Bacterial membrane bilayer as drug target

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ABSTRACT

The widespread emergence of bacterial resistance has led to an urgent need to develop new strategies to regain the efficacy of antibacterials. One of the emerging concept is to target the bacterial membrane bilayer.
Aminoglycosides are among the most potent antimicrobials to treat severe infections. In the search for new antibiotics, we have synthesized derivatives of the small aminoglycoside, neamine in the aim to obtain amphiphilic antibiotics able to disturb bacterial membrane bilayer. One to four hydroxyl functions of neamine were capped with phenyl, naphthyl, pyridyl, or quinolyl rings. The 3',4'-, 3',6- and the 3',4',6-2-naphthylmethylene (2NM) derivatives were active against both sensitive and resistant *S. aureus* strains. The trisubstituted derivative, also showed marked antibacterial activity against Gram (-) bacteria, including resistant strains (1). Regarding its mechanism of action, it showed only a weak and aspecific binding to a model bacterial 16S rRNA as well as a lower ability to decrease ^{3}H leucine incorporation into proteins in *P.aeruginosa*, suggesting it acts through a mechanism probably involving membrane destabilization. To understand the molecular mechanism involved, we determined the ability of 3',4',6-tri-2NM neamine to interact with the bacterial membranes of *P. aeruginosa* or models mimicking these membranes.
Using Atomic Force Microscopy (AFM), 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 3',4',6-tri-2NM neamine derivatives target the membrane of *P. aeruginosa* (2). This should offer promising prospects in the search for new antibacterials against resistant drug or biocide strains.

BACKGROUND

Aminoglycosides are among the most potent antibacterials to eradicate *P. aeruginosa*, a persistent opportunistic pathogen. They act by binding to 16S rRNA, causing mRNA decoding errors, mRNA and tRNA translocation blockage, ribosome recycling inhibition and *in fine* protein synthesis alteration. However the emergence of resistant strains has reduced the potential of these antibiotics leading to treatment failure. In order to develop novel antibacterial drugs, Baussanne et al. have described the synthesis and the antimicrobial property of neamine derivatives carrying hydrophobic groups like naphthylmethylene (2). Among these derivatives, the 3',4',6-tri-2-naphtylmethylene neamine (3',4',6-tri-2NM neamine) showed a very interesting activity against sensitive and resistant *P. aeruginosa* strains as well as *Staphylococcus aureus* strains.

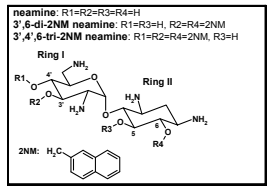
AIM

The aim of the study is to understand the molecular mechanism involved in the mode of action of these modified aminoglycosides. To this end, we investigated 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.

MATERIALS AND METHODS

Derivatives Synthesis: Neamine derivatives were synthesized in three steps from neamine according to our previous reports (1).
Bacterial strains: *P. aeruginosa* [ATCC 27853] was obtained from the Pasteur Institute (Brussels, Belgium; Prof. R. Vanhoof).
Minimal Inhibitory Concentration Determination : The MICs were determined by a geometric microdilution method according to the recommendations of the CLSI norms (2007).
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*^{TM} plasmid (Promega, Leiden, NL) as previously described (3) with modifications.
Atomic Force Microscopy: AFM images were recorded in PBS solution at room temperature, using a Nanoscope V multimode AFM. The 3',4',6-tri-2NM neamine was injected into the AFM liquid cell at 0.5 MIC (4 µg/mL).
Cytoplasmic Membrane Depolarization Assay: The membrane depolarization activity of compounds was determined using the membrane potential-sensitive dye $DiSC_3(5)$ (4).
Fluorescence Displacement Assay for quantifying binding affinities to LPS: The BODIPY-TR-cadaverine displacement assay was used to quantify the affinities of binding of the test compounds to LPS (5).
Liposomal Membrane Permeability Assay: Large Unilamellar Vesicles (LUV) composed of lipids mimicking the composition of lipid membranes of *P. aeruginosa* (POPE, POPG, CL; molar ratio 60:21:11; (Phosphatidylethanolamine [PE], Phosphatidylglycerol [PG] and Cardiolipin [CL])) (6), were prepared by extrusion. Permeabilization of lipid membranes induced by drugs was monitored by following the leakage of entrapped calcein within liposomes (7).
Molecular Modeling and Assembly of Neamine Derivatives with Lipids: The neamine derivative structures were first constructed using Hyperchem 7.0 (Hypercube, Inc). The interaction and insertion of the neamine derivatives within lipids was calculated using two methods, the hypermatrix and the impala method (8).

STRUCTURE



RESULTS

Antimicrobial activity

	P. aeruginosa			*E. coli*			*S. aureus*		
	ATCC 27853	Psa.F03 AAC6'-IIA	PA22 Surexp MexXY	ATCC 25922	PAZ505H8101	06AB003	ATCC 25923	ATCC 33592 HA-MRSA	VRSA-VRS-2
Resistance Mechanism	None	Enzymatic (AAC6'-IIA)	Efflux	None	Enzymatic (AAC6'-IB)	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

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.

Protein synthesis and nanoscale imaging of *P. aeruginosa* cells

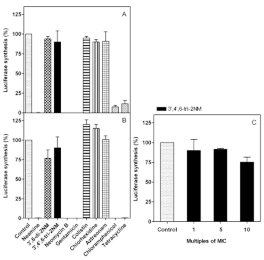
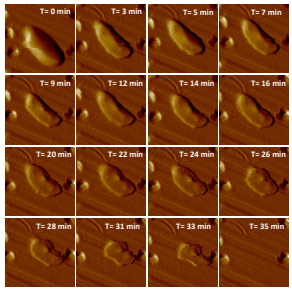


Figure 1. Effect of neamine derivatives and controls on bacterial protein synthesis. Protein translation inhibition by the different compounds is quantified in a coupled transcription/translation assay by using *E. coli* S30 extracts for circular DNA with the pBEST/*luc* plasmid. The compounds are tested at equimolar concentrations (10 µM; panel A) as well as at their MICs against *P. aeruginosa* 27853 (panel B) (neamine [128µg/ml; 397.1 µM], 3',6-di-2NM neamine [128 µg/ml; 212.4 µM], 3',4',6-tri-2NM neamine [8µg/ml; 10.8 µM], neomycin B [64 µg/ml; 104.1 µM], gentamicin [1 µg/ml; 2.1 µM], colistin [1µg/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.



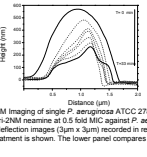


Figure 2. AFM Imaging of single *P. aeruginosa* ATCC 27853 cell following incubation with 3',4',6-tri-2NM neamine at 0.5 fold MIC against *P. aeruginosa* 27853. A series of deflection images (3µm x 3µm) recorded in real time for a single cell prior and after treatment is shown. The lower panel compares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

Effect on bacterial membranes

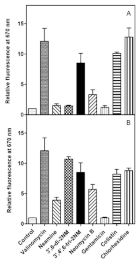


Figure 3. Effect on the fluorescence intensity changes of *P. aeruginosa* ATCC 27853 incubated with $diSC_3(5)$. The experiments are performed at 10 µM (Panel A) and at their MICs against *P. aeruginosa* 27853 (Panel B). Panel C shows the effect of 3',4',6-tri-2NM neamine and 3',6-di-2NM neamine at 0.1, 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.

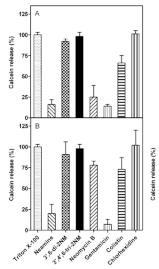


Figure 4. Effect on the release of calcein entrapped within liposomes. Liposomes made of POPE:POPG:CL (60:21:11 molar ratio) are exposed with compound at 10 µM (Panel A) or at their MICs (Panel B) for 1 h at 37°C. Panel C shows the effect of 3',4',6-tri-2NM neamine at 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 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.

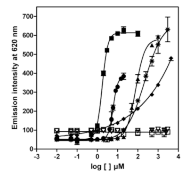


Figure 5. Binding affinity of compounds to LPS determined by the BODIPY-Cadaverine displacement method. Colistin (■) is used as the reference compound in comparison to the effect obtained for 3',4',6-tri-2NM neamine (●), 3',6-di-2NM neamine (▲), neamine (◆), chlorhexidine (✱), imipenem (□) and meropenem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

Molecular modeling of compounds binding

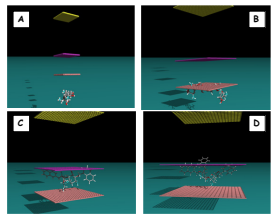


Figure 6. Interaction of neamine (A), neomycin B (B), 3',6-di-2NM neamine (C) and 3',4',6-tri-2NM neamine (D) with the IMPALA membrane Yellow plane = bilayer centre (z= 0); mauve plane = lipid acyl chain/polar headgroup interface at (z= 13.5 Å from the centre); pink plane = lipid/water interface (z =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

molecule	S_{pho}/S_{tot}	Etr_{pho}/Etr_{tot}
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

Table 2. Calculation of the ratios of the hydrophobic (pho) and hydrophilic (phi) surfaces (S; calculated as [72]) and energies (Etr).

molecule	E_{int} (kcal/mol)	$E_{vdW}+E_{elec}$ (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	-284	-134	+72

Table 3. Calculation of the interaction energy for the coumpounds with lipids. E_{int}= total energy (sum of E_{vdw}:VDW and E_{elec}); E_{pho}: hydrophobic energy, E_{VDW}: van der Waals energy, E_{elec}: electrostatic energy. .

3',4',6-tri-2NM neamine:

- ***active against both Gram-positive and Gram-negative strains, including sensitive and resistant bacteria***
- ***unable to inhibit protein synthesis***
- ***induces a decrease of the thickness of P. aeruginosa envelope ⇒ alteration of the cell wall leading to the discharge of most of the intracellular content***
- ***induces P. aeruginosa membrane depolarization***
- ***high potency to displace BODIPY-TR-cadaverine from its binding to lipopolysaccharides***
- ***induces a lipid membrane permeabilization on artificial membranes mimicking P. aeruginosa membrane***
- ***inserts more deeply into the modeled membrane ⇒ derivative more hydrophobic than the controls; interaction is stabilized by hydrophobic and Van der Waals energies***

CONCLUSIONS

This work shows that the introduction of **naphtylmethylene groups on the neamine backbone shifts the mechanism of action from intracellular target mechanism to a membrane target effect**. Such a target is particularly desirable to fight against drug- or biocide-resistant bacterial strains. Amphiphilic neamine derivatives are attractive targets for drug development and relation-structure activity studies should be very helpful to select and design more potent derivative to target both ribosomal RNAs and lipid membranes.

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