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The *Pseudomonas aeruginosa* membranes: A target for a new amphiphilic aminoglycoside derivative?

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ABSTRACT

Aminoglycosides are among the most potent antimicrobials to eradicate *Pseudomonas aeruginosa*. However, the emergence of resistance has clearly led to a shortage of treatment options, especially for critically ill patients. In the search for new antibiotics, we have synthesized derivatives of the small aminoglycoside, neamine. The amphiphilic aminoglycoside 3′,4′,6-tri-2-naphtylmethylene neamine (3′,4′,6-tri-2NM neamine) has appeared to be active against sensitive and resistant *P. aeruginosa* strains as well as *Staphylococcus aureus* strains (Baussanne et al., 2010). To understand the molecular mechanism involved, we determined the ability of 3′,4′,6-tri-2NM neamine to alter the protein synthesis and to interact with the bacterial membranes of *P. aeruginosa* or models mimicking these membranes. Using atomic force microscopy, we observed a decrease of *P. aeruginosa* cell thickness. In models of bacterial lipid membranes, we showed a lipid membrane permeabilization in agreement with the deep insertion of 3′,4′,6-tri-2NM neamine within lipid bilayer as predicted by modeling. This new amphiphilic aminoglycoside bound to lipopolysaccharides and induced *P. aeruginosa* membrane depolarization. All these effects were compared to those obtained with neamine, the disubstituted neamine derivative (3′,6-di-2NM neamine), conventional aminoglycosides (neomycin B and gentamicin) as well as to compounds acting on lipid bilayers like colistin and chlorhexidine. All together, the data showed that naphthylmethyl neamine derivatives target the membrane of *P. aeruginosa*. This should offer promising prospects in the search for new antibacterials against drug- or biocide-resistant strains.

1. Introduction

Pseudomonas aeruginosa is ubiquitous in nature, colonizing soil, industrial surfaces, humans and plants. This bacterium forms a large proportion of the normal flora found at nasal and pharyngeal mucosal surfaces. In normal conditions, *P. aeruginosa* is harmless. However, if the innate immune system is compromised or if the bacterium gains access to submucosal tissues, *P. aeruginosa* can become a persistent opportunistic pathogen. It represents a major cause of morbidity for patients suffering of severe burns [1], cystic fibrosis [2,3] or receiving intensive care [4]. Taking the severity of the illness into account, current guidelines recommend treatment with an aminoglycoside or a fluoroquinolone together with a beta-lactam. However, resistance to such treatment is rapidly emerging resulting in worrisome resistance rates and the appearance of multidrug-resistant *Pseudomonas* strains [5–8]. With the exception of doripenem, no new anti-pseudomonal drugs have reached the market in recent years [9]. Therefore, it is imperative to discover and develop novel anti-*Pseudomonas* drugs to fill a dangerous void in the antibacterial armamentarium of the clinician.

Since aminoglycosides are the cornerstone in the treatment of *P. aeruginosa* infections, the characterization of their action mechanism at the molecular level is critical to synthesize new derivatives active against *P. aeruginosa*, including resistant strains, on a rational basis. Crystallographic and nuclear magnetic resonance (NMR) studies have shown that aminoglycosides target the double helix of the aminoacyl-tRNA decoding site (RNA-A site) [10]. Neamine (Fig. 1), which is prepared by methanolysis of neomycin B, is the main structural element necessary for binding to the aminoacyl-tRNA decoding site [11–13]. Therefore, it is a good candidate as a starting molecule in the synthesis of new potential antibiotics. In naturally occurring aminoglycosides like neomycin or paromomycin, the neamine-core adopts a virtually identical position within the binding site. Ring I is inserted into the helix, where it covers guanine G1491, and binds to adenine A1408.

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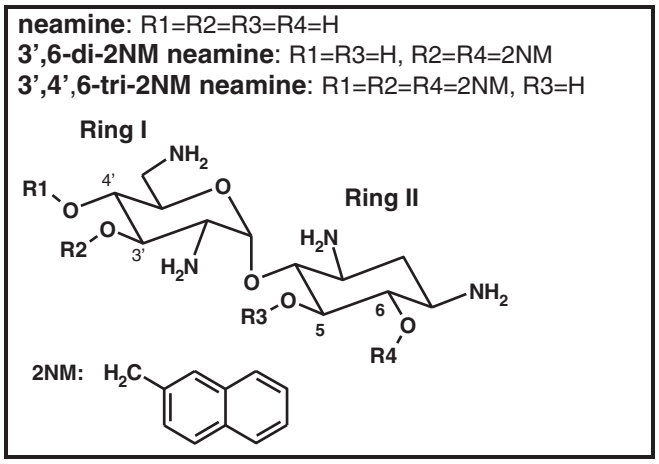

Fig. 1. Structures of neamine, 3′,6-di-2NM neamine, 3′,4′,6-tri-2NM neamine.

The aminogroups of ring II form hydrogen bonds with the universally conserved U1406.U1495 pair as well as with guanine G1494 and a phosphate group of adenine 1493 [14]. Upon binding, the neamine core promotes the stabilization of two adenine bases (A1492 and A1493) of the 16S RNA model in an extrahelical conformation thus increasing their favorable interaction with tRNA. As a result, the ability of the ribosomes to discriminate between cognate and non-cognate tRNA is reduced, which in turn lowers the fidelity of the translation process and leads to the death of the bacterial cell.

In the last few years, several efforts have been done to synthesize new compounds with an enhanced affinity for the RNA-A site [12,15–17]. Modified aminoglycosides have also been designed to induce a dual action towards both protein synthesis and bacterial membranes. The latter are targeted by the addition of lipophilic tails [18], linear lipidic acyl groups [19,20], lipid chains and polyguanidinylated head groups [21], hydrophobic residues in the form of polycarbamate and polyethers [22] to the backbone of aminoglycoside. All the synthesized derivatives have exhibited a strong activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) but they have failed to show activity against Gram-negative bacteria [18,19].

Recently, Baussanne et al. [23] have described the synthesis and the antimicrobial property of neamine derivatives carrying hydrophobic groups like naphthylmethylene. The naphthyl ring has been selected since, as compared with an anthryl- or acridyl ring, it has a higher selectivity for binding to polyA.polyU as an RNA model [24]. Very interestingly, the 3′,4′,6-tri-2-naphthylmethylene (3′,4′,6-tri-2NM) neamine derivative (Fig. 1) has shown antimicrobial activity against *P. aeruginosa* including strains expressing efflux pumps or inactivating enzymes [23]. It is also active against both sensitive and resistant strains of *Escherichia coli, Klebsiella pneumoniae* and *Acinetobacter lwoffii*, including strains expressing r-methylase against which gentamicin, amikacin and tobramycin are totally inactive (MIC>128 μg/ml) [23]. Moreover, the 3′,4′,6-tri-2NM neamine has shown a low MIC (2–4 μg/ml) against sensitive and resistant *S. aureus* strains including MRSA and vancomyin-resistant *S. aureus* (VRSA) strains while clinically used aminoglycosides like amikacin and gentamicin are much less active [23]. This antimicrobial activity is in strict contrast to that of the 3′,6-di-2-naphthylmethylene neamine derivative (3′,6-di-2NM; Fig. 1), which is active against Gram-positive bacteria but fails to eradicate Gram-negative strains. Remarkably, the 3′,4′,6-tri-2-quinolylmethylene neamine derivative (3′,4′,6-tri-2-QN), which differs from the 3′,4′,6-tri-2NM derivative through the replacement of one carbon atom by a nitrogen atom in each naphthyl ring, has appeared to be completely inefficient against Gram(+) and Gram(−) bacteria [23].

The strong activity of 3′,4′,6-tri-2NM neamine against *P. aeruginosa* pushed us to further investigate the mechanism involved. We therefore evaluated its ability to alter protein synthesis as well as to interact with the bacterial membranes of *P. aeruginosa* or models mimicking these membranes. All these effects were compared to those obtained with neamine, the di-substituted neamine derivative (3′,6-tri-2NM neamine), conventional aminoglycosides (neomycin B and gentamicin) as well as to compounds acting on lipid bilayers. Two compounds were selected in this respect, colistin (also called polymyxin E and used as an anti-pseudomonal compound) [25–30] and chlorhexidine (a broad-spectrum biocidal compound) [31,32].

To combine both fundamental studies with potential clinical interest of the new amphiphilic aminoglycosides, the work will be performed using both models of membranes as well as on entire bacteria.

First, on entire bacteria, we took advantage of atomic force microscopy (AFM) to investigate *in situ* the effect of 3′,4′,6-tri-2NM neamine on surface nanostructure of bacteria. In parallel, we characterized the ability of both 3′,6-di-2NM neamine and 3′,4′,6-tri-2NM neamine to depolarize the cytoplasmic membrane of *P. aeruginosa* using $DiSC_3(5)$. We also examined their relative binding toward lipopolysaccharides (LPS) using a fluorescence based displacement assay using BODIPY-TR cadaverine. To obtain further information on the interactions of both derivatives with lipid membranes, we determined their permeabilizing effect in models of bacterial lipid membranes by following the release of calcein entrapped within liposomes. Yet, we carried out molecular modeling of both compounds in interaction with the major lipids found in cytoplasmic membranes of *P. aeruginosa*. All our data suggest that the activity of 3′,4′,6-tri-2NM neamine against *P. aeruginosa* is related to its ability to target lipid membranes.

2. Materials and methods

2.1. Materials

P. aeruginosa [ATCC 27853 and Psa.F03 (PAZ505H8101)] and *E. coli* (PAZ505H8101) were obtained from the Pasteur Institute (Brussels, Belgium; Prof. R. Vanhoof). *P. aeruginosa* (PT629, PA22) was provided by the Department of Bacteriology, University of Franche-Comte, Faculty of Medicine (Besancon, France; Prof. P. Plésiat) and *E. coli* (06AB003), by the Laboratory of Bacteriology, Cliniques Universitaires UCL de Mont-Godinne (Yvoir, Belgium; Prof. Y. Glupczynski). *S. aureus* (ATCC 25923) and *E. coli* (25922) were provided by ATCC-LGC (Middlesex, TW11 OLY, UK). MRSA and VRSA strains were obtained from NARSA (Network on Antimicrobal Resistance in *S. aureus*) via Eurofins Medinet Inc., Herndon, VA).

LPS *E. coli* (0111:B4) was supplied from Quadratech (Epsom, Surrey, UK). Valinomycin was purchased from Calbiochem. Beef heart cardiolipin (CL; Disodium Salt; purity>99%), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (POPG) sodium salt and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. 3,3′ Dipropyl thiadicarbocyanine iodide ($DiSC_3(5)$) and 5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino)pentylamine, hydrochloride (BODIPY-TR-cadaverine) were obtained from Molecular probes, Inc. (Eugene, OR). Neomycin B sulfate and calcein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calcein was purified as described previously [33]. Briefly, calcein was dissolved in 6 N NaOH and subjected to size-exclusion chromatography through a Sephadex^R LH-20 column. The final concentration of calcein solution in 20 mM Tris–HCl was 73 mM with an osmolarity of 434 mOsm/kg (measured by the freezing point technique, using a model 3C2 Advanced Cryomatic Osmometer [Advanced Instruments, Needham Heights, MA]). Other reagents were purchased from E. Merck AG

(Darmstadt, Germany), Sigma-Aldrich-Fluka (Lyon, France) or Acros Organics (Illkirch, France).

2.2. 3′,6-di-2-naphthylmethylene neamine and 3′,4′,6-tri-2-naphthylmethylene neamine synthesis

The 3′,6-di-2NM neamine and the 3′,4′,6-tri-2NM neamine were synthesized in three steps from neamine according to our previous reports (21% and 19% yields, respectively): (i) tritylation of the four amine functions, (ii) alkylation with 2-bromomethylnaphthalene in DMF/THF (1:9) after addition of NaH and (iii) deprotection in TFA/anisole [23,34].

2.3. Minimal inhibitory concentration determination

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic that prevented visible growth after incubation at 37 °C for 18–24 h. All strains were grown on TSA-agar plates (BD Diagnostics, BD, Franklin Lakes, NJ) at 37 °C, overnight. The MICs were determined by a geometric microdilution method according to the recommendations of the CLSI norms (2007) for *P. aeruginosa* [ATCC 27853 (WT), Psa.F03, PA22], *E. coli* [ATCC 25922 (WT), EcPAZ505H8101, Ec06AB003 (Arm)], and *S. aureus* strains [ATCC 25923 (WT), ATCC 33592 HA-MRSA and VRSA VRS-2].

2.4. Luciferase inhibition translation

Inhibition of cell-free translation by the different compounds was quantified by using *E. coli* S30 Extracts System for circular DNA with the pBest*luc*™ plasmid (Promega, Leiden, NL) as previously described [35] with modifications. Briefly, 10 µl of S30 premix, 7.5 µl of S30 extract, 2.5 µl of complete amino acid mixture, 0.25 µg of pBest*luc*™ plasmid and 2 µl of tested compounds at different concentrations were mixed in a total volume of 25 µl. Translation reactions were performed at 37 °C for 60 min, cooled at 4 °C for 5 min and diluted 10-fold with phosphate-buffered saline (PBS) 1× containing 1% BSA. The luminescence was measured immediately after adding 5 µl of the diluted mixture to 25 µl of the Luciferase assay reagent (Promega, Leiden, NL) with a Luminometer (Turner Designs TD 20/20 Luminometer). Data were normalized to untreated reaction mixture assigned to 100%.

2.5. Atomic force microscopy

AFM images were recorded in PBS solution (10 mM Na_3PO_4, 150 mM NaCl, pH 7.4) at room temperature, using a Nanoscope V multimode AFM (Veeco Metrology Group, Santa Barabara, CA, USA). *P. aeruginosa* (ATCC 27853) was filtered onto a porous polycarbonate membrane (Millipore) with a pore size similar to the bacterial cell size (0.6 µm). After filtering a concentrated cell suspension, the filter was gently rinsed with PBS, carefully cut (1 × 1 cm), and attached to a steel sample puck (Veeco Metrology Group) using a small piece of adhesive tape and the mounted sample was transferred into the AFM liquid cell while avoiding dewetting. The 3′,4′,6-tri-2NM neamine was injected into the AFM liquid cell at 0.5 MIC (4 µg/ml).

2.6. Cytoplasmic membrane depolarization assay

The cytoplasmic membrane depolarization activity of neamine derivatives was determined using the membrane potential-sensitive dye $DiSC_3(5)$ [36]. This probe is taken up by bacterial cells according to the magnitude of the electrical gradient of the cytoplasmic membrane and becomes concentrated in the cytoplasmic membrane, where it self-quenches its own fluorescence [36]. Any compound that alters the permeability of the cytoplasmic membrane and thus induces depolarization will lead to the release of $DiSC_3(5)$ and a consequent increase in fluorescence. This assay, which is widely applied to monitor the membrane potential and ion permeability in a variety of cells including red blood cells [36,37], Gram-positive [38] and -negative bacteria [39,40], does not measure membrane destruction or an equivalent lethal event [40].

Briefly, overnight cultures of *P. aeruginosa* (ATCC 27853) were diluted in CA-MHB media and allowed to grow to the exponential phase (OD_{620} = 0.5–0.7). Bacteria were collected by centrifugation, washed 3 times with buffer (5 mM HEPES, pH 7.8) and resuspended in the same buffer added with 0.2 mM EDTA to an OD_{620} of 0.05. The cell suspension was incubated during 20 min at 37 °C under shaking (150 rpm) with 0.4 µM $DiSC_3(5)$ and 0.1 M KCl until dye uptake was maximal (after 20 min a stable reduction in fluorescence due to fluorescence quenching was observed). The desired concentration of the test compound was then added; the fluorescence was monitored under shaking (150 rpm) at 37 °C at an excitation wavelength of 622 nm and an emission wavelength of 670 nm after 15 min (Perkin-Elmer Model LS55, Perkin Elmer Ltd., Beaconsfield, UK). A blank with only cells and the dye was used as background. We previously checked the absence of effect on $DiSC_3(5)$ fluorescence and MICs, of EDTA (0.2 mM) and KCl (0.1 M) used to permeabilize the outer membrane to allow dye uptake and equilibrate the cytoplasmic and external K^+ concentrations, respectively.

2.7. Binding affinities to LPS

The BODIPY-TR-cadaverine displacement assay was previously used to quantify the affinities of binding of the test compounds to LPS [41]. The fluorescence of BODIPY-TR-cadaverine is quenched upon binding to LPS, and the displacement of BODIPY-TR-cadaverine by the test compounds results in dequenching of BODIPY-TR-cadaverine fluorescence. Polymyxin B, a peptide antibiotic known to bind and neutralize LPS [42], served as the positive control. Imipenem and meropenem, acting by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls are used as negative controls. Stock solutions of LPS (5 mg/ml) and BODIPY-TR-cadaverine (500 µM) were prepared in Tris buffer (pH 7.4, 50 mM). 1 ml each of the LPS and BODIPY-TR-cadaverine stocks was mixed and diluted in Tris buffer to a final volume of 100 ml, yielding final concentrations of 50 µg/ml of LPS and 5 µM BODIPY-TR-cadaverine. 40 µl of this BODIPY-TR-cadaverine:LPS mixture was added to each well of the plate. Fluorescence measurements were made at 25 °C on a SPECTRAmax GEMINI XS Microplate Spectrofluorometer (Biocompare), using Spectra Max Gemini XS software. The BODIPY-TR-cadaverine excitation wavelength was 580 nm. Emission spectra were taken at 620 nm. Emission and excitation monochromator bandpasses were setting at 5 nm.

2.8. Large unilamellar vesicles preparation

Large unilamellar vesicles (LUV), composed of lipids mimicking the composition of lipid membranes of *P. aeruginosa* (Phosphatidylethanolamine [PE], Phosphatidylglycerol [PG] and Cardiolipin [CL]) [43,44], were prepared by extrusion.

Lipids (POPE, POPG, CL; molar ratio 60:21:11) were dissolved in $CHCl_3$:CH_3OH (2:1, v/v) and mixed to obtain the desired composition in a round flask. The solvent was evaporated using a Rotavapor system (model R-210, Buchi Labortechnik AG, Flawil, Switzerland). Dried films were maintained under reduced pressure overnight and thereafter hydrated with purified calcein in 20 mM Tris 200 mM NaCl, pH 7.4. LUV were obtained after 5 cycles of freeze/thawing and 10 cycles of extrusion in a 10 ml Thermobarrel Extruder (Lipex Biomembranes, Vancouver, Canada) under a nitrogen pressure of 10 bars through two polycarbonate filters of 100 nm (Nucleopore, Costar

Corporation, Badhoevedorp, The Netherlands). Non-entrapped calcein was removed using minicolumn centrifugation. The size and polydispersity of liposome suspensions were monitored by quasi-elastic light scattering with a Zetasizer Nano SZ (Malvern Instruments, Worcestershire, UK). Lipid concentration on the liposomal suspensions was measured by phosphorous quantification as previously described [45].

2.9. Liposomal membrane permeability assay

Permeabilization of lipid membranes induced by drugs was monitored by following the leakage of entrapped calcein within liposomes. We monitored the increase in fluorescence of the samples, resulting from the dilution and release of self-quenching of this tracer [46] as previously described [33].

2.10. Log P and log D determinations

MarvinSketch software [Marvin 5.1.3, 2010, ChemAxon (http://www.chemaxon.com)] was used for drawing, displaying, and characterizing chemical structures and substructures. The log P plug-in in this software was used to calculate the octanol/water partition coefficient, which is used in QSAR analysis and rational drug design as a measure of molecular hydrophobicity.

2.11. Molecular modeling and assembly of neamine derivatives with lipids

The assembly of neamine derivatives with lipids was studied by molecular modeling. The neamine derivative structures were first constructed using Hyperchem 7.0 (Hypercube, Inc). A preliminary geometry optimization was made by the steepest descent method using the MM + force field. The structures were then submitted to a systematic analysis structure tree applied on the most important torsional angles of each molecule [47]. The most probable structure was then kept for the next steps.

The interaction and insertion of the neamine derivatives within lipids were calculated using two methods, the hypermatrix and the impala method as described previously [48–50].

The Pex method [51] was used to analyze the molecular assemblies and calculations are performed on a Linux station bi-xeon quad core, using Z-Ultim software. Graphs were drawn using WinMGM (Ab Initio technology, Obernai, France).

2.12. Statistical analysis

Statistical analysis was done with GraphPad Prism, version 4.03, for Windows (GraphPad Prism Software, San Diego, CA) using an ANOVA with a Tukey post hoc test.

3. Results

3.1. Antimicrobial activity against sensitive and resistant Gram(−) and Gram(+) bacteria

The antimicrobial activity against sensitive and resistant Gram-negative and Gram-positive bacteria of the tri-substituted (3′,4′,6-tri-2NM neamine), and the di-substituted (3′,6-di-2NM neamine) derivatives, as well as parent compounds (neamine, neomycin B and gentamicin) is shown in Table 1. We also determined the MICs of colistin and chlorhexidine digluconate against *P. aeruginosa* (ATCC 27853). They were 1 μg/ml and 8 μg/ml, respectively.

The tri-substituted derivative has revealed an interesting activity against *P. aeruginosa* ATCC 27853 (MIC 8 μg/ml) as well as against strains expressing modifying enzyme (AAC-6′-IIA) or efflux pump (MexXY) (MICs of 8 and 4 μg/ml, respectively). The di-substituted derivative, as well as neomycin B and neamine, has appeared to be inactive whatever the selected strains. Gentamicin is active against *P. aeruginosa* ATCC 27853 and strain expressing efflux pump (MexXY) with MICs of 1 and 4 μg/ml, respectively but lost all activity against strain expressing modifying enzyme (AAC6′-IIA) (MIC>128 μg/ml).

Moving on enterobacteriacea, the tri-substituted derivative has shown MIC against *E. coli* ATCC 25922 of 16 μg/ml lower than MIC obtained for neamine (32 μg/ml) but higher than MICs found with neomycin B (2 μg/ml) and gentamicin (<0.5–1 μg/ml). MICs of 4 μg/ml have been measured against strains expressing modifying enzyme (AAC6′-IB) or r-methylase, near those obtained with neomycin B (MICs of 4 and 1 μg/ml, respectively). Gentamicin also was found active against *E. coli* expressing AAC-6′-IB (MIC 1 μg/ml) but unfortunately inactive against strain expressing methylase activity (MIC>64 μg/ml). The di-substituted derivative and neamine have appeared to be inactive whatever the strains selected.

Against Gram-positive bacteria, in the presence of the tri-substituted derivative (3′,4′,6-tri-2NM neamine), a MIC of 4 μg/ml has been measured against *S. aureus* ATCC 25923 slightly higher than MICs found for neomycin B (2 μg/ml) or for gentamicin (0.5 μg/ml). Again, the tri-substituted derivative has a strong activity against resistant strains with MICs values ranging between 2 and 4 μg/ml for MRSA and VRSA for which neomycin B lost all activity (MICs>128 μg/ml). Gentamicin is also inactive against VRSA with a MIC of 32 μg/ml but still had an activity against MRSA (MIC 1–2 μg/ml). Neamine was inactive whatever the selected strains and the di-substituted derivative is only moderately active against Gram-positive bacteria (MICs = 8–16 μg/ml).

These results clearly have pointed out the antimicrobial activity of the 3′,4′,6-tri-2NM neamine against both Gram-positive and Gram-negative strains, including sensitive and resistant bacteria.

3.2. Bacterial protein synthesis

Inhibition of protein synthesis is the well-known mechanism of action of aminoglycoside antibiotics. Thus, we first investigated the

Table 1
MIC values (μg/ml) of the compounds used in this study on *P. aeruginosa*-, *E. coli*- and *S. aureus*-sensitive and -resistant strains.

	P. aeruginosa			*E. coli*			*S. aureus*		
	ATCC 27853	Psa.F03 AAC6′-IIA	PA22 Overexpres MexXY	ATCC 25922	PAZ505H8101 AAC6′-IB	06AB003	ATCC 25923	ATCC 33592 HA-MRSA	VRSA-VRS-2
Resistance mechanism	None	Enzymatic	Efflux	None	Enzymatic	16S RNA methylase (arm)	None	Low affinity of target for methicillin	Low affinity for glycopeptides
Neamine	>128	>128	>128	32	>128	32	32	>128	>128
3′,6-di-2NM neamine	128	128	>128	64	64	>128	8	16	16
3′,4′,6-tri-2NM neamine	8	8	4	16	4	4	4	2	4
Neomycin B	64	>128	>128	2	4	1	2	>128	>128
Gentamicin	1	>128	4	<0.5-1	1	>64	0.5	1-2	32

ability of 3′,4′,6-tri-2NM neamine to inhibit the protein synthesis using an *in vitro* transcription/translation assay [35]. The effects on bacterial protein synthesis of all selected compounds were determined at an equimolar concentration of 10 μM (molar concentration equivalent to the MIC of the 3′,4′,6-tri-2NM neamine on *P. aeruginosa* ATCC 27853) and at the MIC. As expected, we confirmed the inhibitory effect of neamine, neomycin B, gentamicin as well as chloramphenicol and tetracycline on bacterial protein synthesis. In contrast, 3′,4′,6-tri-2NM neamine did not inhibit protein synthesis and showed similar profiles as the ones obtained with colistin, chlorhexidine, and aztreonam. Colistin and chlorhexidin are known to act on the membrane [52,53] whereas aztreonam interacts with the bacterial cell wall synthesis and blocks peptidoglycan cross-linking [54]. The absence of an inhibitory effect on the protein synthesis was independent on the number (two or three) of naphthylmethylene groups substituting neamine as well as the concentrations selected (equimolar concentrations (10 μM; Fig. 2A) or MICs against *P. aeruginosa* ATCC 27853 (Fig. 2B)). Even at 10-fold the MIC, the 3′,4′,6-tri-2NM neamine was unable to significantly inhibit the protein synthesis (Fig. 2C).

These results clearly showed that the addition of naphthylmethylene groups to the neamine backbone changes the primary mode of action of aminoglycosides since both the di- and tri-substituted derivatives are unable to inhibit the protein synthesis whatever the conditions used (10 μM or at their MICs).

3.3. Nanoscale imaging of *P. aeruginosa* cells

Since the 3′,4′,6-tri-2NM neamine did not affect protein synthesis, we further investigated the effect of this compound on the surface of *P. aeruginosa* ATCC 27853 using *in situ* AFM imaging [55,56]. Cells immobilized onto polycarbonate membranes were imaged in aqueous solution without any pre-treatment such as air drying or chemical fixation. Fig. 3 shows deflection images for the same bacterium following incubation with 3′,4′,6-tri-2NM neamine at 0.5-fold MIC. Series of deflection images were recorded up to 35 min. As shown in the vertical cross-sections, the bacterium progressively flattened over the course of the experiment. The cell thickness decreased by 50% after 33 min. We interpret such swelling down as evidence for the alteration of the cell wall leading to the discharge of most of the intracellular content. Finally, after 35 min, the remaining bacterium detached from the surface.

3.4. *P. aeruginosa* membrane depolarization

To gain insights on the effect of 3′,4′,6-tri-2NM neamine on the *P. aeruginosa* ATCC 27853 membrane, we investigated the ability of this compound to depolarize the bacterial membrane using $DiSC_3(5)$, a membrane potential-dependent probe [36]. Upon permeabilization and disruption of the membrane, the membrane potential will be dissipated, and $DiSC_3(5)$ will be released into the medium causing a consequent increase in fluorescence [40]. Valinomycin, a K^+ ionophore leading to depolarization of the cytoplasmic membrane was used as positive control. At 10 μM, it increased the fluorescence intensity of $DiSC_3(5)$ more than 10-fold.

When compounds were tested at equimolar concentration (10 μM) (Fig. 4A), the 3′,4′,6-tri-2NM neamine induced membrane depolarization but less than valinomycin (8.5-fold increase in fluorescence versus 12-fold, respectively). In contrast, the 3′,6-di 2NM neamine, neamine and gentamicin showed no effect. Neomycin B induced a small effect, with an increase of relative fluorescence around 3.3-fold.

When the membrane depolarization was measured after addition of compounds at their MICs (Fig. 4B), we found a clear dissipating membrane potential effect induced by the di- and tri-substituted neamine derivatives with a 11- and 8.5-fold increase in relative fluorescence, respectively. Neomycin B and neamine also induced, but in a lesser extent, an increase in relative fluorescence (5.7- and 3.9-fold, respectively). In contrast, gentamicin did not induce membrane depolarization.

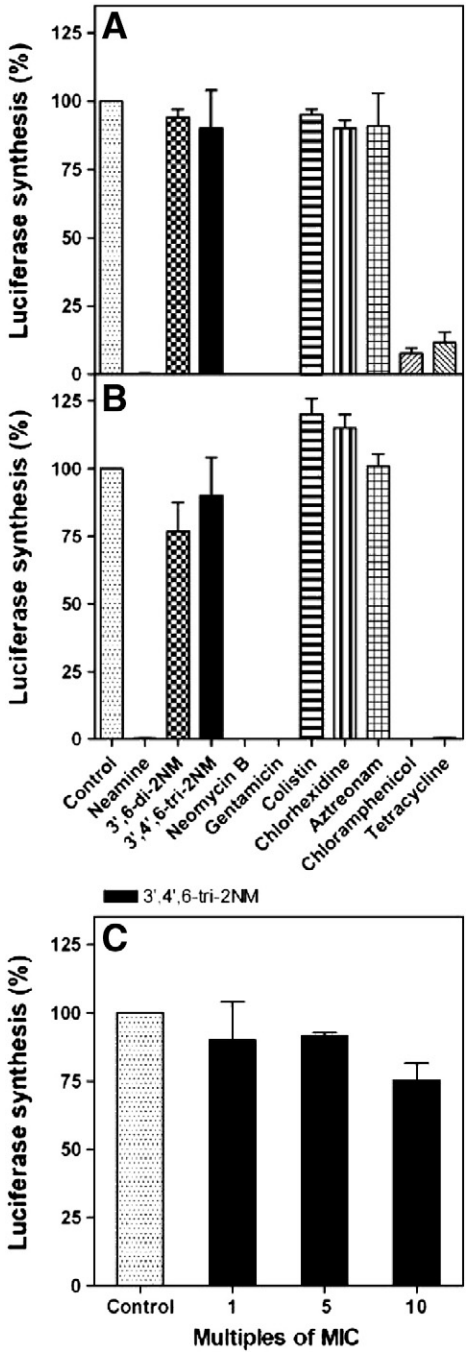

Fig. 2. Effect of neamine derivatives (3′,4′,6-tri-2NM neamine, 3′,6-di-2NM neamine), neamine, neomycin B, gentamicin, colistin and chlorhexidine on bacterial protein synthesis. Chloramphenicol and tetracycline are selected for their known inhibitory potency towards protein synthesis. Aztreonam is chosen for the absence of effect on protein synthesis. Protein translation inhibition by the different compounds is quantified in a coupled transcription/translation assay by using *E. coli* S30 extracts for circular DNA with the pBEST*luc* plasmid. The luminescence is measured immediately after the addition of luciferase assay reagent. The compounds are tested at equimolar concentrations (10 μM; panel A) as well as at their MICs against *P. aeruginosa* 27853 (panel B) (neamine [128 μg/ml; 397.1 μM], 3′,6-di-2NM neamine [128 μg/ml; 212.4 μM], 3′,4′,6-tri-2NM neamine [8 μg/ml; 10.8 μM], neomycin B [64 μg/ml; 104.1 μM], gentamicin [1 μg/ml; 2.1 μM], colistin [1 μg/ml; 0.9 μM], chlorhexidine [8 μg/ml; 8.9 μM], aztreonam [4 μg/ml; 9.2 μM], chloramphenicol [64 μg/ml; 198.1 μM], tetracycline [16 μg/ml; 36.0 μM]). Panel C shows the effect of 3′,4′,6-tri-2NM neamine at 1, 5 and 10 times MIC. Each value is the mean of two independent experimental determinations ± SEM.

To gain further data about the mechanism involved, we compared, at 10 μM and at their MICs, the effect of aminoglycosides to other antibacterial (colistin) or biocidal drugs (chlorhexidine), known to

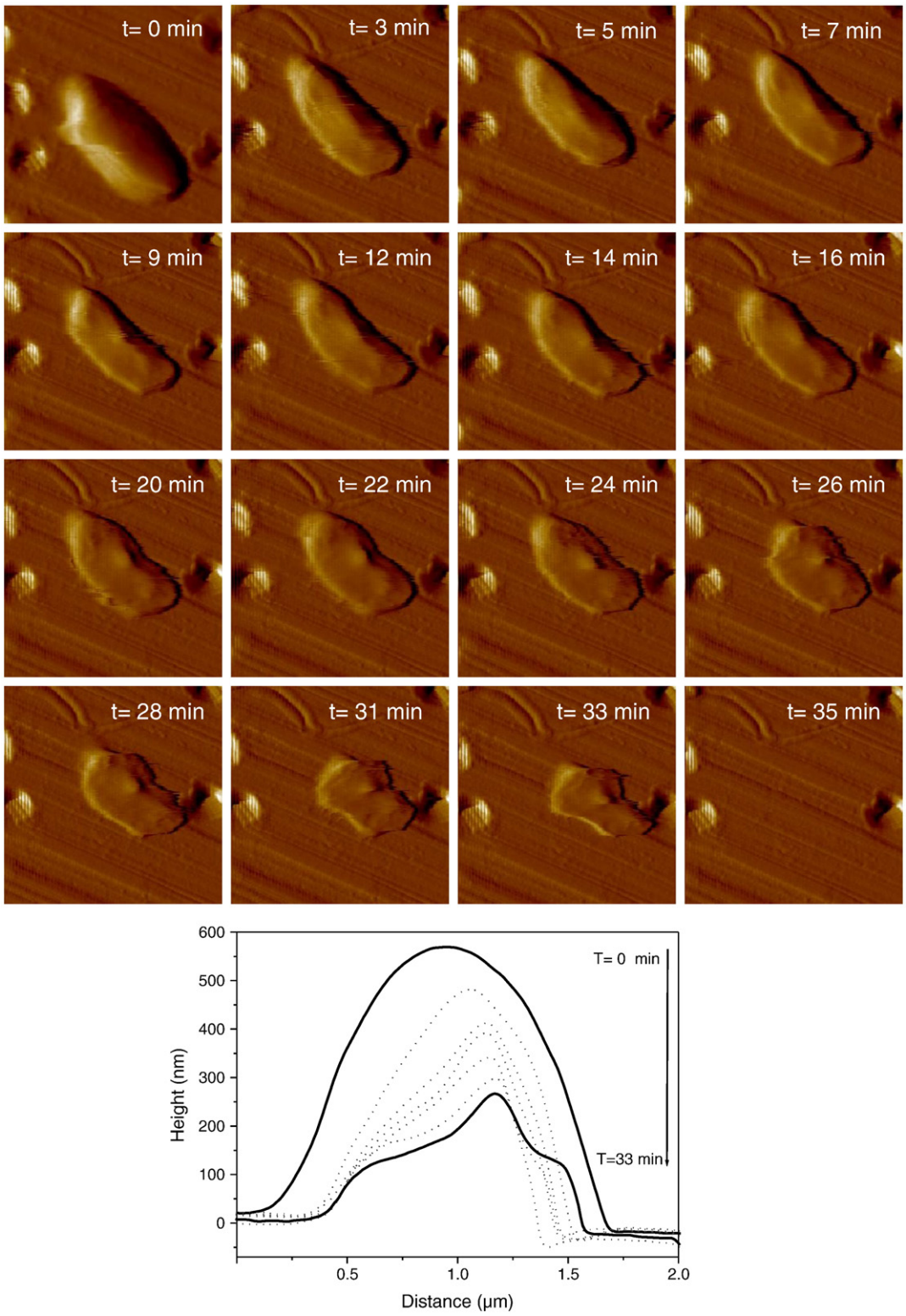

Fig. 3. AFM imaging of single *P. aeruginosa* ATCC 27853 cell following incubation with 3′,4′,6-tri-2NM neamine at 0.5-fold MIC against *P. aeruginosa* 27853. A series of deflection images (3 μm × 3 μm) recorded in real time for a single cell prior and after treatment is shown. The lower panel compares vertical cross-sections.

interact with lipid bilayers. At the MICs (Fig. 4B), colistin and chlorhexidine showed a similar effect to the one observed with 3′,4′,6-tri-2NM neamine whereas at equimolar concentration (10 μM) (Fig 4A), chlorhexidine was more effective than 3′,4′,6-tri-2NM neamine.

Additionally, we investigated the dose-dependent effect in multiples of MICs (Fig. 4C). 3′,4′,6-tri-2NM neamine induced an increase of fluorescence for concentrations ranging from 0.1 to the MIC. A plateau value was observed at 0.25-fold the MIC. The di-substituted derivative induced a more progressive increase of the signal between 0.1- to 1-fold MIC.

All together, 3′,4′,6-tri-2NM neamine clearly induced *P. aeruginosa* membrane depolarization. At equimolar concentration, 3′,6-di-2NM neamine did not modify the membrane polarization.

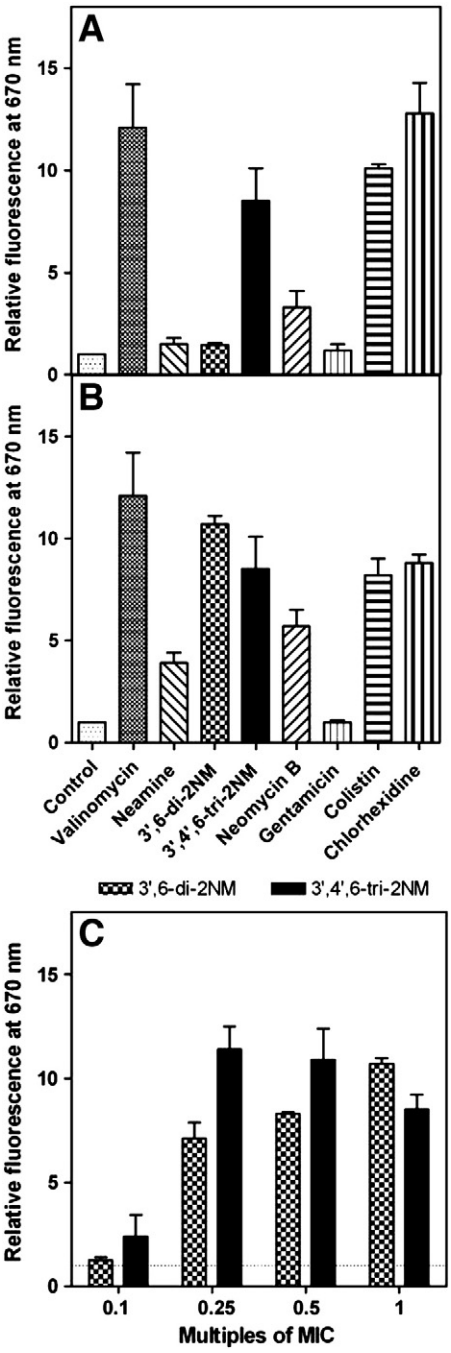

Fig. 4. Effect of Neamine derivatives (3′,4′,6-tri-2NM neamine, 3′,6-di-2NM neamine), neamine, neomycin B, gentamicin, colistin and chlorhexidine on the fluorescence intensity changes of *P. aeruginosa* ATCC 27853 incubated with $DiSC_3(5)$. The experiments are performed at 10 μM (panel A) and at their MICs (see individual values in legend of Fig. 2) against *P. aeruginosa* 27853 (panel B). Panel C shows the effect of 3′,4′,6-tri-2NM neamine and 3′,6-di-2NM neamine at 0.1-, 0.25-, 0.5-, and 1-fold MIC. Results are expressed in relative intensity fluorescence observed at 670 nm as compared to negative control. Valinomycin (10 μM) was used as positive control. Values are mean ± SD of three determinations.

3.5. Binding to LPS

Since LPS is the main outer membrane constituent of Gram-negative bacteria, we examined the ability of 3′,4′,6-tri-2NM neamine to bind to LPS, using a fluorescence based displacement assay with BODIPY-TR-cadaverine [41] (Fig. 5). The effects of neamine, 3′,6-di-2NM neamine, colistin, chlorhexidine, imipenem and meropenem were monitored in parallel. Colistin (polymyxin E) showed

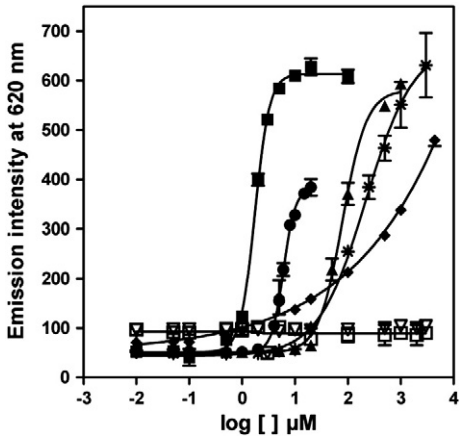

Fig. 5. Binding affinity of compounds to LPS determined by the BODIPY-Cadaverine displacement method. Colistin (■) is used as the reference compound in comparison to the effect obtained for 3′,4′,6-tri-2NM neamine (●), 3′,6-di-2NM neamine (▲), neamine (♦), chlorhexidine (✻), imipenem (□) and meropenem (▽). Experiments are reproduced two times with identical results.

an effect similar to the one observed with polymyxin B, a decapeptide antibiotic, which is known to bind to LPS [40,57,58] and is generally used as a reference compound. Penems, which bind to penicillin binding proteins and thus prevent bacterial cell wall synthesis, did not induce any effect. Naphtylmethyl neamine derivatives, chlorhexidine and neamine showed intermediate effects with the highest potency for the 3′,4′,6-tri-2NM neamine to displace BODIPY-TR-cadaverine from its binding to LPS, followed by the 3′,6-di-2NM neamine, chlorhexidine and neamine. The apparent Kd values obtained for chlorhexidine and colistin are similar to those reported by others [59].

3.6. Lipid membrane permeabilization

Ensuing the marked effect of 3′,4′,6-tri-2NM neamine on membrane properties of *P. aeruginosa,* especially on the membrane thickness and the membrane polarization, we investigated the ability of this compound to permeabilize artificial lipid membranes. This was examined on liposomes mimicking the lipid composition of *P. aeruginosa* membranes (POPE:POPG:CL; 60:21:11) [43,44]. Calcein was entrapped within these vesicles at a self-quenching concentration. Increase of its fluorescence reflects its dequenching due to membrane permeabilization and release of the dye.

At 10 μM, both 3′,4′,6-tri-2NM neamine and 3′,6-di-2NM neamine induced a marked increase in the fluorescence signal which reached a value similar to that obtained with Triton X-100 (Fig. 6A). This permeabilizing effect was rapid since all calcein was released within the first 5 min (data not shown). Only around 20% of the calcein was released by the parent compound, neamine, as well as by the clinically used aminoglycosides, neomycin B and gentamicin.

When measured at the MIC against *P. aeruginosa* ATCC 27853, the release of calcein induced by tri-substituted and di-substituted derivatives was comparable to that of Triton X-100 and slightly higher than that observed with neomycin B. Gentamicin did not induce a release of calcein and neamine allowed a release of approximately 20% of calcein (Fig. 6B).

When we compared the effect of aminoglycosides to those obtained with colistin and chlorhexidine known to induce permeabilization, we observed a release of calcein which reached 100% with chlorhexidine and around 70% with colistin, depending on the experimental conditions (Fig. 6A and B).

For both tri-substituted and di-substituted derivatives, the effect was clearly dose-dependent (between 0.01- and 1-fold the MIC) (Fig. 6C). Systematically, the 3′,4′,6-tri-2NM neamine induced a

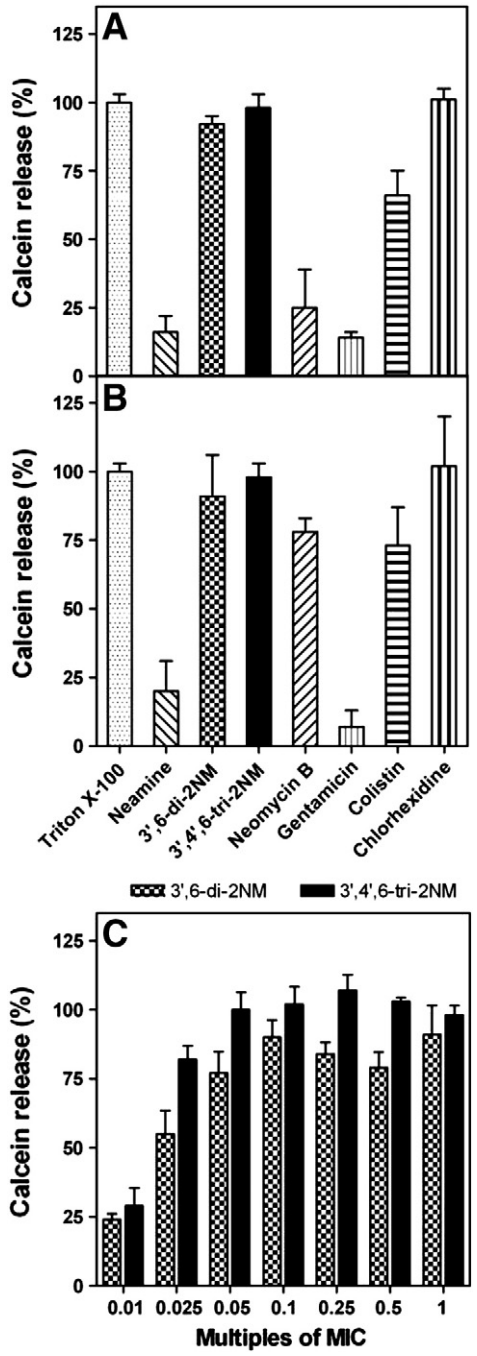

Fig. 6. Effect of neamine derivatives (3′,4′,6-tri-2NM neamine, 3′,6-di-2NM neamine), neamine, neomycin B, gentamicin, colistin and chlorhexidine on the release of calcein entrapped within liposomes. Liposomes (5 μM phospholipids) made of POPE:POPG:CL (60:21:11 molar ratio) are exposed with compound at 10 μM (panel A) or at their MICs (see individual values in legend of Fig. 2) (panel B) for 1 h at 37 °C. Panel C shows the effect of 3′,4′,6-tri-2NM neamine at 0.01-, 0.025-, 0.05-, 0.1-, 0.25-, 0.5- and 1-fold MIC against *P. aeruginosa* 27853. The ordinate shows the maximal percentage of calcein released compared to what was observed after addition of 2% Triton X-100. Each value is the mean of two independent experimental determinations ± SD.

slightly higher increase of calcein release as compared with the 3′,6-di-2NM neamine. For both compounds, the maximal effect was obtained at 0.05-fold the MIC.

These studies therefore suggested that on artificial membranes, both the di- and the tri-substituted derivatives induced a lipid membrane permeabilization at equimolar concentrations as well as at their MICs and even at very low concentrations.

3.7. Interactions with polar heads and interfacial domains of phospholipids

To further characterize the 3′,4′,6-tri-2NM neamine and 3′,6-di-2NM neamine binding to lipids, we calculated the lipid insertion propensity of both compounds by molecular modeling using neamine and neomycin B as reference compounds. The Impala plot (Fig. 7) showed that neamine (Fig. 7A) and neomycin B (Fig. 7B) were located near or on the polar head group/water interface, while 3′,6-di-2NM neamine (Fig. 7C) and 3′,4′,6-tri-2NM neamine (Fig. 7D) inserted more deeply into the modeled membrane. The deepest carbon atom of the di-substituted neamine derivative was at the level of the acyl-chain C4–C5 atom while this was located at the C9 level for the tri-substituted derivative. This correlated with the calculation of the ratio of the hydrophobic (pho)/hydrophilic (phi) surface and of the transfer energy (Table 2), showing that the two amphiplilic derivatives are more hydrophobic than the controls.

The interaction with lipids mimicking those found in membranes of *P. aeruginosa* [phosphatidylethanolamine (POPE), phosphatidylglycerol (POPG) or cardiolipin (CL)] was further investigated for the two neamine derivatives and compared to the interaction of neomycin B. Neamine was not further considered since its interaction with the model membrane was less significant (Fig. 7) in accordance with the inability of this compound to alter bacterial membranes and/or to reach intracellular bacterial targets. The interaction between the drugs and POPE is shown in the Supplementary Material. Again, the interaction of the neamine derivatives occurred at the interface between the polar head groups and the acyl chains of the lipid (panels B and C) with a deeper insertion of 3′,4′,6-tri-2NM neamine (panel C), while neomycin B interacted with the head groups (panel A). Hence, the interaction energy of the whole complex further suggested that the interaction was stabilized by hydrophobic and Van der Waals energies for the neamine derivatives. Taking into account the three positive charges of aminoglycosides, we also determined their interaction with negatively charged lipids like cardiolipin. The contribution of electrostatic energies was the most important for neomycin B, in agreement with its relative membrane location at the phospholipid head group/water interface. This contribution decreased for the di-substituted derivative and even more for the tri-substituted neamine which is in accordance with their deeper insertions in the bilayer. The same was observed when the interaction of the di-substituted derivative with POPG – another negatively charged lipid – as compared to that of the tri-substituted one. In contrast, no major differences in the contribution of electrostatic energies could be found when the di- and tri-derivatives were interacting with POPE, a zwitterionic phospholipid (Table 3).

4. Discussion

Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life-threatening infections. However, despite rigorous patient monitoring, nephro- and oto-toxicities appear as relatively frequent side effects of therapeutic courses [60–62]. Aminoglycosides act by binding to 16S rRNA, causing mRNA decoding errors, mRNA and tRNA translocation blockage, ribosome recycling inhibition and *in fine* protein synthesis alteration [14]. The emergence of resistant strains has somewhat reduced the potential of these antibacterials leading to treatment failure. In the search for new antimicrobial agents and/or new targets, we introduced hydrophobic groups, like arylmethylene groups, on the neamine backbone [23]. Of the sixteen mono-, di-, tri- and tetra-aryl methylene neamine derivatives synthesized, the 3′,4′,6-tri-2-naphthylmethylene neamine (3′,4′,6-tri-2NM neamine) showed a very interesting activity against both Gram-positive and Gram-negative strains. Even if the 3′,4′,6-tri-2NM neamine does not reach the high activity of conventional aminglycosides like gentamicin

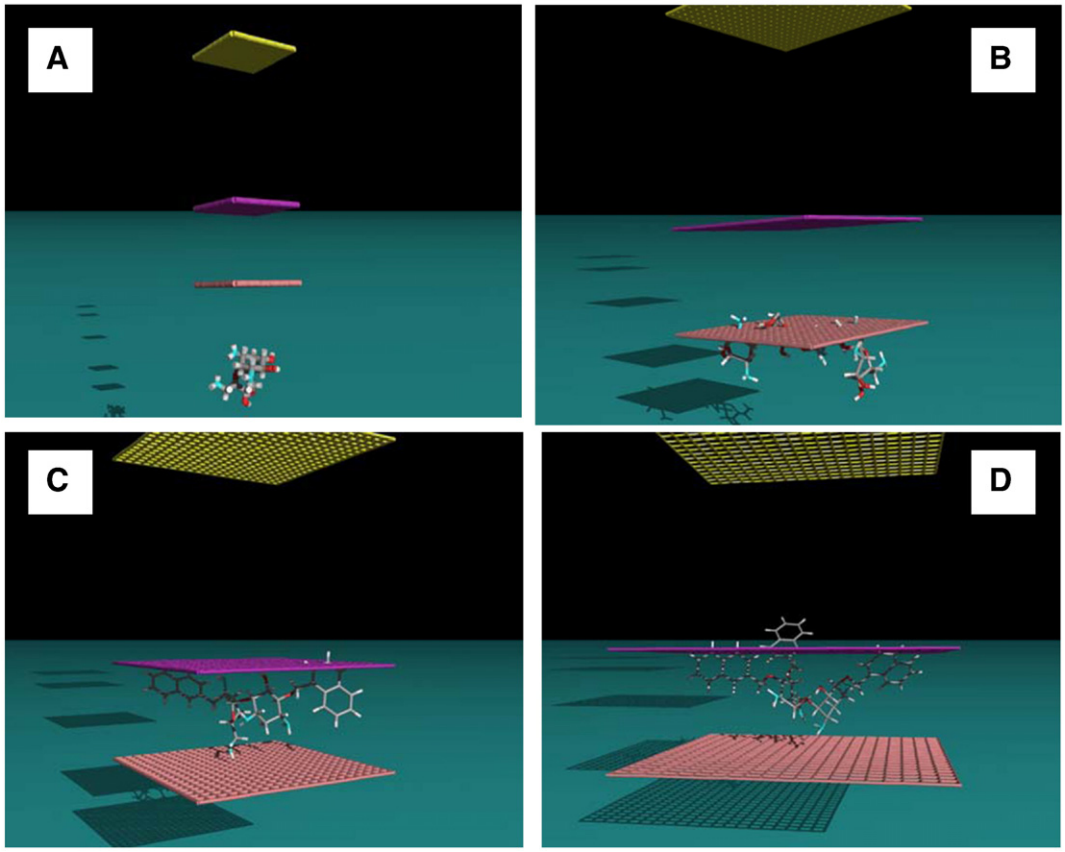

Fig. 7. Interaction of neamine (A), neomycin B (B), 3′,6-di-2NM neamine (C) and 3′,4′,6-tri-2NM neamine (D) with the IMPALA membrane Yellow plane = bilayer center ($z = 0$); mauve plane = lipid acyl chain/polar head group interface at ($z = 13.5$ Å from the center); pink plane = lipid/water interface ($z = 18$ Å).

against *P. aeruginosa*-sensitive strain, it however presents a broad spectrum of activity against resistant strains including bacteria expressing enzymes inactivating aminoglycosides as well as against strains expressing rRNA methylases or *S. aureus* MRSA, VRSA [23]. Interestingly, and as also reported for amphiphilic aminoglycoside-peptide triazole conjugates [63], the 3′,4′,6-tri-2NM neamine exhibited higher or similar activity against *P. aeruginosa*, *E. coli* and *S. aureus* – resistant strains as compared to sensitive strains. The first studies examining the binding of these naphtylmethylene derivatives to a model of bacterial ribosomal A site by microcalorimetry or quantifying the protein synthesis by measuring leucine incorporation [23], suggested a major shift in the primary mode of action of these aminoglycosides.

In this work, by using an *in vitro* transcription/translation assay [35], we confirmed that 3′,4′,6-tri-2NM neamine failed to inhibit the bacterial protein synthesis. This could eventually be caused by the steric hindrance of the naphthylmethylene groups resulting in a decrease of RNA site A recognition [23]. A similar assumption has been drawn by Zhang and collaborators, who synthesized neomycin derivatives bearing linear lipidic acyl groups with various chain lengths (from C7 to C18) at the 5″ position [19].

Table 2
Calculation of the ratios of the hydrophobic (pho) and hydrophilic (phi) surfaces (S; calculated as in Ref. [84]) and energies (Etr) of the 3′,6-di-2NM neamine, 3′,4′,6-tri-2NM neamine, neamine and neomycin B.

Molecule	S_{pho}/S_{phi}	Etr_{pho}/Etr_{phi}
Neamine	0.4	0.25
Neomycin B	0.5	0.35
3′,6-di-2NM neamine	2.3	1.4
3′,4′,6-tri-2NM neamine	3.5	2.3

Because the 3′,4′,6-tri-2NM neamine has shown a very interesting antibacterial effect on Gram-negative bacteria despite its inability to inhibit the protein synthesis, we investigated the mechanism involved. One of the most likely hypotheses could be that the addition of hydrophobic groups on the aminoglycoside backbone alters the mode of action from an intracellular to a membraneous target. To explore this hypothesis, we characterized the effect of 3′,4′,6-tri-2NM neamine on the entire bacteria at the nanoscale using AFM, and on the membrane depolarization using $DiSC_3(5)$ assay. In parallel, we investigated the ability of 3′,4′,6-tri-2NM neamine to bind to LPS, the main component of the external membrane of Gram-negative bacteria, and to permeabilize lipid membranes. We showed that 3′,4′,6-tri-2NM neamine induced a decrease of the thickness of

Table 3
Calculation of the interaction energy for the complexes presented on Fig. 8.

Molecule	E_{Tot} (kcal/mol)	E_{pho}–E_{VDW} (kcal/mol)	E_{elec} (kcal/mol)
Neomycin B/CL	−238	−96	−142
Neomycin B/POPE	−184	−70	−114
Neomycin B/POPG	−240	−120	−120
3′,6-di-2NM neamine/CL	−196	−116	−80
3′,6-di-2NM neamine/POPE	−277	−150	−127
3′,6-di-2NM neamine/POPG	−315	−175	−140
3′,4′,6-tri-2NM neamine/CL	−165	−160	−5
3′,4′,6-tri-2NM neamine/POPE	−283	−156	−127
3′,4′,6-tri-2NM neamine/POPG	−204	−134	−72

E_{Tot} = total energy (sum of E_{pho}-VDW and E_{elec}); E_{pho}: hydrophobic energy, E_{VDW}: van der Waals energy, E_{elec}: electrostatic energy. CL: Beef heart cardiolipin, POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol), POPE:1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol-amine.

P. aeruginosa envelope in parallel with membrane depolarization. It also bound to LPS and induced membrane permeabilization. The importance of each of these alterations for antibacterial activity is still unclear even if for cholic acid-derived antimicrobial agents, membrane depolarization seems play a critical role [64]. Regarding AFM results, further experiments will be necessary to understand the role played by the decrease of the height of bacterial wall for bacterial death since our pictures by AFM did not show the appearance of holes as it was the case with lipoglycopeptides [65]. All together, results indicated that naphthylmethyl neamine derivatives target the membrane of *P. aeruginosa.* They are in accordance with molecular modeling, an approach previously validated by the experimental data obtained with peptides known to interact with membranes [66,67] and which allowed to predict the insertion of tilted peptide [68], to design de novo a fusogenic peptide made of non-natural amino acids [69] and to confirm experimental data related to drug/lipid interactions (e.g., [70]). In this study, calculations showed that 3′,4′,6-tri-2NM neamine was inserted deeply into the modeled membrane and was prone to interact strongly with lipids present in *Pseudomonas* membranes.

Comparing the di- (3′,6-di-2NM neamine) and the tri- (3′,4′,6-tri-2NM neamine) substituted derivatives, at equimolar concentration (10 μM), only the tri-substituted derivative is able to depolarize *P. aeruginosa* membrane. Moreover, using Bodipy cadaverine fluorescent probe displacement, the tri-substituted neamine derivative was 13-fold more effective to interact with LPS as compared with the di-substituted neamine derivative. This could be related to the microbiological effects which showed that only the tri-substituted derivative was active against Gram-negative bacteria. The tri-substituted derivative probably inserted more deeply into adjacent LPS molecules and acted as a spacer in the plane of the bilayer, reducing the short-range attractive forces between LPS saccharide cores and inducing release of LPS. This has previously been described for biosurfactants like rhamnolipids, which solubilize hydrophobic compounds within micellar structures and cause the cell surface of *P. aeruginosa* to become more hydrophobic through release of LPS [71].

At a glance, the effect of the 3′,4′,6-tri-2NM neamine could therefore be due to the release of LPS, the accumulation of the compound within the membrane and the subsequent destabilization of the lipid membrane. Electrostatic interactions are probably critical at the initial stage of bacterial recognition. The positive charges of the amphiphilic aminoglycosides conceivably target the anionic environment of Gram-negative bacteria due to the anionic bisphosphorylated sugar head groups of LPS as well as the negatively charged lipids like cardiolipin and POPG. These results are in agreement with our molecular modeling and the values of energy. Our data are also in accordance with the model designed for dicationic linear molecules interacting with LPS. This model hypothesized distances between charged amino groups inter NH_2 distances from 5 to 16 Å allowing interactions with the negatively charged phosphates of lipid A [72] and decrease of the area per hydrocarbon chain of LPS [73]. Our calculations suggested a maximum distance of 8.5 Å between the furthest cationic amino functions of 3′,4′,6-tri-2NM neamine. The hydrophobic interaction between the amphiphilic aminoglycosides and LPS could play an additional critical role for the specificity and antibacterial activity. This correlates with the hydrophobic/hydrophilic balance of 3′,4′,6-tri-2NM neamine and 3′,6-di-2NM neamine as shown by their log P/log D. The calculated log P and log D (at pH 7) of 3′,4′,6-tri-2NM-neamine and 3′,6-di-2NM neamine are −9.3 and −0.4, and −12.7 and −4.1, respectively. These differences in interactions with phospholipids between the di- and tri-substituted neamines could explain the decrease of the contribution of electrostatic energies for 3′,4′,6-tri-2NM neamine in interaction with negatively charged lipids like cardiolipine or POPG in comparison of the energies found for 3′,6-di-2NM neamine.

Regarding the interactions with lipid membranes as well as on LPS, the tri-naphthylmethylene neamine derivative seems to act on lipid bacterial membranes as do polymyxins like colistin. Polymyxins are polycationic peptides with D- and L-amino acids in a cyclic heptapeptide ring carrying a tripeptide chain and a fatty acid chain. Colistin (polymyxin E) is distinguished from polymyxin B through the replacement of D-phenylalanine by D-leucine in the polypeptide ring [25–29,74,75]. In addition to the well-known binding to LPS, colistin is able to interact with lipid membranes by inducing molecular contacts between the inner and outer membranes of the bacteria resulting in lipid exchange, unequal pressure forces, rearrangement to the inner membrane and formation of a transient hole in the membrane [25–29]. As compared to the requirement of the naphthyl groups to observe membraneous effect and binding to LPS for amphiphilic aminoglycosides, the role of the lipophilic tail is critical since treatment of polymyxins with ficin or related enzymes that remove the lipid moiety (the 6-methyoctanoyl or 6-methylheptanoylresidue) induced a loss of antibacterial activity [76].

The biological relevance of this study is another important question to answer. Considering the drug/lipid ratio, the molecular modeling suggested that one molecule of drug is surrounded by 6 to 8 molecules of lipids, depending on the lipid type, a ratio close to that previously reported for kanamycin, gentamicin and slightly lower than that reported for antimicrobial peptides [77]. In MIC experiments, about 10^5 bacteria are present in 1 ml of media and the accepted approximate number of lipids per cell is 2.2×10^7 to 2.5×10^7. These values lead to a total lipid concentration of 3.65×10^{-3} μmol/l. Considering the MIC of the 3′,4′,6-tri-2NM neamine is around 4 μg/ml, this translates to a surprisingly high drug/lipid ratio of 1/0.001 at this concentration. Even if the cell density is 10^7 to 10^9 per ml, the drug/lipid ratio is still high. This calculation suggests that in the biophysical studies with model membranes the drug/lipid ratio used is drastically below of a standard MIC experiment. Therefore, the drug/lipid ratios used in biophysical assays are extremely conservative, in that very small drug concentrations (far below the MIC) can cause significant membrane perturbation. Thus, it is likely that the perturbation occurring at these low drug/lipid ratios will also occur at the MIC [78]. Considering the time-frame of the cellular alterations (bacterial death, membrane depolarization and permeabilization), we cannot extrapolated from the present data. In addition, literature suggested that drug-induced loss of viability is not simply a consequence of membrane depolarization [79].

Finally, the potential interaction with mammalian membranes and the potential cell toxicity remain to be evaluated. The concept of a characteristic lipid composition for a given cell membrane is well accepted, although changes in lipid composition may occur depending on environmental conditions [80,81]. One feature that distinguishes the membranes of prokaryotic organisms from those of eukaryotic organisms is that the former harbor more negatively charged lipids in the outer leaflet of the plasma membrane. Most Gram-negative bacteria contain ~25% of negatively charged lipids such as phosphatidylglycerol or cardiolipin and ~75% of phosphatidylethanolamine as their most common zwitterionic lipid. In contrast, the outer leaflet of the asymmetric eukaryotic cells, the erythrocyte membrane bilayer is devoid of anionic lipids and composed of ~25% cholesterol, ~33% phosphatidylcholine, ~18% sphingomyelin, and ~9% phosphatidylethanolamine. On the premise that the composition of membrane phospholipids differs between the microbial and human cells, design of new amphiphilic neamine derivatives should take into account these differences to improve the selectivity for the targeted bacterial membranes.

In this respect, the number and position of the substituents as well as the hydrophilic/hydrophobic balance of the synthesized derivatives have to be carefully designed. We showed here that limited modifications in the structure of the neamine derivatives, i.e., the addition of one naphthyl ring from the di-substituted derivative, allows to target Gram-negative bacteria even if the number and the

position of positive charges are the same. The position of the naphthyl groups in the di-2NM derivatives is also critical since the 3′,4′-di-2NM neamine and 3′,6-di-2NM neamine are much more efficient against *S. aureus* (ATCC 25923) than the 4′,5-di-2NM neamine and 4′,6-di-2NM neamine. Yet, the 3′,4′,6-tri-2-quinolylmethylene neamine derivative (3′,4′,6-tri-2QN) which differs from the 3′,4′,6-tri-2NM derivative through the simple replacement in each naphthyl rings of one carbon by a nitrogen atom, is inefficient against Gram(+) and Gram(−) bacteria [23], suggesting that minor structural changes can have large consequences on the biological effects.

In a nutshell, this work showed that the introduction of naphthylmethylene groups on the neamine backbone shifts the mechanism of action from an intracellular target mechanism to a membrane target effect. Such a target is particularly desirable since it is unlikely to be susceptible to existing mechanisms of bacterial resistance. Relation–structure activity studies should be very helpful to select and design derivatives with appropriate hydrophobic/hydrophilic balance as well as a steric hindrance to improve the selectivity for bacterial membranes and LPS or to target both ribosomal RNAs and lipid membranes. Such strategy has successfully been followed for lipoglycopeptides like telavancin and oritavancin [65,82] and is now in progress for aminoglycosides.

In conclusion, this work suggests that amphiphilic neamine derivatives are attractive targets for drug development. This can be extended to other cationic amphiphiles inspired by the natural antimicrobial scaffold [83]. However, before the usefulness of the drug in clinical settings, extensive relation–structure activity studies, work to overcome potential pharmacokinetic shortcomings including formulation difficulties and assessment of preclinical toxicity studies remain to be established.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamem.2011.01.014.

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