) when compared to peptide-based APTCs suggesting that APTCs may exhibit reduced in vitro toxicity.[28]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.03.116.

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