Motuporamine Derivatives as Antimicrobial Agents and Antibiotic Enhancers against Resistant Gram-Negative Bacteria

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Dihydromotuporamine C and its derivatives were evaluated for their in vitro antimicrobial activities and antibiotic enhancement properties against Gram-negative bacteria and clinical isolates. The mechanism of action of one of these derivatives, MOTU-N44, was investigated against *Enterobacter aerogenes* by using fluorescent dyes to evaluate outer-membrane depolarization and permeabilization. Its efficiency correlated with inhibition of dye transport, thus suggesting that these molecules inhibit drug transporters by de-energization of the efflux pump rather than by direct interaction of the molecule with the pump. This suggests that depowering the efflux pump provides another strategy to address antibiotic resistance.

Introduction

Antimicrobial resistance threatens the prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses, and fungi. An increasing number of governments around the world are devoting efforts to this problem, which is so serious that it threatens the achievements of modern medicine. Far from being an apocalyptic fantasy, a post-antibiotic era in which common infections and minor injuries can kill is a real possibility for the 21st century. A recent WHO report makes a clear case that resistance to common bacteria has reached alarming levels in many parts of the world, and that in some settings few, if any, of the available treatment options remain effective for common infections. Another important finding of the report is that surveillance of antibacterial resistance is neither coordinated nor harmonized and there are many gaps in information regarding bacteria of major public health importance.[1]

The intensive use of antibiotics for the treatment of numerous bacterial infections is one of the biggest healthcare advances in modern times. Nevertheless, their widespread use has led to an increasing number of antibiotic-resistant bacteria.[2] In particular, the emergence of Gram-negative multidrug-resistant (MDR) bacteria, such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, has prompted efforts to develop new classes of antibiotics and chemosensitizers (molecules to promote an increase in the internal antibiotic concentration in resistant strains). Thus, diseases caused by MDR Gram-negative bacteria are increasing worldwide,[3,4] and the emergence of pan drug-resistant (PDR) bacteria (resistant to all classes of antibiotics and to quaternary ammonium disinfectants)[5] appears to have reached a point of no return.[6,7] We have noticed great concern in the medical community, as numerous recent clinical reports have confirmed that Gram-negative bacteria have developed resistance to polymyxins, the last efficient therapy against PDR Gram-negative bacteria.[8–10]

An appealing target is the unique structure of the bacterial membrane, which is highly conserved among most species of Gram-negative bacteria, and forms an effective barrier to many types of antibiotics.[11] Indeed, the acquisition of resistance to membrane-active antibiotics has likely required major changes in membrane structure. Ironically, modifications to the bacterial membrane to escape membrane-targeting antibiotics might increase the permeability of the barrier and actually increase the susceptibility of the bacteria to hydrophobic antibiotics.

It is well established that most immune responses to Gram-negative bacteria involve recognition of lipopolysaccharides (LPS) and their lipid A anchors, which constitute the major components of the outer membrane.[12–17] The permeability barrier of the outer membrane is due to the cross-bridging electrostatic interactions between lipid A molecules and divalent cations such as calcium or magnesium.[12] We speculated that cationic peptides[18] and polyamines[19] could out-compete these divalent cations for their membrane binding sites and disrupt the outer membrane organization, thereby increasing

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permeability. Because of the promising applications of polyamine derivatives in medicine,[20–22] we evaluated a series of hydrophobic polyamine derivatives for their ability to target the membrane stability of Gram-negative bacteria and increase the sensitivity of these bacteria to known antibiotics.

The motuporamines (originally isolated from the marine sponge *Xestospongia exigua*)[23] were selected because their amphiphilic architectures comprise a large hydrophobic macrocycle with an appended polyamine motif (1–3, Scheme 1). A series of motuporamine derivatives (4–6) was prepared[24, 25] along with a series of related anthracenyl-polyamine derivatives (7a–d). These amphiphilic polyamines have large hydrophobic substituents to facilitate interaction with the bacterial membrane.

Here, 4–6 and 7a–d were screened for their in vitro antimicrobial activities and antibiotic-enhancement properties against resistant Gram-negative bacteria. We also explored the mechanism of action of this class of derivatives against *Enterobacter aerogenes* (EA289) by using fluorescent dyes, in order to evaluate changes in outer-membrane depolarization and permeabilization.

Results and Discussion

Our investigations began with the determination of the minimum inhibitory concentrations (MICs) of 4–7 in Gram-positive and -negative species, in order to identify the concentrations that produce a direct antibacterial effect and allowed us to rank their relative potencies. We included two Gram-negative bacteria encountered in hospitals, *P. aeruginosa* and *Klebsiella pneumonia*, and multidrug-resistant *E. aerogenes* EA289

(Table 1). Several compounds showed MICs of 100–200 μM for these bacterial strains. The anthracenyl compounds 7a–d had relatively weak antimicrobial activities, whereas their related motuporamine derivatives 4a–b, 5a–b, and 6a–b showed MICs of 1.56–50 μM. Specifically, 6a (MOTU-CH₂-33) and 6b (MOTU-CH₂-44) exhibited excellent antimicrobial activities against many species, including the multidrug-resistant *E. aerogenes* EA289.

As stated previously, the development of chemo-sensitizing agents, which enhance the intracellular antibiotic concentration in resistant strains (or by other mechanisms) is an attractive approach to overcome bacterial resistance. Thus, we investigated the use of these polyamine derivatives as adjuvants in combination with antibiotics. Success here would provide an exciting approach to increase the potency of current antibacterial drugs, even for strains that have developed resistance.

We investigated whether these polyamine agents could restore the potency of the antibiotic doxycycline at significantly below its MIC. For example, in our hands the MIC of doxycycline against *P. aeruginosa* PAO1 was 16 μg mL⁻¹, so we investigated the use of doxycycline at a significantly lower concentration (2 μg mL⁻¹, corresponding to its pharmacokinetic properties in humans)[6] in the presence of the polyamine derivatives. We speculated that the polyamine agents would disrupt bacterial membrane integrity and increase antibiotic delivery to the bacteria and thus increase doxocycline potency. Rewardingly, even at this low doxycycline concentration, eight of the polyamine derivatives restored doxycycline activity against *E. aerogenes* EA289, *P. aeruginosa* PAO1, and *K. pneumoniae* KPC2-ST258; no improvement was observed for 7b (ANT4) or 7a (ANT-N-butyl) even at 40 μM (Table 2). The fact that this effect
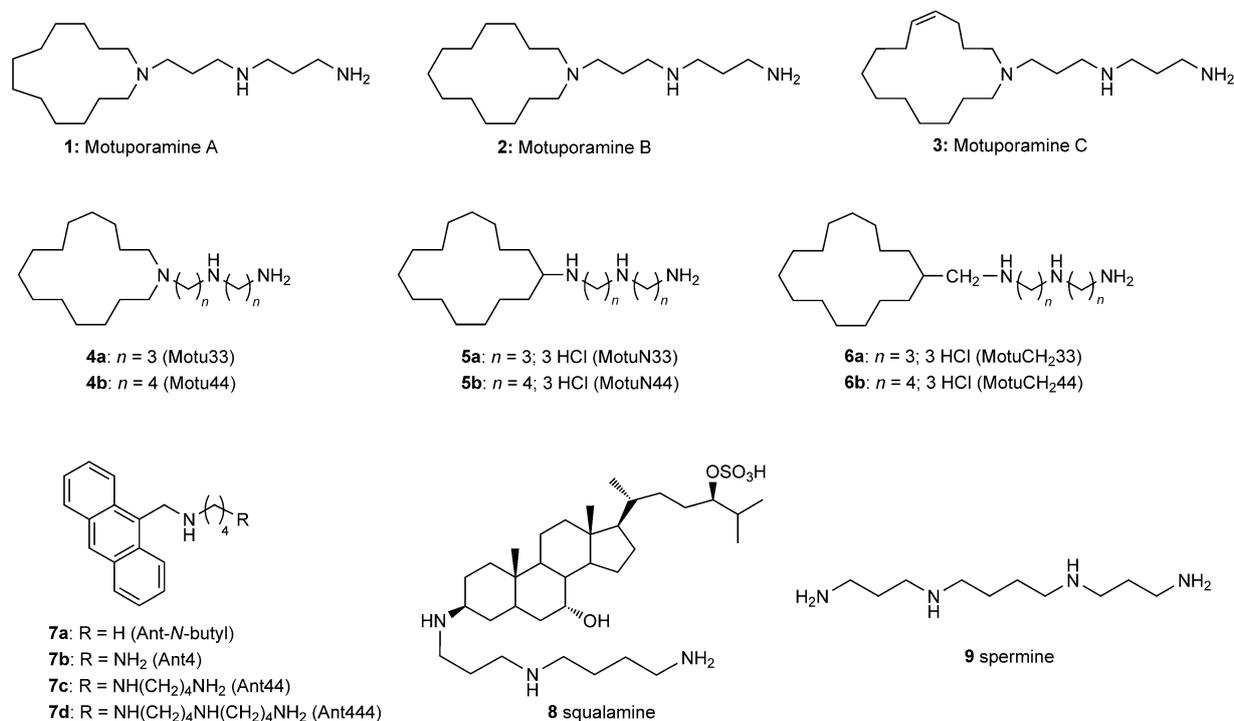
Scheme 1. Motuporamine compounds 1–6, anthracenyl compounds 1–7 squalamine 8, and spermine 9.

Table 1. MIC of motuporamine derivatives against various bacterial strains.							
Compound	MIC [μM]						
	S. aureus ATCC25923	*S. intermedius* 1051997	*E. faecalis* ATCC29212	*E. coli* ATCC28922	*P. aeruginosa* PAO1	*E. aerogenes* EA289	*K. pneumoniae* KPC2-ST258
7b, ANT4	>200	200	>200	200	200	100	100
7c, ANT44	50	200	>200	50	100	200	>200
7d, ANT444	12.5	25	200	25	100	100	100
7a, ANT-*N*-butyl	>200	200	>200	>200	>200	>200	>200
6a, MOTU-CH$_2$-33	1.56	3.125	3.125	1.56	6.25	50	100
5a, MOTU-N33	3.125	1.56	12.5	3.125	12.5	100	100
6b, MOTU-CH$_2$-44	1.56	1.56	3.125	1.56	12.5	50	100
4b, MOTU44	100	50	>200	100	100	50	100
4a, MOTU33	50	50	100	50	50	100	100
5b, MOTU-N44	1.56	1.56	6.25	6.25	25	50	50

was compound-specific was intriguing and ruled out a non-specific detergent effect, especially because no cell lysis was observed.

Several of the effective compounds also acted synergistically with chloramphenicol and erythromycin, particularly against PAO1, but weakly against EA289 and KPC2-ST258 (Table 3). Thus, we identified two groups of compounds: one (**7a** and **7b**) displayed weak or no activity, and the second (e.g., **5a** and **5b**) increased the antibiotic susceptibility effectively against PAO1. Overall, **5a** and **5b** appeared the most promising adjuvants for use with doxycycline; **5b** (MOTU-N44) was chosen to investigate the mechanism of action of this molecular class.

Within the motuporamine series (**4**–**6**) several compounds exhibited moderate to good antibacterial activity as well as potent synergy with different antibiotics against Gram-negative bacteria. We explored the mechanism of action of these compounds and focused on two possible pathways: permeabilization and/or disruption of the outer membrane, and inhibition of an efflux pump.

First, we determined the effect of **5b** on *Staphylococcus aureus* ATCC25923 by measuring ATP release for 1 min: there was dramatic disruption of the bacterial membrane, similar to that by squalamine (positive control; Figure 1).[26] Conversely, no significant effect was found for the polyamine spermine (negative control).

As we observed different compound performance in the assays with *S. aureus* in Table 1, we speculated that some of these molecules might achieve lethality by increasing the rate

Table 2. Concentration of motuporamine derivatives necessary to restore doxycycline activity (2 μg mL^{-1}) against EA289, PAO1 and KPC2 ST258 Gram-negative bacterial strains.			
Compound	Concentration of motuporamine derivative [μM]		
	EA289	PAO1	KPC2 ST258
7c, ANT44	10	5	5
7d, ANT444	1.25	2.5	1.25
4a, MOTU33	2.5	1.25	1.25
4b, MOTU44	1.25	2.5	1.25
5a, MOTU-N33	2.5	2.5	2.5
5b, MOTU-N44	5	1.25	2.5
6a, MOTU-CH$_2$-33	5	5	2.5
6b, MOTU-CH$_2$-44	2.5	2.5	1.25
7b, ANT4	40	>40	40
7a, ANT-*N*-butyl	>40	>40	>40

MICs of doxycycline against PAO1, EA289, KPC2ST258: 40 μg mL^{-1} (90 μM), 20 μg mL^{-1} (45 μM), and 10 μg mL^{-1} (22.5 μM), respectively.

of transport of molecules across the cytoplasmic membrane, whereas others might not. We surmised that compounds like **5b** might induce a smaller membrane breach, modestly affect the permeability barrier of the cytoplasmic membrane and cause membrane depolarization. Indeed, a small breach would allow the passage of electric current (thereby causing membrane depolarization) without allowing the passage of larger molecules. This alternative mechanism seemed plausible because depolarization would de-energize the efflux pump and also lead to increased potency of the antibiotic agent. Therefore, we investigated whether these molecules generated

Table 3. Concentration of the motuporamine derivative [μM] required to restore chloramphenicol, erythromycin, and cefepime activity (2 μg mL^{-1}) against EA289, PAO1, and KPC2 ST258.									
Compound	PAO1			EA289			KPC2 ST258		
	CHL	ERY	FEP	CHL	ERY	FEP	CHL	ERY	FEP
4a, MOTU33	5	20	n.t.	40	40	40	40	>40	>40
4b, MOTU44	5	40	n.t.	40	>40	>40	40	>40	>40
5a, MOTU-N33	2.5	10	n.t.	20	20	20	20	40	>40
5b, MOTU-N44	5	>40	n.t.	>40	40	>40	40	>40	40
6b, MOTU-CH$_2$-44	2.5	10	n.t.	20	20	>40	20	>40	>40

CHL: chloramphenicol, ERY: erythromycin, FEP: cefepime, n.t.: not tested. MIC of FEP against PAO1: 10 μg mL^{-1}. All other antibiotic/strain combinations: >100 μg mL^{-1}.

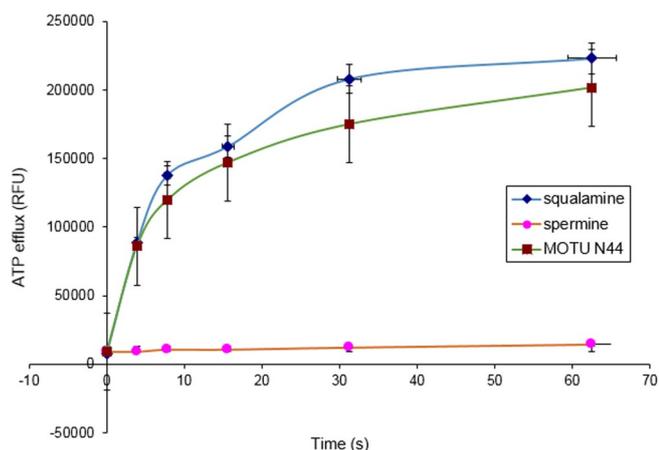**Figure 1.** The effect of squalamine (100 µg mL⁻¹), spermine (100 µg mL⁻¹), and **5 b** (MOTU-N44, 100 µg mL⁻¹) on ATP release kinetics for Gram-positive bacteria *S. aureus*.

a smaller breach of the permeability barrier of the cytoplasmic membrane.

Fluorescent cyanine dyes are excellent probes to monitor membrane depolarization. These dyes lose fluorescence intensity when in polarized membranes and become highly fluorescent once polarization is lost.[27] Thus, one can use changes in dye fluorescence to monitor change in membrane polarization. Interestingly, strong depolarization of *S. aureus* membranes was observed after 21 minutes as a strong increase in relative fluorescent units (RFU) of the cyanine dye (Figure 2) in the presence of **5 b**. This suggests that **5 b** facilitated membrane depolarization.

Next, **5 b** was investigated for its ability to alter the cell outer membrane integrity of *E. aerogenes* EA289, by using nitrocefin, a chromogenic β-lactam that is efficiently hydrolyzed by periplasmic β-lactamases, thereby resulting in a significant color change from yellow to red.[28, 29] Thus, colorimetric changes were used to monitor outer membrane integrity. Even
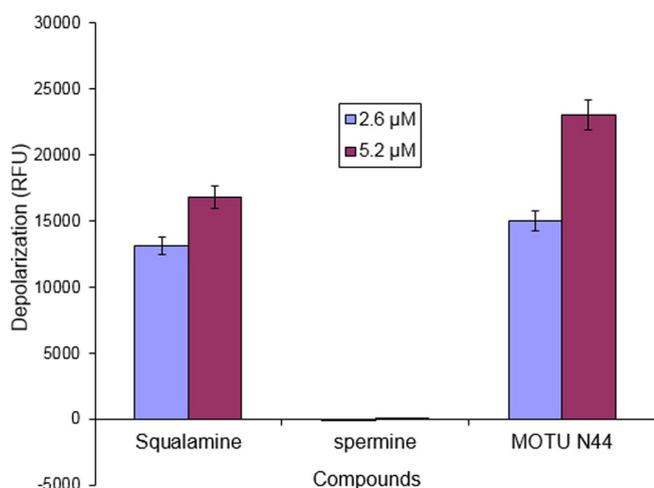

Figure 2. Depolarization of the bacterial membrane of *S. aureus* in the presence of 2.6 and 5.2 µM squalamine, spermine, or **5 b** (MOTU-N44).

at a low concentration (3.9 µM), **5 b** increased the rate of nitrocefin hydrolysis compared to the spermine-treated or untreated control (Figure 3 a). The behavior was similar to that of the positive control polymyxin-B (PMB) which also produced an increase in nitrocefin hydrolysis. All these data suggest that **5 b** is able to permeabilize or disrupt the outer membrane of Gram-negative bacteria as no cell lysis was observed.

The drug-resistant bacterium EA289 overexpresses the AcrAB-TolC pump,[30] which belongs to the RND efflux pumps and uses the proton gradient across the inner membrane as an energy source. In order to determine if **5 b** could act as a disruptor of the transmembrane potential, we used the membrane-potential-sensitive probe DiSC₃(5) which concentrates at the inner membrane and self-quenches its fluorescence.[31] When a compound impairs the membrane potential, the dye is released into the growth medium thus leading to a fluorescence increase. Treatment with **5 b** resulted in dose-dependent depolarization after 10 min of incubation (Figure 3 b), thus suggesting disruption of the proton gradient and an ability to affect efflux pumps from the RND family such as AcrAB-TolC.

A similar outcome was observed when using a bioluminescence method to determine the release of intracellular ATP. Addition of **5 b** caused dose-dependent permeabilization (Figure 3 c). Interestingly, 10 µg mL⁻¹ **5 b** caused 11 % ATP release into the medium after a few seconds, thus suggesting rapid disruption.

In general, efflux systems employ an energy-dependent mechanism (active transport) to pump out unwanted substances such as toxins, antibiotics, or dyes, through specific efflux pumps.[32] Some efflux systems are drug-specific, whereas others eject multiple drugs, and thus contribute to MDR. Efflux pumps are proteinaceous transporters in the cytoplasmic membrane of bacteria and are active transporters; thus they require a source of chemical energy. Some are primary active transporters that use ATP hydrolysis as a source of energy, whereas in others (secondary active transporters) transport is coupled to an electrochemical potential difference created by pumping protons or sodium ions from or to the outside of the cell. The transport of a known transport substrate can be used to directly monitor the function of efflux pumps, and **5 b** was thus tested for its ability to inhibit efflux.

After loading EA289 bacteria with the dye 1,2′-dinaphthylamine (1,2′-diNA), which is a substrate of the AcrAB-TolC efflux pump,[33] the bacteria fluoresced. Bacteria were then incubated with and without **5 b** at different concentrations before addition of glucose as an energy source. In the absence of **5 b**, rapid active transport of more than 80 % of the dye was observed (Figure 3 d, black line). When **5 b** was present, significant dose-dependent inhibition was observed (>80 % retention at up to 25 µM **5 b**; Figure 3 c, orange line). These results suggest that **5 b** inhibits the AcrAB-TolC efflux pump.

A time-kill assay (Figure 4) and a cell viability assay (Figure 5) were performed in order to evaluate the bactericidal or bacteriostatic behavior of this compound. As shown in Figure 4, a time kill analysis was performed against the EA289 bacterial strain by using **5 b** at a four times the MIC: 99.9 % death (detection limit) occurred by 2 h.
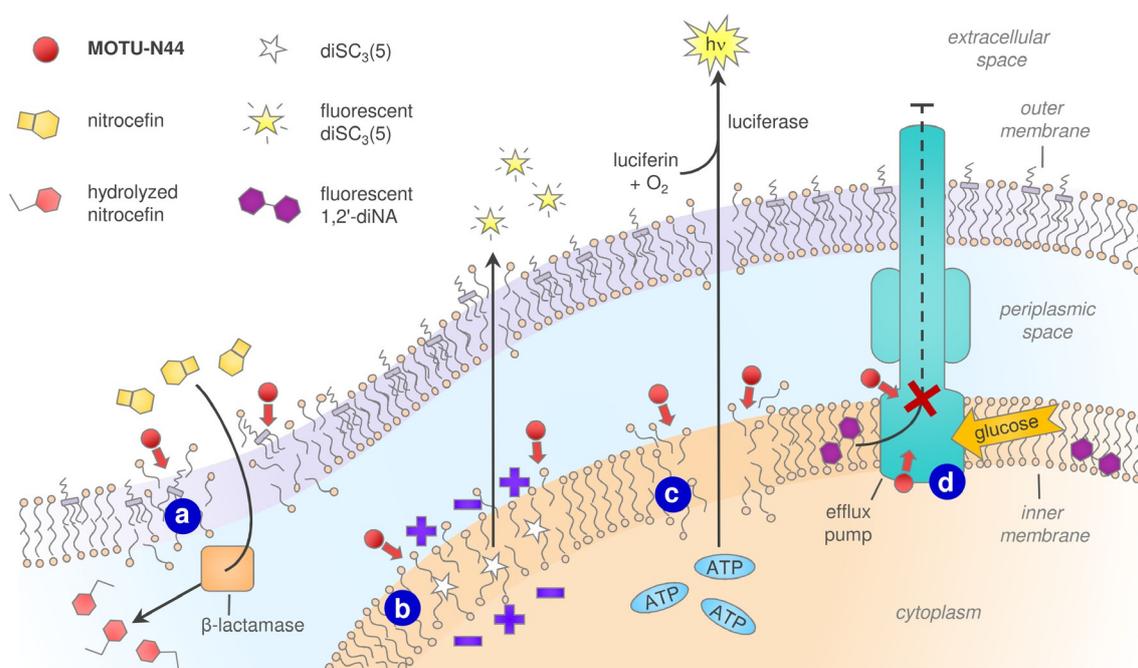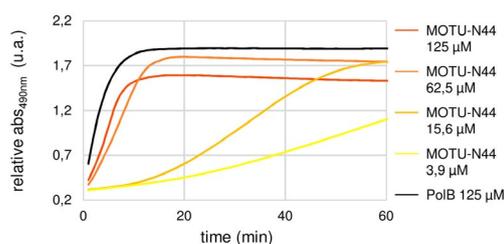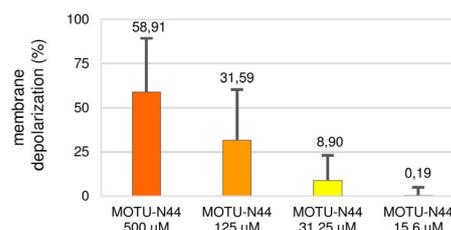
Figure 3. MOTU-N44 (**5 b**) has multiple effects on the cell membrane of the Gram-negative bacterium *E. aerogenes* EA289. a) Outer-membrane permeabilization detected by nitroceflin hydrolysis, in a dose-and time-dependent manner. b) Dose-dependent inner-membrane depolarization quantified by the release of DiSC$_3$(5). c) Membrane disruption revelaed by APT efflux. d) Inhibition of glucose-triggered 1,2'-diNA release via effux pumps.

A cell viability assay (Figure 5) was performed by monitoring the irreversible reduction of blue resazurin to red resorufin by viable cells. This conversion is an oxidation–reduction indica-tion in cell viability assays and can serve as an aerobic respira-tion measurement for bacteria.[34] When using **5 b** at four times the MIC, there was clearly no cell viability.
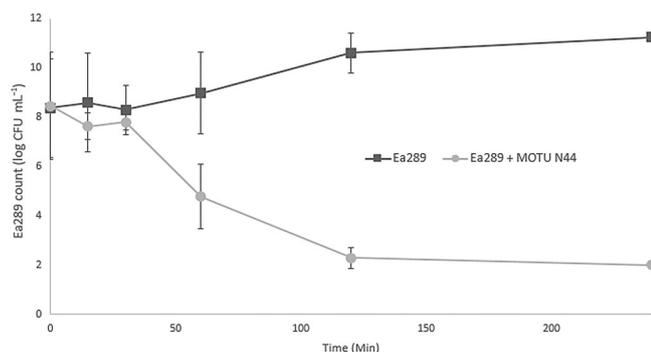
Figure 4. Time-kill curves of **5b** (MOTU-N44, 4×MIC) over 4 h against EA289 bacteria.
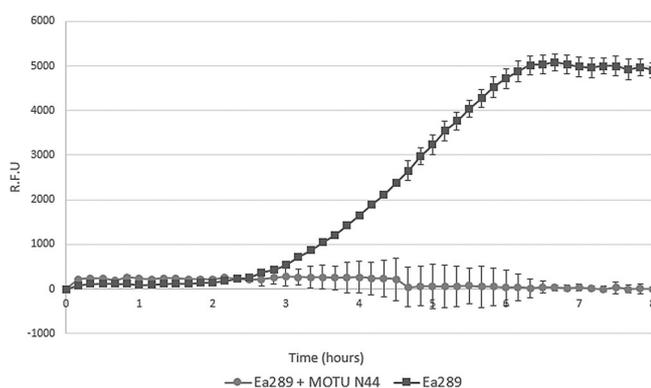

Figure 5. Cell viability of EA289 in the presence of **5b** (MOTU-N44, 4×MIC).

Thus, the time-kill experiment shows that **5b** at four times MIC (200 μм) led to a decrease in live bacteria after 30 min. When the cells were incubated for 60 min at this concentration, the cell viability assay demonstrated total inhibition of respiratory metabolism allowing us to conclude that this decrease in bacterial count correlates highly with cell death.

The real-time assay demonstrated the ability of **5b** to inhibit efflux transport to around 60% by using a sub-inhibitory concentration (10 μм, MIC/4). The results from the time-kill assay allow us to state that the cells remain viable in the efflux assay conditions (≤30 min) and that the inhibition of the dye transport is a consequence of a specific action of the compound.

On the other hand, the nitrocefin hydrolysis and membrane depolarization assays suggest that efflux inhibition is probably due to disruption of membrane integrity thereby leading to proton-motive force dissipation. Indeed, the hydrolysis kinetics observed at a low concentration of **5b** demonstrated a slight effect on the membrane, thus correlating with the results obtained for the depolarization assay. We noted that outer-membrane permeation increased with increasing **5b** concentration, and this is likely responsible for cell death at high levels. We also note that the real-time assays required higher concentrations than those for fixed incubation times to generate a quantifiable signal.

Wang et al. recently described a similar action of the substituted diamine, 1,13-bis (((2,2-diphenyl)-1-ethyl)thioureido)-4,10-diazatridecane.[35] This diamine compound was also shown to

depolarize the cytoplasmic membrane and provide enhanced permeabilization of the outer bacterial membrane. Further structure–activity relationship studies revealed that the central diamine nitrogens were key to bioactivity. In contrast to the N-substituted systems, the unsubstituted diamines (putrescine and cadaverine) had no antibacterial activity, did not affect membrane permeability, and did not cause membrane rupture. Both of the higher polyamines (spermidine and spermine) were found to be inactive against *S. aureus* RN4220, *P. aeruginosa* PAO1and *E. coli* ANSI. This, when coupled to our findings, suggests that either mono- or di-substituted polyamine systems can serve as antibacterial agents, whereas the unsubstituted native polyamine systems do not. Taken together, our studies also suggest that the presence of hydrophobic N-substituents is key to the ability of these compounds to target bacterial membranes and elicit a bacteriocidal response.

Conclusion

Several polyamine derivatives were investigated for their intrinsic antimicrobial activities against Gram-positive and Gram-negative bacteria. Derivatives **5a** and **5b** showed excellent activities (MIC 1.56–100 μм). In addition, **5b** dramatically affected the antibiotic susceptibility of *E. aerogenes*, *P. aeruginosa*, and *K. pneumoniae* MDR strains. We conclude that changes in the transmembrane electrical potential in *E. aerogenes EA289* correlate with permeabilization of the cell membrane by motuporamine derivatives, thereby leading to (or concomitantly facilitating) an altered proton homeostasis. Finally, motuporamine derivatives such as **5b**, that are able to disrupt the proton gradient, effectively de-energize the efflux pump and can be considered as efflux-pump inhibitors.

Experimental Section

Bacterial strains: Eight bacterial strains (Institut Pasteur and personal collection) were used in this study. Gram-positive bacteria (*S. aureus* ATCC25923, *S. intermedius* 1051997, *Enterococcus faecalis* ATCC29212) and Gram-negative bacteria (*E. coli* ATCC28922, *P. aeruginosa* PAO1, *E. aerogenes* EA289, a Kan derivative of the MDR clinical isolate Ea27,[30] and *K. pneumoniae* KPC2 ST258) were stored at −80°C in glycerol (15%, *v/v*). Bacteria were grown in Mueller–Hinton (MH) broth at 37°C.

Antibiotics: All the antibiotics were purchased from Sigma–Aldrich except fordoxycycline, which was purchased from TCI Europe (Zwijndrecht, Belgium). All antibiotics were dissolved in water. The susceptibility of bacterial strains to antibiotics and compounds was determined in microplates by the standard broth dilution method, according to the recommendations of the Comité de l'Antibio-Gramme de la Société Française de Microbiologie (CA-SFM).[36] Briefly, MICs were determined with an inoculum of 10^5 CFU in of MH broth (200 μL) containing twofold serial dilutions of each drug. MIC was defined as the lowest concentration to completely inhibit growth after incubation for 18 h at 37°C. Measurements were repeated in triplicate.

Determination of antibiotic MIC in the presence of compounds: Briefly, restoring enhancer concentrations were determined with an inoculum of 5×10^5 CFU in MH broth (200 μL) containing twofold

serial dilutions of each derivative and antibiotic (chloramphenicol, doxycycline, cefepime, or erythromycin; 2 µg mL^{-1}). The lowest concentration of the polyamine adjuvant that completely inhibited growth after incubation for 18 h at 37 °C was determined. Measurements were repeated in triplicate.

Membrane depolarization assays: Bacteria were grown in MH broth for 24 h at 37 °C and centrifuged (3 600 g, 20 °C). The pellet was washed twice with buffered HEPES (pH 7.2) sucrose (250 mM) and magnesium sulfate (5 mM). The fluorescent dye 3,3′-diethyl-thiacarbocyanine iodide was added (3 µM) and allowed to penetrate into bacterial membranes by incubation for 1 h of at 37 °C. Cells were then washed to remove the unbound dye before adding **5 b** at different concentrations. Fluorescence measurements were performed on a FluoroMax 3 spectrofluorometer (Horiba; slit widths 5/5 nm). The relative corrected fluorescence (RFU) was recorded at 0, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 min. Maximum RFU was that recorded with a pure solution of the fluorescent dye in buffer (3 µM).

Nitrocefin hydrolysis assay: Outer membrane permeabilization was measured by using nitrocefin, a chromogenic substrate of periplasmic β-lactamase. MH broth (10 mL) was inoculated with of an overnight culture (0.1 mL) of EA289 and grown at 37 °C to OD$_{600}$ = 0.5. The remaining steps were performed at room temperature. Cells were recovered by centrifugation (3600 g, 20 min) and washed once with potassium phosphate buffer (PPB; 20 mM, pH 7.2) containing MgCl$_2$ (1 mM). After another centrifugation, the pellet was resuspended in PPB (100 µL) and adjusted to OD$_{600}$ = 0.5. Then, either Polymyxin B (positive control; Sigma–Aldrich) or **5 b** (50 µL) was added to the cell suspension (100 µL) to final concentrations of 0.98–500 µM. Nitrocefin (50 µL, 50 µg mL^{-1}; Oxoid) was added, and its hydrolysis was monitored spectrophotometrically by measuring the increase in absorbance at 490 nm. Assays were performed in 96-well plates with an M200 Pro spectrophotometer (Tecan).

Glucose-triggered 1,2′-diNA efflux assays: Bacteria were grown to stationary phase, collected by centrifugation, and resuspended to OD$_{600}$ = 0.25 in PPB (20 mM, pH 7.2) supplemented with carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 5 µM; Sigma–Aldrich), and incubated overnight with 1,2′-dinaphthylamine (1,2′-diNA, 32 µM; Sigma–Aldrich) at 37 °C. Before addition of compound **5 b** (100 µM), the cells were washed with phosphate buffer. Glucose (50 mM) was added after 300 s to initiate bacterial energization. Release of membrane-incorporated 1,2′-diNA was followed by monitoring the fluorescence (λ_{ex} = 370 nm; λ_{em} = 420 nm) every 30 s at 37 °C in an Infinite M200 Pro plate reader (Tecan). Assays were performed in 96-well plates (half area, black with solid bottom, 100 µL per well; Greiner Bio-One).

Measurement of ATP efflux: Squalamine were prepared in doubly distilled water at different concentrations. A suspension of growing S. aureus or E. aerogenes (EA289) in MH broth was incubated at 37 °C. The suspension (90 µL) was added to squalamine solution synthesized in our laboratory according reported procedures (10 µL), and the mixture was vortexed for 1 s. Luciferin–luciferase reagent (Yelen, France; 50 µL) was immediately added, and luminescence was quantified with an Infinite M200 microplate reader (Tecan) for 5 s. ATP concentration was quantified by addition of a known amount of ATP (1 µM). A similar procedure was performed for spermine (100 µg mL^{-1}) and for **5 b** (200 µM, i.e., 4×MIC).

Time-killing assay: Mid-log phase cultures of EA289 with an inoculum of 10^7 CFU mL were incubated with **5 b** (4×MIC, 200 µM) at 37 °C with 160 rpm shaking. Bacterial counts were performed after

0, 15, 30, 90, 120 and 240 min by spreading appropriate dilutions on MH agar plates (detection limit 10^2 CFU mL^{-1}). The plates were incubated overnight at 37 °C before colonies were counted. The curves from two independent experiments were averaged and expressed as logarithms (mean ± SE).

Cell viability assay: An overnight culture of EA289 was diluted 100-fold into MHII broth. An inoculum of 10^7 CFU mL^{-1} was incubated in the presence or absence of **5 b** (4×MIC, 200 µM) for 1 h at 37 °C with shaking at 160 rpm. The fluorescence of the cell suspension was monitored after addition of CellTiter-Blue reagent (10%, v/v; Promega). Measurements were performed by using a 96-well Greiner film-bottom black microplate (Greiner Bio-One) and an Infinite M200 microplate reader (Tecan; λ_{ex} = 568 nm and λ_{em} = 660 nm). The curves from two independent experiments were combined (mean ± SE).

Synthesis of compounds 4–7: The synthesis of **4–7** was previously reported.[15,37–41]

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