

## REVIEW



Cite this: *Med. Chem. Commun.*,  
2016, 7, 586

# Bacterial lipid membranes as promising targets to fight antimicrobial resistance, molecular foundations and illustration through the renewal of aminoglycoside antibiotics and emergence of amphiphilic aminoglycosides†

Marie-Paule Mingeot-Leclercq<sup>a</sup> and Jean-Luc Décout<sup>b</sup>

Hereunder, we highlight bacterial membrane anionic lipids as attractive targets in the design of antibacterial drugs which can be effective against both Gram-positive and Gram-negative resistant bacteria. In this approach, first, molecular foundations and structure–activity relationships are laid out for membrane-targeting drugs and drug candidates from the structure and physicochemical properties of the main membrane targets, describing, as well, the corresponding identified resistances. Second, this approach is illustrated by the history of the emergence of antibacterial and antifungal amphiphilic aminoglycosides (AAGs) which are active against Gram-positive and Gram-negative resistant bacteria. AAGs have resulted from intensive medicinal chemistry development of a group of old antibiotic drugs known as aminoglycosides (AGs), which target ribosomal RNA. The aforementioned AAG's are being used towards discovering new antibiotics which are less toxic and less susceptible to resistance. The recent results in the field of AAGs are described and discussed in terms of structure–activity relationships and mechanism of action.

Received 2nd November 2015,  
Accepted 8th February 2016

DOI: 10.1039/c5md00503e

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<sup>a</sup> Louvain Drug Research Institute, Université catholique de Louvain, Unité de Pharmacologie Cellulaire et Moléculaire, avenue E. Mounier 73, B1.73.05, Brussels, Belgium. E-mail: [marie-paule.mingeot@uclouvain.be](mailto:marie-paule.mingeot@uclouvain.be)

<sup>b</sup> Département de Pharmacochimie Moléculaire, Université Grenoble Alpes/CNRS, UMR 5063, ICMG FR 2607, 470 rue de la Chimie, BP 53, F-38041 Grenoble, France. E-mail: [jean-luc.decout@univ-grenoble.fr](mailto:jean-luc.decout@univ-grenoble.fr)

† The authors declare no competing interests.

## Introduction

Bacterial resistance to antibiotics is increasingly leading to a pressing healthcare problem since treatment options for patients have become very limited, in particular, against bacteria inducing life-threatening infections. Today, efforts towards old



**Marie-Paule Mingeot-Leclercq**

*Prof. Marie-Paule Mingeot-Leclercq received Masters in Chemistry (1979) and Pharmacy (1982), Ph.D. degree (1988) and habilitation (2000) from Université catholique de Louvain (UCL, Belgium). Prof. Mingeot-Leclercq's research interest encompasses biophysics and cellular and molecular pharmacology. Her research team develops multiple projects in anti-bacterial, anticancer and toxicological areas. From 2007 to 2009, she was in charge of the*

*School of Pharmacy and, since 2009, she is in charge of the Louvain Drug Research Institute (<http://www.uclouvain.be/en-ldri.html>) at UCL.*



**Jean-Luc Décout**

*Prof. Jean-Luc Décout received Ph.D. degrees from University of Lille (France) in chemistry and physicochemistry of polymers in 1976 and in bioorganic chemistry in 1983. He was researcher at "Centre National de la Recherche Scientifique" (CNRS) in Lille and in Grenoble. He spent one year from 1983 in Montreal at Mac Gill University. In 1998, he joined as Professor the University Grenoble Alpes at the Faculty of Pharmacy and the*

*Research Department of Molecular Pharmacology (<http://dpm.ujf-grenoble.fr/>) and was in charge of his Department during ten years. His research works are focused on the search for new drugs targeting nucleic acids and membranes.*

drug revisiting, drug repositioning or for novel antibacterial discoveries appear to be picking up speed.<sup>1</sup> This includes orthogonal approaches for systematic research of compounds that enhance the activity of existing antibiotics against resistant bacteria, block resistance enzymes, perturb efflux, design new targets. A thorough analysis of the property space from existing antibiotics has shed light on therapeutics that are driven by the unique architecture of bacterial envelopes and biophysical properties of bacterial membranes. In addition to the well-known inhibitors of peptidoglycan synthesis (*e.g.*  $\beta$ -lactams, cephalosporins, glycopeptides) or agents acting on bacterial lipid membranes like polymyxins, the concomitant modulation of membrane protein conformation, localisation and in turn activity, is also an important factor to consider for antibacterial activity.

Membrane-active antibacterials present several benefits including (i) a target which is essential and preserved among various bacterial species cultures, (ii) the capacity to be active against slow-growing or dormant bacteria as well as on biofilms, (iii) a low potential for the development of resistance due mostly to their multiple targeted mode of action, (iv) a favorable pharmacokinetic profile based on low, non-specific binding to human serum proteins, stability in serum and high tissue penetration and (v) a potential to serve as a chemosensitizer able to increase the activity of other antibiotics.

However, major concerns in the development of clinically useful membrane-active antibacterial agents for biomedical applications have been identified including (i) their ability to access the cytoplasmic membrane of the bacteria and (ii) their capacity to achieve selectivity toward mammalian cells over bacterial cells. Moreover, how the multi-target mode of action, contributes to the low potential for the development of resistance is unknown or insufficiently exploited.<sup>2</sup> An additional, but more general challenge is to shift from a “hit” scaffold to a “lead compound” through structural changes allowing optimization of the potency, efficacy and safety.

In this review, we attempt to address the issues related to the development of clinically useful membrane-active antibacterial agents through examination of the molecular parameters involved in the interaction between membrane-active agents and bacterial lipid membranes as well as their consequences.

We will highlight bacterial membrane anionic lipids as attractive targets in the design of antibacterial drugs active against Gram-positive and Gram-negative resistant bacteria. In this approach, first, molecular foundations and structure–activity relationships will be laid out for membrane-targeting drugs and drug candidates from the structure and physicochemical properties of their main membrane targets and we will also describe the corresponding identified resistances. Second, this approach will be illustrated by the history of the emergence of amphiphilic antibacterial and antifungal aminoglycosides (AAGs) active against Gram-positive and Gram-negative resistant bacteria. AAGs have resulted from intensive medicinal chemistry development of a group of old

antibiotic drugs called aminoglycosides (AGs), which target ribosomal RNA. The aforementioned AAG's are being used towards discovering new antibiotic AGs which are less toxic and less susceptible to resistance. The recent results in the field of AAGs will be described and discussed in terms of structure–activity relationships and mechanism of action.

## Part I. Lipid membranes as target for antibiotics

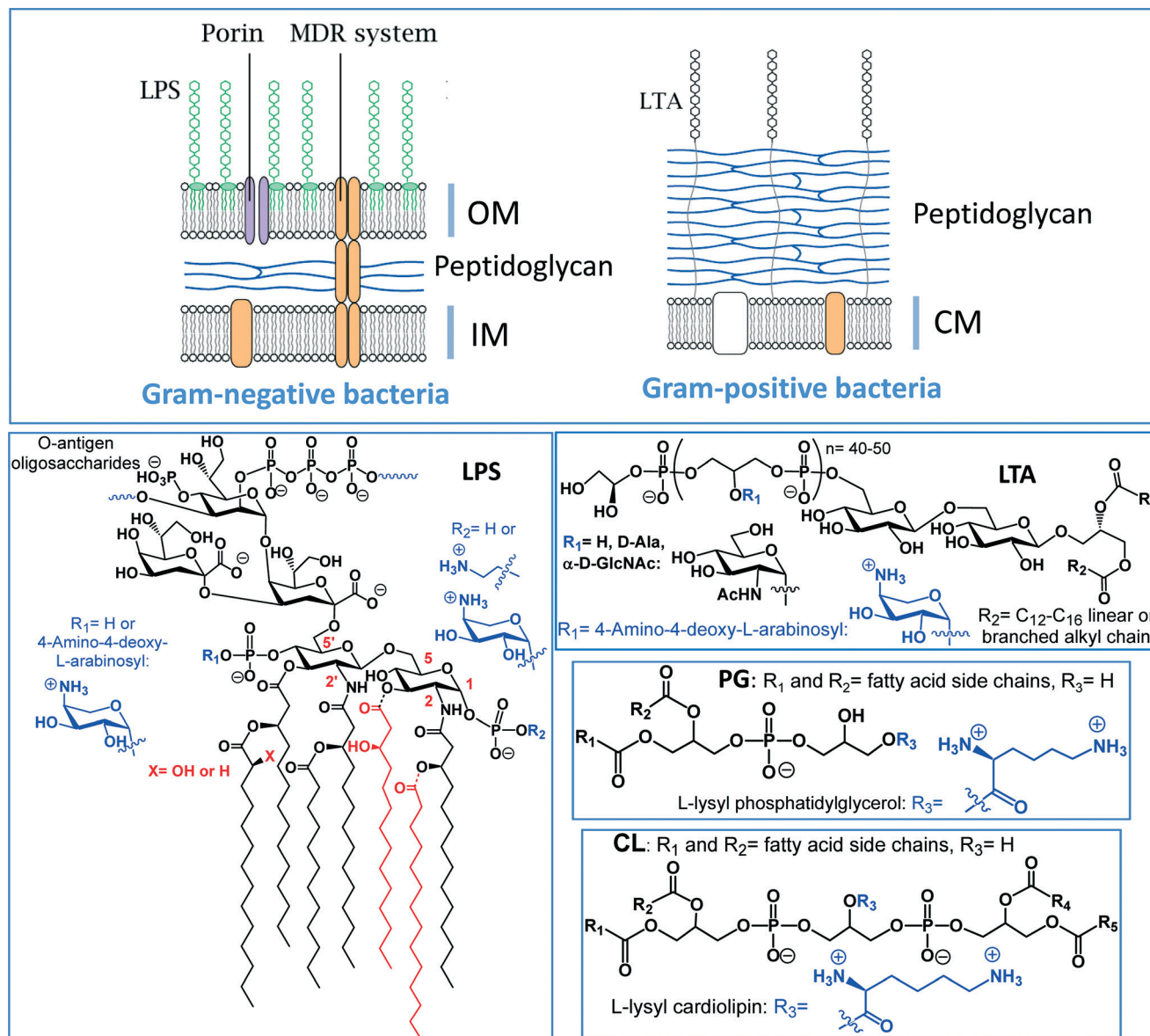
### Bacterial lipid membranes diversity

Most membrane-damaging agents interfere with multiple targets including (i) interaction of a lipophilic moiety with the bacterial membrane (causing disruption of membrane architecture and functional integrity), (ii) conformation and/or localization of membrane embedded proteins and (iii) alteration of the proton motive force (PMF). All these effects are highly dependent upon the diversity in lipid membrane composition.

Selectivity of membrane-active agents for toxicity against bacteria as compared to mammalian cells is mainly mediated by differences in membrane organization as well as lipid composition. Negatively charged phospholipids like phosphatidylglycerol (PG) and cardiolipin (CL) and/or lipopolysaccharides (LPS) and lipoteichoic acids (LTA) are characteristic from bacteria. In contrast, phosphatidylcholine (PC), a zwitterionic phospholipid is more abundant in membranes from mammalian cells.<sup>3</sup> Another critical difference between lipids from bacterial and mammalian cells is the hopanoids.<sup>4</sup> They are pentacyclic triterpenoid lipids that are analogues of cholesterol in prokaryotic membranes.<sup>5</sup>

Within bacteria in themselves, a great diversity is observed. The envelope of Gram-negative and Gram-positive bacteria (Fig. 1, top) greatly differs in their individual architecture, with that of the Gram-negative bacteria being the most complex one since it is composed of two distinct lipid membranes: an outer membrane (OM) and an inner membrane (IM) separated by a thin layer of peptidoglycan.

The OM is an asymmetric lipid bilayer, which significantly slows down passage of drugs. Inside the OM there are inserted porins. These are large water-filled channels, which allow the diffusion of hydrophilic molecules into the periplasmic space. The inner leaflet of the OM is composed of glycerophospholipids, whereas the outer leaflet is composed of LPS that are largely responsible for the low permeability characteristic.<sup>6</sup> LPS is composed of three modules: lipid A, a core oligosaccharide and a highly variable O-antigen constituted of repeating oligosaccharide units. The core is covalently linked to lipid A and can be further divided into inner and outer core. The inner core contains at least one residue of 3-deoxy-D-manno-octulosonic acid (Kdo).<sup>7</sup> In addition to glycerophospholipids and LPS, the OM also contains a unique set of transmembrane proteins that adopt a  $\beta$ -barrel architecture. Many OM proteins carry out functions in nutrient transport but also in secretion and adhesion. They are synthesized at the cytoplasmic protein synthesis machinery



**Fig. 1** Top. Schematic presentation of the membrane architecture of Gram-negative and Gram-positive bacteria. Bottom. Modulation induced through activation of transcriptional regulatory genes by bacteria to avoid the activity of antibiotics-active on lipid bacterial membranes. The main modifications are those involving amphiphilicity and charge of LPS, LTA, cardiolipin (CL) and phosphatidylglycerol (PG). Modifications of polar groups and hydrophobic moieties are drawn in blue and red, respectively.

and then transported across the IM before being incorporated into the OM.<sup>8</sup>

The IM is composed of glycerophospholipids in both its inner and outer leaflets (mainly phosphatidylethanolamine (PE), PG and CL<sup>3</sup> as well as integral and peripheral membrane proteins. The IM carries out a variety of functions typically assigned to both the plasma membrane and specific organelles in eucaryotes. These include protein export, solute import, cell signaling, cell division, biosynthesis, electron transport, and maintenance of a proton motrice force (PMF) and ATP synthesis.<sup>9</sup>

In comparison with the envelope of Gram-negative bacteria, that of Gram-positive bacteria is far less complex. It consists of a cytoplasmic plasma membrane (CM) surrounded by

the cell wall, a thick layer of peptidoglycan and LTA. The latter is an anionic macroamphiphile that contains glucose and D-alanine substituted polyglycerol phosphate attached to a glycopeptide which anchors the Gram-positive bacterial cytoplasmic membrane<sup>10</sup> in a manner similar to the interaction between the lipid A moiety of LPS and OM of Gram-negative bacteria.<sup>11</sup> Although LPS and LTA show structural relatedness (amphiphilicity, negative-charge), they are structurally quite different from each other and one might expect that they are also recognized by different receptors of the innate immune system, the so called toll-like receptors 4 and 2 (TLR4 and TLR2), respectively.<sup>12</sup> The cell wall of Gram-positive bacteria is permeable and typically does not restrict the penetration of antibiotics into the cell. Regarding the lipid composition, the

phosphatidylethanolamine (PE) content is lower in Gram-positive bacteria as compared with Gram-negative strains.<sup>3</sup> As for Gram-negative bacteria, the predominant anionic lipids are PG and CL.<sup>3</sup>

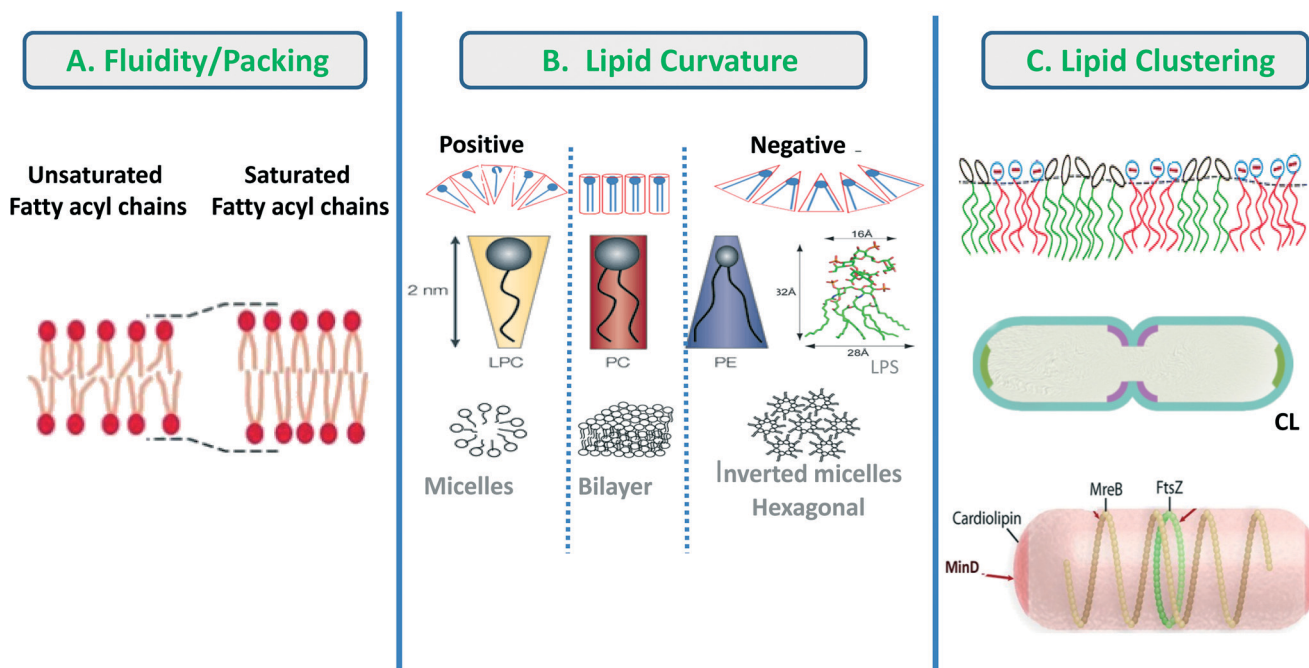
Research performed on Gram-negative and Gram-positive bacteria emphasizes how the regulation of each bacterium must be assessed on a species-specific basis. Different bacteria possess distinctive biochemical and transcriptional regulatory checkpoints to control the rate of lipid synthesis. The ability to change the lipid composition is crucial for survival in the wide range of environmental conditions where bacteria thrive. Globally speaking, a critical question is to know if differences in the phospholipid composition in the cytoplasmic/inner membranes of different bacterial species<sup>13</sup> are responsible for the response of Gram-positive and Gram-negative bacteria to membrane-active antibiotics.

### Design of membrane-active antibiotics from diversity of bacterial lipid membranes

Diversity in lipid composition and membrane structure and organization results in specific biophysical and physicochemical properties. In addition to amphiphilicity, three biophysical parameters of lipid bacterial membranes are known to govern the activity/selectivity of membrane-active

compounds like daptomycin, lipoglycopeptides or polymyxins. They include (i) fluidity/packing, (ii) curvature and (iii) clustering of negatively-charged lipids. These are critical for the medicinal chemists who have to design new antibiotics targeting bacterial membranes with the aim to eradicate bacteria including those resistant to conventional agents.

The considerable metabolic flexibility including in the synthesis of LPS/LTA or of lipids of bacterial membranes is linked to the activation of transcriptional regulatory genes that in turn lead to critical modifications for the recognition between cationic amphiphilic antibacterials as well as bacterial envelopes and/or for antibiotic diffusion throughout the lipid membranes. These modifications mainly include (i) alterations in the amphiphilicity by addition (or structural changes) in acyl chains, (ii) decrease in the negative charge of LPS/LTA or PG/CL by addition of positively-charged substituents (*e.g.* lysyl)<sup>14–16</sup> (Fig. 1 bottom), and (iii) modifications in the degree of saturation/unsaturation of fatty acyl chains (Fig. 2A). Changes in the lipid composition and in the relative content of individual lipids might be also critical, in relation with the specific functions of membranes. This includes bacterial division that is related to membrane curvature and lipid clustering (Fig. 2B and C) and OM proteins folding which are highly dependent upon PE and PG head



**Fig. 2** Physicochemical parameters of lipid bilayers (fluidity/packing, curvature, lipid clustering) involved in the activity of antibiotics acting against bacterial lipid membranes from sensitive and resistant bacterial strains. (A) Fluidity and packing: effect of unsaturation on overall packing and thickness of lipid bilayers; (B) lipid curvature: lipids with a bulky polar head and only one acyl chain like lysophosphatidylcholine (LPC) have a molecular shape that resembles to an inverted cone. They induce a positive curvature and favor the formation of micelles. Lipids such as phosphatidylcholine (PC) have similar cross-sectional area for the polar head and hydrophobic region. They form cylinders. Lipids with a small polar head such as phosphatidylethanolamine (PE) have a molecular shape that resembles a truncated cone as also observed for LPS. They induce a negative curvature strain and tend to adopt structures like inverted micelles or hexagonal phases; (C) lipid clustering/cluster of negatively-charged lipids: distribution of cardiolipin (CL) in regions characterized by a high degree of curvature as well as the tubulin homologue Ftsz, actin homologue MreB and MinD, a component of the Min system whose function is dependent on and regulated by CL (adapted from Govindarajan *et al.*<sup>87</sup>).

groups. The kinetic retardation of the folding of OM proteins places a strong negative pressure against spontaneous incorporation of OM proteins into inner bacterial membranes, which would dissipate the proton motive force (PMF) and undoubtedly kill bacteria.<sup>6</sup>

Keeping in mind the aforementioned metabolic flexibility, we review hereunder the main physicochemical properties of bacterial lipid membranes in relation with the activity of membrane-active antibiotics, including those acting on resistant strains.

**Amphiphilicity and membrane-active antibiotics.** The main physicochemical property of lipids that composed bacterial membranes (and those of eukaryotic cells as well) is their amphiphilic character. Lipids present within membrane bilayers including the most predominant lipids from bacteria, glycerophospholipids, share this property. The latter are defined as acylated derivatives of *sn*-glycerol-3 phosphate composed of two hydrophobic fatty acid chains, a glycerol unit and a phosphate group linked to a polar head group as illustrated for PG (Fig. 1).<sup>17</sup>

To optimize the interaction between drugs and lipid membranes and the passage of antibiotics that have intracellular targets (*e.g.* ribosomes, DNA gyrase, topoisomerases), synthesized molecules have to show an optimal ratio between hydrophilic and hydrophobic behaviors. This is especially critical for antibacterials active against Gram-positive bacteria since the uptake of antibiotics is mostly dependent upon their molecular weight and amphiphilicity and against Gram-negative bacteria if they are unable to pass through porin (*e.g.* molecules with high molecular weight). Furthermore, this is also important for compounds designed to destabilize the bacterial membranes in order to enhance the uptake of other antibiotics with intracellular activity. The hydrophobic/hydrophilic ratio can be estimated by the partition coefficients of the molecule in different solvents from 1-octanol and water system ( $\log P$ ). This parameter relates to the difference of the hydrogen bonding capability of the compound for a solvent and 1-octanol. If the amphiphilic compound has some ionizable functions, the partition of ion-pair complexes to 1-octanol cannot be neglected and the distribution coefficient (apparent hydrophobicity) at a particular pH ( $\log D$ ) is determined. For better mimicking the biological situation, the parallel artificial membrane permeation assay (PAMPA)<sup>18</sup> may be used.<sup>19</sup> This is a high throughput *in vitro* assay system that evaluates transcellular permeation. In PAMPA, a 96-well microtiter plate completely filled with aqueous buffer solutions is covered with a hydrophobic filter coated with lipids in an organic solvent solution in a sandwich construction. Various lipids dissolved in organic solvents, mimicking *e.g.* the lipid bacterial membrane composition can be used.

Antimicrobial peptides (AMPs) are a well-known class of antibacterials characterized by hydrophobic and hydrophilic portions that interact with lipid part and hydrophilic negatively-charged heads in bacterial membranes, respectively. Many linear antimicrobial peptides adopt amphipathic  $\alpha$ -helical conformations with the hydrophobic side chains

arranged along one side of the helical structure and the hydrophilic side chains organized on the opposite side. This arrangement results in the ideal amphipathic helical structures. Some antimicrobial peptides adopt an amphipathic  $\beta$ -sheet conformation to interact with cell membrane.<sup>20</sup>

The polymyxin B (Fig. 3) is a typical amphiphilic molecule, with hydrophobic and polar domains, namely the N-terminal fatty acyl chain and position 6–7 motif (hydrophobic) on the one hand, and L- $\alpha$ , $\gamma$ -diaminobutyric acid (Dab) and Thr residue segments (polar) on the other hand. The cyclic heptapeptide and linear tripeptide provide an integral scaffolding function that involves maintaining the optimal distances between each domain, thereby giving the structure its amphipathicity, a property that is indispensable for polymyxin antibacterial activity.<sup>21</sup> Modulation of amphiphilicity<sup>22,23</sup> can be achieved by different ways, including (i) tandemly repeated sequences of alternating cationic (Lys) and nonpolar (Val or Phe) residues,<sup>24</sup> (ii) modification of N-terminal fatty acyl chain<sup>25</sup> and (iii) substitution of the Dab residue. Polymyxin B derivatives with polar side chain on modified Dab residue showed better antimicrobial activity than polymyxin B and broadened the antibacterial spectrum.<sup>26</sup> Moreover, increase of the lipophilic character of polymyxin B by introducing substituted benzyl groups leads to derivatives with enhanced activity against *S. aureus* and reduced activity against *E. coli*.<sup>27</sup> Moving on N-lipidated peptide dimers which are active against Gram-negative bacteria, including carbapenem-resistant Enterobacteriaceae, Koh *et al.*<sup>25</sup> showed the critical role of amphiphilicity with an optimized lipid length (6–10 carbon atoms) for membrane permeabilization.

Two other families of antibiotics are also characterized by an amphiphilic structure. They are the lipopeptides like daptamycin and the lipoglycopeptides such as teicoplanin, telavancin, oritavancin and dalbavancin. They are active against Gram-positive bacteria microbials and their structures are depicted in Fig. 3 where the hydrophobic moiety is in red and the polar groups in blue.

Due to the critical role of amphiphilicity for interactions between cationic amphiphilic peptides and lipid membranes, changes in the amphiphilicity of lipids from bacterial membranes are a common strategy for generating resistance to these membrane-active antibiotics. One peculiar example is the increase of the hydrophobicity of LPS (Fig. 1, bottom). Hydrophobic lipid chains, added either to lipid A phosphates, to glucosamine backbone or to existing acyl chains, serve to increase LPS saturation and decrease overall permeability, preventing cationic amphiphilic peptides from inserting into the membrane.<sup>28</sup> In *Salmonella*, acyl chains are added by PagP, a palmitoyl transferase, to the glucosamine backbone and phosphates of lipid A.<sup>29</sup> Enhanced acylation of lipid A (PagP dependent or independent) is a general process reported in *E. coli*,<sup>29</sup> *Yersinia enterocolitica*,<sup>29</sup> *Bordetella pertussis*,<sup>30</sup> *Acinetobacter baumannii*.<sup>31</sup> PagP deletion mutants exhibited increased membrane permeability and were nearly 4 times more susceptible to the antimicrobial peptide.

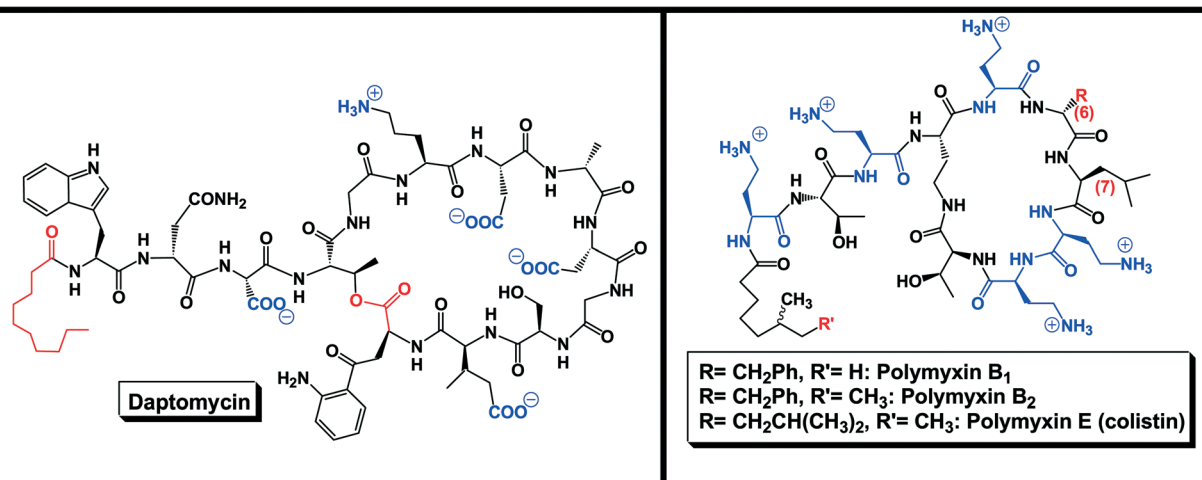
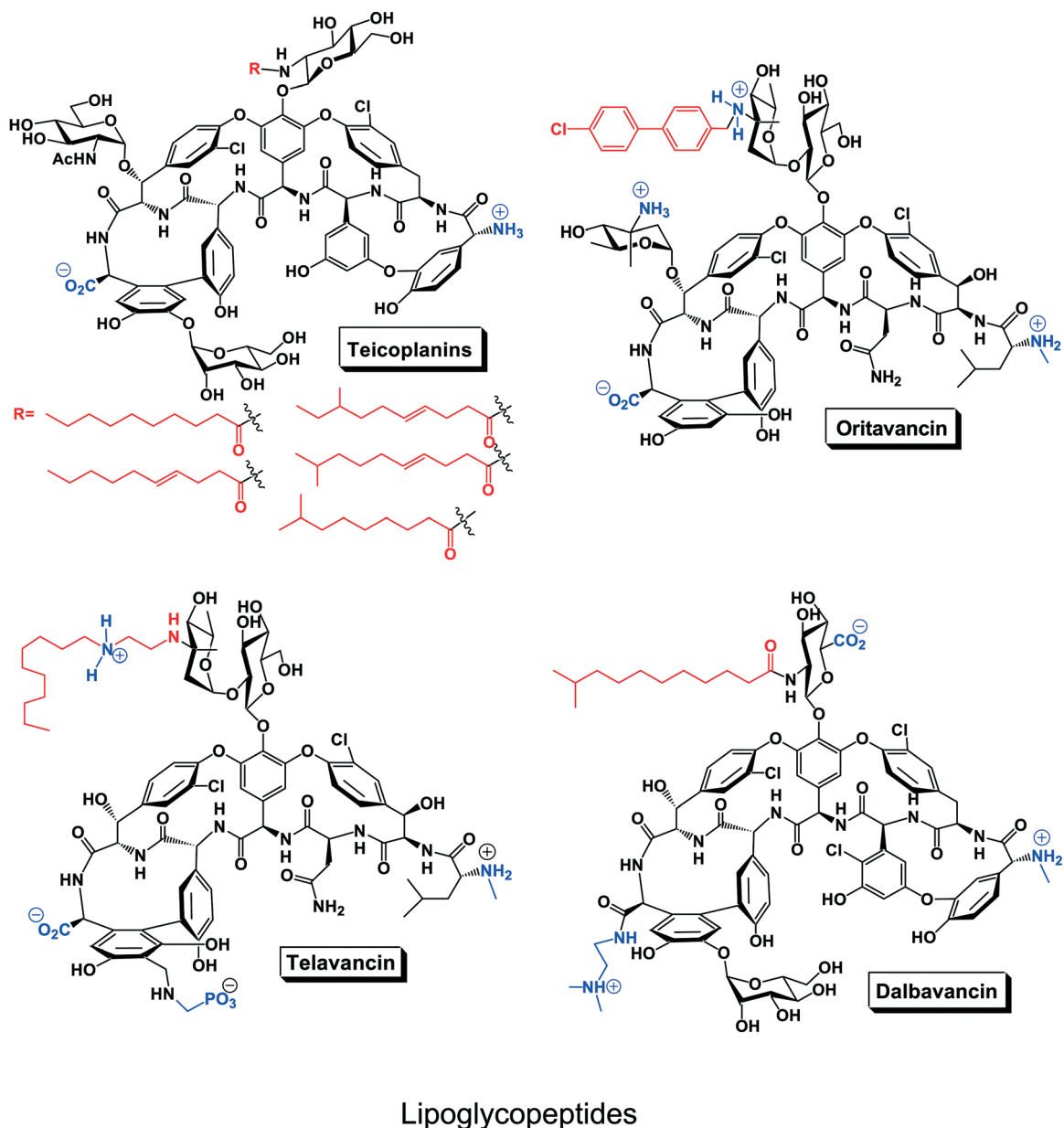


Fig. 3 Chemical structures of lipoglycopeptides (teicoplanins, oritavancin, telavancin, dalbavancin) and lipopeptides (daptomycin and polymyxins). The hydrophobic and hydrophilic moieties are in red and blue, respectively.

Under-acylation may also explain the increased polymyxin susceptibility of *K. pneumoniae* strains. Under-acylation appears to facilitate the integration of the *N*-terminal fatty-acyl chain of polymyxin into the OM, resulting in an increased susceptibility to its antimicrobial activity/activities.<sup>32</sup>

Focusing on LTA (Fig. 1 bottom), acyl chains are also critical for its function, and small variations in acyl chains can alter biological properties of LTA,<sup>33</sup> leading again to a decrease of interaction between membrane-active antibacterials and Gram-positive bacteria.

**Negative-charges and membrane-active antibiotics.** In lipid membrane, an assortment of different polar head groups can be attached to the phosphatidic acid, the basic structure for glycerophospholipids, creating the optimum surface charge for membrane.<sup>34</sup> The membrane charge depends on the ratio between zwitterionic glycerophospholipids, PE, and glycerophospholipids with anionic head groups, PG or CL (Fig. 1 bottom). Mutations in the genes responsible for the production of anionic phospholipids (*pgsA* phosphatidyltransferase and *cls2* synthase involved in biosynthesis of PG and CL, respectively)<sup>35,36</sup> were correlated with changes in charge of the membrane as well as the cell wall thickness in the *S. aureus* clinical isolates.<sup>35</sup>

Other mechanisms involved in the modulation of the charge of bacterial membranes have been described. Among them, changes as lysinylation of PG present at the cytoplasmic membrane and alanylation of cell wall teichoic acids through proteins encoded by *S. aureus* *mprF* and *dlt* operon, respectively<sup>37–39</sup> may induce an enhancement of the net positive charge of the cell surface envelope. MprF (multiple peptide resistance factor) is a large integral membrane protein responsible for translocating lysyl-PG to the outer cytoplasmic membrane bilayer leading to changes of charges at the surface of bacterial membranes.

These changes in charge of bacterial membrane result in a modification of the ability for many integral membrane proteins to adopt the correct steric conformation in the cell membrane<sup>34</sup> or the attended localization of division proteins associated with the bacterial membrane including *e.g.* MinD and FtsZA<sup>40</sup> (Fig. 2C). Moreover, the decrease of the transmembrane potential ( $\Delta\phi$ ) leads to an increase in membrane permeability that in turn disturbs the bacterial physiology and simultaneously facilitates the penetration of free radicals secreted by macrophages of the host immune system.

In addition to the critical role of charges for cell bacterial physiology, it is obvious that negatively-charged lipids are critical for the action of membrane-active antibiotics including *e.g.* daptomycin, dalbavancin, polymyxins. First, negatively-charged lipids like PG and CL are critical for the ability of daptomycin to oligomerize inside of the bacterial cell membrane<sup>41</sup> and to form oligomeric transmembrane pores induced by the daptomycin–calcium complexes.<sup>42</sup> Second, ionic character as observed after derivatization or conversion of the carboxy group into an ester, amide or hydrazide<sup>43</sup> highly modulates the relative activity of teicoplanin-type glycopeptides like dalbavancin, especially against

coagulase negative *Staphylococcus*. Third, it has long been recognized that the positive charge ranging from +2 to +9 is the driving force for the electrostatic interaction between antimicrobial peptides and the negatively-charged bacterial membranes.<sup>44</sup> In addition, the positive charge of the compound might play a critical role in toxicity. So, polymyxin derivatives NAB739 and NAB7061, which carry only three cationic charges bind to the isolated brush border membrane of rat kidney cells at an affinity which is only 1/7 to 1/5 of that of parent compound, polymyxin B, which carries five positive charges. This results in a decrease of nephrotoxic potential of NAB739 and NAB7061.<sup>45</sup>

Changes in the content of negatively-charged lipids in bacterial membrane and modifications of the negative charge of (i) LTA, (ii) LTS and/or (iii) PG/CL (Fig. 1 bottom) contribute to the emergence of resistance to cationic agents in relation with a decrease of the interaction between the cationic agents and the negatively-charged bacterial lipid membranes as illustrated by the following examples.

First at all, any significant reduction in PG content is associated to the development of daptomycin resistance in both *E. faecalis* and *E. faecium*.<sup>16,46</sup>

Focusing on the negative charge of LPS, the addition of galactosamine (*Francisella novicida*), glucosamine (*Bordetella pertussis*, *Bordetella bronchiseptica*) or aminoarabinose (*P. aeruginosa*, *Salmonella thyphimurium*) to lipid A contribute to the increase in the net positive surface charge (Fig. 1 bottom). Addition of 4-amino-4-deoxy-L-arabinose commonly observed in polymyxin resistant strains block the electrostatic interaction between the lipid A phosphates and the positively-charged Dab residues.<sup>47</sup> Addition of 4-amino-4-deoxy-L-arabinose and of PE is mediated by PhoP–PhoQ regulatory system encoded by *phoP* locus. Activated by PhoP–PhoQ, the PmrA–PmrB encoded by *pmrCAB* operon is the major regulator to mediate the LPS modification in Gram-negative bacteria. PmrA dependent modification can occur on each of the three distinct LPS domains, namely, lipid A, core polysaccharide, and O-antigen chain.<sup>48</sup> These modifications result in a decrease of the negative charge of LPS, resulting in decrease of the interaction of cationic antibiotics with membrane and in an increase in resistance to polymyxin.<sup>49,50</sup>

One alternative developed by bacteria is to modify the global negative charge of glycerophospholipids, through lysinylation of PG and its translocation to the outer leaflet of the membrane. Both processes are mediated by MprF<sup>51,52</sup> which represents a particularly interesting antimicrobial drug target because of its presence in both Gram-positive and Gram-negative bacteria. Point mutations in the protein MprF appear to cause a gain-in-function, thus resulting in the accelerated translocation of lysyl-PG (Fig. 1 bottom). In turn, this results in a decrease of the net negative charge which may repel calcium–daptomycin complexes.<sup>16,53</sup> MprF point mutations or alterations in lysyl-PG content became notorious for spontaneous resistance of *S. aureus* to cationic antimicrobial peptides including daptomycin.<sup>53,54</sup> *A contrario*, the loss of

lysyl-PG in MprF mutants also led to cationic amphiphilic peptides susceptibility in *Listeria monocytogenes*, *Bacillus anthracis*, and *Rhizobium tropici*, thereby demonstrating a general role of MprF in bacterial immune evasion.<sup>55</sup>

In addition to adding positive charge to counteract the negative residues on LTA, LPS, PG or CL, some bacteria remove negative residues as an alternative mechanism of mitigating overall negative charge. In *F. tularensis*, the 4' lipid A phosphate is removed by the phosphatase LpxF, leaving only one phosphate group on lipid A.<sup>56</sup>

If surface charge is critical for antibacterial activity of membrane-active antibiotics, it is likely that other factors are also involved. This could explain why there is an inverse relationship between daptomycin resistance and surface charge of *S. aureus*.<sup>39</sup> One candidate would be the extent of cell wall glutamate amidation, which previously has been linked to glycopeptide resistance in methicillin-resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* (VISA) strains.<sup>57</sup> Moreover, the relationship between the surface charge and the resistance appears highly modulated by the structure of the antibacterial. A narrow window has to be defined for each family of membrane-active agents as we observed for the lipophilicity of neamine derivatives.<sup>58</sup>

In addition to amphiphilicity and surface charges, the activity of membrane-active antibiotics is highly modulated by fluidity/packing of lipids as well as their curvature and ability to cluster.

**Fluidity/packing of lipids and membrane-active antibiotics.** The fluid lipid bilayer supports the functional machinery of receptors, channels and pumps that are associated with the membrane. Fluidity also favors the diffusion allowing intracellular delivery of compounds bound to the outer leaflet of lipid bilayers.<sup>59</sup>

The length and the degree of unsaturation of fatty acid chains have a profound effect on membrane fluidity (Fig. 2A) as unsaturated lipids create a kink, preventing the fatty acids from packing together as tightly, thus decreasing the melting temperature (increasing the fluidity) of the membrane. As an example, to increase membrane rigidity, *Pseudomonas* produce more saturated fatty acids whereas when higher fluidity is needed, unsaturated fatty acids are synthesized.<sup>60</sup> Degree of unsaturation also modifies the thickness of lipid bilayers (Fig. 2A). In addition, the presence or not of hopanoids,<sup>61</sup> that are thought to be bacterial surrogates for eukaryotic sterols might also regulate membrane fluidity.<sup>62,63</sup> Fluidity and/or perturbation of the LPS assembly in the outer membrane, might be critical for growth at low temperatures<sup>64</sup> as well as for efficacy of antibacterials<sup>61</sup> including amphiphilic neamine derivatives.<sup>65</sup> From the point of view of the antibacterial, both its structure, and especially, its flexibility, have also to be taken into account for explaining its activity.

The role of fluidity for bacterial resistance is far more complex since class IIa bacteriocin-resistant *L. monocytogenes* is related to increased amounts of unsaturated and short-acyl chain PG with an increase in fluidity whereas nisin-resistant *L. monocytogenes* have a more rigid membrane.<sup>66</sup> Fluidity

membrane adaptation is likely only one of several mechanisms involved in resistance to antibiotics.

**Curvature and membrane-active antibiotics.** Any vesicles can be defined by an area/volume ratio, thus giving rise to the parameter of curvature. Membrane curvature is closely related with lipid polymorphism and molecular shape of lipids (Fig. 2B).<sup>67</sup> A striking feature of CL and PE is their molecular shape which originates from having small cross section head groups relative to large cross section of tail groups. The resulting molecules, with a large intrinsic curvature, are characterized by unique physical properties.<sup>68</sup> The inner membrane of Gram-negative *E. coli* is richer in these negative intrinsic curvature lipids as compared to the lipid membrane from Gram-positive *S. aureus*. The *Pseudomonas* lipidome<sup>69</sup> also reveals a high content in PE. Both PE and CL are able to form reversed non-lamellar structures like the hexagonal phase (formed by inverted tubules, with the fatty acyl chains pointing toward the outside of tubules and the polar head groups toward the center establishing an aqueous channel) as to increase lateral pressure and introduce curvature stress. Specific ratio between lipids that organize in bilayer or non-lamellar (e.g. hexagonal or cubic phases) structures is highly regulated.<sup>70</sup>

A curvature-mediated model has been hypothesized as critical for membrane structure remodeling like cell elongation and cell division<sup>71</sup> as well as for peptidoglycan synthesis.<sup>72</sup> These processes are mediated by the activity of specific proteins, sensitive to CL, including e.g. MreB, FtsZ, MinD, and MurG<sup>40,73,74</sup> (Fig. 2B and C).

Additionally, bivalent cations like Ca<sup>2+</sup> or Mg<sup>2+</sup> may induce a very complex structural polymorphism of lipid A from LPS, which is sensitively dependent either on the particular chemical primary structure, the particular on the acylation pattern and/or on the number of phosphate groups at the diglucosamine backbone.<sup>75</sup>

Antimicrobial peptides have been shown to induce non-lamellar lipid phases which may be intimately linked to their proposed mechanism of action. They may alter lipid phase behavior in three ways, by inducing positive membrane curvature, negative membrane curvature and cubic lipid phases.<sup>76,77</sup> As such, the idea of non-bilayer structures is not new and indeed it has been proposed to explain structural changes induced by small amphiphilic molecules in membranes.<sup>78–80</sup> Interestingly, the ability to induce cubic phase formation is correlated with the antimicrobial activity.<sup>81</sup>

**Lipid clustering and membrane-active antibiotics.** Two major breakthroughs have revisited the assumption of lipid homogeneity inherent in the fluid mosaic model of Singer and Nicholson. There are (i) patchy or compartmentalized distributions of specific lipids and proteins and (ii) lipid domains as organizers of proteins.<sup>82–84</sup> Just as mammalian cells, and despite their tiny and the scarcity of membrane bound organelles, bacterial membranes are able to sort lipids and proteins to distinct subcellular domains<sup>85,86</sup> optimizing functionality of vital processes like cell division (Fig. 2C).<sup>87</sup>



Especially, enriched-CL domains<sup>86,88</sup> are attractive targets for developing new antibacterials. CL preferentially localizes in the inner bacterial membranes at both the pole and division sites of rod-shaped bacterial cells (Fig. 2C) including *e.g.* *E. coli*,<sup>82,89,90</sup> *B. subtilis*<sup>91</sup> and *P. putida*.<sup>92</sup> Short range interactions between CL molecules in bacterial membranes are thermodynamically favorable and may lead to the formation of CL-enriched microdomains. These enriched CL domains participate in the formation and maintenance of dynamic lipid-protein and protein-protein interactions.<sup>93</sup>

Besides CL, hopanoids can also potentially confer the ability to subcompartmentalize membranes into functional domains. Evidence for these lipid-dependent functional domains has been reported for *B. subtilis*,<sup>94</sup> and there is evidence for lateral membrane heterogeneity in *Gloeobacter violaceus*,<sup>95</sup> a hopanoid-producing cyanobacterium.

The preferential interaction of cationic antimicrobial peptides with anionic lipids can result in the segregation of anionic lipids into a membrane domain from bilayers containing both anionic and zwitterionic lipids. The domain of anionic lipids would be surrounded by an interface with the rest of the membrane that would be under line tension and consequently less stable. Another mechanism would be the formation of a pore.<sup>96</sup> A similar effect has been demonstrated with homologous antimicrobial cationic arginine-rich non peptides, with some antimicrobial amphipathic cationic peptides, as well as with oligo-acyl-lysines<sup>13</sup> on membranes mimicking the lipid composition of typical Gram-negative bacteria.

The emergence of daptomycin non susceptibility in vancomycin-resistant *E. faecalis* is associated with an initial cell membrane CL microdomain redistribution caused by an amino acid deletion in LiaF that diverts the antibiotic away from the division septum to other CL-rich areas in the cytoplasmic membrane.<sup>41</sup> This prevents the antibiotic from interacting with its main septal target<sup>41</sup> and, perhaps, from reaching the inner leaflet of the cytoplasmic membrane.<sup>97</sup> The remodeling of CL microdomains was associated with a deletion of isoleucine in position 177 of LiaF.<sup>41</sup> Substitutions in LiaFSR appear to be the first pivotal event in the evolution of daptomycin resistance. Possibly, membrane adaptations, interfering with the LiaFSR response may provide a novel strategy to restore and preserve the activity of antimicrobials (*e.g.*, daptomycin) and potentiate the innate immune clearance of resistant microorganisms. Such an approach may become a viable antimicrobial strategy against multidrug-resistant Gram-positive organisms in the future.<sup>98</sup>

At a glance, design and synthesis of membrane-active small molecules have to be thought taking into account the main physicochemical and/or biophysical properties of the bacterial lipid membranes.<sup>99</sup> This helps to explain one of the reasons for the difference in activity of certain antimicrobial agents against different bacterial species.<sup>13</sup> Successful use of the membrane-active antibiotics like lipopeptides (daptomycin) or lipoglycopeptides (telavancin, oritavancin, dalbavancin) indicate that bacterial specificity is achievable.

An additional challenge, however, is to characterize the relationship between membrane-active agents and the main physicochemical parameters governing the biophysical properties of the membrane. This is particularly important for fighting bacteria resistant to conventional antibiotics.

#### Diversity of bacterial lipid membranes: an opportunity to avoid resistance?

Canonical mechanisms involved in antibiotic resistance include (i) modification of cellular targets, (ii) physical removal of an antibiotic from the cell through efflux pumps, (iii) reduced cellular uptake, and (iv) enzymatic inactivation of the antibiotic. They can spontaneously evolve in the laboratory, and whole genome sequence analysis suggests that the picture is far more complex.

As previously described, transcriptional regulatory genes were frequently mutated during evolution of antibiotic resistance.<sup>100,101</sup> Such mutations influence synthesis of lipids of bacterial membranes and production of LPS or LTA with a reduction of the net negative charge. This reduction might be one possible reason for the increased resistance against aminoglycosides simply due to a reduced electrostatic interaction in the first stage of uptake. As transport across the membrane into the cell requires energy and involves proton motive force (PMF), any mutation or event that leads to defective electron transport chain components will also confer resistance.<sup>102</sup>

In this context, modulating or disrupting the bacterial membrane bilayer and the activity of integral proteins needed for membrane function is a valuable strategy for increasing collateral sensitivity of antibiotic resistant bacteria (chemosensitization) and for eradicating resistant or persister cells. Combining two or more antimicrobials including one acting on bacterial lipid membrane (Fig. 4B and C) is also interesting to pursue, especially for the eradication of multidrug resistance bacteria since mutations that provide resistance to both drugs are exceptionally rare.

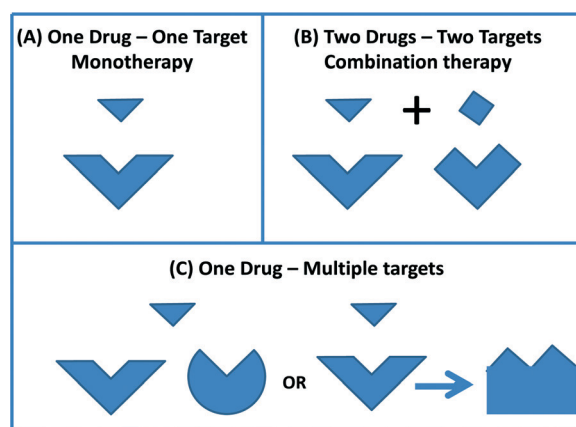


Fig. 4 Therapeutic strategies from monotherapy to combination therapy and multitargeting to increase activity and decrease resistance. (A) Monotherapy: drug acting on a single target, (B) combination therapy: two drugs acting on two targets. (C) Multitargeting: one drug acting on two targets (inspired from Oldfield and Feng, 2014).

**Increasing collateral sensitivity of antibiotic resistant bacteria.** Alteration of membrane potential of bacterial membranes is responsible for increasing collateral sensitivity of antibiotic resistant microbes. Collateral sensitivity refers to mutations that caused multidrug resistance in bacteria which can simultaneously enhance sensitivity to many other unrelated drugs. Regarding aminoglycoside antibiotics, for example, their cellular uptake demands an active proton motive force (PMF).<sup>103</sup> PMF is also critically involved in the activity of many multidrug efflux pumps since the free energy released by moving protons to the cytoplasm is used to expel drug molecules from the cell.<sup>104</sup> This constitutes a typical example of collateral sensitivity. Resistance to aminoglycosides, is achieved partly by the reduction of the proton motive force (PMF) through membrane-potential-altering mutations.<sup>104,105</sup> In turn, reduction of PMF in aminoglycoside-resistant lines will diminish the activity of PMF-dependent major efflux pumps, leading to susceptibility to several unrelated classes of antibiotics. Generally speaking, this could explain why strains evolved for aminoglycoside became more sensitive to various antibiotics.

Alteration of bacterial membrane potential can be therefore viewed as a double-edged sword as it modulates intracellular antibiotic concentrations in an antagonistic manner. Combination therapy could take benefit from this collateral sensitivity of antibiotic resistant microbes. However, before direct therapeutic implication of collateral-sensitivity network and design of treatments in which multiple antibiotics are cycled over time, it is critical (i) to study the frequency of PMF-altering mutations in clinical isolates, (ii) to understand the molecular mechanism beyond this process and (iii) to decipher the long-term impact of collateral sensitivity on resistance evolution.<sup>104,106,107</sup>

**Eradicating persister bacteria.** Persister cells are tolerant to antimicrobials and considered to be a subpopulation of stochastically produced, non-growing (dormant) cells present in biofilm and planktonic bacterial cultures. Persister cells account for  $10^{-6}$  to  $10^{-4}$  of the total cell population of mid-exponential-phase cells and up to 1% of the total cell population of stationary-phase cells and biofilms. Persister infections are hard to treat with antibiotics that target biosynthetic processes in growing cells. Transcriptional regulatory genes involved in energy generation and oxidative phosphorylation including *e.g.* NADH dehydrogenase, ATP synthase, and cytochrome ubiquinol oxidase have been shown to be downregulated in persister bacteria.<sup>108</sup> Well-known clinical examples of such infections are the staphylococcal biofilms that result in endocarditis and other medical device-related infections, *P. aeruginosa* infections of the lungs of patients with cystic fibrosis, streptococcal otitis media, ischemic osteomyelitis containing slow-growing microorganisms, and tuberculous granulomas that contain latent *Mycobacterium tuberculosis*. The potential therapeutic benefit of membrane-acting agents arises from the fact that membrane is vital to both active and metabolic inactive pathogens and quiescent bacteria. Even in absence of growth, maintenance of redox

homeostasis and cellular energy is required. In this context, targeting bacterial membrane function appears as an under-exploited mechanism for treating persister infections.<sup>109</sup>

**Design of compounds showing a dual action.** Multi-target inhibition or combination of drugs is a logical approach developed to control the appearance of resistance.<sup>2</sup> The dual or multiple actions arise from the use of multiple drugs (Fig. 4B) or from one compound that inhibit multiple biological targets (Fig. 4C). Even such an approach will not solve the issue of resistance, this might help to delay the onset and constitutes an inviting prospect in antibacterial research.

First, antibacterial poly-pharmacology (Fig. 4B) is an alternative approach for overcoming the limitations of both monotherapy and combination therapy by targeting multiple proteins/processes in the disease associated network.<sup>110</sup> Indeed if the probability of resistance due to mutations developing in target A is PA and in target B is PB, the likelihood that resistance develops in both targets will be given by the conditional probability PA PB, a small number.<sup>2</sup> As a well-known example, inhibitors of early cell wall synthesis like teicoplanin and vancomycin, at sub-inhibitory concentrations exhibit significant synergy with  $\beta$ -lactam antibiotics with a high level of reduction in MRSA. Another example is given by the hybrid tobramycin–ciprofloxacin hybrid adjuvants that rescue the activity of fluoroquinolone antibiotics against multi drug resistant (MDR) strains and extremely drug-resistant *Pseudomonas aeruginosa* isolates *in vitro* and enhance fluoroquinolone efficacy *in vivo*. Mechanistic studies indicate that the antibacterial modes of ciprofloxacin are retained while the role of tobramycin is limited to destabilization of the outer membrane.<sup>111</sup> In addition, the development of adjuvants that either directly target resistance mechanisms or indirectly target resistance by interfering with bacterial signaling pathways, could also help in the fight for eradicating multidrug resistant bacteria.<sup>112</sup>

Second, if lipid targeting is critical, lipids need not be the only targets in multi-target inhibition (Fig. 4C). Indeed, it is likely that purely-membrane-targeting antibiotics will eventually lose efficacy as a result of changes in cell lipid-membrane composition. Targeting both a lipid membrane and protein (or other) target is therefore key. This is exactly what it has been achieved with SQ109,<sup>113</sup> a new agent against tuberculosis which (i) targets the trehalose monomycolate transporter MmpL3 (mycobacterial membrane protein large 3), (ii) inhibits two enzymes involved in menaquinone biosynthesis (MenA and MenG), and (iii) collapses the proton motive force PMF through distortion of  $\Delta\text{pH}$  and  $\Delta\varphi$  where  $\Delta\text{pH}$  is the pH gradient and  $\Delta\varphi$ , the membrane potential.<sup>114</sup>

Regarding the amphiphilic compounds, the lipid membrane is often the primary target, but additional effects are expected including (i) cell wall synthesis, (ii) cell division or (iii) immune system sensitization (may be essential since this places an organism under less selective pressure for the development of resistance<sup>115</sup>). Multiple targets are likely the reason why little *in vitro* resistance to cationic amphiphilic antibiotics has been observed or why a trend towards

increased daptomycin resistance has not been reported, although several cases of daptomycin non-susceptibility have been described.<sup>42</sup> This also explains why amphiphilic aminoglycosides, by interacting with LPS but also with CL domains localized in the IM of Gram-negative strains (Sautrey *et al.*, in revision), and modifying the activity of proteins involved in septum formation and cell division, are promising compounds. Regarding the amphiphilic tobramycins, they combine direct antibacterial effects with the induction of immunomodulatory responses in host immune cells, and specifically the recruitment of neutrophils efficacy leading to superior efficacy against multi drug resistant strains.<sup>116</sup>

### Examples of marketed membrane targeting antibiotics

Nowadays, a few membrane-active antibiotics are already approved including the multi-target lipopeptide (daptomycin), lipoglycopeptides (teicoplanin, oritavancin, telavancin, dalbavancin), and cyclopeptide antibiotics such as polymyxin B or colistin (polymyxin E) (Fig. 3).

**Lipopeptides.** Daptomycin is a cyclic lipopeptide constituted from a hydrophilic depsipeptide attached to a decanoyl fatty acid chain. It has a negative net charge and shows rapid bactericidal activity against Gram-positive cocci.<sup>117</sup> Daptomycin has been approved for the treatment of complicated skin infections and for bacteraemia caused by *S. aureus*, including endocarditis. Originally described as inhibitor of peptidoglycan synthesis,<sup>118</sup> daptomycin was later demonstrated to cause calcium-dependent membrane depolarization.<sup>119,120</sup> Although globally polar, daptomycin undergoes a conformational change in the presence of Ca<sup>2+</sup> that enhances its amphiphilicity by assembling the charged amino acids on one side of the molecule and exposing its lipophilic tail on the other side.<sup>121</sup> Ca<sup>2+</sup> also favors daptomycin oligomerization in micelle-like structures, with this lipid tails pointing inwards. In the presence of PG,<sup>122</sup> daptomycin micelles go through a second structural transition that enables the interaction of the lipophilic tail with the membrane. Daptomycin insertion in the bacterial membrane induces the leakage of cytosolic content causing a rapid bactericidal effect.<sup>123</sup> Daptomycin is one example of drug with dual action. Indeed, in addition to an effect on membranes, inhibition of the biosynthesis of the cell wall, protein, DNA, RNA, and lipoteichoic acid<sup>124</sup> has been reported.

**Lipoglycopeptides.** Lipoglycopeptides act against Gram-positive bacteria by dual interaction with peptidoglycan precursors and with membrane lipids. The peptide backbone of the glycopeptides mimics the D-alanyl-D-alanine binding site, which is an absolute requirement for antimicrobial activity of natural glycopeptides. To keep intact the binding pocket with the aim to promote a dual action, new molecules have been designed with removal or substitutions of sugars, derivatization of functional groups. Four molecules have emerged teicoplanin, oritavancin, telavancin, and dalbavancin.

Teicoplanin is produced by fermentation of *Actinoplanes teichomyceticus* as a complex formed by five closely related

glycopeptides characterized by different fatty acid chains of ten and eleven carbon atoms in addition with minor quantities of related substances.<sup>125</sup> The fatty acid chains serve as a membrane anchor and help to localize the antibiotic at the bacterial cell surface. This membrane localization may account for the improved activity of teicoplanin and has been suggested to be responsible for the VanB activity.<sup>126,127</sup>

Three other lipoglycopeptides have reached the market, oritavancin, telavancin and dalbavancin. Oritavancin differs from the parent compound, vancomycin, by the addition of a 4-epi-vancosamine sugar, which increases dimer formation<sup>128</sup> and a chlorobiphenyl side-chain, which ensures membrane anchoring.<sup>129</sup> Telavancin differs from vancomycin by the presence of a lipophilic decylaminoethyl tail and a phosphonomethyl-aminomethyl substituent<sup>130</sup> modifying the hydrophobic/hydrophilic properties. Telavancin has a dual mechanism of action: inhibition of the transglycosylation process of peptidoglycan cell wall synthesis by the formation of a complex with the D-alanyl-D-alanine precursors and depolarization of the bacterial membrane.<sup>131–133</sup> The latter effect requires both the presence of lipid II as well as an interaction between telavancin and D-alanyl-D-alanine residues.<sup>134</sup> Dalbavancin is a teicoplanin-like glycopeptide A 40926 which shows similar spectrum of *in vitro* activity to the other glycopeptides, but it is more potent than teicoplanin. The monoamide (dimethylaminopropyl) substituent at the peptide carboxy group is responsible for increased potency against staphylococci, particularly against coagulase negative staphylococci. The relative activity of teicoplanin-type glycopeptides largely depends on their ionic character.<sup>43</sup> The most innovative feature of dalbavancin is its pharmacokinetics. Due to the presence of the *N*-acylglucosamine, it exhibits excellent tissue penetration, particularly in the skin, and a long half-life that allows once-weekly administration.<sup>135,136</sup>

**Cyclopeptide antibiotics.** Cyclic cationic lipopeptide antibiotics like polymyxin B and colistin (polymyxin E) were produced by *Bacillus* and *Paenibacillus* spp.<sup>137</sup> The polymyxin molecule consists of five key structural features: (i) the hydrophobic N-terminal fatty acyl chain; (ii) the positive charge of the five Dab residues (at physiological pH); (iii) the linear tripeptide segment; (iv) the hydrophobic motif at positions (6) and (7) (Fig. 3) in the cyclic heptapeptide ring; and (v) the heptapeptide backbone.<sup>23</sup> Polymyxin E differs from polymyxin B only by the substitution of D-leucine (Leu) for D-phenylalanine (Phe) as one of the amino acids in the cyclic part of the structure. Polymyxin B as well as polymyxin E contains four major components, differing only in their fatty acid moiety. Polymyxins were first identified in the late 1950s. These molecules are cyclized at the C-terminus by an ester or amide bond and the lipid tail is incorporated through acylation of the N-terminal amino acid by nonribosomal peptide synthetases. The overall cationic charge usually comes from the incorporation of multiple residues of the nonproteogenic amino acid Dab. Numerous medicinal chemistry modifications have been made to the polymyxin core structural domains.<sup>50</sup> They were used in both

human and veterinary medicine.<sup>138</sup> Their primary role was to treat serious infections caused by Gram-negative bacteria. Toxicity, reported as early as 1965, limited the use of these agents for several decades. The drugs are now used as a treatment of last resort for infections caused by multi drug resistant Gram-negative pathogens.<sup>139</sup> Different surface charge of colistin-susceptible and resistant *A. baumannii* cells have been measured as a function of growth phase and colistin treatment.<sup>140</sup> The molecular mechanism is thought to involve the competition for the binding of divalent interactions leading to enhanced lateral diffusion of LPS.<sup>139</sup> The resulting destabilization of LPS layer allows the penetration of polymyxin B into the periplasm, providing essentially a “self-promoted uptake pathway” to reach its target, the cytoplasmic membrane. Then the fatty acid tail on polymyxin B allows it to permeabilize the inner membrane, leading to its antibacterial effect.<sup>141</sup> An alternative mechanism, called vesicle-vesicle contact has also been proposed.<sup>142,143</sup>

Despite extensive structure activity relationships done to optimize the original scaffold, more studies are required to fine-tune the parameters governing amphiphilicity or charge of the molecule. Indeed, subtle structural modifications have a major impact on the antibacterial effect<sup>144</sup> due to the multiple factors acting on bacterial lipid membranes, such as their lipid composition.

### Drugs in development

Eventhough further critical analysis of the physicochemical properties of promising derivatives has to be done regarding the biophysical properties of the bacterial membranes for development of new antibiotics targeting bacterial membranes and showing dual activity (*e.g.* inhibition of cell wall, cell division), some drugs have already been marketed and several pre-clinical or experimental membrane-active antibiotics are in ongoing investigations. They include DCAP,<sup>145</sup> benzophenone derivatives,<sup>146</sup> porphyrin antibacterial agents like XF-70 and XF-73,<sup>147</sup> reutericyclin, a membrane-active antibiotic from *Lactobacillus reuteri*,<sup>148</sup> small quinoline-derived compound like HT61,<sup>149</sup> acylated derivatives of epigallocatechin gallate,<sup>150</sup> dirhamnolipid,<sup>151</sup> squalamine and aminosterol derivatives,<sup>152</sup> WAP-8294 A2,<sup>153</sup> trehalose lipids,<sup>154</sup> xanthone analogues.<sup>155</sup> Extensive work performed on antimicrobial peptides (see recent reviews<sup>86,156–158</sup>) has inspired new approaches for the development of amphiphilic antibiotics<sup>159</sup> which avoid the drawbacks of antimicrobial peptides including degradation by proteases and toxicity. Other than cationic amphiphilic peptides,<sup>160</sup> ceragenins and amphiphilic aminoglycoside derivatives,<sup>58,97,161,162</sup> are promising candidate antimicrobial agents. Ceragenins are cholic acid-derived antimicrobial agents designed to mimic the activities of antimicrobial peptides. They target bacterial cytoplasmic membranes inducing membrane depolarization<sup>163</sup> and adopt a cationic facially amphiphilic structures in the presence of lipids. Since the hydroxyl groups of bile acid are oriented on one face of the molecule resulting in facially amphiphiles

with one hydrophobic side formed by the sterane ring and the positive charges of the aminogroups forming the hydrophilic face of the molecule. The most promising derivative, CSA-13, shows very interesting activity against carbapenem-resistant *A. baumannii* strains<sup>164</sup> and persistent infections involving biofilms formed by *P. aeruginosa*.<sup>165</sup> CSA-13 retained potent antibacterial activity against *S. aureus* over the course of 30 serial passages. Resistance generated in Gram-negative bacteria correlates with modifications to the outer membranes of these organisms<sup>166</sup> with membrane lipids. Regarding amphiphilic aminoglycosides (AAGs) and their rationale development for fighting bacterial resistant strains, this constitutes the second part of this review.

## Part II: a continuum in medicinal chemistry from antibiotic aminoglycoside drugs targeting ribosomal RNA to amphiphilic aminoglycosides targeting bacterial membranes

Antibacterial amphiphilic aminoglycosides (antibacterial AAGs) are derived from aminoglycosides (AGs) that are potent bactericidal antibiotic drugs endowed with broad-spectrum activity. Discovered from 1943 to 1963, these antibiotics, including streptomycin, neomycins, paromomycin, kanamycins, tobramycin, gentamicins... (Fig. 5) are produced by Gram-positive bacteria of the *Actinomycete* group such as *Streptomyces* and *Micromonospora* that have elaborated sophisticated biochemical pathways for antibiotic production to reduce the number of competing organisms.<sup>167</sup> AGs, which are, at physiological pH, polycationic pseudo-oligosaccharides, strongly bind to bacterial ribosomal RNA (rRNA) mainly to the A site of 16S rRNA and cause *in fine* protein synthesis alteration.<sup>168–172</sup>

AGs are mainly used against Gram-negative bacteria in hospitals. Amikacin (Fig. 5) is a semi-synthetic AG antibiotic derived from kanamycin A by introduction of the (*S*)-4-amino-2-hydroxybutanoyl (AHB) group and is most often used for treating severe, hospital-acquired infections with MDR Gram-negative bacteria such as *P. aeruginosa*, *Acinetobacter* and *Enterobacter*. Apramycin (Fig. 5) is a structurally unique natural antibiotic made of a fused bicyclic sugar moiety that stands out among AGs for its mechanism of action based on translocation blockage and its ability to also bind significantly to the eukaryotic decoding site.<sup>171</sup> As a consequence, apramycin is used only for the treatment of bacterial infections in veterinary practice.

A review article published in 2014 describes some of the new trends observed in the use of AGs in the past decade, along with the current understanding of their mechanisms of action in various bacterial and eukaryotic cellular processes.<sup>172</sup> An overview of the mechanisms by which bacteria

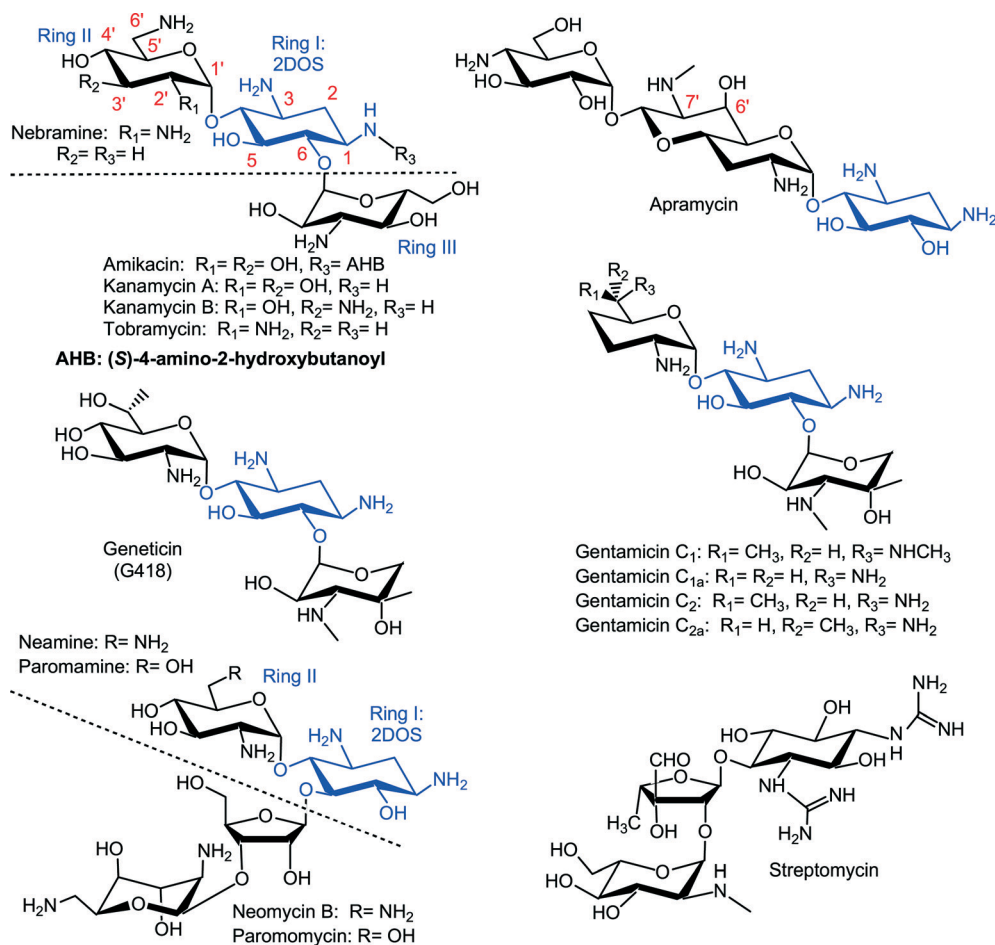


Fig. 5 Structure of the natural or hemi-synthetic AG drugs made of the 2-deoxystreptamine (in blue) or streptomycin core (alphabetic order from the first compound drawn in each series).

become resistant to AGs was also published recently in which their potential and prevalence for clinical relevance are discussed.<sup>173</sup> A non-conventional review article was published in order to point out the renaissance on AG development/application by summarizing all patents filed on AGs from 2011–2015 and highlighting some related publications on the most recent works done on AGs to overcome resistance and improving their therapeutic use.<sup>174</sup>

Side toxicities, mainly nephrotoxicity and ototoxicity, have been observed in antibiotherapy with AGs such as gentamicins (Fig. 5).<sup>172,174–181</sup> These antibiotics are associated with hearing loss and vestibular dysfunction due to hair cell loss.<sup>179,180</sup> However, AGs are used in the treatment of Ménière's disease of the inner ear characterized by recurring attacks of disabling vertigo, hearing loss, and tinnitus.<sup>172,182</sup>

AGs such as gentamicins and amikacin (Fig. 5) allow, *via* binding to human rRNA, the readthrough by the translation complex of disease-causing nonsense mutations and, therefore, the synthesis of full-length active proteins.<sup>172,183–186</sup> This read-through activity is being evaluated for the treatment of genetic diseases such as cystic fibrosis resulting from mutations in the gene coding for the protein cystic fibrosis transmembrane conductance regulator.

AGs bind strongly to different RNA<sup>187–195</sup> and DNA<sup>196,197</sup> sequences such as several HIV RNA sequences<sup>189–195</sup> and, were used as key tools in biochemical and biological studies involving nucleic acids. They are also able to cleave RNA and DNA sequences *in vitro* under physiological conditions and/or in the presence of copper ions.<sup>181,198–205</sup> Therefore, AGs constitute a major family of antibiotic drugs of high diversity and a large family of nucleic acid binders having multiple biological and medicinal effects.<sup>172,187,189,196</sup>

Decades of widespread clinical use of AGs strongly reduced their clinical efficacy through the selection of resistant bacteria.<sup>172–174,206–219</sup> As for other classes of antibiotic drugs, four modes of bacterial resistance to AGs have been identified: reduction in the intracellular concentration of the antibiotics<sup>212–215</sup> (i) by surexpression of efflux pump proteins and/or (ii) through reduction of membrane permeability, (iii) deactivation by AG-modifying enzymes,<sup>206–211,218,219</sup> and (iv) structural modifications of the 16S ribosomal RNA binding site that lead to reduced target affinity.<sup>173,219</sup>

Amphiphilic AGs (AAGs) have received many applications of medicinal interest.

They are emerging antibacterial agents discovered between 2007–2010 in the search for new AGs targeting rRNA

and less susceptible to resistance causing enzymes.<sup>161,162,172–174,217–256</sup> AAGs have also been demonstrated to be efficient intracellular delivery vehicles for biologically relevant molecules<sup>258</sup> such as genes,<sup>259–261</sup> siRNA.<sup>262</sup> Amphiphilic conjugates of AGs to polyamide nucleic acids (PNAs) that strongly target complementary viral RNA sequences showed high bioavailability in human cells found both in the cytosol and the nucleus, whereas PNAs alone are not able to enter human cells.<sup>263–265</sup> Attachment of the neamine core (Fig. 5) to a 16 mer PNA targeting HIV-1 TAR RNA has led to amphiphilic conjugates soluble in water which are endowed with good antiviral activity in CEM cells and also with a unique RNA cleavage property particularly specific to the target site.<sup>263</sup>

In the history of the emergence of antibacterial AAGs, the works of Hanessian and collaborators constitute an example of strategy that preludes to the discovery of antibacterials AAGs.<sup>229–232</sup> They designed and synthesised antibacterial 2'' ether analogues of paromomycin based on new site-selective introduction of *O*-alkyl side groups. A new mode of binding in the A-site rRNA was highlighted for these derivatives.<sup>229,230</sup> With few exceptions, all of the new analogues synthesized showed potent inhibitory activity equal or better than paromomycin against a sensitive strain of *S. aureus*. Low MIC values were also obtained against *E. coli* with some derivatives carrying side alkyl and alkylaryl groups containing polar or basic end groups.<sup>230,231</sup> Two analogues showed excellent survival rate in a mouse septicemia protection assay against *S. aureus*. Preliminary studies showed no overt signs of toxicity while controls with neomycin B were toxic at lower doses.<sup>230</sup> Incorporation of a hydrophobic 2-(phenethylamino)-ethyl ether at C2'' of *N*1-((*S*)-4-amino-2-hydroxybutanoyl)-3',4'-dideoxyparomomycin led to a novel analog with an excellent antibacterial profile against a panel of resistant bacteria (among them *S. aureus* and *E. coli* resistant strains).<sup>231</sup> The synthesised 2'' ethers of paromomycin were also capable of inhibiting both AG-deactivating enzymes APH(3')-IIIa and AAC(6')-II with  $K_i$  values in the low micromolars.<sup>232</sup>

Several groups, ours included, contributed to the antibacterial AAG emergence leading to the identification of strong antibacterials acting on the bacterial membranes, not only against AG-resistant bacteria but also against MDR bacteria, offering a promising direction for the development of novel antibiotics.<sup>58,65,111,159,161,162,233–256</sup> Many AAGs were discovered to be strongly active against sensitive and resistant Gram-positive bacteria and a few also active against sensitive and resistant Gram-negative bacteria.

AAGs that are antifungal, but not antibacterial, and that inhibit the growth of fungi by perturbation of plasma membrane functions were also recently discovered.<sup>257</sup> Their low toxicities against plant and mammalian cells, their specificity for the fungal plasma membrane offer new perspectives in the fight against fungal pathogens in medicine and agriculture.

The main results obtained in the discovery and development of antibacterial and antifungal AAGs were reviewed in

this journal in 2014 (ref. 172, 174, 254, 255) and in the chapter of a book in 2015.<sup>256</sup>

Here, our report is mostly limited to the recent highlights in the field of antibacterial AAGs and focuses mainly on the delineation of structure–activity relationships in regard to the recent progress in the understanding of their mode of action. Perspectives in the field are also discussed at the end of this report.

## Chemistry

Many groups have worked on the chemistry of AGs in the search for antibacterial and antiviral agents as well as tools useful in biochemical and biological studies involving RNA and DNA.<sup>172–174,183,189,196,216–228</sup> Nowadays, the chemistry developed for the synthesis of modified AGs allows the selective modification of different amine and hydroxyl functions in the main AG drugs.

AAGs can result from introduction of one or several lipophilic groups on the AG amine and/or hydroxyl functions. Natural AG drugs carry a large number of amine and alcohol functions (5–6 amino and 5–8 hydroxyl groups) and the selective modification of these functions involves a series of protection and deprotection that can be long. In order to modify one or more hydroxyl groups, amine functions were often converted to azido groups. The shortest routes used for preparing AAGs were developed for the selective introduction of one lipophilic group from an aminomethylene or a primary hydroxyl group, when present alone and/or being the most reactive in the structure. Introduction of one or two lipophilic groups from hindered amines or from secondary alcohol functions required longer procedures. In the search for antibacterial AAGs from smaller AGs than natural AGs drugs, the latter drugs were selectively cleaved. For instance, neamine and paromamine (Fig. 5) carrying three and four amine functions were obtained by methanolysis of neomycin B and paromomycin, respectively, and nebramine (Fig. 5) derivatives were prepared from tobramycin after conversion of the amine functions to azido groups.

For the attachment of the lipophilic groups to AGs, amide groups were formed as junctions from the amine functions,<sup>159,238,243</sup> and, from hydroxyl groups, amide,<sup>233,234,238,244</sup> amine,<sup>241</sup> carbamate,<sup>235,238</sup> ether,<sup>58,161,230,231,237,238,246,247,250</sup> thioether,<sup>159,250–252</sup> sulfoxide and sulfone<sup>159,248</sup> junctions were generated. Click chemistry was also used for attachment of side chains and lipophilic groups *via* triazole ring(s), for instance in the neomycin B series (Fig. 6).<sup>233,241,243,245,248</sup>

On the one hand, the fact that carbamate junctions can be partially hydrolyzed *in vivo* to lead to the parent AGs may be seen as an advantage, the corresponding AAGs and their AG metabolites targeting bacterial membranes and rRNA, respectively. On the other hand, the aforementioned fact may also be seen as a disadvantage due to the decrease of AAG concentration and to possible side effects. Thioether junctions can be oxidized in sulfoxides and sulfones *in vivo*. However, efforts should be made in the design of new antimicrobials in

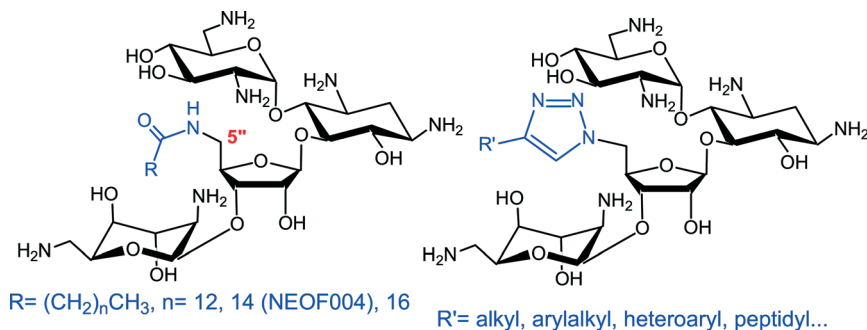


Fig. 6 Examples of amphiphilic 5''-monoalkyl neomycin B derivatives synthesised.<sup>233,234,238,241</sup> Many derivatives showed strong activity against sensitive and resistant Gram-positive bacteria and a few displayed good activity against several sensitive Gram-negative bacteria (compounds on the left in blue among them NEOF004: MIC = 4–16  $\mu\text{g mL}^{-1}$ ).<sup>234,254</sup>

order to favor their metabolic and environmental degradations and/or transformation leading to inactive and nontoxic products in order to minimize the selection of resistant bacteria after treatment in the environment.

The conversion of amines to amides decreases the number of positive charges carried by the resulting AAGs. In order to keep enough positive charges for strong binding to the anionic components of bacterial membranes, alcohols functions were modified in order to preserve the amine functions protonated at physiological pH, for instance in the modification of neomycin B<sup>233,234,238,241</sup> (Fig. 6) and of the small pseudo-disaccharidic AGs neamine, paromamine and nebramine.<sup>58,161,251,252</sup>

Other antibacterial AAGs derived from kanamycins, neomycin B, paromomycin, tobramycin, and which present a structure made of three or four rings, carrying one lipophilic group were synthesised and evaluated. Additionally, poly-*O*-benzylated AGs,<sup>249,251</sup> di-, tri-alkyl ethers,<sup>58,161,251,252</sup> dialkylcarbamates and tetraphenylcarbamates<sup>239</sup> of neamine, di-, tri-alkyl ethers of paromamine<sup>58</sup> and its parent AG paromomycin,<sup>249</sup> dithioethers of tobramycin and nebramine were also synthesised and evaluated.<sup>251,252</sup> AAGs in which amine functions are converted to guanidine groups were prepared in the kanamycin A and neomycin B series.<sup>240</sup>

Various lipophilic groups of different sizes and lengths were introduced on AGs such as alkyl, arylalkyl, alkylcycloalkyl groups carrying or not hydrophilic function(s) and

incorporating or not heteroatom(s). AAGs bearing fluoroalkyl groups having both hydrophobic and lipophobic characters were also prepared.<sup>244</sup>

#### Update: new antibacterial and antifungal AAGs described since 2014

Here, we highlight the main recent results described in the field of antibacterial AAGs since publication in 2014 of the recent review articles in these fields.<sup>172,174,254,255</sup>

In 2015, Schweizer and collaborators reported that 5-*O*-alkyl tobramycin derivatives (Fig. 7), exhibiting good activity against sensitive and tobramycin-resistant Gram-positive bacteria (MIC = 2–16  $\mu\text{g mL}^{-1}$ ) and reduced activity against Gram-negative bacteria (MIC = 16–256  $\mu\text{g mL}^{-1}$ ), can, not only, boost the innate immune response, specifically the recruitment of immune cells such as neutrophils, but can also selectively control inflammatory responses to prevent septic shock.<sup>246</sup> Many antibacterials have showed anti-inflammatory effects<sup>266</sup> and, for the first time, AAGs showing good activity against Gram-positive bacteria were proved to induce immunomodulatory responses at concentrations that are non-toxic to host cells.

In 2015, Fridman and collaborators described the synthesis and the antibacterial evaluation of twenty three 4',5-di- and 4',5,6-tri-alkylated nebramine derivatives and penta-*O*-alkyl tobramycins from the penta-azido tobramycin and from

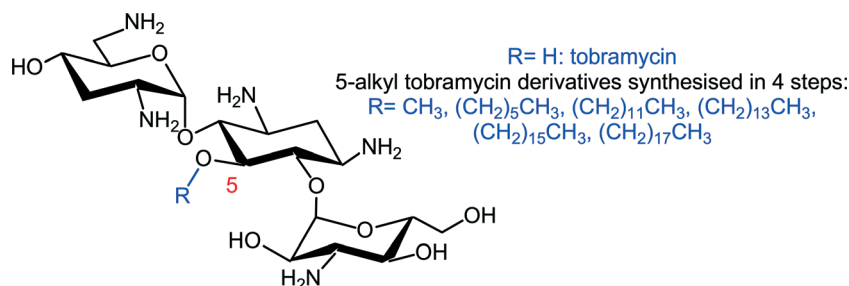


Fig. 7 Amphiphilic tobramycin derivatives synthesised in the search for antibacterial AAGs and selective modulators of endotoxin-induced inflammatory responses. The tetradecyl (R = C<sub>14</sub>H<sub>29</sub>) and hexadecyl (R = C<sub>16</sub>H<sub>33</sub>) derivatives showed good anti-Gram-positive activity and were found able to boost the innate immune response.<sup>246</sup>

the tetra-azido derivative of its pseudo-disaccharide constitutive element nebramine (Fig. 8).<sup>251</sup> Only one amphiphilic nebramine derivative carrying three heptyl chains was found to be effective against all of the tested strains of Gram-positive and Gram-negative bacteria (MRSA, *E. coli*, *K. pneumonia*, *P. aeruginosa* strains). Ten derivatives showed a good activity against sensitive and resistant Gram-positive bacteria.

In November 2015, the same group reported that di-*N*-methylation of all primary amine functions present in the previously identified anti-Gram-negative triheptyl nebramine and other nebramine, tobramycin and paromomycin amphiphilic derivatives, only active against Gram-positive bacteria, results in a significant and general enhancement of the antimicrobial activity leading to AAGs acting against both Gram-positive and Gram-negative bacteria.<sup>252</sup>

At the end of 2015, Garneau-Tsodikova and collaborators reported the design and synthesis of seven kanamycin B derivatives.<sup>253</sup> Two of these compounds, with a C<sub>12</sub> and C<sub>14</sub> aliphatic chain attached at the 6''-position through a thioether linkage, exhibited good anti-Gram-positive and antifungal activity, and were found to be poorer substrates than kanamycin B for several AG-modifying enzymes. They were both relatively less hemolytic than the known membrane targeting antibiotic gramicidin and the known antifungal agent amphotericin B and were not toxic at their antifungal MIC values. Their oxidation to sulfones was also demonstrated to have no effect on their activities. Moreover, they both acted synergistically with posaconazole, an azole currently used in the treatment of human fungal infections.

#### Delineation of structure–activity and structure–cytotoxicity relationships for AAGs and mechanisms of action mainly against Gram-negative bacteria

In our first report in the field, in 2010, we proposed for the first time bacterial membranes as targets for explaining the antibacterial activity observed with the di- and tri-2-naphthylmethylene (2NM) nebramine derivatives (Fig. 9).<sup>161</sup> The

3',4'- and 3',6-di2NM derivatives have shown good activity against sensitive and resistant *S. aureus* (for instance MRSA and vancomycin-resistant *S. aureus*) whereas the 3',4',6-tri2NM derivative has appeared to be strongly active against both sensitive and resistant Gram-positive and Gram-negative bacteria (*A. baumannii*, *E. coli*, *K. pneumonia*, *P. aeruginosa*...). We have observed for this trialkyl compound and the 3',6-di2NM derivative a weak and aspecific binding to a bacterial 16S rRNA model in comparison to neomycin B. The observed low ability of 3',4',6-tri2NM neamine to decrease <sup>3</sup>H leucine incorporation into proteins in *P. aeruginosa* confirmed that antibacterial AAGs can exert a different mode of action compared to the parent AG.<sup>161</sup>

In 2013, through the delineation of lipophilicity/activity and lipophilicity/cytotoxicity relationships, we described the determination of best number of alkyl chains to attach to the neamine core and their optimal lipophilicity for obtaining strong and broad-spectrum antibacterial effects and low cytotoxicity in eukaryotic cells.<sup>58</sup> Dialkyl neamines (Fig. 9) were found to be better than 3',4',6-trialkyl neamines mainly in regard to their lower cytotoxicity in eukaryotic cells which can be related to a higher selectivity for bacterial membranes. The favourable windows of lipophilicity for a good activity

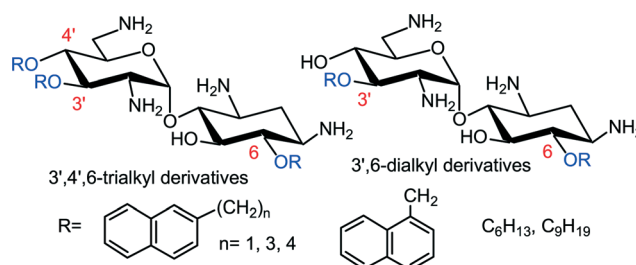


Fig. 9 Structure of the main 3',6-dialkyl and 3',4',6-trialkyl nebramine derivatives which showed good to strong activity against both sensitive and resistant Gram-positive and Gram-negative bacteria (trialkyl series: R = 1- or 2-naphthylmethylene, hexyl; dialkyl series: R = 2-naphthylpropyl, 2-naphthylbutyl and nonyl).<sup>58,65,161,162</sup>

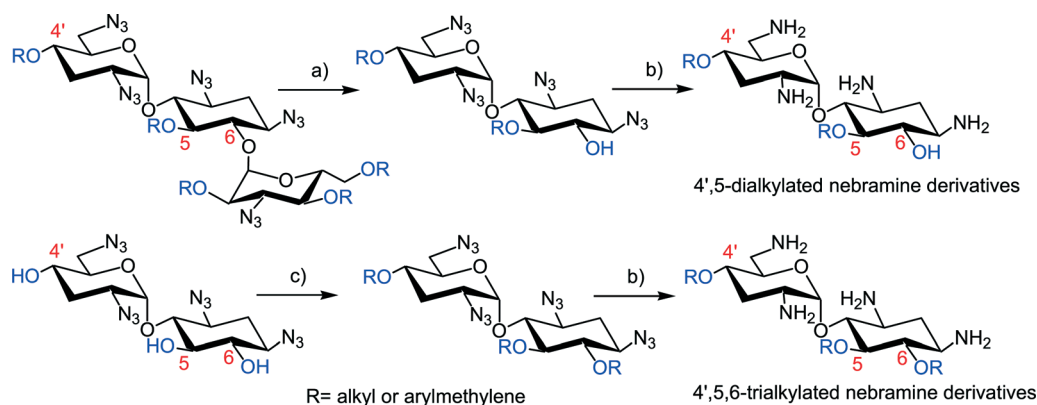


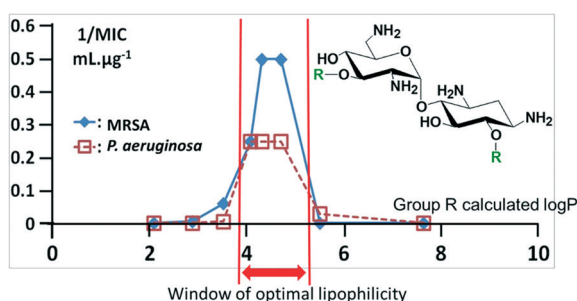
Fig. 8 Synthesis of 4',5-di- and 4',5,6-trialkylated nebramine derivatives from penta-azido tobramycin and tetra-azido nebramine, respectively, and prepared from tobramycin: a) 1.5 M H<sub>2</sub>SO<sub>4</sub> in MeOH, reflux, 40–96%; b) 1.0 M P(Me)<sub>3</sub> in THF, H<sub>2</sub>O/THF (1 : 9), 0.1 M NaOH; 77–99%; c) alkyl bromide or alkyl chloride; NaH; TBAI; DMF.<sup>251</sup>



against different bacteria strains were delineated in the di- and in the tri-alkyl series of neamine derivatives. When compared in a same serie, di- or tri-alkyl derivatives, against sensitive and resistant Gram-positive and Gram-negative bacteria, the windows of entire lipophilicity of the compounds or the windows of the substituent lipophilicity appeared to be close (Fig. 10). Such a close proximity in the location and width of these windows for Gram-positive and Gram-negative bacteria is unexpected. When compared both series, di- and the tri-alkyl series, the windows of lipophilicity for a same bacteria appeared to be different with overlapping areas.

Among the synthesised 3',6-dialkyl neamines, three compounds, the 3',6-di-*O*-[(2"-naphthyl)propyl] (3',6-di2NP), 3',6-di-*O*-[(2"-naphthyl)butyl] (3',6-di2NB), and 3',6-di-*O*-nonyl (3',6-diNn) (Fig. 9) displayed strong activity against susceptible and resistant *S. aureus* (MICs = 0.25 to 8  $\mu\text{g mL}^{-1}$ ) and good activity against susceptible and resistant Gram-negative bacteria (*E. coli*, *P. aeruginosa*...; MICs = 2 to 16  $\mu\text{g mL}^{-1}$ ) and low toxicity in eukaryotic cells at 10  $\mu\text{M}$ .<sup>58</sup> These amphiphilic neamine derivatives were also active against clinical colistin-resistant *P. aeruginosa* strains (MICs = 2 to 8  $\mu\text{g mL}^{-1}$ ) and the most active one, 3',6-diNn neamine, was bactericidal at its MIC and inhibited *P. aeruginosa* biofilm formation at 2-fold its MIC.<sup>65</sup>

In the study of the mechanism of action of these antibacterial AAGs against Gram-negative bacteria, we reported in 2011 and 2014 their ability to interact with LPS and to alter the bacterial outer membrane.<sup>65,162</sup> Their effects on LPS micelles suggested changes in the cross bridging of LPS and disordering in the hydrophobic core of the micelles. The molecular shape of the 3',6-dialkylated neamine derivatives induced by the nature of the grafted hydrophobic moieties (naphthylalkyl instead alkyl) and the flexibility of this hydrophobic moiety are critical for their fluidifying effect and their ability to displace cations bridging LPS. Grafting long and linear alkyl chains (nonyl) optimized binding to LPS and outer membrane permeabilization.<sup>65</sup> We demonstrated also their capacity to interact with lipids like CL, a lipid mostly located within the inner membrane of *P. aeruginosa* (Sautrey *et al.*, in revision). We characterized (i) the interaction of 3',6-



**Fig. 10** Values of  $1/\text{MIC}$  ( $\mu\text{g mL}^{-1}$ ) against ATCC 33592 HA-MRSA (blue diamonds) and *P. aeruginosa* ATCC 27853 (open red squares) as a function of calculated  $\log P$  of the lipophilic substituent carried by the synthesised neamine derivatives (calculated  $\log P$  of the corresponding alkanes).<sup>58</sup>

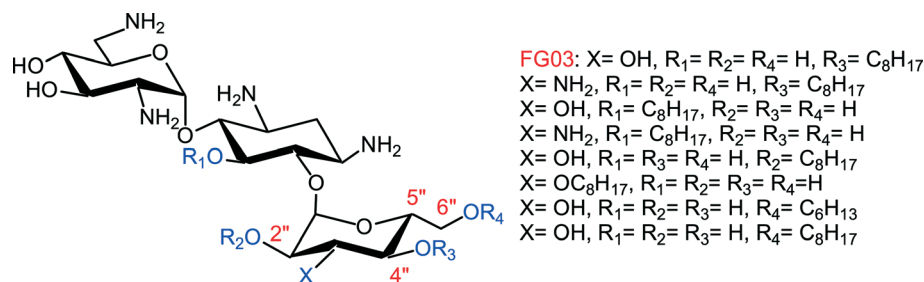
dinonyl neamine with CL and the impairment in the lateral segregation of CL (giant unilamellar vesicles (GUVs) labelled with fluorescent CL, Langmuir isotherm compression), and (ii) the hemifusion process resulting in membrane permeabilization. The modulation in the lateral phase distribution of CL induced by 3',6-dinonyl neamine could affect curvature stress in the bilayer potentially responsible for an hemifusion process and pore formation.

The antibacterial activities of 1-naphthylmethylene (1NM) and 2NM paromamine and neamine derivatives (Fig. 9) were also compared.<sup>58</sup> We observed higher antibacterial activities, mainly against Gram-negative bacteria, of the neamine derivatives in comparison to their paromamine homologues with the exception of sensitive *A. lwoffii* against which the 3',4',6-tri-1NM or -2NM paromamine derivatives were found strongly active (MICs = 1–2  $\mu\text{g mL}^{-1}$ ).<sup>58</sup> These results showed the key role in the antibacterial effects of the 6'-amine function of the neamine core that is protonated at physiological pH. Electrostatic interactions at the 6'-position appeared to be critical at the early stage of the *E. coli* and *P. aeruginosa* recognition.

We did not obtain significant antibacterial effects with monoalkyl neamines in a lipophilicity range including the lipophilicity of the identified active di- and tri-alkyl neamines whereas many antibacterial AAGs carrying one long alkyl chain attached to AG scaffolds larger than the neamine one and bearing more amino groups have been described.

From the parent AG of neamine, neomycin B, the Chang and Schweitzer groups have synthesised neomycin B-based AAGs showing good activity against both sensitive and resistant Gram-positive and Gram-negative bacteria (Fig. 6).<sup>233,234,238,254</sup> Such alkyl neomycin derivatives lack antifungal activity, whereas kanamycin derivatives showed good antifungal activity. For instance, introduction of one octyl group at the *O*-4" position of kanamycin B converted this antibiotic AG to a novel antifungal agent (Fig. 11).<sup>254,257</sup> In 2015, in the delineation of the structure–activity relationships (SAR) for antifungal activity, the synthesis of a library of kanamycin B analogs alkylated at various hydroxyl groups was described.<sup>237</sup> A lead AAGs FG03 with a hydroxyl group replacing the 3"-NH<sub>2</sub> group of kanamycin B was identified. SAR studies from the obtained library revealed that the *O*-4" position is the optimal site for attaching a linear alkyl chain, and that the 3"-NH<sub>2</sub> and 6"-OH groups of the kanamycin B parent molecule are not essential. Tested for growth inhibitory activities against *S. aureus* and *E. coli*, all of the amphiphilic kanamycin B analogs, regardless of the positions of alkylation, were inactive (MIC > 32  $\mu\text{g mL}^{-1}$ ). These works well demonstrate that antifungal and antibacterial activities of AAGs can be differentiated and possible selectivity of AAGs for fungal and bacterial membranes that remains to understand. In our opinion, it also points out that the neomycin scaffold and its neamine core are better than the kanamycin scaffold for the design of antibacterial AAGs.

The structure of nebramine is closely related to that of neamine (Fig. 5) from which antibacterial AAGs active against



**Fig. 11** Examples of amphiphilic kanamycin B derivatives synthesised in the search for antifungal AAGs and delineation of structure–activity relationships.<sup>257,254,257</sup> FG03 was the lead compound identified<sup>257</sup> after the discovery of one broad-spectrum fungicide analogue of FG03, FG08, in which the 5''-CH<sub>2</sub>OH group is converted to a methyl group.<sup>254,257</sup>

both sensitive and resistant Gram-positive and Gram-negative bacteria have been obtained. In nebramine, a hydrogen atom replaces the 3'-OH group found in neamine. In the search for antibacterial AAGs from tobramycin and its pseudo-disaccharide element nebramine (Fig. 8), among 23 AAGs synthesised, Fridman and collaborators identified one amphiphilic nebramine derivative carrying three heptyl chains active against sensitive and resistant Gram-positive (MIC = 1–4  $\mu\text{g mL}^{-1}$ ) and Gram-negative bacteria (MIC = 4–8  $\mu\text{g mL}^{-1}$ ) (MRSA, *E. coli*, *K. Pneumonia*, *P. aeruginosa*... strains).<sup>251</sup> Ten derivatives showed a good activity against sensitive and resistant Gram-positive bacteria and all of the most active derivatives fell into a narrow log*P* range of 5.4 to 6.1. As seen from our previous results in the neamine series, this suggests that log*P* may be used as a consideration for the molecular-design of antibacterial AAGs. However, on the contrary to the neamine derivatives, the window of lipophilicity delineated in this study for obtaining an anti-Gram-positive activity does not correlate with the lipophilicity of the unique anti-Gram-negative triheptyl nebramine derivative identified. Clearly, in the nebramine and tobramycin series, the value of AAG lipophilicity does not allow on its own the design of AAGs targeting Gram-negative bacteria.

The MIC values of the triheptyl nebramine against *E. coli* and *S. pyogenes* were measured in the presence of *E. coli* LPS in a range of concentrations from 0 to 100  $\mu\text{g mL}^{-1}$  in comparison to the antimicrobial cationic lipopeptide PMX. An increase in LPS concentration led to an increase in the MIC values of the nebramine derivative and PMX against *E. coli* ATCC 25922 of up to eightfold and a similar effect was observed against *S. pyogenes*. The observed increase in MIC values in the presence of LPS may be explained by a binding of the triheptyl nebramine derivative to LPS that was confirmed by competitive displacement of the LPS binding fluorescent dye bodipy-cadaverine by this derivative. Similar results were obtained with a derivative of the active triheptyl nebramine only active against Gram-positive bacteria.

The triheptyl nebramine appeared to be the most hemolytic derivative of the synthesised derivatives in PBS buffer. However, in a brain-heart infusion (BHI) broth, it was significantly less hemolytic than in PBS due to the presence of negatively charged species in the BHI broth. Experiments performed by bright-field epi-fluorescence microscopy with

Gram-positive *B. subtilis* showed the loss of fluorescent cytosolic produced YFP-protein in the presence of the antibacterial AAGs prepared having relatively low hemolytic activity. These experiments confirmed the expected disruption of the Gram-positive bacterial membrane structure in the presence of the synthesised compounds.

The membrane disrupting effects of the good antibacterial neomycin B derivative NEOF004 carrying one hexadecanoyl (C16) group at the 5'' position (Fig. 9) have been also investigated by Chang and collaborators using a fluorogenic dye, SYTOX, that cannot penetrate the intact bacterial membrane.<sup>236</sup> If the membrane is damaged by membrane disrupting agents, SYTOX dye can bind to nucleic acid and emit strong fluorescence. In experiments conducted with *S. aureus*, NEOF004 caused damage to the bacterial membrane in contrast to neomycin B for which no sign of membrane disruption was detected as expected from a traditional AG.

These results, together with those of Fridman and collaborators<sup>159,249,251</sup> demonstrate that the strong activity of AAGs against Gram-positive bacteria is related to their membrane-disrupting effects and confirm the novel antibacterial mode of action of AAGs in comparison to AGs as demonstrated previously against *P. aeruginosa*.<sup>65,162</sup>

## Discussion and perspectives

To the best of our knowledge, no AAGs able to target both the bacterial membranes and rRNA are presently described. In the search for AGs targeting rRNA, some previously reported antibacterial lipophilic AGs could have bacterial membranes as targets instead of rRNA but have been previously considered as acting directly on rRNA.

The classification of antibacterial AGs in two separate families, AGs and amphiphilic AGs (AAGs), is related to the lipophilicity of these compounds and, thus, to their mode of action, either on rRNA or bacterial membranes, that is related to the AG scaffold chosen for modification and to the AAG structure, with key parameters which are the numbers of amine functions and lipophilic groups introduced and their positioning.

It seems possible to obtain antibacterial AAGs from many natural antibiotic AG drugs and their constitutive components. Antibacterial amphiphilic derivatives of amikacin,

kanamycins, neomycin B, paromomycin and tobramycin, and of the smaller antibiotic AG elements, neamine, paromamine and nebramine, targeting, probably all, bacterial membranes were identified.

Most of them showed strong effects against both sensitive and resistant Gram-positive bacteria and a limited number of AAGs exhibited good to significant activity against sensitive and resistant Gram-negative bacteria ( $2 \leq \text{MIC} \leq 16 \mu\text{g mL}^{-1}$ ). They belong mainly to the neomycin series with (i) 5'-alkylamido neomycin derivatives that are monoalkylated neomycins (Fig. 6)<sup>237,254</sup> and (ii) di- and tri-*O*-alkyl derivatives of neamine.<sup>58,65,161,162</sup> The triheptyl nebramine derivative synthesised from tobramycin was also found to be effective against both sensitive and resistant Gram-positive ( $\text{MIC} = 1\text{--}4 \mu\text{g mL}^{-1}$ ) and Gram-negative bacteria ( $\text{MIC} = 4\text{--}8 \mu\text{g mL}^{-1}$ ) (MRSA, *E. coli*, *K. Pneumonia*, *P. aeruginosa*... strains).<sup>251</sup>

Comparison between di-alkylated C5–C7 paromomycins and a mono-alkylated C16 paromomycin indicated that the di-alkylation strategy, leads to both an improvement in antimicrobial activity against sensitive and resistant Gram-positive bacteria, like the one observed by us in the neamine series, and to a dramatic reduction in undesired red blood cell hemolysis caused by many AG-based cationic amphiphiles.<sup>249</sup>

Chang, Takemoto and collaborators pointed out that the attachment of a long alkyl chain (e.g. C14–C18) to ring III of tobramycin and neomycin B yields to strongly active antibacterial derivatives (mainly against Gram-positive bacteria) that for the neomycin derivatives lack antifungal activities.<sup>234,237,254</sup> Two of the good antibacterial neamine derivatives have also been found inactive against clinical isolates of filamentous fungi (*Aspergillus* species) and yeasts (*Candida* species) (unpublished results). Therefore, it is clear that kanamycin-based AAGs can have a selective antifungal action without antibacterial activity with low eukaryotic cytotoxicity and *vice versa* in the neomycin series including neamine derivatives.

More recently, at the end of 2015, two kanamycin B-based AAGs with a C<sub>12</sub> and C<sub>14</sub> aliphatic chain attached at the 6'-position through a thioether linkage were reported to exhibit good anti-Gram-positive and antifungal activity.<sup>253</sup> Therefore, it is also possible to obtain AAGs having both, good antibacterial and antifungal activity.

Different works demonstrate that antibacterial AAGs act through binding in bacterial membranes causing membrane permeabilization. As for other antibacterial cationic amphiphiles, the selectivity of AAGs for bacterial membranes *versus* eukaryotic membranes has to be improved to limit their cytotoxicity. The non-specific interactions of AAGs, for instance with hydrophobic and/or anionic proteins, such as serum albumin, and, their hemolytic properties, that appeared to be strongly dependent on the biochemical environment,<sup>251</sup> can limit their antibacterial interest. However, the non-specific interactions and effects could be minimized in decreasing and optimizing the lipophilicity and its distribution. Examples of antibacterial AAGs with reduced hemolytic activity

and reduced affinity for bovine serum albumin have been described in the kanamycin, neomycin B, paromomycin and tobramycin series.

All antibacterial AAGs should act on Gram-negative bacteria through binding to LPS inducing membrane depolarization and permeation as showed for the dialkyl and trialkyl neamines and the triheptyl nebramine derivative. The pseudo-flexibility of the AG scaffold, the distribution of the positive charges, the structure and the positioning of the lipophilic groups are key parameters in the binding to LPS and in its consequences.

Antibacterial AAGs should have several targets in Gram-negative bacteria as revealed by the observed affinity of the 3',6-dinonyl neamine derivative for the anionic phospholipid CL and its effects on artificial membrane structures. Such a pluri-activity should limit the emergence of resistance and is an advantage for an antibacterial drug in the fight against resistant and persistent bacteria. It should be noted here that the fully protonated form of AAGs is perhaps not the most active form according to the presence of amino groups with pK<sub>a</sub> values near to 7. Most of the AG drugs carry at physiological pH protonated and unprotonated amino groups.<sup>267–269</sup> For instance, neomycin B (Fig. 5) bears six amino groups with pK<sub>a</sub> values ranging from 6.9 to 9.6.<sup>267</sup>

A significant and general enhancement of the antimicrobial activity of the previously identified anti-Gram-positive and -Gram-negative triheptyl nebramine derivative and anti-Gram-positive nebramine, tobramycin and paromomycin AAGs was reported in 2015.<sup>255</sup> AAGs acting against a panel of Gram-positive and Gram-negative bacteria were obtained by di-*N*-methylation of all amine functions of the corresponding AAGs. Their antibacterial activities and their strong hemolytic effects were related to a strong increase in lipophilicity in comparison to the parent non-*N*-dimethylated AAGs (increase of the calculated log *P* by about a 3 order of magnitude). The synthesised di-*N*-methylated AAGs were also found to be considerably more hemolytic than the parent AAGs supporting the hypothesis that di-*N*-methylation increases the erythrocyte and antibacterial membrane disruption efficacy through an increase in the non-specific van der Waals interactions with membrane lipids. Cetrimonium and Gramicidin D which are both in antibacterial topical clinical use, appeared to be significantly more hemolytic than all studied antibacterial AAGs. These results suggest that di-*N*-methylation of AAGs is a general direction for the development of potent and broad-spectrum AAG antibacterial membrane-disrupting agents with high potential for the treatment of persistent topical infections.

The recent report of Schweizer and collaborators showing for the first time that antibacterial AAGs can boost the innate immune response and induce immunomodulatory responses<sup>246</sup> opens also new perspectives in the field especially for the treatment of persistent dormant infections.

In conclusion, the discovery of antimicrobial AAGs revives the interest for the old family of aminoglycosides antibiotics and offers an alternative strategy for discovering novel

antimicrobials through chemical modification of aminoglycosides.

New antibacterial AAGs acting against MRSA and VRSA, against both Gram-positive and Gram-negative resistant bacteria strains to AG drugs or surexpressing efflux pumps and also against MDR bacteria are emerging. The new mode of action of antibacterial AAGs in comparison to AGs should be different from those of useful membrane-acting antibacterial drugs as suggested by the good activity of the 3',6-dialkyl neamine derivatives against *P. aeruginosa* strains resistant to colistin.<sup>65</sup> Antibacterial AAGs can have a spectrum of activity limited to sensitive and resistant Gram-positive bacteria or a broad-spectrum acting on both sensitive and resistant Gram-positive and Gram-negative bacteria. Such a duality, their external mode of action on the bacterial membranes and their potential different targets in these membranes are all advantages in the fight against resistant bacteria. Indeed, due to their interactions with key membrane targets present in a great number of copies, biochemical modifications of the targets should have a high cost for bacteria and result in a limited emergence of resistances and in a high sensitivity to antibiotic drugs of other classes. We have now to demonstrate their low toxicity and their strong antibacterial potential in animal models of infection.

Regarding the new mode of action of AAGs in comparison to AGs, we would like to investigate new aspects and open new perspectives about the effect of amphiphilic drugs on lipid-protein interactions, as consequences of the interactions between antibiotic AAGs and lipids (CL) and alterations of domains enriched in CL. In turn, these effects could alter the localization/activity of proteins, sensitive to the formation of CL clusters and involved in bacteria elongation, division or peptidoglycan synthesis.

## General conclusion

The development of antimicrobial drugs acting on lipid membranes constitutes a promising way of research in the fight against resistant and persistent bacteria. This review article describes the molecular foundations useful in the design of new antimicrobials acting on bacterial lipid membranes. The possible targets of antibiotic drugs and developed drug-candidates are discussed in terms of structure-activity relationships, specificity and emergence of resistance. With this in mind, the understanding at the molecular level of the role of the lipidic composition of bacterial membranes in the cell life appears to be central for an efficient development of new antimicrobial drugs. The outlined continuum in medicinal chemistry leading to antibacterial amphiphilic aminoglycosides acting on bacterial membranes from aminoglycosides acting by binding to ribosomal RNA illustrates the interest of membrane function targeting. Continuous and persevering works within the scientific community have led to a revival and reorientation of old drugs. This resurgence also highlights the relevance of complementary approaches including chemistry, biophysics and microbiology in the

search for new antimicrobial drugs active against resistant and persistent bacteria.

## Abbreviations

AG	Aminoglycoside
AAG	Amphiphilic aminoglycoside
CM	Cytoplasmic membrane
Dab	L- $\alpha$ , $\gamma$ -Diaminobutyric acid
ATP	Adenosine triphosphate
CL	Cardiolipin
IM	Inner membrane of Gram-negative bacteria
LPS	Lipopolysaccharides
LTA	Lipotechoic acids
MDR	Multi drug resistant
MRSA	Methicillin-resistant <i>S. aureus</i>
OM	Outer membrane of Gram-negative bacteria
PAMPA	Parallel artificial membrane permeation assay
PG	Phosphatidylglycerol
PMF	Proton motive force
MDR	Multidrug-resistant
VISA	Vancomycin-intermediate <i>S. aureus</i>
VRSA	Vancomycin-resistant <i>S. aureus</i>

## Acknowledgements

Our works were supported by the Belgian Funds for Scientific Research (Fonds National de la Recherche Scientifique, grants no. 3.4.588.10, 3.4.578.12 and T.1003.14), the French National Research Agency (ANR) "Physique et Chimie du Vivant", the Indo-French Centre for the Promotion of Advanced Research (IFCPAR, project no. 3405-1), the Région Rhône-Alpes (ARC 1, grant no. 12-00887201), the "Programme Labex" (ARCANE project no. ANR-11-LABX-003). We gratefully acknowledge all people who worked on this topic, introduced us to specific fields (e.g. biofilms, characterization of colistin-resistant strains) or who gave us clinical strains.

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