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Dimers of Nostocarboline with Potent Antibacterial Activity

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Key Words
Cationic antibacterials · Chlorhexidine · Mode of action

Abstract
Objectives: In this study, the in vitro antimicrobial activity and spectrum of new dimeric compounds derived from the cyanobacterial alkaloid nostocarboline were investigated. The mechanism of action and selectivity to bacteria were studied and compared to the cationic antiseptic chlorhexidine. ***Methods:*** Minimal inhibitory concentrations were determined against clinical isolates and against a panel of microbial reference strains using the CLSI microdilution method. Bacterial membrane damage was addressed by measuring ATP leakage and the mode of action was investigated in *Escherichia coli* reporter strains. Selectivity was tested by a cytotoxicity assay using MTS. ***Results:*** The antimicrobial potency of dimers varied with length of the hydrophobic linker. The most potent compounds, NCD9 and NCD10, had a C10 and C12 linker, respectively, and showed strong activity against Gram-positive bacteria, notably methicillin-resistant *Staphylococcus aureus* strains. Similar to chlorhexidine, these compounds showed a rapid concentration-dependent bactericidal effect, which correlated with membrane damage as indicated by ATP leakage. NCD9, in contrast to NCD10 and chlorhexidine, lacked activity against yeast strains and showed low cytotoxicity in CHO cells indicating a high degree of selectivity. In *E. coli* reporter strains, NCD9 induced the DegP response pathway as well as the SOS response, suggesting interaction with both the cell envelope and DNA metabolism. ***Conclusions:*** The results presented in this report indicate the potential of this new class of cationic antimicrobial compounds for the design of potent and selective antibacterials with low cytotoxicity.

Introduction

The problem of microbial drug resistance has reached a global dimension and an alarming magnitude. Epidemic antibiotic resistance has been reported in numerous pathogens, including the global pandemic of methicillin-resistant *Staphylococcus aureus* (MRSA) infection, and epidemic increases of multidrug-resistant and increasingly pan-resistant strains of Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [1–3]. The systemic or topical treatment of the increasing number of infections caused by resistant or multiresistant bacteria demands new antibiotics, preferably from new structural classes and with new mechanisms of action. However, the difficulty of finding and developing

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Table 1. MICs (mg/l) by microdilution method against a panel of bacterial and yeast strains

	Compounds												
	NCD1	NCD2	NCD3	NCD4	NCD5	NCD6	NCD7	NCD8	NCD9	NCD10	NC	CIP	CHL
S. aureus ATCC 29213	16	4	32	8	>32	32	8	2	0.25	0.25	>32	0.25	0.25
S. aureus A-798 (MRSA)	16	8	>32	8	32	8	16	8	0.5	0.5	>32	>32	0.25
E. faecium A-949 (VRE)	32	8	32	8	32	32	32	16	2	1	>32	>32	2
S. pneumoniae ATCC 49619	>32	>32	>32	32	>32	>32	>32	32	4	4	>32	1	4
H. influenzae A-921	>32	>32	>32	>32	>32	>32	>32	>32	8	32	>32	≤0.03	0.25
E. coli ATCC 25922	32	>32	>32	>32	>32	>32	32	8	8	8	>32	≤0.03	0.125
E. coli Ec2085	2	8	8	>32	>32	1	1	0.5	0.5	1	>32	0.004	0.125
A. baumannii T-6474	32	32	>32	>32	>32	>32	>32	32	16	8	>32	>32	16
P. aeruginosa ATCC 27853	32	>32	>32	>32	>32	>32	>32	>32	32	>32	>32	0.5	4
S. cerevisiae A-136	>32	>32	>32	>32	>32	>32	>32	>32	>32	1	>32	>32	4
C. albicans T-3419	>32	32	>32	>32	>32	>32	>32	>32	>32	0.5	>32	>32	4

VRE = Vancomycin-resistant *Enterococcus*; NC = nostocarboline; CIP = ciprofloxacin; CHL = chlorhexidine.

new antibacterial agents is underlined by the fact that only two new chemical classes were brought into medical use over the past 35 years: the oxazolidinones (with linezolid as the only representative so far) and daptomycin [4].

Natural products have historically been a rich source of antimicrobial compounds and roughly 50% of all agents on the market addressing infectious diseases are natural products or derivatives thereof [5]. Cyanobacteria have been shown to be a prolific source of bioactive compounds [6–8]. The cyanobacterial metabolite nostocarboline has potent algicidal activity, while antibacterial activity is weak or absent [7–9]. This secondary metabolite, originally isolated from the freshwater cyanobacterium *Nostoc* 78–12A [9], has been demonstrated to inhibit the growth of other algae [7], a feature (alloleopathy) that is commonly found in cyanobacterial metabolites [8]. Recently, it was demonstrated that nostocarboline is active against the parasite *Plasmodium falciparum* and homodimers of nostocarboline with potent antiplasmodial activity were synthesized [10, 11]. The mode of action of nostocarboline against *Plasmodium* was speculated to be correlated to its algicidal activity, which is unclear at present. Dimerization of cationic natural products often leads to enhanced biological activity and the resulting bisbicationic compounds are clinically used as antimicrobial agents [10]. Here, we report on the antibacterial activity and spectrum of a series of nostocarboline dimers as well as on investigations on the mode of action and selectivity of these compounds. Since dimerization of nostocarboline leads to bisbicationic compounds, we chose the membrane-acting bisbiguanide antiseptic chlorhexidine as a comparator. In addition, ciprofloxacin was used as a reference antibiotic and as an example of an antibacterial with a distinct intracellular target.

Material and Methods

Antibacterial Compounds

The synthesis of nostocarboline and its homodimers has been reported elsewhere [10, 11].

Ciprofloxacin and chlorhexidine were obtained from Sigma-Aldrich.

Microbial Strains

Strains used in this study were from the strain collection of Actelion Pharmaceuticals Ltd. Most of the clinical isolates were collected between 2001 and 2006 from various European and US hospitals. Reference strains were obtained from the American Type Culture Collection (ATCC). All strains were stored at –80°C in 20% glycerol cultures. A primary panel of quality control strains from the ATCC as well as selected clinical isolates were used (table 1). Selected compounds were also tested against a panel of 20 MRSA clinical isolates. Genetically modified *Escherichia coli* strains were constructed in-house and used in reporter assays (see below). The stress response due to either DNA damage or damage to the cell envelope was assayed in strains derived from MG1655 (CGSC 7740). The *luxCDABE* operon of *Photorhabdus luminescens* [12] was linked to the trimethoprim resistance marker *dfrA1* from ColE1::Tn7 (DSMZ 3872) and then integrated in single copy in the chromosome using standard genetic procedures at the *lac* locus generating a Δ*lacZ*::*(luxCDABE-dfrA1)* strain. The *sulA* or *degP* promoter region were PCR-amplified from the chromosome and fused by overlapping PCR to the gentamicin resistance cassette *aph*(2')-*aac*(6') from *Enterococcus faecalis* (ATCC 51299). Finally, the promoter region upstream of *luxC* was re-

placed with either the *sulA* or *degP* promoter following the procedure by Datsenko and Wanner [13]. The reporter strains were also made more susceptible to the effect of small molecules by introducing *tolC* [14] or *rfaC* disruptions (Actelion strain collection) using generalized P1 transduction [15] resulting in strains Ec2376 [Δ*tolC* Δ*rfaC* Δ*lacZ*::*(sulA'-'luxCDABE)*] and Ec2297 [Δ*tolC* Δ*lacZ*::*(degP'-'luxCDABE)*]. An isogenic Δ*tolC* Δ*rfaC* mutant (Ec2085) was used for the determination of minimal inhibitory concentrations (MICs).

Determination of MICs

Stock solutions of compounds were made in DMSO (5 mg/ml). MICs were determined by a broth microdilution assay following the guidelines of the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) [16]. Cation-adjusted Mueller-Hinton II broth (CA-MHB) was used as the basic test medium (Becton Dickinson and Co., Sparks, Md., USA), which was supplemented as appropriate for fastidious organisms [16].

Time-Kill Assays

Exponential-phase *S. aureus* ATCC 29213 was diluted in fresh CA-MHB at approximately 10^7 colony-forming units (cfu) per ml. After 1 h of incubation with shaking at 37°C, compounds were added at a concentration matching 4× and 16× the MIC. After further incubation for 1 and 4 h, viable bacterial counts were determined by plating appropriate dilutions on Mueller-Hinton agar plates (Becton Dickinson and Co., Sparks, Md., USA), followed by determination of the cfu after 24 h incubation at 37°C.

ATP Release Assay

Leakage of cellular ATP was measured to investigate the effect of compounds on membrane integrity [17]. Briefly, mid-exponential-phase *S. aureus* cells were centrifuged and resuspended in PBS containing antibacterial compounds. After incubation for 20 min at 37°C with shaking, suspensions were centrifuged again and the ATP concentration in the supernatant was determined using the BacTiter-Glo microbial cell viability assay kit (Promega, Madison, Wisc., USA). Untreated cultures served as the negative control while 0.1% (w/w) SDS was used as the positive (membranolytic) control.

Stress Response Assays

For the stress response assays, plates (Costar 3882, Corning, Mass., USA) were prepared as described for MIC assays, except that 10^8 cells/ml were added to the compounds. Luminescence was read after 2 h of incubation at 30°C with the compounds and normalized to the cells present in the wells (nRLU). The induction of the stress response was expressed as the ratio of nRLU measured with compounds and nRLU with 1% DMSO.

Cytotoxicity

Toxicity to eukaryotic cells was measured by using CHO-K1 cells and an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] reduction assay (Promega, Madison, Wisc., USA). Briefly, 60,000 cells/well were grown in flat 96-well plates for 4 h. Compounds were added to the cells and incubated at 37°C. Cell viability was measured after 24 h by adding MTS Reagent (10%). After 2 h, optical density (OD) was measured at 490 nm with 630 nm as the reference wavelength using a spectrophotometer.

Table 2. In vitro activity against a set of 20 clinical isolates of MRSA (MICs in mg/l)

Compound	MIC 50%	MIC 90%	Range
NCD9	0.25	2	0.125–2
NCD10	0.25	0.5	0.125–0.5
Ciprofloxacin	32	>32	0.06 to >32
Chlorhexidine	0.125	0.5	0.06–0.5

Results

Ten nostocarboline dimers (fig. 1), synthesized with the aim of evaluating linker size and antibacterial properties, were tested against a panel of bacterial and yeast strains (table 1). Monomeric nostocarboline, the biscationic antiseptic chlorhexidine and the fluoroquinolone antibiotic ciprofloxacin were used as comparators. While the nostocarboline monomer was not active at the tested concentrations, all dimers showed antimicrobial activity against at least one strain. Potency and antimicrobial spectrum varied with length and structure of the linker used to connect the monomers. Activities of dimers NCD1–NCD8, with linkers of equal or shorter than eight carbon units, were relatively weak. In contrast, NCD9 and NCD10, with linear C10 and C12 linkers, respectively, showed potent activity with MICs of less than 1 mg/l for many strains. Against Gram-positive bacteria *(S. aureus, Enterococcus faecium, Streptococcus pneumoniae),* these compounds were as potent as chlorhexidine, while against Gram-negative bacteria *(Haemophilus influenzae, A. baumannii, E. coli, P. aeruginosa),* they were less active. Potency increased again significantly in a permeable and efflux-deficient *E. coli* mutant (Ec2085). Interestingly, only NCD10 with the longest linker showed potent activity against the yeast strains *Saccharomyces cerevisiae* and *Candida albicans*.

The two most active dimers, NCD9 and NCD10, were further challenged against a collection of 20 recent clinical isolates of MRSA, most of which were resistant to fluoroquinolones (table 2). All strains were susceptible to NCD9 and NCD10 with MICs in the range of 0.125–2 mg/l and 0.125–0.5 mg/l, respectively, similar in potency to chlorhexidine.

Time-dependent antibacterial killing was investigated in an MRSA strain (table 3). At a concentration of 16× the MIC, both NCD9 and NCD10 showed a rapid reduction of viable bacteria (>99% within 4 h), while at 4× the MIC, the effect was only static. A similar concentration-

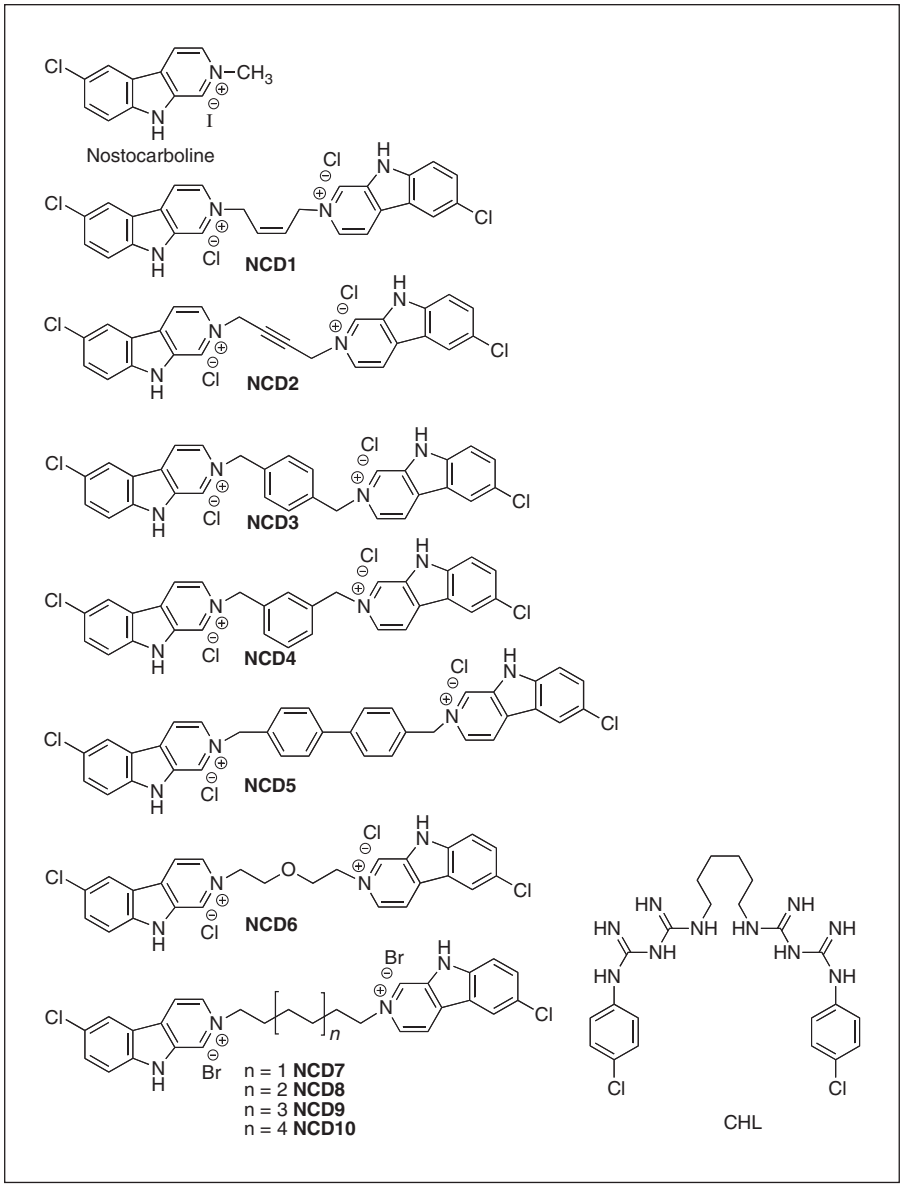

Fig. 1. Chemical structures of nostocarboline, nostocarboline homodimers and chlorhexidine (CHL).

dependent killing effect was also observed with chlorhexidine.

Although the mode of action of chlorhexidine and other polycationic antiseptics is not clear in all details, disruption of the plasma membrane seems to be an important factor for their bactericidal effect [18, 19]. Bacterial membrane damage exhibited by nostocarboline dimers was examined by measuring the leakage of cellular ATP (table 3). Short-term exposure (20 min) of cultures of *S. aureus* to NCD9 and NCD10 at 16× the MIC led to strong ATP release (30–40% of SDS-treated positive control), while at 4× the MIC, only a minor effect was observed compared to the untreated control. Chlorhexidine also exhibited a concentration-dependent ATP leakage, while ciprofloxacin, as expected, had no short-term effect on membrane integrity. The observed concentration-dependent ATP leakage exhibited by NCD9 and NCD10 goes parallel with the rapid concentration-dependent bactericidal effect and suggests that interference with plasma membrane integrity is at least one of the reasons for their bactericidal effect.

To test specificity for bacterial cells, the two most active nostocarboline dimers were tested for cytotoxicity in CHO cells (table 4). Cytotoxic activities of the nostocar-

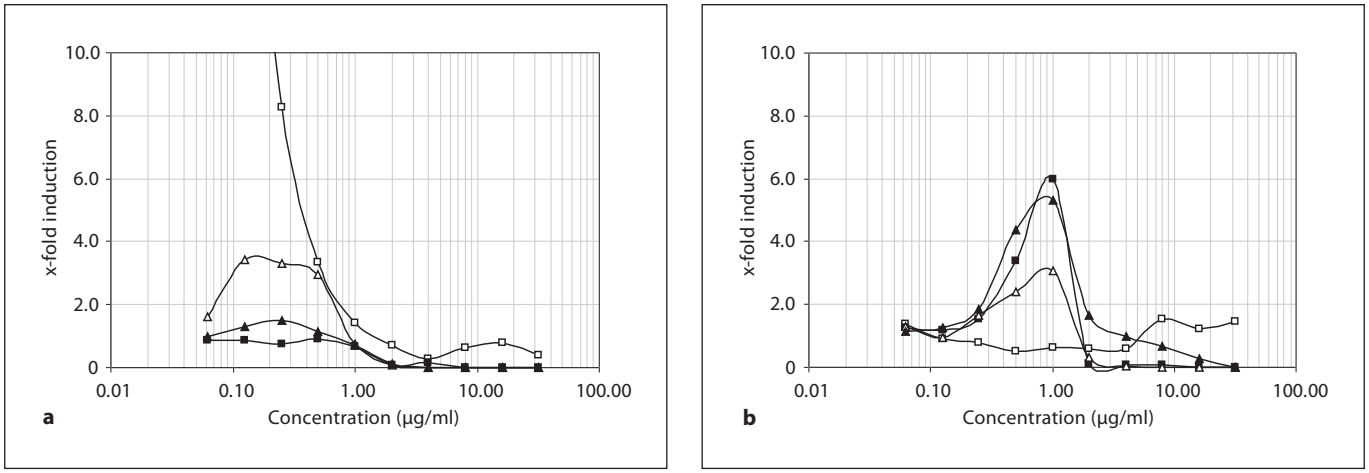

Fig. 2. Induction of stress response reporters by antibacterial compounds ciprofloxacin (CIP; open squares), chlorhexidine (CHL; filled squares), NCD9 (open triangles) and NCD10 (filled triangles). SOS induction measured in Ec2376 [Δ*tolC* Δ*rfaC* Δ*lacZ*::(*sulA*'-'*luxCDABE)*] 2 h after addition to compound (**a**) and induction of cell-envelope stress response in Ec2297 [Δ*tolC* Δ*lacZ*::(*degP*'-'*luxCDABE*-)] (**b**). Three independent experiments were performed and representative data is shown.

Table 3. Bactericidal effect and release of cellular ATP in *S. aureus*

Compound	log CFU change		Release of cellular ATP, % of SDS-lysed control
	1 h	4 h	
Untreated control	+0.84	+2.07	0.5
NCD9 (4× MIC)	–0.11	–0.15	4.4
NCD9 (16× MIC)	–1.31	–2.61	41.1
NCD10 (4× MIC)	+0.25	+0.01	2.6
NCD10 (16× MIC)	–0.96	–3.28	33.2
Ciprofloxacin (4× MIC)	–0.7	–2.8	0.8
Ciprofloxacin (16× MIC)	–1.5	–2.6	0.7
Chlorhexidine (4× MIC)	–0.14	–1.26	2.3
Chlorhexidine (16× MIC)	–0.9	–4.1	9.8

ATP in supernatant was measured by luminescence assay. 0.1% SDS was used as a membranolytic control and relative light units obtained were arbitrarily set as 100%.

Table 4. Cytotoxicities of selected dimers of nostocarboline compared to chlorhexidine

Compound	Cytotoxicity in CHO cells (IC_{50} in mg/l)	
	24 h	48 h
NCD9	>50	23.8
NCD10	25.7	14.2
Chlorhexidine	13.9	1.7
Ciprofloxacin	>50	>50

Data are averages of three determinations.

boline dimers were relatively weak compared to the MIC against *S. aureus* strains. NCD9 was significantly less toxic than NCD10 and both dimers were clearly less toxic than chlorhexidine. This is in agreement with the finding that NCD10 (and chlorhexidine) is active against yeast cells while NCD9 is not. This observation suggests that NCD9 with a C10 linker has better selectivity for prokaryotic cells than NCD10 with a C12 spacer.

The mechanism of action of NCD9 and NCD10 was also tested in *E. coli* reporter strains lacking the multidrug resistance TolC pump. The *sulA* fusion strain Ec2376 was used to detect induction of the SOS response following DNA damage as described previously [20]. Additionally, the DegP reporter strain Ec2297 was used to monitor the cell envelope stress response [21, 22]. Only NCD9 induces an SOS response, albeit at a much weaker degree than ciprofloxacin (fig. 2a), whereas NCD10 (and, more weakly, NCD9) elicited cell envelope stress in a similar fashion as chlorhexidine (fig. 2b).

Conclusion

Cationic antimicrobials are widely used as disinfectants and preservatives and in topical antimicrobial products [18]. On the other hand, cationic antimicrobial peptide antibiotics, such as the polymyxins, have also proved to be useful for systemic antibacterial treatment and colistin (polymyxin E) is considered as a last resort drug in the treatment of infections involving some multidrug-resistant bacteria [23]. While the mechanism of antibacterial action is not elucidated in all details, early damage to the cytoplasmic membrane leading to lethal effects is considered to be the common theme for this class of compounds [19]. One or more strong positive charges, together with a hydrophobic region, are the basic structural requirements for membrane interaction. However, there is still controversy whether other interactions including intracellular targets are involved, leading to the final lethal effect [24]. Furthermore, the mechanism of action may vary considerably within this class of compounds [25, 26]. The low propensity for resistance development due to multiple target sites, the rapid bactericidal effect and the potentially broad antimicrobial spectrum are properties that make this antimicrobial class attractive for future treatment of bacterial infections, including bacteria resistant to all currently available antibiotics [26, 27]. However, there are several problems associated with the development of such compounds for (systemic) therapeutic use, e.g. the lack of specificity and consequently potentially toxic side effects, poor bioavailability and stability, and lack of systemic in vivo activity [26–28]. In this report, we show that some homodimeric compounds (biscarbolines) created from the natural alkaloid nostocarboline display potent antibacterial activity. Structurally, the nostocarboline dimers can be compared to bisbiguanide antiseptics, such as chlorhexidine, i.e. bipolar positive charges separated by a hydrophobic spacer region. Antimicrobial potencies and spectra of the dimers varied with length of the linker used to connect the two carbolinium moieties: the derivatives with linear C10- and C12-linkers were highly potent against Gram-positive bacteria while compounds with a shorter linker had only modest activities and the nostocarboline monomer was essentially inactive. The length of the hydrophobic spacer is also important for antibacterial activity of the bisguanides and a six carbon bridge seems to be ideal for activity [19]. In contrast, quaternary ammonium antiseptics are monocationic with a long hydrophobic tail. However, these compounds show dimerization in solution and, consequently, the dimeric structure may be responsible for antibacterial activity.

The two most active nostocarboline dimers, NCD9 and NCD10, showed potent Gram-positive activity especially against *S. aureus*, including multi-resistant clinical MRSA isolates. On the other hand, activities against Gram-negative bacteria were relatively weak, in particular against *P. aeruginosa*. Poor penetration through the outer membrane and/or intensive efflux are possible reasons for that finding. This notion is underscored by the strongly increased activities in *E. coli* efflux k.o. and outer membrane permeability mutants. Interestingly, NCD9 with a C10 linker seems to act in a more specific way than NCD10 with a C12 spacer as indicated by lower cytotoxicity and absence of activity against yeast strains. It is tempting to speculate that selectivity to bacteria versus eukaryotic cells may depend on the length of the linker. Preliminary investigations addressing the mode of action in bacteria suggest that the nostocarboline dimers act on Gram-positive bacteria in a similar way as chlorhexidine, i.e. by concentration-dependent membrane damage leading to leakage of intracellular material followed by rapid cell death. In reporter strains of efflux-deficient *E. coli,* we found induction of DegP response pathway, consistent with the data above suggesting damage to the cell envelope including the proton motive force. It has been shown that the DegP expression is regulated by the CpxR regulator and depends on σE, which in turn can sense misfolded proteins in extracellular compartments or defects in the biogenesis of outer membrane proteins, respectively [21]. It has also been shown that membrane-active compounds that abolish the proton motive force induced the DegP-dependent stress response pathway [22]. Therefore, it was quite intriguing to find that NCD9, but not NCD10, also induced the SOS response, as this would suggest that the compound could interact with a cytoplasmic target as well.

Nostocarboline and its dimers have also shown potent anti-plasmodial activity [10], but the structure activity relationship is different from the one observed in this study. In particular, nostocarboline was determined to possess submicromolar activity against *P. falciparum*. The dimers showed consistent activity below 100 nM for dimers NCD6-NCD10 against this parasite. This different structure/activity relationship suggests that different modes of action are involved.

The results presented in this report indicate the potential of this new class of cationic antimicrobial compounds for the design of potent and selective antibacterials with low cytotoxicity. Further studies should involve the evaluation of antibacterial activity and toxicity in in vivo models and the design of new compounds with improved Gram-negative activity while optimizing selectivity.

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Disclosure Statement

H.H.L., D.R., P.P., M.G., A.K., D.S. and S.S. are employees of Actelion Pharmaceuticals Ltd., Allschwil, Switzerland and own/have owned stocks or shares of Actelion.

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