MedChemComm

RSC Publishing

CONCISE ARTICLE

View Article Online
View Journal | View Issue

Design of membrane targeting tobramycin-based cationic amphiphiles with reduced hemolytic activity†‡

Cite this: *Med. Chem. Commun.*, 2013, **4**, 120

Ido M. Herzog,[a] Mark Feldman,[a] Anat Eldar-Boock,[b] Ronit Satchi-Fainaro[b] and Micha Fridman*[a]

Received 21st June 2012
Accepted 20th July 2012

DOI: 10.1039/c2md20162c

www.rsc.org/medchemcomm

Tobramycin-based cationic amphiphiles differing in the chemical bond linking their hydrophobic and hydrophilic parts were synthesized and biologically evaluated. Several compounds demonstrated potent antimicrobial activities compared to the parent drug. One analogue exhibited a significant reduction in red blood cells hemolysis, demonstrating that it is possible to maintain the antimicrobial potency of these molecules while reducing their undesired hemolytic effect through chemical modifications.

Membranes and cell walls are essential constituents required for the viability of bacterial cells, and therefore serve as attractive targets for the development of antibiotics. Amongst cell-wall-targeting antibiotics are several families of peptidoglycan biosynthesis inhibitors, including β-lactams that irreversibly inhibit the activity of the peptidoglycan trans-peptidation biosynthetic step,[1,2] glyco-peptide antibiotics such as vancomycin that competitively inhibit the trans-peptidation step, and the glyco-lipid antibiotic agent moenomycin A that inhibits the peptidoglycan trans-glycosylation step.[3–6] To date, disruption of the bacterial membrane bi-layer has been poorly exploited as a strategy for the development of antibiotics. Bacterial membrane-disrupting antibiotics offer several advantages over antimicrobial agents that target intracellular bacterial targets: first, membrane disruption is not dependent on the bacterial cell cycle state and is therefore a promising strategy for the eradication of dormant bacteria and treatment of persistent infections.[7] Second, antimicrobial agents that act in the extracellular bacterial environment evade intracellular resistance mechanisms and are expected to maintain prolonged clinical efficacy. Finally, cell permeability consideration, which is often a significant challenge for drug designers, is not necessary for the design of membrane-targeting antibiotics. Although peptidoglycan exists solely in bacteria, membranes composed of lipid bi-layers are common to all cells; therefore, avoiding cytotoxicity to eukaryotic cells through non-selective membrane disruption is a major challenge. In contrast to most eukaryotic cell membranes, both Gram-positive and Gram-negative bacterial membranes are highly negatively charged due to high content of anionic lipids such as cardiolipin and phosphatidylglycerol.[8,9] Gram-negative bacterial membranes also have the negatively charged core of lipopolysaccharide (LPS), while negatively charged techoic acids are major constituents of Gram-positive bacterial cell walls.[10,11]

Hence, both Gram-positive and Gram-negative bacterial membranes attract positively charged organic compounds through ionic interactions. LPS that constitutes the Gram-negative outer membrane leaflet is unique to bacteria and

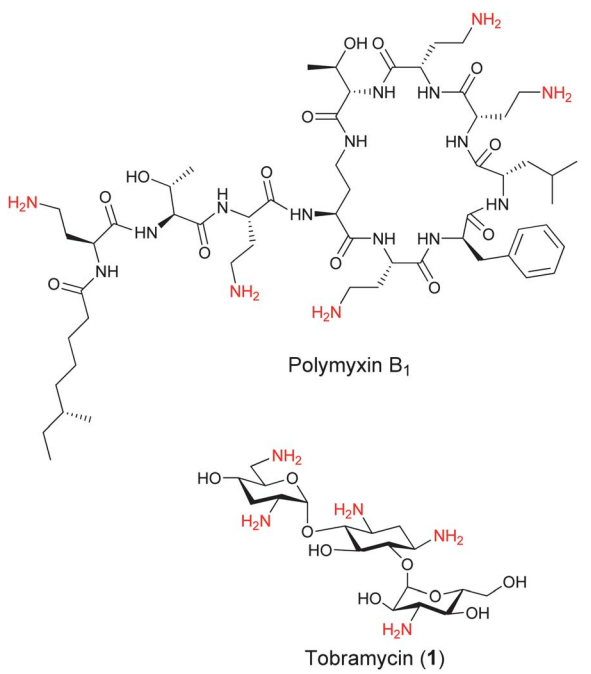


Fig. 1 Structures of the Gram-negative bacteria targeting polymyxin B_1 and the bacterial ribosome targeting aminoglycoside tobramycin.

[a]*School of Chemistry, Tel Aviv University, Tel Aviv 69978, Israel. E-mail: mfridman@post.tau.ca.il; Fax: +972 3-6409293; Tel: +972 3-6408687*

[b]*Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*

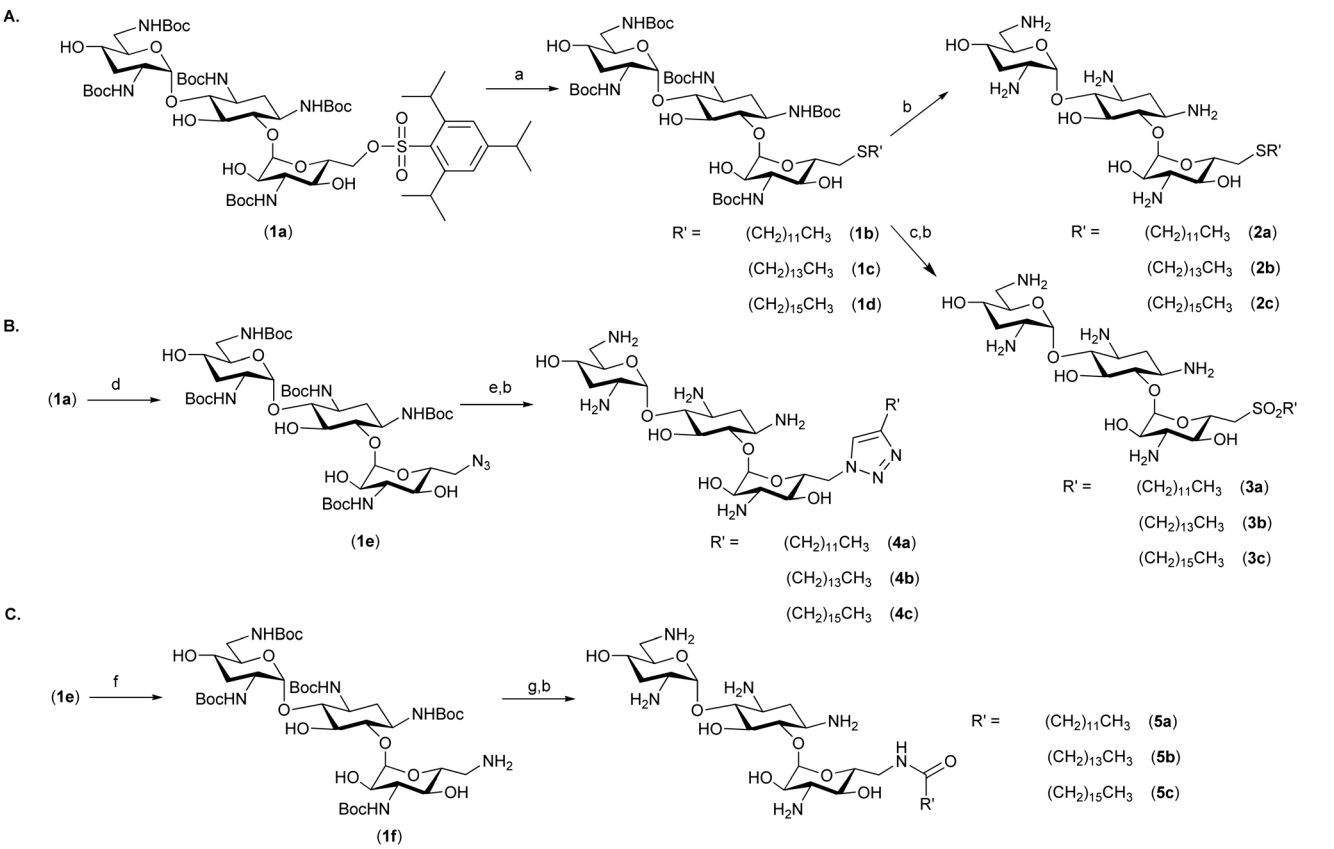
† This article is part of a MedChemComm 'New Talents' issue highlighting the work of outstanding rising scientists in medicinal chemistry research.

‡ Electronic supplementary information (ESI) available: Synthetic protocols, ^{1}H, ^{13}C NMR assignments and spectra, HRMS, MIC, MBIC, hemolysis assay procedures are provided. See DOI: 10.1039/c2md20162c

serves as a target for the antimicrobial agent polymyxin B_1 (Fig. 1). Polymyxin B_1 composed of a cyclic cationic decapeptide with an N-terminal hydrophobic residue is a potent and clinically used antibiotic that binds to the negatively charged LPS core and disrupts the outer membrane of Gram-negative bacteria.[12,13] The potency and broad-spectrum activity of polymyxin B_1 against Gram-negative bacteria demonstrate the potential that lies in the development of membrane-targeting antibiotics. In recent years, several studies have demonstrated the potential of positively charged aminoglycosides (AGs) as scaffolds for the development of membrane-targeting cationic amphiphilic antimicrobial agents by the attachment of hydrophobic residues to one or more positions on the AG.[14–17]

We have been particularly interested in tobramycin (**1**) based cationic amphiphiles since similar to polymyxin B_1, this AG also contains five primary amines which are positively charged under physiological conditions (Fig. 1). We recently demonstrated that the attachment of aliphatic chains to the 6″-position of tobramycin resulted in potent antimicrobial agents and provided evidence for their membrane-disruption activity.[18]

The most potent and broad-spectrum antimicrobial activity was observed for thioether analogues containing C_{12}-, C_{14}-, and C_{16}-linear alkyl chains (Scheme 1A, **2a–c**). The aliphatic chain length affected not only the antimicrobial activity but also the level of undesired red blood cell (RBC) hemolysis; the C_{12} analogue had the least hemolytic activity. We hypothesized that the aliphatic alkyl chains and the AG scaffold are required for optimal antimicrobial activity but that altering the link between these two segments should not have a dramatic effect on the antimicrobial performance, yet may affect the specificity of these compounds towards different membranes. To test this hypothesis, we chose to evaluate several types of chemical bonds between the aliphatic chain and tobramycin (**1**). We compared the thioether-linked analogues (Scheme 1A, **2a–c**) to sulfone-linked analogues (**3a–c**), triazole ring-linked analogues (Scheme 1B, **4a–c**), and amide bond-linked analogues (Scheme 1C, **5a–c**). The thioethers **2a–c** were prepared from the penta-*NH*-Boc-6″-O-trisyl tobramycin (Scheme 1A, **1a**) as previously reported.[18,19] Oxidation of the protected thioether analogues (**1b–d**) using *m*CPBA followed by the removal of the *NH*-Boc protecting groups in neat TFA yielded the sulfone analogues (**3a–c**). The 6″-O-trisyl group of **1a** was replaced by an azide to yield compound **1e**,[20] which served as a precursor for the preparation of the triazole analogues (Scheme 1B, **4a–c**). Microwave-heated click reaction using **1e** and terminal alkynyl aliphatic chains, followed by the removal of the *NH*-Boc groups, yielded the desired triazole analogues **4a–c**. Reduction of the 6″-azido group of **1e** under the Staudinger reaction conditions resulted in superior yields (80%) of the 6″-amino tobramycin analogue **1f** (Scheme 1C) compared to the reduction of the azide



Scheme 1 Synthesis of amphiphilic tobramycin analogues: *Reagents and conditions*: (a) R′SH, Cs_2CO_3, DMF, 25–60 °C, 63–92%; (b) neat TFA, rt; (c) *m*CPBA (3 equiv.), $CHCl_3$, rt; (d) NaN_3, DMF, 60 °C, 12 h, 91%; (e) R′CCH, $CuSO_4 \cdot 5H_2O$ (0.1 equiv.), sodium ascorbate (0.2 equiv.), DMF, microwave irradiation, 87–94%; (f) PMe_3 (1 M in THF, 1.1 equiv.), 0.01 M aqueous NaOH/THF: 1/20, rt, 80%; and (g) R′COOH, HBTU, DIEA, DMF, 71–86%.

under catalytic hydrogenation conditions (H_2, Pd/C, MeOH). Compound **1f** served as the precursor for the preparation of the amide analogues (Scheme 1C); **1f** was coupled to linear aliphatic carboxylic acids using HBTU (71–86% yield), and the *NH*-Boc groups were removed to yield the amide-linked analogues (Scheme 1C, **5a–c**).

The minimum inhibitory concentrations (MICs) of the semi-synthetic tobramycin amphiphiles were determined for 11 Gram-positive and Gram-negative strains (Table 1). Amongst the Gram-positive bacteria were pathogenic strains such as *Streptococcus pyogenes* M12 (strain *A*), a hospital isolate of methicillin-resistant *Staphylococcus aureus* (MRSA; strain B), and vancomycin-resistant *Enterococcus* (VRE; strain *D*) with high levels of resistance to tobramycin (MIC = 64 μg mL^{-1} for strain *A* and >128 μg mL^{-1} for strains *B* and *D*). Amongst the Gram-negative isolates were the pathogenic and highly tobramycin resistant (MIC > 128 μg mL^{-1}) *Pseudomonas aeruginosa* (ATCC33347; strain *I*) and *Shigella sonnei*, which is responsible for the severe foodborne disease shigellosis. Two types of *S. sonnei* were tested: O-antigen positive (strain *J*), and O-antigen negative (strain *K*).[21] In general, analogues with a C_{14} linear aliphatic chain (**2b**, **3b**, **4b**, and **5b**) exhibited the most potent antimicrobial activity, which was in most cases one to two double dilutions more potent than the activity of the corresponding C_{12} and C_{16} linear aliphatic chain analogues. The chemical links between the AG and the aliphatic chain did not have significant effects on MIC values against the tested strains with the exception of the sulfone linked analogues **3a–c**. These analogues were less potent than the corresponding un-oxidized thioether analogues **2a–c**. Some of the amphiphilic tobramycin analogues demonstrated high potency against strains that were highly tobramycin resistant: the MIC of tobramycin against *S. pyogenes* M12 (strain *A*) was 64 μg mL^{-1}; the thioether **2b**, triazole **4b**, and the amide analogue **5b** were 16 to 32 times more potent against this strain (2 μg mL^{-1} for **2b**, and 4 μg mL^{-1} for **4b** and **5b**). A significant improvement in antimicrobial activity of the semi-synthetic analogues compared to that of tobramycin was also observed in the case of *S. mutans* UA159 and *S. epidermidis* ATCC35984 (strains *C* and *G*, respectively). Although most of the synthetic analogues were not active against the tested *P. aeruginosa* (strain *I*), the C_{12} chain triazole analogue **4a** and amide analogue **5a** demonstrated improved antimicrobial activity against this strain relative to tobramycin (MICs = 64 and 32 μg mL^{-1}, respectively, and MIC > 128 μg mL^{-1} for tobramycin).

The antibacterial activity of tobramycin and six out of the 12 synthetic analogues was better against O-antigen positive *S. sonnei* (strain *J*) than against the corresponding O-antigen negative (strain *K*). This difference may be explained by the higher overall negative charge of the membrane of the O-antigen positive *S. sonnei*, which contains the negatively charged 2-acetamido-2-deoxy-L-altruronic acid.[22]

It was previously demonstrated that low micromolar concentrations of saturated fatty acids inhibit the formation of biofilms formed by *S. aureus* and *Listeria monocytogenes* strains.[23] The most potent biofilm growth inhibitors were C_{12}–C_{14} aliphatic chain carboxylic acids. We therefore determined the minimal biofilm inhibition concentration (MBIC) values for each of the C_{12} and C_{14} chain tobramycin analogues (Table 2). MBIC tests were performed using *S. mutans* UA159 and *S. epidermidis* ATCC35984 grown under biofilm-forming conditions. Compared to tobramycin (MBIC range of 64–128 μg mL^{-1}), the tested analogues demonstrated improved biofilm growth inhibition properties (MBIC range of 4–32 μg mL^{-1}) against the tested strains (Table 2). However, the MBIC values of the tested compounds were identical or no more than one double dilution lower than their MIC values against strains *C* and *G*. We therefore conclude that these compounds have no specific biofilm growth inhibition properties against the tested strains, and that their MBIC values result from their antibacterial activity.

Table 1 MIC values (μg mL^{-1}) of tobramycin and its amphiphilic analogues

	MICs (μg mL^{-1}) for tested bacterial strains[b]										
AG[a]	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*
1	64	>128	128	>128	>128	16	128	<1	>128	16	32
2a	8	64	8	64	128	16	8	8	128	32	64
2b	2	32	2	16	64	8	4	4	128	16	32
2c	4	64	4	64	128	16	8	4	>128	32	64
3a	32	>128	16	64	>128	64	64	128	>128	>128	>128
3b	4	64	4	32	64	16	16	16	128	32	64
3c	8	32	8	32	64	8	16	16	>128	32	128
4a	8	128	4	32	128	16	8	16	64	64	64
4b	4	64	4	32	128	8	8	4	128	32	32
4c	16	32	8	16	128	16	8	4	>128	128	64
5a	16	128	4	64	128	16	8	8	32	128	128
5b	4	32	4	32	64	8	4	4	128	16	32
5c	8	32	4	32	128	8	4	4	128	32	32

[a] AG = aminoglycoside. [b] MIC values were determined against Gram-positive bacterial strains: *A*, *S. pyogenes* serotype M12 (strain MGAS9429); *B*, MRSA; *C*, *S. mutans* UA159; *D*, VRE; *E*, *E. faecalis* ATCC29212; *F*, *S. aureus* ATCC9144; *G*, *S. epidermidis* ATCC35984; *H*, *S. epidermis* ATCC12228 and Gram-negative bacterial strains: *I*, *P. aeruginosa* ATCC33347; *J*, *S. sonnei* clinical isolate 6831 (O-antigen positive); and *K*, *S. sonnei* clinical isolate 6831 (O-antigen negative). All strains were tested by using the double-dilution method (from a starting concentration of 128 μg mL^{-1}). All experiments were performed in triplicate, and results were obtained from two different sets of experiments.

Table 2 Biofilm growth inhibition. MBIC values (μg mL^{-1}) of the amphiphilic tobramycin analogues and tobramycin (**1**)

AG[a]									
Strain[b]	**1**	**2a**	**2b**	**3a**	**3b**	**4a**	**4b**	**5a**	**5b**
C	64	4	4	16	4	4	4	8	4
G	128	16	8	32	8	16	16	8	8

[a] AG = aminoglycoside. [b] *S. mutans* UA159, *C*; *S. epidermidis* ATCC35984, *G*. All strains were tested by using the double-dilution method (starting from 128 μg mL^{-1}). *S. mutans* biofilm was grown in BHI + sucrose 2%, at final dilution 1 : 100. *S. epidermidis* biofilm was grown in TSB + glucose 1%, at final dilution 1 : 100. Biofilms were stained using crystal violet. All experiments were performed in triplicate and results were obtained from two different sets of experiments.

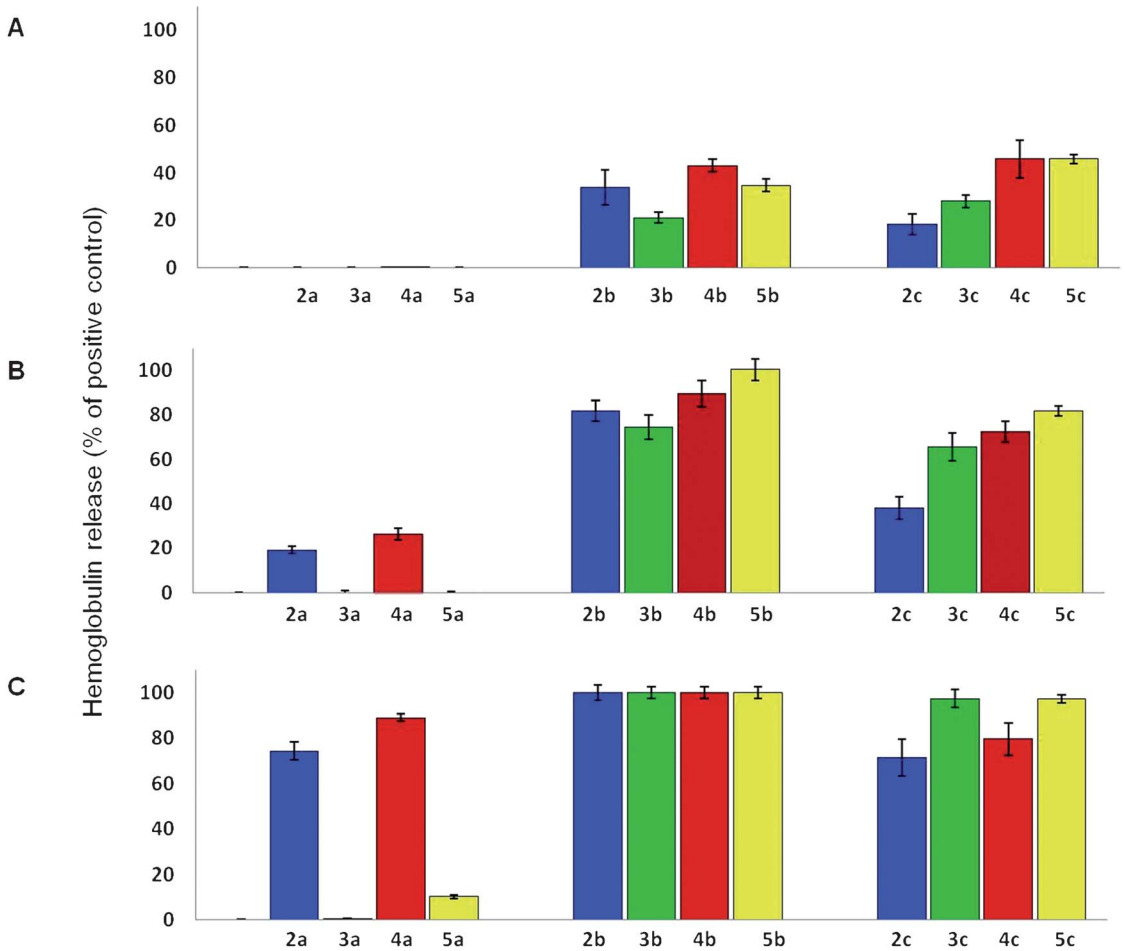


Fig. 2 Laboratory rat RBC hemolysis assay. Amphiphilic tobramycin analogues were incubated with RBCs isolated from a laboratory rat at concentrations of (A) 32 μg mL^{-1}, (B) 64 μg mL^{-1}, and (C) 128 μg mL^{-1} for 1 hour at 37 °C. All experiments were performed in triplicate, and results are the average from two different sets of experiments using blood samples from two laboratory rats.

Finally, the hemolytic activity was determined using a hemolysis assay using laboratory rat RBCs (Fig. 2A–C).[18] The MIC and MBIC values were significantly lower than the concentrations required for 100% hemolysis for some of the analogues (Fig. 2). In most cases, the MIC range of analogues with the C_{14} aliphatic chain was 2–32 μg mL^{-1}; these analogues caused significant hemolysis (~23 to 43%) at 32 μg mL^{-1}. All of the C_{14} aliphatic chain analogues caused extensive hemolysis (74.4 ± 5.5% to 100%) at a concentration of 64 μg mL^{-1}. The C_{16} aliphatic chain analogues also caused high levels of hemolysis at 64 μg mL^{-1} (37.9 ± 5.1% to 81.8 ± 2.3%).

No direct correlation between the antibacterial potency and the hemolytic activity was detected for the thioether, triazole, or amide analogues. As initially hypothesized, the hemolytic activity of the different tobramycin analogues was affected by the type of bond between the aliphatic chain and the AG scaffold. The most dramatic effect was observed for the C_{12} aliphatic chain analogues. At 64 μg mL^{-1}, the triazole C_{12} aliphatic chain analogue **4a** demonstrated the highest hemolytic effect (26.3 ± 2.7%) of the C_{12} aliphatic chain tobramycin analogues. The C_{12} aliphatic chain amide analogue **5a** caused almost no hemolysis at the same concentration (0.0 ± 0.4%). At 128 μg mL^{-1}, the triazole analogue **4a** caused extensive hemolysis (89.1 ± 1.6%), the thioether **2a** caused 71.6 ± 8.3% hemolysis, yet the amide analogue **5a** caused significantly less hemolysis (10.2 ± 0.8%). The lowest hemolytic activity at all of the tested concentrations was observed for the C_{12} sulfone analogue **3a**, however, this compound had poor antimicrobial activity against the tested strains. In contrast, while the C_{12} amide analogue **5a** was potent against several of the tested bacterial strains, and was the most potent analogue against the tested *P. aeruginosa* (strain *I*), it caused the lowest levels of hemolysis at a concentration which was 16–32 times higher than the MIC values of this compound against several of the tested strains.

Conclusions

In conclusion, 12 6″-aliphatic chain tobramycin analogues differing in the chemical linkage between the AG and the hydrophobic chain (thioether, sulfone, triazole, and amide bonds) and in the length of their hydrophobic linear aliphatic chain (C_{12}, C_{14}, and C_{16} chains) were synthesized and

evaluated for their antimicrobial activity against 11 bacterial strains. Of the three chain lengths tested, the C_{14} aliphatic chain analogues were the most potent antimicrobial agents, and were in most cases one or two double dilutions more potent than the corresponding C_{12} and C_{16} chain analogues. In some cases, the antimicrobial activity was at least 32-fold more potent than that of the parent AG tobramycin (**1**). Finally, RBC hemolysis tests revealed that there was no linear correlation between the antimicrobial potency and the hemolytic activity of the amphiphilic tobramycin analogues. Both the aliphatic chain length and the type of chemical linkage between the hydrophilic and hydrophobic parts of the molecule affect the specificity towards bacterial membranes. The C_{12} linear aliphatic chain 6″-amide analogue **5a** is of particular interest. This analogue was significantly more potent than tobramycin and caused little measurable hemolysis of laboratory rat RBCs at concentrations up to 32 times higher than the MIC values of this compound against some of the tested bacterial strains. The results of this study demonstrate that the choice of the hydrophobic segment and the chemical group that links the hydrophobic region to the AG is an important factor in the design of such membrane targeting antibiotics. Hence, further improvement in the selectivity of these compounds towards bacterial membranes through chemical modifications is worth pursuing.

Acknowledgements

This work was supported by the FP7-PEOPLE-2009-RG Marie Curie Action: Reintegration Grants (grant 246673). We thank Profs Itzhak Ofek, Dani Cohen (Tel Aviv University) and Doron Steinberg (The Hebrew University of Jerusalem) for the gift of bacterial strains.

Notes and references

1 H. S. Chung, Z. Yao, N. W. Goehring, R. Kishony, J. Beckwith and D. Kahne, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 21872–21877.
2 J. F. Fisher, S. O. Meroueh and S. Mobashery, *Chem. Rev.*, 2005, **105**, 395–424.
3 V. L. Healy, I. A. D. Lessard, D. I. Roper, J. R. Knox and C. T. Walsh, *Chem. Biol.*, 2000, **7**, R109–R119.
4 M. Ge, Z. Chen, H. R. Onishi, J. Kohler, L. L. Silver, R. Kerns, S. Fukuzawa, C. Thompson and D. Kahne, *Science*, 1999, **284**, 507–511.
5 Y. Yuan, S. Fuse, B. Ostash, P. Sliz, D. Kahne and S. Walker, *ACS Chem. Biol.*, 2008, **3**, 429–436.
6 S. Fuse, H. Tsukamoto, Y. Yuan, T.-S. A. Wang, Y. Zhang, M. Bolla, S. Walker, P. Sliz and D. Kahne, *ACS Chem. Biol.*, 2010, **5**, 701–711.
7 J. G. Hurdle, A. J. O'Neill, I. Chopra and R. E. Lee, *Nat. Rev. Microbiol.*, 2011, **9**, 62–75.
8 J. Weghuber, M. C. Aichinger, M. Brameshuber, S. Wieser, V. Ruprecht, B. Plochberger, J. Madl, A. Horner, S. Reipert, K. Lohner, T. Henics and G. J. Schutz, *Biochim. Biophys. Acta, Biomembr.*, 2011, **1808**, 2581–2590.
9 R. F. Epand, P. B. Savage and R. M. Epand, *Biochim. Biophys. Acta, Biomembr.*, 2007, **1768**, 2500–2509.
10 T. J. Silhavy, D. Kahne and S. Walker, *Cold Spring Harbor Perspect. Biol.*, 2010, **2**, 000414.
11 J. G. Swoboda, J. Campbell, T. C. Meredith and S. Walker, *ChemBioChem*, 2010, **11**, 35–45.
12 H. Tsubery, I. Ofek, S. Cohen, M. Eisenstein and M. Fridkin, *Mol. Pharmacol.*, 2002, **62**, 1036–1042.
13 H. Tsubery, I. Ofek, S. Cohen and M. Fridkin, *J. Med. Chem.*, 2000, **43**, 3085–3092.
14 M. Ouberai, F. El Garch, A. Bussiere, M. Riou, D. Alsteens, L. Lins, I. Baussanne, Y. F. Dufrene, R. Brasseur, J.-L. Decout and M.-P. Mingeot-Leclercq, *Biochim. Biophys. Acta, Biomembr.*, 2011, **1808**, 1716–1727.
15 S. Bera, G. G. Zhanel and F. Schweizer, *J. Med. Chem.*, 2010, **53**, 3626–3631.
16 I. Baussanne, A. Bussiere, S. Halder, C. Ganem-Elbaz, M. Ouberai, M. Riou, J.-M. Paris, E. Ennifar, M. P. Mingeot-Leclercq and J.-L. Decout, *J. Med. Chem.*, 2010, **53**, 119–127.
17 S. Hanessian, K. Pachamuthu, J. Szychowski, A. Giguere, E. E. Swayze, M. T. Migawa, B. Francois, J. Kondo and E. Westhof, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 7097–7101.
18 I. M. Herzog, K. D. Green, Y. Berkov-Zrihen, M. Feldman, R. R. Vidavski, A. Eldar-Boock, R. Satchi-Fainaro, A. Eldar, S. Garneau-Tsodikova and M. Fridman, *Angew. Chem., Int. Ed.*, 2012, **51**, 5652–5656.
19 K. Michael, H. Wang and Y. Tor, *Bioorg. Med. Chem.*, 1999, **7**, 1361–1371.
20 M. D. Disney and O. J. Barrett, *Biochemistry*, 2007, **46**, 11223–11230.
21 J. G. Shepherd, L. Wang and P. R. Reeves, *Infect. Immun.*, 2000, **68**, 6056–6061.
22 B. Liu, Y. A. Knirel, L. Feng, A. V. Perepelov, S. N. Senchenkova, Q. Wang, P. R. Reeves and L. Wang, *FEMS Microbiol. Rev.*, 2010, **34**, 606.
23 U. T. Nguyen, I. B. Wenderska, M. A. Chong, K. Koteva, G. D. Wright and L. L. Burrows, *Appl. Environ. Microbiol.*, 2012, **78**, 1454–1465.

