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Article

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Alkyl-guanidine compounds as potent broad-spectrum antibacterial agents: chemical library extension and biological characterization.

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KEYWORDS

Antibacterial; broad-spectrum; dimer; guanidine; polycationic; bactericidal; drug-resistant bacteria; haemolytic assay.

ABSTRACT

Nowadays, the increasing of multi-drug-resistant pathogenic bacteria represents a serious threat to public health and the lack of new antibiotics is becoming a global emergency. Therefore, the research in the antibacterial field is urgently needed to expand the currently available arsenal of drugs. We have recently reported an alkyl-guanidine derivative (**2**), characterized by a symmetrical dimeric structure, as a good candidate for further developments, with a high antibacterial activity against both Gram-positive and Gram-negative strains. In this study, starting from its chemical scaffold, we synthesized a small library of analogues. Moreover, biological and *in vitro* pharmacokinetic characterizations were conducted on some selected derivatives, revealing notable properties: broad-spectrum profile, activity against resistant clinical isolates and appreciable aqueous solubility. Interestingly, **2** seems neither to select for resistant strains nor to macroscopically alter the membranes, but further studies are required to determine the mode of action.

INTRODUCTION

The availability of effective antimicrobial chemotherapies is critical in many areas of medicine, being fundamental to treat or prevent nosocomial infections, especially during invasive surgery, in

cancer chemotherapy and in elderly or immune-compromised patient treatments. However, the rise of antibiotic resistance, in part due to overuse or misuse of antibiotics, is now compromising the effectiveness of such common and relatively cheap drugs.[1] Today antibiotic-resistant bacteria accounts for 700,000 infections worldwide, and are responsible for over 25,000 deaths per year in the European Union only, which World Health Organization (WHO) identified as a major public health issue of the 21st century.[2,3]

Antibiotic resistance has been developing worldwide in major clinical bacterial pathogens causing common hospital-acquired infections, such as pneumonia, or urinary tract and bloodstream infections. Critical issues are represented by methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), or multidrug-resistant Gram-negative bacteria, including *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* or *Acinetobacter baumannii*.[4] The WHO Global Report on Surveillance of Antimicrobial Resistance 2014[5] reported that Gram-negative pathogens such as *Escherichia coli* and *Klebsiella pneumoniae* have developed resistance to more than 50% of commonly used antibacterial drugs.[6–10] Concomitantly, the investment in the discovery and development of novel antibacterials were largely considered unattractive for big pharma,[11] since unlike chronic pathologies, drugs against infectious diseases were used for therapeutic treatments that take a limited period. This lack of interest concerns not only the developing countries but also the developed ones.[12] This limited the introduction of new antibiotics in the clinical practice especially those active against Gram-negative bacteria. Carbapenems and, subsequently, colistin became essential last resort drug for the treatment of infections caused by multi-drug resistant bacteria but are gradually losing their efficacy, as we are now facing the emergence of extensively- or pan-drug resistant isolates. Only very recently several new antibacterial agents (mainly new β-lactam-β-lactamase inhibitor combinations such as ceftazidime-avibactam or meropenem-vaborbactam) were introduced in the clinical practice. However, not only resistance to these new generation drugs have already been detected, but they are not active against all antibiotic-resistant bacteria (such as for instance, New Delhi Metallo-β-lactamase-1 (NDM-1)-producing *K. pneumoniae*).[13–15]

The majority of current drugs have the same well-understood target. For this reason, there is an urgent need to expand our currently available arsenal of antibiotics, possibly with some new classes of compounds showing a novel mechanism of action.[16] Only a few drugs showing the latter property and active on Gram-negative pathogens are actually in the stage of clinical development and include murepavadin and ACHN-745, which both target the bacterial lipopolysaccharide biosynthesis/transport machinery.

In the literature polyguanidines have attracted considerable attention for their antibacterial activity.[16,17] The supposed mechanism of action of guanidine compounds is based on the strong electrostatic interaction between their positive charges and the electronegative envelope of bacteria. This immediate binding to the components of the cytoplasmic membrane or the cell wall leads to the loss of bio-functions of phospholipids or to the disruption of the membrane and eventually the cell death.[18–21]

Our research group has investigated a series of alkyl-guanidines as potent antibacterial agents against both Gram-positive and Gram-negative clinically relevant bacterial pathogens.[22] In our recent study[23] on dimeric and trimeric isomers of a linear alkyl-guanidine derivative (compound **1**, **Figure 1**)[23] we identified compound **2** as the most potent derivative, with Minimum Inhibitory Concentration (MIC) values ranging from 1 to 8 μg/mL on representative Gram-positive and Gram-negative bacterial strains.

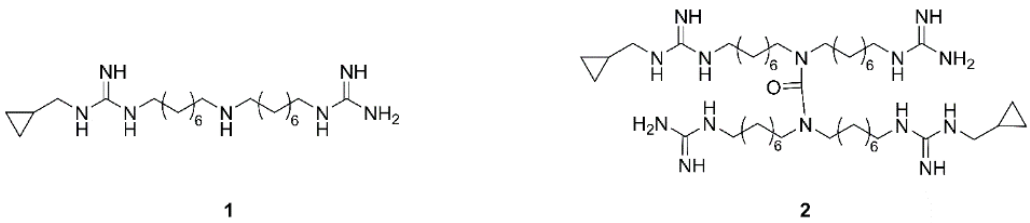


Figure 1. Structures of monomer (**1**) and its dimeric derivative (**2**).

Encouraged by these good results obtained we decided to better investigate the chemical class of dimers, structurally analogous to compound **2** (**Figure 1**) in order to obtain a small chemical library that would allow us to perform some preliminary structure-activity relationship (SAR) considerations.

With this purpose, we designed some possible modifications by changing the length of the alkyl chains and the substituent on the guanidine moieties.

RESULTS

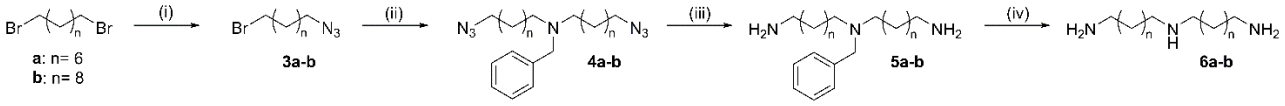
Chemistry.

The design of the new class of dimeric antibacterial agents consisted in the connection between two (bis)guanidinic building blocks: a monomer and an appropriate carbamoyl derivative, as reported in our previous work.[23] Our first synthetic strategy started from a commercial product, namely Bis(octamethylene)triamine, that was subsequently bis-guanylated with the appropriate guanylating agents, providing the desired monomers.[22] Unfortunately this reagent was no longer commercially available, so an alternative synthetic approach that bypassed its use was developed.[23]

Aimed to the generation of a new series of dimers, a more efficient and more convenient synthetic pathway was set up, in order to obtain an important intermediate, the triamine derivative (**Scheme 1**), with few and high yielded steps. However, its preparation represented a great challenge and it was not trivial because, despite its simple chemical structure, this compound is highly polar for the presence of a secondary and two primary amino groups that make the purification step very difficult. Two previously described strategies for the synthesis of bis(8-aminooctyl)amine (**6a**) start both from 1,8-diaminooctane and use Raney Nickel[23] or nitric acid at high temperature,[24] respectively, to perform the dimerization of the starting material. Unfortunately, they are hardly practicable in an academic laboratory, also for economic and sustainability reasons. Taking into account all the disadvantages of these pathways, a new cheap and convenient method was set up in our research group. In this way, the whole synthetic process became more accessible and versatile. It is based on a straightforward four-steps route and represents a modification of a recently published procedure.[25] We preferred azido groups, in place of the cyano moiety used by Zhang et al., in order to insert the terminal amino functions. We thus obtained the final product under milder conditions, higher yields and avoiding the use of a high amount of dangerous sodium cyanide and expensive Raney Nickel,

used as a catalyst for cyano groups reduction. The development of this synthetic approach is fairly significant considering its versatility. On confirmation of that, it was easily readapted for the 10 C atoms derivative. A bis-alkylation of benzylamine with bromoazide **3a-b** (as already described in our previous work)[23] was performed to obtain diazide derivatives **4a-b**. Reduction of the azide moieties was accomplished by Staudinger reaction, giving compounds **5a-b**. This very mild protocol involves the formation of a triphenyl-phosphazide intermediate, by treatment with triphenylphosphine, followed by loss of nitrogen gas with the formation of an iminophosphorane. Aqueous work up leads to the amine **5a-b** and the very stable phosphine oxide. [26] Then, a debenzylation of the central amine through an acid-catalyzed hydrogenation over Palladium followed, affording the triamines **6a-b**,[25] as shown in **Scheme 1**.

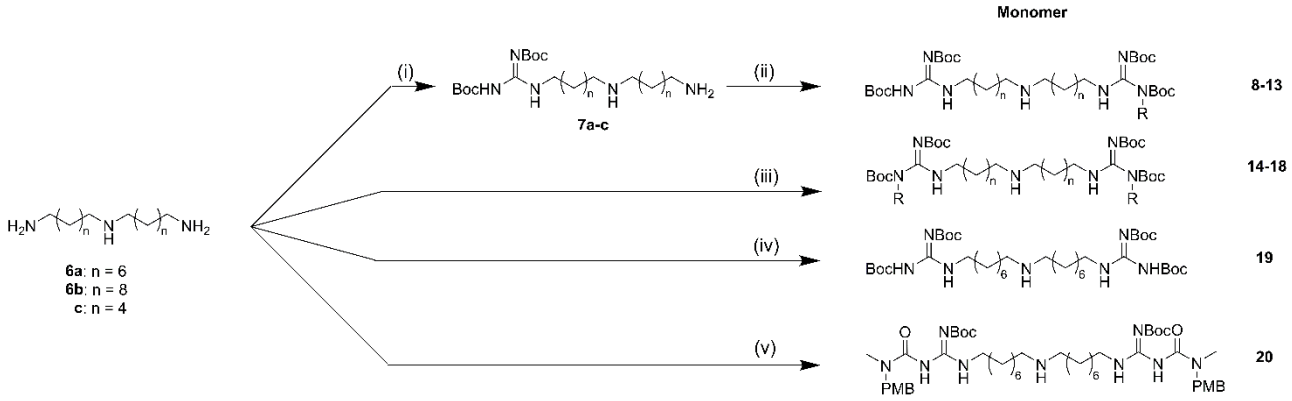
Scheme 1. Synthesis of triamines 6a-b.[a]



[a]*Reagents and conditions*: (i) NaN_3, DMF, 50 °C, 16 h; (ii) Benzylamine, KI, K_2CO_3, 1-BuOH, 115 °C, 20 h; (iii) PPh_3, H_2O, THF, r. t., 16 h; (iv) H_2, Pd/C, AcOH, *i*-PrOH, r. t., 16 h. Where **a** indicates compounds with n = 6 and **b** indicates compounds with n = 8.

The obtained triamines **6a-b** or the commercial bis(hexamethylene)triamine were thus guanylated as shown in **Scheme 2**: the appropriate guanylating agents (**21a-d** or **24**) were used in the right stoichiometric ratio, in order to obtain monomers with the alkyl substitution on one (i, ii), two (iii) or no (iv) guanidine functions or an *N*-methyl amidinourea moiety (v in **Scheme 2**). The synthesis and characterization of *N,N'*-Di-Boc-*N*-alkyl-pyrazole-1-carboxamidine **21a-d** have been already described,[22,27] but reported again, together with the procedure for 24, in *Supporting Information* **Scheme S 1** and **S 2** respectively.

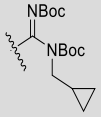
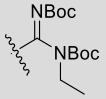
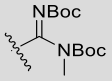
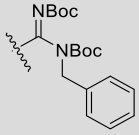
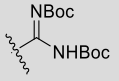
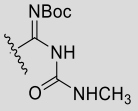
Scheme 2. Synthesis of monomers.[a]



[a]R*eagents and conditions*: (i) 1,3-Bis(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea, THF/MeOH, 50 °C, 16 h; (ii) appropriate guanylating agent **21a-d**), DIPEA, THF, r.t., 16 h; (iii) *N,N'*-Di-Boc-*N*-alkyl-pyrazole-1-carboxamidine, THF/MeOH, r.t., 16 h; (iv) 1,3-Bis(tert-butoxycarbonyl)-2-methyl-2-thiopseudourea, THF/MeOH, r. t., 16 h; (v) 23 , DIPEA, THF/MeOH, r. t., 16 h.

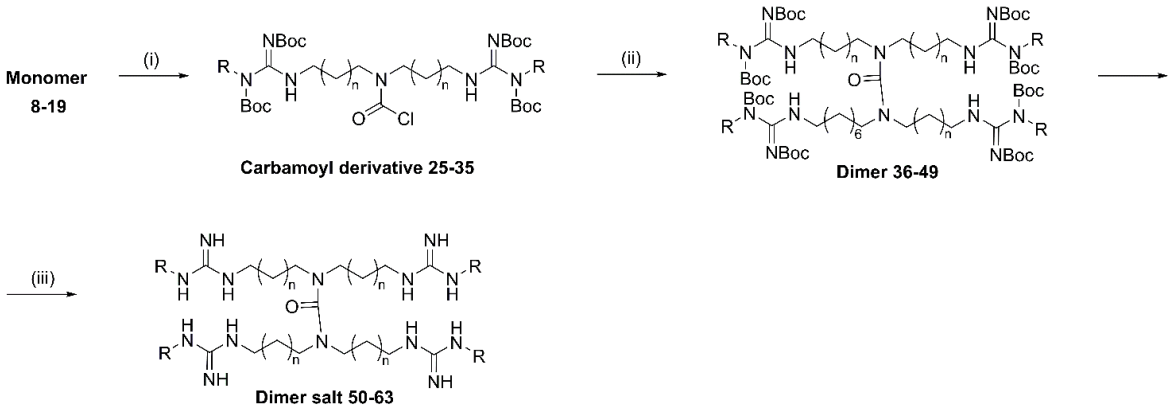
A coding system to identify monomers, carbamoyl derivatives and dimers is adopted, each monomer being named with an alphanumeric code consisting in three parts. The first number (6, 8 or 10) indicates the number of C atoms in the alkyl chains, while the following two letters identify the substituents (legend shown in **Table 1**) present on the two guanidine moieties of the monomer. The carbamoyl derivatives are identified through the code of the corresponding monomer with an asterisk; the dimers with the respective codes of their constituting building blocks separated by a slash and the deprotected final compounds followed by the letter "s" (for "salt") after the dimeric code. In *Supporting Information*, **Table S 1**, the correspondence between each compound and its code is shown, e.g., compound **2** is identified with the code: 8CH*/8CH s.

Table 1. Legend of the symbol identifying each substituent.

Symbol	C	E	M	B	H	U
R						

The resulting monomers were converted into the corresponding carbamoyl derivatives, the dimerization occurred and the dimers were deprotected under acidic conditions, as previously described in Zamperini et al[23] and reported in **Scheme 3**.

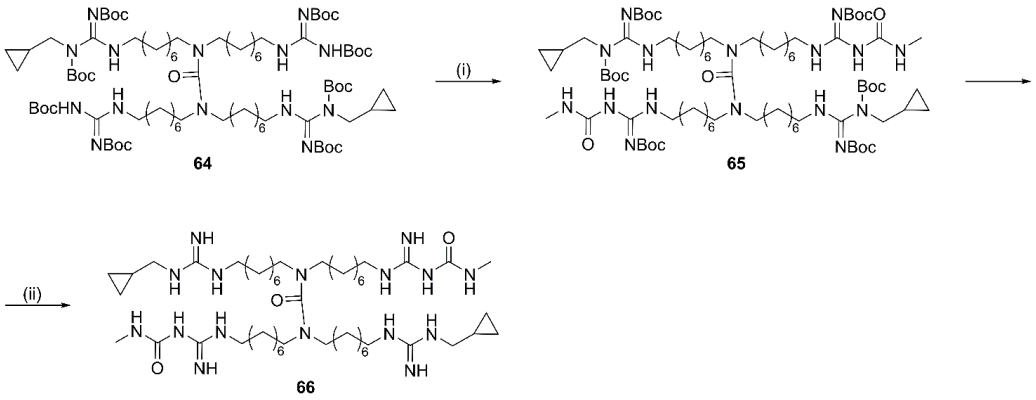
Scheme 3. Synthesis of dimers.[a]



[a]*Reagents and conditions*: (i) Triphosgene, DIPEA, dry THF, 0 °C to r.t., 1 h; (ii) appropriate monomer 8-20, DIPEA, NaI, dry DCM, 40 °C, 48 h; (iii) TFA, dry DCM, r. t.; 7 h.

Compound **66** with *N*-methyl amidinourea moieties on the two terminals was synthesized as reported in **Scheme 4**. The reaction involves the carbonyl group of the Boc on the two non-substituted guanidines and the methylamine as the nucleophilic reagent, starting from the intermediate **64** (Boc-protected compound **2**), already described by Zamperini et al.[23]

Scheme 4. Synthesis of amidinourea compound **66**. [a]

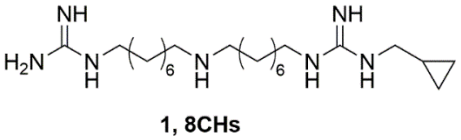


[a]*Reagents and conditions:* (i) CH_3NH_2 aq., DIPEA, THF, 80 °C, 24 h; (ii) TFA 10%, dry DCM, r.t., 7 h.

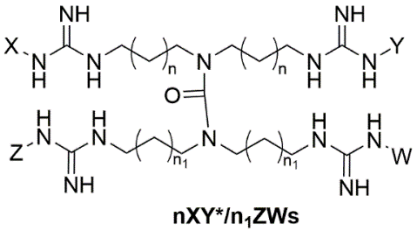
Evaluation of antibacterial activity of new substituted alkyl-guanidines.

The antibacterial activity of the newly synthesized compounds **50-63**, **66** and the intermediate **45** was evaluated on a panel of 8 representative organisms, including four Gram-positive and four Gram-negative type strains (as reported in **Table 2**). Although the compounds synthesized in this series were not enough to conduct an exhaustive SAR analysis, data reported in **Table 2** allowed us to do some preliminary but interesting observations, in terms of the length of the alkyl chains, the type and the number of the substituents on the guanidine functions and other significant modifications.

Table 2. MICs of the dimer chemical library and control antibiotics (colistin, vancomycin and daptomycin) on representative Gram-positive and Gram-negative bacteria.[a]



1, 8CHs



nXY*/n$_1$ZWs

n, n$_1$: 4, 6, 8

X, Y, Z, W:

C: cyclopropylmethyl;

H: hydrogen; **E**: ethyl;

M: methyl; **B**: benzyl;

U: $CONHCH_3$

*: carbamoyl intermediate

s: trifluoroacetate salt

Cpd	Cpd Code	*B. subtilis* ATCC 6633	*E. faecalis* ATCC 19433	*S. aureus* ATCC 25923 SEP	*S. pyogenes* ATCC 12344	*A. baumannii* ATCC 17978	*E. coli* CCUG^T	*K. pneumoniae* ATCC 13833	*P. aeruginosa* ATCC 27853
1	**8CHs**	64	>64	>64	>64	>64	64	>64	>64
2	**8CH*/8CHs**	0.5	2	2	1	8	2	2	8
50	**10CH*/10CHs**	2	4	8	4	64	16	8	64
51	**6CH*/6CHs**	4	32	4	2	128	16	32	256
52	**8EH*/8EHs**	1	1	1	0.5	16	2	2	16
53	**8MH*/8MHs**	0.5	1	0.5	0.5	8	1	4	8
54	**8BH*/8BHs**	2	2	2	2	8	4	2	16
55	**8CC*/8CCs**	8	4	16	2	8	16	4	64
56	**8EE*/8EEs**	0.5	1	0.5	0.25	16	2	4	32
57	**8BB*/8BBs**	4	4	8	1	16	4	4	8
58	**8HH*/8HHs**	32	2	2	1	16	4	32	32
59	**8CC*/8HHs**	2	8	8	4	16	4	4	16
60	**6CC*/6CHs**	4	16	4	2	64	8	16	128
61	**6EE*/6EHs**	16	64	32	4	256	64	128	>256
62	**8CC*/6CCs**	4	8	2	1	64	4	4	128
63	**8CC*/8UUs**	2	4	2	2	8	4	4	16
66	**8CU/8CUs**	8	16	8	16	16	8	8	32
Colistin		-	-	-	-	1	0.5	0.5	0.5
Vancomycin		0.5	1	1	0.5	-	-	-	-

Daptomycin	1	1	0.5	0.12	-	-	-	-

[a] - : not determined. MICs (μg/mL) are expressed as median values calculated from experiments performed at least in triplicate. Data for compounds **1** and **2** are from Zamperini et al.[23]

With regard to the alkyl chains, we increased the length of the alkyl chains to 10 C atoms or decreased it to 6 C atoms, synthesizing homologous of **2** (**50** and **51**, respectively). Observing the MIC values of compounds **2, 50** and **51**, we could identify 8 as the optimal number of carbons in the alkyl chains. In fact, whereas the increasing of two carbon atoms (**50**) determined a modest reduction of the activity, the decreasing to 6 carbon atoms (**51**) resulted in a complete loss of antibacterial potency, in particular against Gram-negative strains, where high MIC values were often recorded.

Then, the cyclopropylmethyl substituents of **2** were replaced with ethyl, methyl or benzyl groups (compounds **52, 53** and **54** respectively): ethyl and methyl groups were chosen as substituents because of their smaller alkyl size whereas benzyl moiety was inserted to hinder and make more lipophilic the *N*-termination of the guanidine moiety. These compounds retain the activity trend of **2**. Thus, the nature of the alkyl substituent did not appear to affect considerably the antibacterial profile. In particular, by comparing the MIC values of these compounds, we observed that in general the activities of these compounds were a bit worse than the one of **2**, but the difference was never higher than one or two dilutions. The activity of **52** and **53** against the Gram-positive *Staphylococcus aureus* resulted to be higher than the one of **2**, as also seen for the activity of **54** against the Gram-negative *Burkholderia cepacea*. Again, we detected a significant improvement of activity for **53** and **54** against the Gram-negative *Acinetobacter faecalis* with a MIC value lower than 0.125 μg/mL.

Moreover, in order to understand the influence of the number of guanidine substitutions on the antibacterial properties and starting from **2**, we synthesized compounds characterized by four alkyl substituents, such as cyclopropylmethyl (**55**), ethyl (**56**) or benzyl (**57**) groups, or no one (**58**) and an analogous compound of **2** with a symmetry axis instead of a plane, yielding a dimer composed by two different monomers (**59**). Comparing their MIC values, we observed some interesting SARs: both

55 and **58** (with the cyclopropylmethyl substituent on all or no guanidine functions, respectively) showed a moderate activity. Compound **59** was synthesized through the dimerization of two symmetric monomers, one completely substituted, while the other no, to evaluate if this structural inversion would affect the activity. Despite our expectations, the comparison between the antimicrobial profiles of **2** and **59** revealed that the inversion compromised the activity of the hit compound, although their superficial charge was the same. Moreover, it was evident that **57** (with four benzyl substituents) was only a little more active than **55** (with four cyclopropylmethyl substituents) and **56** (with four ethyl substituents), while **58** was less active against almost all the tested bacterial strains. The obtained data highlighted that the increasing of the number or lipophilicity of substituents did not lead to any improvement of antibacterial activity, thus leading to the conclusion that the optimal number of substitutions was two.

Compounds with three, instead of two, substituted guanidines (**60** and **61**) were also evaluated and despite maintaining a significant antibacterial activity on Gram-positive pathogens (MIC values ranging from 2 to 64 μg/mL), compound **61** (three ethyl substituents) showed a very poor activity on Gram-negatives.

The hybrid dimer **62**, characterized by monomers with different length of carbon chains, exhibited, when compared to compound **2** a narrower spectrum of activity on Gram-negatives, with a higher activity (MIC range, 8-16 μg/mL) on *Enterobacteriaceae*.

Furthermore, we assumed that a decreasing in pKa value could improve the selectivity towards bacterial cells, so we designed two compounds with a lower predicted pKa characterized by the transformation of the guanidine functions into *N*-methyl amidinoureas (**63** and **66**). This required the development of a new synthetic strategy. These two dimers showed to be active against almost all tested strains, even if a decrease of the antibacterial potency was found in particular against the Gram-negative *Acinetobacter baumannii* and *Pseudomonas aeruginosa.*

Then, in order to understand if a Boc-cleavage occurred in bacterial cells, the intermediate **45** was also tested. However, it was found to be inactive with MIC values ≥ 256 μg/mL for all the tested bacterial strains.

Little chemical modifications were performed furnishing a library of compounds active against almost all the tested bacterial strains, showing in some cases a broad-spectrum activity. Eventually, we did not observe a significant change of the antibacterial profile and the hit compound **2** still remains the most potent compound, except for *S. aureus* ATCC 25923 and *A. faecalis* 424/98, on which **53** shows better MIC values.

Further biological characterization of selected compounds.

The antibacterial properties of selected analogues of compound **2** were further investigated on recent antibiotic-resistant clinical isolates, including pandrug-resistant *Klebsiella pneumoniae* (**Table 3**). Most interestingly, all compounds retained much of their activity on specific pathogens regardless of the resistance phenotype, and particularly colistin-resistant *K. pneumoniae*. This indicates that, despite the polycationic nature of both colistin (a polymyxin antibacterial peptide) and our alkyl-guanidines, the latter likely show a different mechanism of action, and are not susceptible to alterations of the Lipopolysaccharide (LPS) layer determining a decrease of the negative net charge on the bacterial cell surface.[28]

Table 3. MICs of selected compounds on Gram-negative antibiotic-resistant clinical isolates.[a]

Cpd	**2**	**52**	**53**	**54**	**55**	**56**
Cpd Code	**8CH*/8CHs**	**8EH*/8EHs**	**8MH*/8MHs**	**8BH*/8BHs**	**8CC*/8CCs**	**8EE*/8EEs**
***A. baumannii* AC-54/97**	2	-	4	-	8	8
***B. cepacia* SI-R2**	16	32	32	16	32	32
***E. cloacae* VA-417/02**	1	4	4	4	4	2
K. pneumoniae* SI-081	2	4	8	8	4	8
***S. maltophilia* 634/08**	16	16	16	8	8	32

[a] -: not determined; * clinical isolate with pandrug-resistant phenotype. MICs (μg/mL) are expressed as median values calculated from experiments performed at least in triplicate.

The Minimal Bactericidal Concentration (MBC) values of most active compounds, although measured only for *E. coli* and *A. baumannii*, resulted identical to that of the MIC, indicating a strong bactericidal activity of these compounds (data not shown). This was further confirmed by a time-kill curve analysis, in which the bactericidal activity of dimer **2** (4 μg /mL, 2 x MIC) was shown to be ≥4 log10 in one hour and thus superior to that of colistin (*Supporting Information*, **Figure S 1**).

Furthermore, in order to estimate the possible effect of plasma protein binding (PPB) on antibacterial activity of alkyl-guanidine derivatives, compound **2** was tested on *E. coli* MG1655 in Mueller Hinton Broth (MHB) supplemented with 10% complement-free human serum (HS). *E. coli* MG1655 was completely inhibited by 2 μg/mL of compound **2** (MIC; **Table S 2** in *Supporting Information*) while 1 μg/mL did not affect the growth profile (1/2 MIC; **Figure S 2** in *Supporting Information*). The addition of 10% HS to MHB moderately increased its MIC value (2-fold), although

growth *E. coli* MG1655 was clearly retarded in MHB-HS supplemented with 2 μg/mL of compound **2** (MIC; **Figure S 2A** in *Supporting Information*). This result indicates that HS component(s) can reduce, but not abrogate, the efficacy of compound **2**. An obvious candidate is human serum albumin (HSA), the most abundant plasma protein (concentration 35 – 50 g/L,[29] which acts as a trap for a variety of ligands. Since bovine serum albumin (BSA) shares 76% sequence identity with HSA,[30] the susceptibility of *E. coli* MG1655 to compound **2** was tested in MHB supplemented with increasing concentrations of BSA. Remarkably, the addition of BSA (5 to 40 mg/mL) to MHB did not alter the MIC value of compound **2** (**Figure S 2B** in *Supporting Information*), suggesting that other serum proteins (e.g. alpha-1-acid glycoprotein, AGP) could eventually affect the activity of compound **2** in HS. In fact, the binding assays performed on HSA and AGP (reported in ADME paragraph) confirmed a high affinity to this latter, without compromising the antibacterial activity. These results are compatible with a future development of the alkylguanidine derivatives as antibacterial agents. In fact, also if incremented, the observed MIC value for compound **2** on the tested *E. coli* strain in presence of plasma proteins can be considered a reliable prediction of a maintained antibacterial activity *in vivo* studies.

Considering the polycationic nature of our compounds, a potential mechanism of action could be represented by the impairment of the structural integrity of the bacterial cytoplasmic (inner) membrane or the outer membrane of Gram-negative organisms. Thus, the first hypothesis was tested using a simple enzyme-based whole assay, in which the activity of a cytoplasmic enzyme on a poorly permeable chromogenic substrate was evaluated. Indeed, the chromogenic substrate *o*-nitrophenyl-*β*-D-galactoside (ONPG), colourless, releases the yellow *o*-nitrophenol (ONP) upon enzymatic hydrolysis but only reaches the cytoplasm following permeability defects, such as those acting on membrane integrity, such as colistin and other antimicrobial peptides (AMP).[31,32] The assay was conducted for some rationally selected compounds (**1, 2, 50, 55** and **58**) in three bacterial strains characterized by physiological or induced high-level of intracellular production of β-galactosidase. Interestingly, as shown in **Figure 2**, none of the tested compounds, besides sodium dodecyl sulfate

(SDS) which was used as a positive control, apparently induced significant levels of OPNG conversion, indicating that they did not cause a macroscopic alteration of the bacterial membrane. However, this assay does not allow to exclude the possibility that the tested compounds could generate very small, microstructural, damages in the inner membrane.

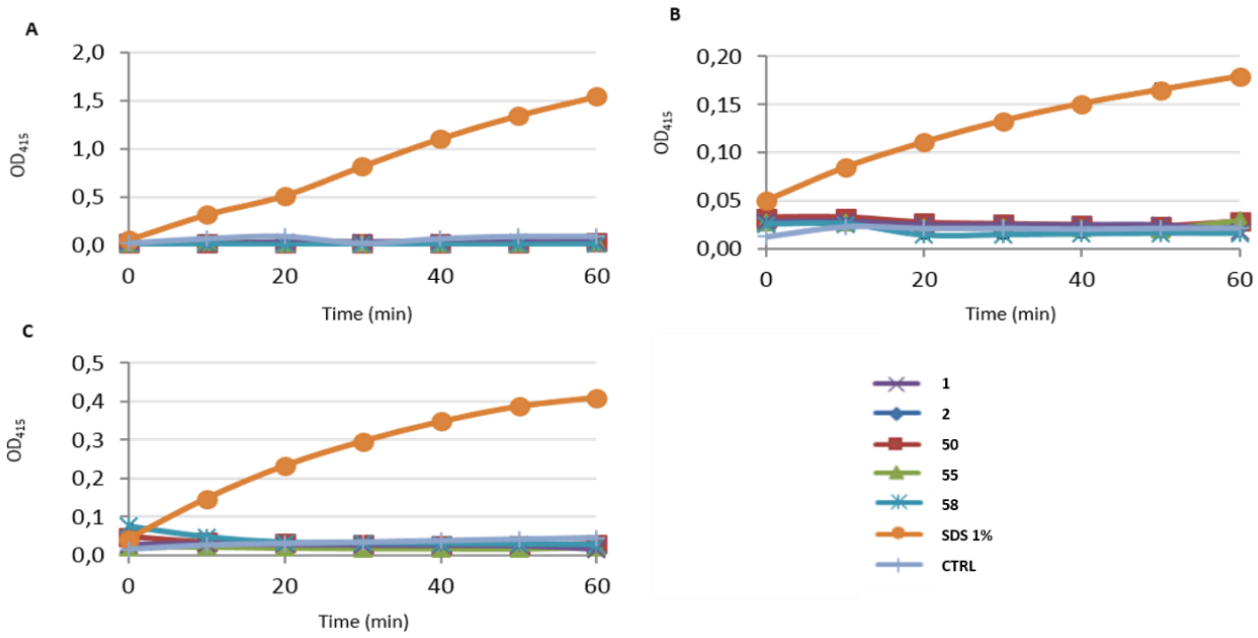


Figure 2. Effect of selected compounds on bacterial permeability. Time-depending production of *o*-nitrophenol from ONPG by A) *Escherichia coli* MG1655; B) *Acinetobacter baumannii* ATCC19606T harboring plasmid pMP220::PrrnB; C) *Pseudomonas aeruginosa* ATCC15692 harboring plasmid pMP220::PrrnB, untreated (negative control) and upon exposure to 256 µg/mL of each compound or 1% of SDS (positive control), as indicated.

The possibility to affect the outer membrane integrity of Gram-negative species was investigated by an additional specific fluorescence assay. Three Gram-negative bacteria were exposed to increasing concentrations of the selected compounds, colistin or dimethylsulfoxide (DMSO), as positive and negative controls, respectively. Then, the addition of 1-*N*-phenylnaphthylamine (NPN) followed. This lipophilic dye weakly fluoresces in aqueous environments but it emits strong fluorescence in the hydrophobic ones, thus enabling to detect a potential outer membrane damage. In

fact, the LPS external layer of the outer membrane of Gram-negative bacteria acts as a permeability barrier to lipophilic substances like NPN. Disruption of outer membrane integrity makes phospholipids of the internal outer membrane layer accessible to NPN, allowing fluorescence emission by the probe.[33] Notably, all the tested compounds caused a dose-dependent increase in the NPN fluorescence emission for all Gram-negative species tested (**Figure 3**). Interestingly, compounds **2, 50** and **55** caused higher fluorescence emission than colistin, whereas any fluorescence was detected in the presence of DMSO. In contrast, compound **1** showed the lowest activity on the outer membrane. These results indicate that all compounds perturb the Gram-negative outer membrane, although to a different extent.

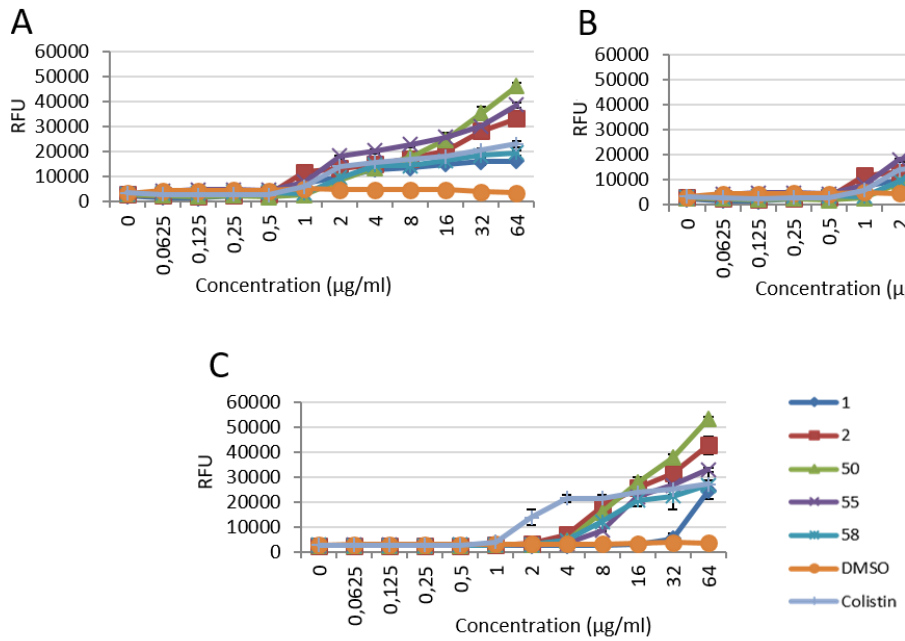


Figure 3. Effect of selected compounds on Gram-negative outer membrane integrity. The uptake of NPN (measured as relative fluorescence unit, RFU) by A) *E. coli* MG1655, B) *A. baumannii* ATCC19606^T and C) *P. aeruginosa* ATCC15692 in the presence of increasing concentrations of each selected compound. Colistin or DMSO were used as positive and negative controls, respectively. Data are the mean ± standard deviation of triplicate experiments.

As another potential mechanism of action, the depolarization of the cytoplasmic membrane (thus affecting the proton motive force) was also investigated. Thus, compound **2** was tested for its ability to depolarize *E. coli* membranes using the fluorescent dye 3,3'-dipropylthiacarbocyanine ($DiSC_3$-5), essentially as described by Breeuwer and Wand.[18,34] In this assay, compound **2** was unable to restore any fluorescence after incubation of bacterial cells at a concentration 2 x MIC (4 μg/mL), thus suggesting that membrane depolarization was likely not the prevalent mechanism accounting for its fast bactericidal activity.

Biofilm is a very complex microbial architecture that allows bacteria to attach to surfaces and to form aggregates, encasing them into a matrix composed of self-produced extracellular polymers. Biofilm makes microorganisms more resistant to antibacterial treatments, harsh environments and human immunity. One of the current strategies to prevent the formation of biofilms is the treatment with AMPs like colistin, polymixins and gramicidin. [35,36] These molecules are able to bind electrostatically the negative charged LPS through their cationic amino acids (lysine and arginine) and to stabilize this binding through hydrophobic interactions between the hydrophobic amino acids of the peptide and fatty acyl chains of LPS.[37] Our compounds, being polycationic and amphiphilic, bear both the polar and nonpolar functions to let us hypothesizing a similar trend. For these reasons, in-depth analyses were performed to evaluate their behaviour toward the biofilm, revealing that they are able neither to inhibit the formation of biofilm (assessed through the crystal violet assay) nor to alter the three-dimensional structure of biofilm (**Table S 2** and **Figures S 3** and **S 4** in *Supporting Information*).

Furthermore, considering that our monomer derivatives are endowed with the inhibition of Maize Poly-Amino Oxidase (mPAO) with a micromolar activity, as already published,[38] we strongly hypothesize a possible involvement of the dimeric derivatives in the bacterial polyamine pathways. Indeed, we supposed that our dimers could be a possible substrate of some poly-amino transporters, present in all kind of cells, also in bacteria ones,[39,40] since they show an amphiphilic structure like the polyamines. Thus, compound **2** was already tested on *E. coli* mutants in which the genes encoding

the main components of polyamine transport were inactivated (mutants *potA*$^-$, *potB*$^-$, *potC*$^-$, *potD*$^-$, *potE*$^-$, *potF*$^-$, *potG*$^-$, *potH*$^-$ and *potI*$^-$ mutants from the Keio library).[41] Comparing to the activity on *E. coli* CCUG^T, the MIC values on the mutant strains were unaffected. This does not completely invalidate our hypothesis because the assay results could be explained in two ways, being the polyamine transport systems different for spermidine (potABCD) and putrescine (potGHIF): dimer **2** could interact with neither potABCD nor potGHIF or could interact with both the systems so when one is abolished by a single mutation, it can bind the other one. Unfortunately, a limited literature in the field of bacterial polyamines is available so far, but ours is just a preliminary assumption and further studies are certainly needed to better investigate this issue.

In order to evaluate the propensity of dimer **2** to select for resistant mutants, and potentially investigate the resistance mechanism(s), *E. coli* CCUG^T (starting inoculum, 10^6 – 10^7 CFU/mL) was subcultured sequentially in MHB containing increasing concentrations of compound **2** (4-32 μg/mL corresponding to 2-16 x MIC). No growth could be observed at concentrations higher than 32 μg/mL, after up to 48 hours. Cells recovered from the various cultures were plated on agar plates containing the same concentration of compound **2**. Surprisingly, the phenotypic analysis of these isolates did not show the acquisition of a stable resistant phenotype, as the 2 MIC values measured for 25 randomly-selected isolates was essentially identical to that of the parental strain. This would indicate that **2** could induce the formation of persisters in the bacterial population, rather than selecting stable mutants with an acquired mutation, but that this tolerance phenomenon could only promote survival at concentrations equal or below to 32 μg/mL.

Haemolytic activity and cytotoxicity of representative compounds.

The same rationally selected compounds (**50, 55** and **58**), in addition to **1** and **2**, have been investigated about their behaviour towards human membranes. Haemolysis of red blood cells is often used as a tool to study the effect of compounds on mammalian cell integrity.[42] Each compound was tested at 64 μg/mL on human erythrocytes recovered from healthy donors with different blood groups;

0.2% Triton X-100 and 1.6 % DMSO were used as positive and negative controls, respectively. As shown by data reported in *Supporting Information*, **Table S 3**, the assay revealed that there was no evidence of haemolysis, with the exception of compound **50**, that showed a significant percentage of haemolysis ranging from 41 to 54%, depending on the blood group. This is interesting considering that the only compound with haemolytic activity showed a low antibacterial activity, indicating that the length of the alkyl chain could be very important for selectivity. For the other compounds, the obtained values are in a range of 0-7%, in agreement as preliminary described for **2** in a previous assay.[23] Only minor blood group-dependent haemolytic differences were observed for all compounds.

The potential cytotoxicity of compound **2** was further evaluated on the commercially available HeLa cells. When tested at 16 μg/mL, it showed only a minimal cytotoxicity after 24 hours of incubation. Cytotoxicity was more evident at 256 μg/mL, although it did not exceed 60% after 24 hours (**Figure 4**), indicating that the selectivity towards bacterial cells should be improved.

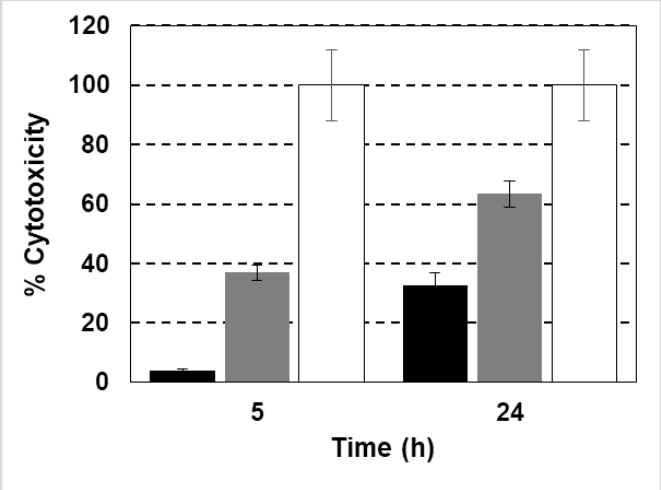


Figure 4. Cytotoxicity of compound **2** on HeLa cells. The figure shows the cytotoxicity (%) after 5 and 24 hours of incubation when **2** was tested at 16 μg/mL (black) or 256 μg/mL (grey). The cytotoxicity control is shown in white.

Preliminary ADME characterization of hit compound **2**.

In the end, *in vitro* preliminary absorption, distribution, metabolism, excretion (ADME) properties for the most active compound **2** was evaluated (**Table 4**). It was found to be metabolically stable, as measured by means of human liver microsomal proteins, and very soluble in pure water, probably thanks to its positive charges at physiological pH (7.4), allowing a future intravenous administration development. Moreover, the distribution constant logD measurement at pH 7.4 revealed a good value, comparable to the ones of daptomycin and vancomycin and is in agreement with the experimental solubility found. The indirect fluorescence method was used for PPB assays. This protocol was validated by using known drugs (see *Experimental session*) characterized by variable interaction with HSA and AGP. The main goal of drug binding assays is the determination of plasma-bounded drug fraction at therapeutic concentrations. During the discovery stage of a research project, this information could help to classify drugs as high, intermediate or poorly bound to plasmatic proteins. The drugs that are highly bound, such as warfarin and diazepam, show dissociation rate constant (K_d) of less than 100 μM, while the poorly bound drugs, as paracetamol, are characterized by a K_d value higher than 1 mM. Compound **2** shows a good affinity to the AGP, as expected for molecules that bear positive charges at physiological pH but it has a low percentage of bond (B_{max}) values, as reported in **Table 4.**

Thus, a low maximal percentage of bond and a slow dissociation rate of compound **2** are considered as permissive conditions to the predicted *in vivo* ADME.[43–45] Moreover, as above-reported, kill curve experiments in presence of albumin or serum, showed that the PPB does not impair restrictively the antibacterial activity.

Table 4. Preliminary ADME assays result for compound **2**

Metabolic stability (%)[a]	**Solubility [g/L][b]**	**logD (pH 7.4)[c]**	**HSA**		**AGP**	
			K_d [μM][d]	B_{max} (%)[d]	K_d [μM][d]	B_{max} (%)[d]
> 99	0.292 ± 0.022	-0.79	23.27 ± 14.6	14.0	0.11 ± 0.06	30.2

[a]The human liver microsome stability is expressed as a percentage of the unmodified parent drug. [b]Aqueous solubility was determined by means of the LC-MS method. [c]Distribution coefficient 1-octanol/ TRIS buffer pH 7.4 . [d]K_d and B_{max} values are measured by means of the indirect fluorescence method.

The parallel artificial membrane permeability assay (PAMPA), validated with known drugs (rifamixin and chloramphenicol)[22] revealed a low apparent permeability (P_{app}) at physiological pH, as expected since ionic compounds cannot pass across the membrane (**Table 5**): dimer **2** is, in fact, a trifluoroacetic salt with four net positive charges on the guanidine moieties and no pH adjustments have been performed during the assay. Since this experiment, from Kansy et al,[46] replaces cellular membranes (phospholipids, enzymes, proteins, sterols) with a barrier made of only phospholipids (phosphatidylcholine in our case), it correlates only with passive diffusion and can give false results for compounds that are substrate of membrane enzymes or transporters (active permeability). In fact, it is known from the literature that organic cations, such as endogenous substances bearing guanidine and amine functions, can cross biological membranes through apical transport mechanisms, like H^+ antiports and P-glycoprotein G (P-gp), and the less studied basolateral ones. In general, a family of organic cation transporters (OCT) has been cloned and studied to give more information about absorption and secretion of cationic compounds.[47–49] To evaluate if compound **2** is a substrate of this kind of transporters, in-depth permeability experiments were conducted. In this frame, we opted for the Caco-2 permeability assays that are based on the use of a polarized cell monolayer. It is the gold

standard *in vitro* test system, since it is quicker to use, convenient, and it produces more reproducible data than animal studies, giving an effective assessment of the permeability (P_{app}) of new compounds. Caco-2 is the best known immortal human colon carcinoma cell line as a model of the epithelial cell layer permeability barrier that compounds encounter in the small intestine. This cell line is characterized by a particular morphology that allows multiple permeability mechanisms and it provides the opportunity to investigate various permeability pathways. This model resembles the morphology of gastrointestinal epithelial cells for the presence of microvilli on the apical surface and for the expression of certain enzymes and membrane transporters, such as P-gp, breast cancer resistance protein (BCRP), and multidrug resistance protein 2 (MRP2). These characteristics make Caco-2 cells the most suitable and frequent used line to conduct permeability experiments and to rank compounds, according to the Biopharmaceutics Classification System (BCS) by Food and Drug Administration (FDA), as low, medium or high permeability compounds.[50–53] We calculated the P_{app} apical-to-basolateral and basolateral-to-apical by means of two different experiments, placing the test compound in the apical or in the basolateral side buffers. The first value provides information about the permeability in the absorptive direction, furnishing a quite reliable model of the gastrointestinal absorption. Compound **2** showed a very low permeability, in accordance with PAMPA results. In fact, the low $P_{app(A>B)}$ found (**Table 5**) indicates that it is not absorbed through passive diffusion (as already showed by PAMPA experiment), but also paracellular permeation and active transport are not involved in its absorption. This corresponds to a low oral bioavailability, that can be overcame by intravenous administration, as it happens for the majority part of natural drugs, as example those antibiotics active against chemo-resistant organisms, such as colistimethate[54] (prodrug of colistin, indicated for treatment of infection by multi-drug resistant Gram-negative strains, often used as a last resort), daptomycin (for treatment of nosocomial Gram-positive infections including MRSA and VRE strains) and vancomycin (for treatment of Gram-positive bacterial infections). The opposite experiment was performed to study the permeability of compound **2** by cell membrane transporters in the efflux direction. Also in this case, we obtained a very low P_{app}. In **Table 5**, we reported the

ratio between P_{app} in the B>A and the A>B directions: this value, known as efflux ratio, is 0.65, because the permeability values are approximatively the same (in the same range), highlighting that compound **2** could permeate only by passive or paracellular diffusions, not being substrates of active transporters for the cellular uptake or efflux. In fact, in the first case it should have been A>B/B>A) ≥2 and in the second one B>A/A>B) ≥2.[45,55,56] Considering that the efflux is a serious issue for an antibiotic, not allowing it to reach the appropriate concentration in the cell (MIC) to have antibacterial efficacy and provoking the rise of resistant strains, a low efflux is a relevant finding.

Table 5. Apparent Permeability Coefficients obtained by PAMPA and Caco-2 cell line experiments and efflux ratio for dimer **2**, atenolol (characterized by a low permeability) and propranolol (classified as a highly permeable compound), as reference compounds.

Cpd	**Apparent Permeability (PAMPA)** P_{app} **[10^{-6} cm/s]**	**Caco-2 Permeability**[a] $P_{app(A>B)}$ **[10^{-6} cm/s]**	**Efflux ratio**[b] $P_{app(A>B)}$ **/** $P_{app(B>A)}$
2	1.60 ± 0.43	0.19 ± 0.09	0.65
Rifamixin	0.06 ± 0.01	-	
Chloramphenicol	0.30 ± 0.50	-	
Atenolol	-	0.13 ± 0.04	0.37
Propranolol	-	22.4 ± 5.4	0.40

[a]A, apical; B, basolateral; $P_{app(A>B)}$ is the Papp in the apical-to-basolateral direction.

[b]B>A/A>B is the ratio of the basolateral-to-apical and the apical-to-basolateral permeation rate. Results are obtained from the average values of P_{app} (A>B) and (B>A).

CONCLUSION

Recent findings[23] highlighted a notable antibacterial activity of alkyl-guanidine oligomers, serendipitously originated from a monomeric compound. In particular, the symmetric dimer 2 was

identified as a potent broad-spectrum bactericidal. In this work, we present a chemical library of fifteen derivatives (**50-63** and **66**) which have been tested allowing us to perform some preliminary SAR considerations. Indeed, it clearly appears that the optimal length of the aliphatic linker should include 8 carbon atoms, whereas the nature and the number of the substituents on the guanidine moieties do not considerably affect the antibacterial properties of these compounds. Thus, the biological data indicate that almost all the synthesized derivatives show a strong bactericidal activity against a panel of Gram-positive and in some cases against Gram-negative pathogens. Despite the structural changes, dimer **2** still remains the best compound in antibacterial activity terms. Moreover, **2** and some rationally selected derivatives turn out to be active also against drug-resistant clinical isolates, acting through a bactericidal behavior similar to that of colistin, i.e. by not causing a macroscopic permeabilization of bacterial membranes or cell lysis. They were also investigated about their behavior towards human cell membranes, through haemolytic assays, revealing that there is no evidence of haemolysis, with the exception of 50. Furthermore, extended biological investigations of dimer **2** were performed, such as the evaluation of a possible interaction with polyamine transport systems and the propensity to select for resistant mutants, revealing the induction of persisters generation, without selecting stable mutants. The nature of these molecules strongly suggests that they could act on the membranes by electrostatic interactions, even if macroscopic alterations of the bacterial membrane were not observed, we cannot exclude the generation of microstructural damages. In fact, they can perturb the Gram-negative outer membrane and affect neither the proton motive force nor the ability of bacteria to form the biofilm. Finally, the *in vitro* ADME properties of dimer **2** was investigated: it was found to be metabolically stable, very soluble in pure water and it showed a low binding to plasma proteins that does not impair the activity and low passive and active permeability.

In summary, we synthesized a small library of alkyl-guanidine symmetric dimers as bactericidal agents, in some cases characterized by a broad-spectrum activity, including on drug-resistant clinical isolates. These compounds are generally not haemolytic, moderately toxic and do not apparently

select for stable resistant mutants, as found for the hit compound **2**. Notably, ADMET properties of **2** make it suitable to proceed with further optimization, including the improvement of selectivity.

EXPERIMENTAL SECTION

1. CHEMISTRY

1.1 General Chemistry.

All commercially available chemicals and solvents were used as purchased. DCM was dried over calcium hydride and THF was dried over sodium and benzophenone prior to use. Anhydrous reactions were run under a positive pressure of dry nitrogen. Chromatographic separations were performed on columns packed with silica gel (230-400 mesh, for flash technique). ^{1}H NMR and ^{13}C NMR were recorded at 400 and 100 MHz respectively on a Bruker AC200F spectrometer and are reported in parts per million (δ scale) and internally referenced to the $CDCl_3$ or CD_3OD signal, respectively at δ 7.24 ppm and 3.31 ppm. Chemical shifts for carbon are reported in parts per million (δ scale) and referenced to the carbon resonances of the solvent ($CDCl_3$ at δ 77.00 and CD_3OD at δ 49.00 ppm). Data are shown as following: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet and/or multiplet resonances, br = broad), coupling constant (*J*) in Hertz (Hz) and integration. Mass spectra (LC-MS) were acquired using an Agilent 1100 LC-MSD VL system (G1946C) by direct injection with a 0.4 mL/min flow rate using a binary solvent system of 95/5 MeOH/H_2O. UV detection was monitored at 254 nm. Mass spectra were acquired in positive mode scanning over the mass range 105-1500 *m/z*, using a variable fragmentor voltage of 10-70 mV.

1.2 Determination of the purity.

The purity of final products (**50-63** and **66**) was 95% or higher and it was assessed by HPLC-UV-MS, using an Equivalence 3 C18 column (ACE EQV-8977: 150 x 4.6 mm, 5 μm particle size) at a flow rate of 0.6 mL/min with a linear gradient elution from 100/0 to 50/50 v/v CH_3CN (formic acid 0.1% v/v)/H_2O (formic acid 0.1% v/v). UV detection was monitored at 210 nm. Mass spectra were

acquired in positive mode scanning over the mass range 105-1500 *m/z*, using a fragmentor voltage of 70 mV.

1.3 Synthetic procedures for compounds 3-49 are reported in *Supporting Information.*

1.4 General procedure for synthesis of dimer salts 50-63.

Protected dimeric compound **36-49** (0.046 mmol) was dissolved in dry DCM (3.6 mL) and TFA (10%, 0.4 mL) was added. The reaction mixture was stirred at room temperature for 7.5h. Then the solvent was evaporated and the crude was dissolved and evaporated several times first with MeOH to remove TFA residue. No further purification followed, the product was obtained a colourless oil.

1.4.1 1,3-bis(10-carbamimidamidodecyl)-1,3-bis({10-[*N′*-(cyclopropylmethyl)carbamimidamido]decyl})urea trifluoroacetate salt (10CH*/10CHs, 50). **^{1}H NMR** (CD_3OD) δ (ppm): 0.26 (m, 4H); 0.57 (m, 4H); 1.05 (m, 2H); 1.34 (m, 48H); 1.50 (m, 8H); 1.56 (m, 8H); 2.20 (bs, 1H); 3.05 (d, 4H, *J* = 6.8 Hz); 3.15 (m, 12H); 3.30 (m, 4H). **^{13}C NMR** (CD_3OD) δ (ppm): 2.41; 9.53; 26.23; 26.60; 27.51; 28.40; 28.48; 28.86; 29.05; 29.10; 29.20; 40.99; 41.10; 45.77; 46.87; 47.08; 47.30; 47.51; 47.73; 47.94; 48.15; 156.01; 166.00. **LCMS** m/z (ES+) = 479.1 $[M + 2H]^{2+}$; 319.8 $[M + 3H]^{3+}$; 240.1 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.2 1,3-bis(6-carbamimidamidohexyl)-1,3-bis({6-[*N′*-(cyclopropylmethyl)carbamimidamido]hexyl})urea trifluoroacetate salt (6CH*/6CHs, 51). **^{1}H NMR** (CD_3OD) δ (ppm): 0.27 (q, 4H, *J* = 4.8 Hz); 0.58 (q, 4H, *J* = 5.6 Hz); 1.06 (m, 2H); 1.34 (m, 16H); 1.55 (m, 16H); 3.05 (d, 4H, *J* = 7.2 Hz); 3.16 (m, 16H). **^{13}C NMR** (CD_3OD) δ (ppm): 4.36; 6.09; 9.53; 22.71; 26.05; 26.28; 27.46; 28.38; 29.26; 41.02; 42.16; 45.80; 155.60; 165.40. **LCMS** m/z (ES+) = 367.1 $[M + 2H]^{2+}$; 245.1 $[M + 3H]^{3+}$ **YIELD:** quantitative

1.4.3 1,3-bis(8-carbamimidamidooctyl)-1,3-bis[8-(*N′*-ethylcarbamimidamido)octyl]urea trifluoroacetate salt (8EH*/8EHs, 52). **^{1}H NMR** (CD_3OD) δ (ppm): 1.20 (t, 6H, *J* = 7.2 Hz); 1.33 (m, 32H); 1.50 (m, 8H); 1.55 (m, 8H); 3.14 (m, 12H); 3.21 (q, 4H, *J* = 7.2 Hz); 3.30 (m, 4H). **^{13}C NMR** (CD_3OD) δ (ppm): 12.96; 22.71; 26.16; 26.52; 27.49; 28.35; 28.43; 28.82; 28.92; 29.18; 29.24;

29.40; 35.91; 40.97; 41.02; 46.87; 59.65; 62.04; 62.89; 124.64; 164.88; 165.23. **LCMS** m/z (ES+) = 793.0 $[M + H]^+$; 397.1 $[M + 2H]^{2+}$; 265.1 $[M + 3H]^{3+}$; 199.2 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.4 1,3-bis(8-carbamimidamidooctyl)-1,3-bis[8-(*N′*-methylcarbamimidamido)octyl]urea trifluoroacetate salt (8MH*/8MHs, 53). ^{1}H NMR (CD_3OD) δ (ppm): 1.34 (m, 32H); 1.51 (m, 8H); 1.56 (m, 8H); 2.83 (s, 6H); 3.14 (q, 12H, *J* = 7.2 Hz); 3.30 (m, 4H). **^{13}C NMR** (CD_3OD) δ (ppm): 26.17; 26.53; 26.74; 27.50; 28.38; 28.92; 29.95; 40.97; 128.09; 156.74; 157.17; 161.28; 165.83. **LCMS** m/z (ES+) = 383.0 $[M + 2H]^{2+}$; 255.8 $[M + 3H]^{3+}$; 192.1 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.5 1,3-bis[8-(*N′*-benzylcarbamimidamido)octyl]-1,3-bis(8-carbamimidamidooctyl)urea trifluoroacetate salt (8BH*/8BHs, 54). ^{1}H NMR (CD_3OD) δ (ppm): 1.33 (m, 32H); 1.53 (m, 16H); 3.13 (q, 8H, *J* = 7.2 Hz); 3.19 (t, 8H, *J* = 6.8 Hz); 4.41 (s, 4H); 7.30 (m, 6H); 7.36 (m, 4H). **^{13}C NMR** (CD_3OD) δ (ppm): 22.30; 26.25; 26.59; 26.93; 27.61; 28.46; 28.87; 28.99; 29.30; 29.50; 41.16; 44.44; 126.60; 127.42; 127.86; 128.38; 136.60; 139.10; 155.90; 157.15; 162.00. **LCMS** m/z (ES+) = 459.0 $[M + 2H]^{2+}$; 306.4 $[M + 3H]^{3+}$; 230.1 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.6 1,1,3,3-tetrakis({8-[*N′*-cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (8CC*/8CCs, 55). ^{1}H NMR (CD_3OD) δ (ppm): 0.27 (q, 8H, *J* = 4.8 Hz); 0.57 (q, 8H, *J* = 8.0 Hz); 1.05 (m, 4H); 1.34 (m, 32H); 1.51 (m, 8H); 1.58 (m, 8H); 3.05 (d, 8H, *J* = 7.2 Hz); 3.06 (t, 8H, *J* = 7.2 Hz); 3.17 (t, 8H, *J* = 7.2 Hz). **^{13}C NMR** (CD_3OD) δ (ppm): 2.41; 9.53; 26.19; 26.55; 27.52; 28.46; 28.86; 28.95; 29.24; 29.39; 41.09; 45.78; 124.09; 155.78; 163.66; 163.75; 167.03. **LCMS** m/z (ES+) 477.5 $[M + 2H]^{2+}$; 318.7 $[M + 3H]^{3+}$; 239.3 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.7 1,1,3,3-tetrakis[8-(*N′*-ethylcarbamimidamido)octyl]urea trifluoroacetate salt (8EE*/8EEs, 56). ^{1}H NMR (CD_3OD) δ (ppm): 1.20 (t, 12H, *J* = 7.2 Hz); 1.34 (m, 32H); 1.50 (m, 8H); 1.55 (m, 8H); 3.13 (t, 8H, *J* = 7.2 Hz); 3.16 (t, 8H, *J* = 7.2 Hz); 3.22 (q, 8H, *J* = 7.2 Hz). **^{13}C NMR** (CD_3OD) δ (ppm): 12.97; 26.18; 26.55; 27.51; 28.44; 28.84; 28.94; 29.24; 29.39; 35.91; 41.03; 124.63; 155.88; 164.88; 165.23. **LCMS** m/z (ES+) = 425.1 $[M + 2H]^{2+}$; 284.0 $[M + 3H]^{3+}$; 213.4 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.8 1,1,3,3-tetrakis[8-(*N′*-benzylcarbamimidamido)octyl]urea trifluoroacetate salt (8BB*/8BBs, 57). **^{1}H NMR** (CD_3OD) δ (ppm): 1.29 (m, 32H); 1.51 (m, 8H); 1.56 (m, 8H); 3.13 (t, 8H, *J* = 7.2 Hz); 3.19 (t, 8H, *J* = 7.2 Hz); 4.41 (s, 8H); 7.30 (m, 12H); 7.37 (m, 8H). **^{13}C NMR** (CD_3OD) δ (ppm): 22.71; 26.13; 26.55; 26.88; 27.54; 28.41; 28.83; 28.94; 29.26; 29.42; 41.16; 44.38; 126.73; 127.48; 127.98; 128.42; 136.39; 139.07; 155.93; 158.15; 161.14; 165.09. **LCMS** m/z (ES+) = 549.1 $[M + 2H]^{2+}$; 366.5 $[M + 3H]^{3+}$; 275.0 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.9 1,1,3,3-tetrakis(8-carbamimidamidooctyl)urea trifluoroacetate salt (8HH*/8HHs, 58). **^{1}H NMR** (CD_3OD) δ (ppm): 1.34 (m, 36h); 1.53 (m, 12H); 3.14 (q, 8H, *J* = 7.2 Hz); 3.26 (m, 8H). **^{13}C NMR** (CD_3OD) δ (ppm): 26.68; 27.14; 27.97; 28.20; 28.84; 29.08; 29.21; 29.48; 49.67; 79.08; 82.87; 153.22; 156.01; 163.53; 165.78. **LCMS** m/z (ES+) = 369.5 $[M + 2H]^{2+}$; 246.6 $[M + 3H]^{3+}$ **YIELD:** quantitative

1.4.10 3,3-bis(8-carbamimidamidooctyl)-1,1-bis({8-[*N′*-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (8CC*/8HHs, 59). **^{1}H NMR** (CD_3OD) δ (ppm): 0.26 (m, 4H); 0.57 (m, 4H); 1.06 (m, 2H); 1.34 (m, 32H); 1.51 (m, 8H); 1.58 (m, 8H); 3.05 (d, 4H, *J* = 6.8 Hz); 3.15 (m, 16H). **^{13}C NMR** (CD_3OD) δ (ppm): 2.39; 9.53; 26.17; 26.54; 27.51; 28.36; 28.45; 28.83; 28.93; 29.24; 40.97; 41.09; 45.78; 46.87; 47.08; 47.30; 47.51; 47.72; 47.93; 48.14; 155.60; 165.40. **LCMS** m/z (ES+) = 423.3 $[M + 2H]^{2+}$; 282.5 $[M + 3H]^{3+}$; 212.1 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.11 1-(6-carbamimidamidohexyl)-1,3,3-tris({6-[*N′*-(cyclopropylmethyl)carbamimidamido]hexyl})urea trifluoroacetate salt (6CC*/6CHs, 60). **^{1}H NMR** (CD_3OD) δ (ppm): 0.26 (q, 6H, *J* = 4.8 Hz); 0.57 (q, 6H, *J* = 8.0 Hz); 1.05 (m, 3H); 1.29 (m, 16H); 1.56 (m, 16H); 3.01 (d, 8H, *J* = 7.2 Hz); 3.06 (m, 16H). **^{13}C NMR** (CD_3OD) δ (ppm): 4.36; 6.09; 9.53; 22.71; 26.05; 26.28; 27.46; 28.38; 29.26; 41.02; 42.16; 45.80; 155.60; 165.40. **LCMS** m/z (ES+) = 787.8 $[M + H]^{+}$; 394.6 $[M + 2H]^{2+}$; 263.3 $[M + 3H]^{3+}$ **YIELD:** quantitative

1.4.12 1-(6-carbamimidamidohexyl)-1,3,3-tris[6-(*N′*-ethylcarbamimidamido)hexyl]urea trifluoroacetate salt (6EE*/6EHs, 61). **^{1}H NMR** (CD_3OD) δ (ppm): 1.19 (t, 9H, *J* = 6.8 Hz); 1.32

(m, 16H); 1.56 (m, 16H); 3.15 (q, 14H, *J* = 8.0 Hz); 3.22 (q, 6H, *J* = 6.8 Hz); 3.30 (m, 2H). **^{13}C NMR** (CD_3OD) δ (ppm): 12.57; 13.21; 26.01; 26.22; 27.42; 28.42; 29.23; 35.92; 40.95; 155.94; 157.32; 162.53; 165.79. **LCMS** m/z (ES+) = 355.1 $[M + 2H]^{2+}$; 237.1 $[M + 3H]^{3+}$; 178.2 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.4.13 3,3-bis({6-[*N′*-(cyclopropylmethyl)carbamimidamido]hexyl})-1,1-bis({8-[*N′*-(cyclopropylmethyl)carbamimidamido]octyl})urea trifluoroacetate salt (8CC*/6CCs, 62). ^{1}H NMR (CD_3OD) δ (ppm): 0.26 (q, 8H, *J* = 4.8 Hz); 0.58 (q, 8H, *J* = 8.0 Hz); 1.03 (m, 4H); 1.34 (m, 24H); 1.55 (m, 16H); 3.05 (d, 8H, *J* = 7.2 Hz); 3.17 (m, 16H). **^{13}C NMR** (CD_3OD) δ (ppm): 3.40; 11.18; 26.31; 26.43; 26.66; 28.87; 29.35; 30.37; 42.26; 44.73; 50.48; 157.82; 164.54. **LCMS** m/z (ES+) 449.0 $[M + 2H]^{2+}$; 299.0 $[M + 3H]^{3+}$ **YIELD:** quantitative

1.4.14 1,1-bis({8-[*N′*-(cyclopropylmethyl)carbamimidamido]octyl})-3,3-bis[8-({[(methylcarbamoyl)amino]methanimidoyl}amino)octyl]urea trifluoroacetate salt (8CC*/8UUs, 63). ^{1}H NMR (CD_3OD) δ (ppm): 0.26 (q, 4H, *J* = 4.8 Hz); 0.58 (q, 4H, *J* = 8.0 Hz); 1.06 (m, 2H); 1.34 (m, 32H); 1.52 (m, 8H); 1.60 (m, 8H); 2.76 (s, 6H); 3.05 (d, 4H, *J* = 7.2 Hz); 3.06 (t, 8H, *J* = 4.0 Hz); 3.18 (t, 4H, *J* = 6.8 Hz); 3.27 (t, 4H, *J* = 7.2 Hz). **^{13}C NMR** (CD_3OD) δ (ppm): 3.45; 11.16; 26.66; 26.79; 28.85; 29.30; 30.34; 41.56; 42.23; 44.78; 50.47; 155.44; 156.59; 157.80; 164.51. **LCMS** m/z (ES+) 959.3 $[M + H]^{+}$; 480.1 $[M + 2H]^{2+}$; 320.1 $[M + 4H]^{4+}$ **YIELD:** quantitative

1.5 Procedure for synthesis of dimer salt 66.

1.5.1 *tert*-butyl *N*-{[(8-{[(8-{[(tert-butoxy)carbonyl]({[(tert-butoxy)carbonyl]imino}[(cyclopropylmethyl)amino]methyl)amino}octyl)({7-[({[(tert-butoxy)carbonyl]imino}[(methylcarbamoyl)amino]methyl)amino]heptyl})carbamoyl]({8-[({[(tert-butoxy)carbonyl]imino}[(methylcarbamoyl)amino]methyl)amino]octyl})amino}octyl)amino]({[(tert-butoxy)carbonyl]imino})methyl}-*N*-(cyclopropylmethyl)carbamate (8CU/8CUs, 65).

Compound **64**, already described by Zamperini et al.[23] (30.0 mg, 0.02 mmol) was solubilized in THF (1.0 mL) in a tube, then, DIPEA (6.0 µL, 0.04 mmol) and a 2.0 M solution of Methylamine in

THF (19.0 μL, 0.54 mmol) were added. The tube was sealed with a screw cap and heated at 80 °C for 24 h. Then solvent was evaporated and the crude was purified by flash chromatography (DCM/MeOH 97/3). **^{1}H NMR** ($CDCl_3$, 400 MHz): δ 0.24 (d, *J* = 4.8 Hz, 4H); 0.45 (d, *J* = 7.6 Hz, 4H); 1.05 (m, 2H); 1.29 (m, 48H); 1.48 (s, 54H); 1.63 (m, 8H); 2.77 (s, 6H); 3.07 (t, 4H, *J* = 7.0 Hz); 3.28 (m, 4H); 3.54 (m, 4H); 7.96 (br, 2H); 12.14 (br, 2H). **^{13}C NMR** ($CDCl_3$, 100 MHz): δ 3.41; 8.33; 26.60; 26.75; 28.42; 29.36; 30.34; 41.53; 42.20; 50.30; 50.49; 79.71; 82.53; 149.10; 154.21; 156.56; 158.07; 158.54; 184.58. **LCMS** m/z (ES+) = 780.3 $[M + 2H]^{2+}$; 791.5 $[M + Na + H]^{2+}$ **YIELD:** 34%

1.5.2 1,3-bis({8-[*N*'-(cyclopropylmethyl)carbamimidamido]octyl})-1-[7-({[(methylcarbamoyl)amino]methanimidoyl}amino)heptyl]-3-[8-({[(methylcarbamoyl)amino]methanimidoyl}amino)octyl]urea trifluoroacetat salt (8CU/8CUs, 66). Same procedure to obtain compounds **50-63**: To a solution of compound **65** in dry DCM (13 mM) TFA (10%) was added. The reaction mixture was stirred at room temperature for 7.5 h. Then the solvent was evaporated and the crude was dissolved and evaporated several times first with MeOH to remove TFA residue. No further purification followed, the product was obtained a colourless oil. **^{1}H NMR** (CD_3OD, 400 MHz): δ 0.26 (m, 4H); 0.57 (m, 4H); 0.87 (m, 2H); 1.34 (m, 32H); 1.50 (m, 8H); 1.60 (m, 8H); 2.76 (s, 6H); 3.05 (d, 4H, *J* = 6.8 Hz); 3.15 (m, 12H); 3.30 (m, 4H). **^{13}C NMR** (CD_3OD, 100 MHz): δ 3.40; 11.12; 26.68; 26.79; 28.82; 29.35; 30.34; 41.58; 42.27; 44.76; 50.42; 155.46; 156.55; 157.81; 164.59. **LCMS** m/z (ES+) 480.0 $[M + 2H]^{2+}$; 320.0 $[M + 3H]^{3+}$; 240.7 $[M + 4H]^{4+}$ **YIELD:** quantitative

2. BIOLOGY

2.1 Antibacterial activity testing. Bacterial strains, including representatives of both Gram-positive and Gram-negative bacteria, were obtained from the American Type Culture Collection (ATCC) or Culture Collection of University of Gothenburg (CCUG) culture collections. Antibiotic-resistant clinical isolates were collected from various Italian hospitals during surveillance studies and characterized elsewhere.[57,58] Compounds were re-suspended in DMSO at a final concentration of 50

or 100 mg/mL and subsequently diluted in the culture medium. The MICs of the compounds were determined using the microdilution broth method using MHB as recommended by the Clinical Laboratory Standards Institute (CLSI)[59]. Bacterial inoculum was 5×10^4 CFU/well. MICs were recorded after 18 h of incubation at 35 °C. Potential mechanisms of acquired resistance were investigated by subculturing *E. coli* CCUG^T (starting inoculum, $10^6 – 10^7$ CFU/mL) in the presence of increasing concentrations of compound **2** (starting from 2 μg/mL). Each culture was incubated for 24 h at 37 °C and cells subsequently diluted in fresh medium containing a concentration of compound 2-fold higher than that of the parental culture. Aliquotes of the cultures were plated on MH plates containing the same concentration of compound **2**, to allow the selection of single colonies, that were randomly selected and whose susceptibility to compound **2** was investigated as described above.

2.2 Antibacterial activity in presence of BSA and HS. To test the effect of HS and BSA on the antibacterial activity of compound **2**, *E. coli* MG1655 was inoculated at ca. 5 x 10^5 CFU/mL in MHB supplemented with increasing concentrations of BSA (Sigma-Aldrich) or with 10% HS. Human serum was collected from 80 healthy donors, pooled, filtered, and inactivated (30 min, 56 °C), as previously described.[60] *E. coli* MG1655 growth was monitored for up to 24 h using a SPARK 10M (TECAN) reader.

2.3 Inner membrane integrity assay. The ability of individual compounds to mine the bacterial membrane integrity was investigated through an assay based on the activity of β-Galactosidase, a cytoplasmic enzyme which hydrolyzes β-D-galactosides, has been performed. The chromogenic substrate ONPG, that is colourless, has been used as the substrate: the enzyme converts it to galactose and ONP,[32,61] that is yellow and can be measured by its absorption at 415 nm. If the ONPG concentration is high enough, ONP produced is proportional to the amount of β-galactosidase present and to the time the enzyme reacts with the ONPG. Through this enzymatic reaction, the inner membrane integrity can be determined: if the bacterial membrane is intact the ONPG cannot enter in the bacterial cell and consequently no absorption was detected at 415 nm, whereas agents that compromise the membrane integrity allow ONPG to diffuse into bacterial cells, where it is converted

to ONP and its concentration can be measured as A_{415}. The β-galactosidase activity was tested in the three reference bacterial species and strains, namely *E. coli* MG1655 (ATCC47076), *A. baumannii* ATCC19606^T and *P. aeruginosa* PAO1 (ATCC15692), after the treatment with the compound.[31] Since both *A. baumannii* and *P. aeruginosa* are unable to ferment the lactose because they do not produce β-galactosidase, to perform the assay, these two reference strains were transformed with a plasmid carrying the β-galactosidase gene under the control of rrnB promoter, that allows a high-level intracellular production of β-galactosidase (plasmid pMP2200::PrrnB). On the contrary, in *E. coli* the β-galactosidase (*lacZ*) gene is chromosomally-located and inducible with Isopropyl β-D-1-thiogalactopyranoside (IPTG). ONP production by the three organisms in the presence of each compound (256 μg/mL), SDS 1% or nothing as positive and negative controls respectively was monitored for 1 hour at 10-min intervals. Bacteria were grown to stationary phase (16 h) at 37°C in Mueller Hinton broth. To induce the expression of the β-galactosidase, *E. coli* MG1655 (ATCC47076) was grown in the presence of 1 mM IPTG. Bacterial cells were washed and diluted in 10 mM tris(hydroxymethyl)aminomethane (TRIS) buffer pH 7, to an OD_{600} of 0.35 for *E. coli* and of 1.0 for *P. aeruginosa* ATCC15692 and *A. baumannii* ATCC19606^T. A 15-μL aliquot of the washed bacterial suspension was further diluted in 135 μL of 10 mM TRIS buffer containing 2.5 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and 256 μg/mL of each compound. The assay has been performed in a final volume of 150 μL in 96-well microtiter plates. Bacterial suspensions in 10 mM TRIS buffer containing 2.5 mM ONPG with or without 1% of SDS were used as positive and negative controls, respectively. The hydrolysis of ONPG to ONP in each well was detected by measuring the absorbance at 415 nm at 10-min intervals for 60 min using Wallac 1420 Victor3V multilabel plate reader (Perkin Elmer).

2.4 Outer membrane permeabilization assay. NPN is a non-polar probe which fluoresces weakly in aqueous environments and strongly in a glycerophospholipid environment, such as lipid bilayers of biological membranes, so it is used to assess the outer membrane integrity in Gram-negative bacteria.[62] A 4 mM stock solution of NPN (Santa Cruz) was prepared in acetone and stored in the

dark at 4°C until used. The NPN assay was performed as previously described,[62] using *A. baumannii* ATCC19606^T, *P. aeruginosa* ATCC15692 (PAO1) and *E. coli* MG1655 (ATCC47076) as test organisms. Briefly, cells from mid-exponential cultures were washed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer 5 mM (Sigma-Aldrich) and diluted in the same buffer to an OD_{600}=1.0. Then, 100 µL aliquots of bacterial suspensions were mixed in a black 96-well microtiter plate with 50 µL of the selected compounds (**1, 2, 50, 55, 58**) and incubated at 37°C. After 30 min, 50 µL of NPN (40 µM in 5 mM HEPES buffer) was added to each well and the fluorescence was detected in a Tecan spark 10 M microtiter plate reader, (excitation 355 nm, emission 460 nm).

2.5 Cytoplasmic Membrane Depolarization Assay. The membrane depolarization activity of compound **2** was determined using a fluorescent probe $DiSC_3$-5 (purchased from Sigma Aldrich), as previously described [18,32] with minor modifications. *E. coli* CCUG^T was grown in LB medium until $A^{600} \approx 0.5$-0.8. Cells were harvested, washed, and re-suspended in 5 mM HEPES buffer (pH 7.2) containing 5 mM glucose and 100 mM KCl. The cell suspensions ($5 \times 10^7 – 2 \times 10^8$ CFU/mL) were incubated in 96-well black Optiplate (Perkin Elmer, Waltham, Mass.) with 0.8 µM $DiSC_3$-5 in darkness until a stable fluorescence signal was obtained (excitation wavelength, 622 nm; emission wavelength, 675 nm; Envision microplate reader equipped with quadruple monochromators, Perkin-Elmer). After the addition of compound **2** (at a concentration of 16 µg/mL, equivalent to 4 × MIC), fluorescence was continuously monitored at 25 °C. 1 mM 2,4-Dinitrophenol (DNP) (Sigma-Aldrich) was used as a positive control.

2.6 Cytotoxicity. Cytotoxicity was evaluated on HeLa cells. The cells (1.0 x 10^4 cells/well) were seeded into 96-well plates containing 100 µL Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in 5% CO_2 for 24 h. The cells were exposed to 16 and 256 µg/mL of compound **2** dissolved in DMSO. Each concentration and control were assayed in triplicates. The cytotoxicity of the compounds was calculated as a percentage reduction in viable cells with respect to the control culture (cells treated with DMSO only) using the

CellTox™ Green Cytotoxicity Assay commercial kit, as recommended by the manufacturer (Promega).

2.7 Interference compounds assessment. The behavior of all the tested compounds (**45**, **50-61** and **66**) as Pan-Assay Interference Compounds (PAINS) was examined through prediction by the online FAFDrugs4 (Free ADME-Tox Filtering Tool) program.[63,64] Through its tool Bank-Formatter the compound library was prepared and then screened with the three different available filters A, B and C.[65] All the analyzed compounds resulted "accepted" by the software, with the exception of **45** that was not analyzed and was named "large compound".[66]

3. ADME

3.1 Metabolic stability assay. Compound **2** in DMSO solution was incubated at 37 °C for 60 min in 50 mM TRIS buffer (pH 7.4), 5 μL of human liver microsomal proteins (0.2 mg/mL), in the presence of a Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH)-generating system at a final volume of 0.5 mL (the final concentration of compound **2** was 50 μM); DMSO did not exceed 2% of the final solution. The reaction was stopped by cooling in ice and adding 1.0 mL of acetonitrile. The reaction mixtures were then centrifuged, and the parent drug and metabolites were subsequently determined by the previously reported LC-UV-MS method.

3.2 Solubility assay. Compound **2** (1.0 mg) was added to 1 mL of pure water. The sample was shaken in a shaker bath at room temperature for 24 h. The suspension was filtered through a 0.45 μm nylon filter (Acrodisc), and the solubilized compound determined by LC-MS. The determination was performed in triplicate. For the quantification, an LC-MS method with the above reported chromatographic conditions was used.

3.3 logD measurement. Compound **2** was dissolved in methanol (5mM), filtered previously through a 0.2 μm filter and aliquots placed in 2 mL test tube (100 μL). Then, the solvent was evaporated through a flux of nitrogen and to the residue filtered 1-octanol (200 μL) and TRIS buffer 50 mM (200 μL, pH 7.4) were added. After dissolution by vigorous stirring, the two phases were

separated by centrifugation for 5 min at 5000 rpm. Most of the 1-octanol fraction was removed and analyzed by means of the previously reported HPLC method. The residue of octanol was carefully removed and discarded. Thus, an aliquot of the buffer fraction was removed for analysis. logD was calculated by taking the logarithm of the ratio of the HPLC peak area of each compound in 1-octanol to the corresponding peak area in buffer. Consequently, logD value was based on analysis of both aqueous and 1-octanol fractions.

3.4 Binding Fluorimetric Assay. The binding of compound **2** to HSA and AGP was monitored by fluorescence spectroscopy in order to determine the two K_d. A quantitative analysis of the potential interaction was performed by fluorimetric analysis using 96-multiwell plates: in each well, a fixed concentration of HSA or AGP (10 μM in phosphate buffer 1 mM), was added with different amounts of tested compound (0.1 μM to 500 μM by stock solutions in DMSO). Plates were gently shacked and after allowing 30 minutes at room temperature for equilibration, after excitation at 295 nm, spectra were recorded from 300 to 400 nm. The obtained fluorescence quenching percentages were plotted against drug concentrations and the relative K_d values were obtained using GraphPad software (version 6.0). Selected method was first validated with drugs having known K_d (Warfarin, Diazepam, Carbamazepine and Paracetamol for HSA and Chlorpromazine for AGP) and experimental were in good agreement with literature data[67,68] (reported in *Supporting Information,* **Table S 4**).

3.5 Parallel Artificial Membrane Permeability Assay. The apparent permeability (P_{app}) was measured by using the PAMPA. Donor solution (0.5 mM) was prepared by diluting 1 mM DMSO compound stock solution using TRIS buffer (50 mM) at pH 7.4. Filters were coated with 5 μL of a 1% (w/v) dodecane solution of phosphatidylcholine. Donor solution (150 μL) was added to each well of the filter plate. To each well of the acceptor plate, 300 μL of a 50% DMSO in phosphate buffer solution was added. Compound **2** was tested in three different plates on different days. The sandwich was incubated for 5 h at room temperature under gentle shaking. After the incubation time, plates were separated, and samples were taken from both receiver and donor sides and analyzed using LC with UV-MS. The analyses were conducted using a Polaris C18 column (150 x 4.6 mm, 5 μm particle

size) at a flow rate of 0.8 mL min^{-1} with a mobile phase composed of 50% CH_3CN/50% H_2O-formic acid 0.1%. Permeability (P_{app}) was calculated according to the below-reported equation, already described,[41,69] with some modification to obtain values in cm/s.

$$P_{app} = \frac{V_D V_A}{(V_D + V_A)At} - \ln(1 - r)$$

Where V_A is the volume in the acceptor well, V_D is the volume in the donor well (cm^3), A is the effective area of the membrane (cm^2), t is the incubation time (s) and r the ratio between drug concentration in the acceptor and equilibrium concentration of the drug in the total volume (V_D+V_A). Drug concentration was estimated by using the peak area integration.

3.6 Caco-2 permeability assay. Caco-2 cells were cultured in their appropriate complete medium and were maintained in a 37°C incubator with saturated humidity and an atmosphere of 95% air and 5% CO_2. Caco-2 cells were grown for 21-29 days on specific sterile 12 mm transwell® chambers, with 0.4 μm pore polycarbonate membrane insert. Cell monolayer integrity was tested by TEER (transepithelial electrical resistance) measurement and lucifer yellow paracellular permeability assays. Compound **2** and the reference compounds atenolol and propranolol (purchased by Merck-Sigma Aldrich) were added (all at the dose of 10 μM) to the apical or basolateral side of the transwell chamber (containing 500 μL Hank's Balanced Salt Solution, HBSS) in order to calculate $P_{app(A>B)}$ and $P_{app(B>A)}$ respectively and incubated at 37°C in gentle shaking. At times 15, 30, 60, 120 min, 350 μL of HBSS buffer were taken from the acceptor side of the chamber (containing 1500 μL HBSS). At times 0 and 120 min, 100 μL of buffer were taken from the donor side of the chamber, as well. Samples from apical and basal compartments were analyzed by LC-MS/MS to determine the amount of compound **2** and atenolol and propranolol as standard. LC-MS/MS system consisted of a Varian apparatus (Varian Inc) including a vacuum solvent degassing unit, two pumps (212-LC), a Triple Quadrupole MSD (Mod. 320-LC) mass spectrometer with ES interface and Varian MS Workstation System Control Vers. 6.9 software. The instrument operated in positive mode and parameters were: detector 1450 V, drying gas pressure 19.1 psi, desolvation temperature 199.5 °C, nebulizing gas 42.0

psi, needle 5000 V, and shield 600 V. Nitrogen was used as nebulizer gas and drying gas. Collision-induced dissociation was performed using Argon as the collision gas at a pressure of 1.8 mTorr in the collision cell. Capillary voltage was set at 59.0 V and the collision energy was 40 V. A Kinetex Phenomenex C18 100Å (30 X 2.1 mm) with 2.6 μM particle size as the stationary phase and water/acetonitrile gradient with 0.1% volume of formic acid as the mobile phase were used to achieve the chromatographic separation.

ASSOCIATED CONTENT

Supporting Information Available: [Synthesis of guanylating agents: Scheme S 1. Synthesis of N,N'-Di-Boc-N-alkyl-pyrazole-1-carboxamidines as guanylating agents 21a-d, Scheme S 2. Synthesis of guanylating agent 24 with the N-methylamidinourea moiety. Codes for monomer, carbamoyl and dimer derivatives: Table S 1. Compounds 1-2, 6-18, 23-61 with codes. Time-kill curve experiments of dimer 2: Figure S 1. Time-kill curve experiments performed with E. coli CCUGT. Plasma protein binding effect on antibacterial activity of compound 2: Figure S 2. Effect of human serum (HS) and bovine serum albumin (BSA) and on the inhibitory activity of compound 2. Biofilm formation: Table S 2: Minimum inhibitory concentration of selected compounds; Figure S 3. Growth profile of different bacterial species in MHB supplemented with selected compound at ½ MIC; Figure S 4. Effect of selected compounds on biofilm formation by different bacterial species; Experimental procedure for biofilm formation and confocal microscopy analysis. Haemolytic activity of selected compounds: Table S 3. Haemolytic activity of selected compounds for each blood group; Experimental procedure for haemolytic assay. Synthetic procedures for compounds 3-49. Binding studies: Table S 4. Binding studies realized to validate the used fluorimetric assay. References. Molecular Formula Strings.]

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AUTHOR INFORMATION

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Author Contributions.

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. C.P. and I.D. equally contributed to the work. C.P., I.D., D.D.; G.I.T. synthesized and characterized the compounds. C.Z. performed the ADME characterization, F.D., F.S., R.D.P., T.F., D.V, P.V. and J.D.D. performed the biological assays, C.Z., M.B.G. and A.M. performed Caco-2 experiments. I.D., C.P., J.D.D. and M.B. prepared the manuscript. All authors have read and approved the manuscript.

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ABBREVIATIONS

ADME(T), Absorption, Distribution, Metabolism, Excretion, (Toxicity); AGP, α1-acid glycoprotein; AMP, antimicrobial peptide; ATCC, American Type Culture Collection (ATCC); BCRP, Breast Cancer Resistance Protein; BCS, Biopharmaceutics Classification System; B_{max}, maximum number of binding sites; BOC, *tert*-butyloxycarbonyl; BSA, Bovine Serum Albumin; CCUG, Culture Collection of University of Gothenburg; CFU, Colony Forming Unit; CLSI, Clinical Laboratory Standards Institute; CV, Crystal Violet; DCM, dichloromethane; DIPEA, *N,N*-Diisopropylethylamine; $DISC_3$-5, 3,3'-dipropylthiacarbocyanine; DMEM, Dulbecco's Modified

Eagle Media; DMF, *N,N*-Dimethylformamide; DMSO, *N,N*-Dimethylsulfoxide; DNP, dinitrophenol; FBS, fetal bovine serum; FDA, Food and Drug Administration; HBSS, Hank's Balanced Salt Solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; HS, Human Serum; HSA, Human Serum Albumin; HeLa cells, cells of Henriette Lacks; IPTG, isopropyl β-D-1-thiogalactopyranoside; K_d, ligand concentration that binds to half the receptor sites at equilibrium; LC, Liquid Chromatography; logD, distribution constant; LPS, Lipopolysaccharide; MBC, Minimal Bactericidal Concentration; MHB, Mueller Hinton Broth; MIC, Minimal Inhibition Concentration; MoA, Mechanism Of Action; mPAO, Maize Polyamine-Oxidase; MRP2, Multidrug Resistance Protein 2; MRSA, Methicillin-Resistant *Staphylococcus aureus*; MS, Mass Spectrometry; NADPH, Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; NDM, New Delhi Metallo-β-lactamase; NMR, Nuclear Magnetic Resonance; NPN, 1-*N*-phenylnaphthylamine; OCT, organic cation transporters; ONP, *o*-nitrophenol; ONPG, *o*-nitrophenyl-β-D-galactoside; PAINS, Pan-Assay Interference Compounds, PAMPA, Parallel Artificial Membrane Permeability Assay; PAO, Polyamine Oxidase; P_{app}, apparent permeability; P-gp, P-glycoprotein; pKa, acid dissociation constant; RFU, relative fluorescence unit; SAR, Structure-Activity Relationship; SDS, sodium dodecylsulfate; TEER, Transepithelial Electrical Resistance; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TRIS, tris(hydroxymethyl)aminomethane; VRE, Vancomycin-Resistant *Enterococci*; WHO, World Health Organization.

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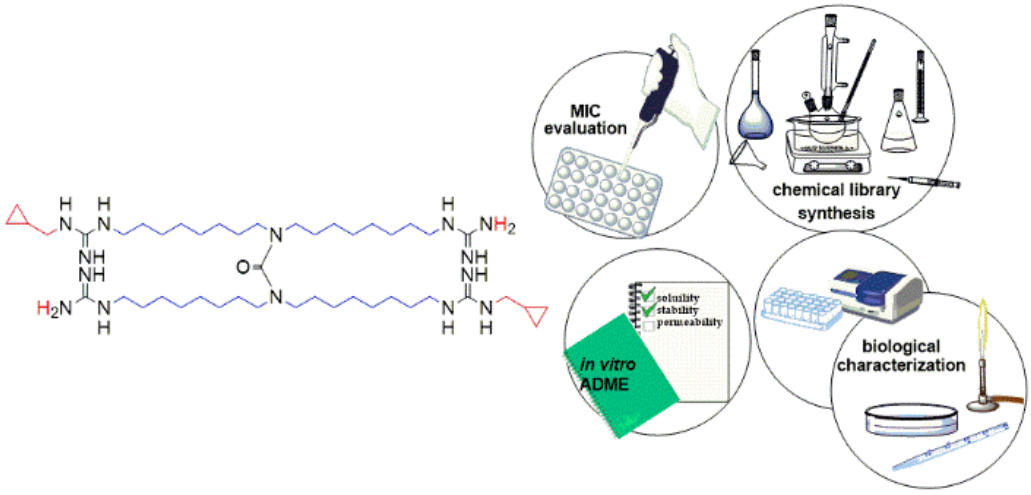
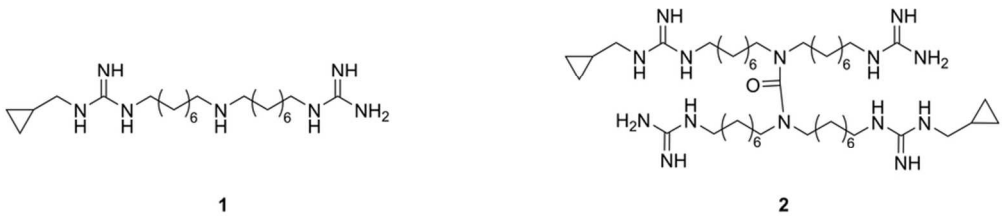


Table of Contents graphic.



1 2

Figure 1. Structures of monomer (1) and its dimeric derivative (2).

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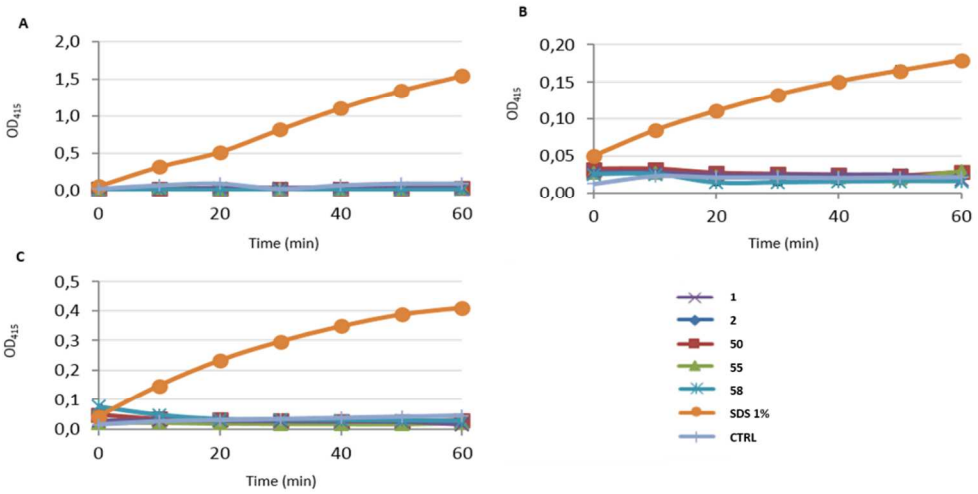

Figure 2. Effect of selected compounds on bacterial permeability.

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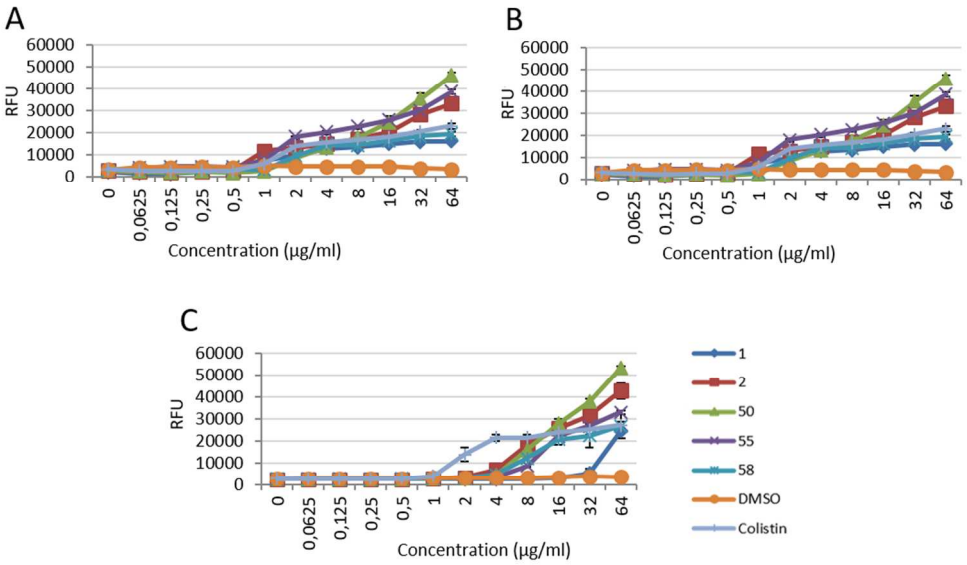

Figure 3. Effect of selected compounds on Gram-negative outer membrane integrity.

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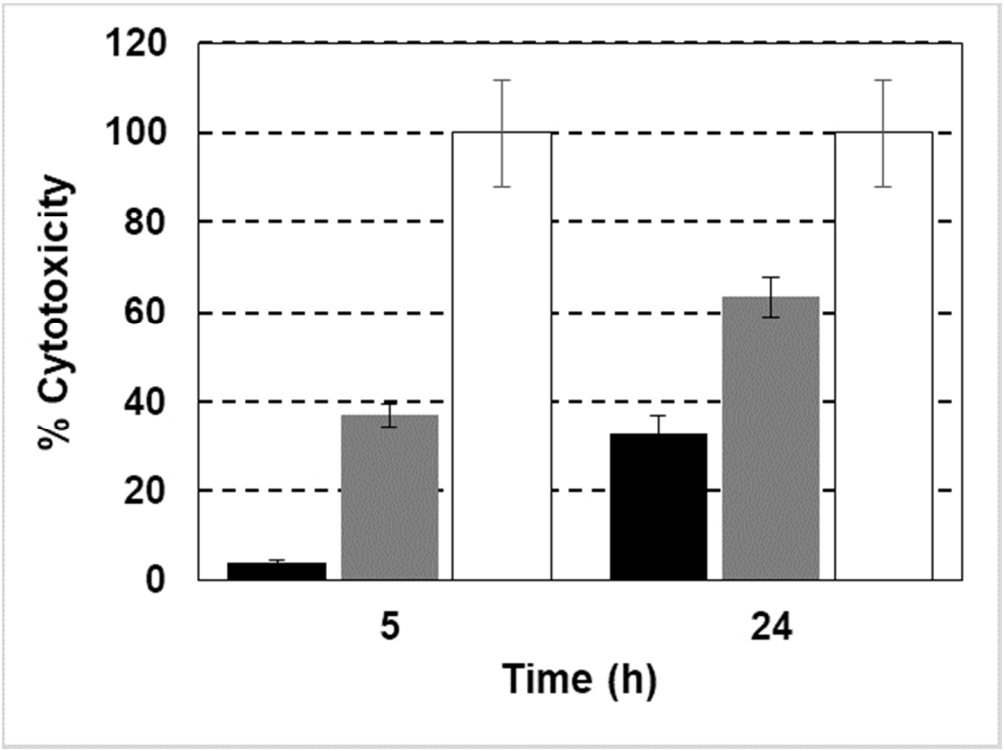

Figure 4. Cytotoxicity of compound 2 on HeLa cells.

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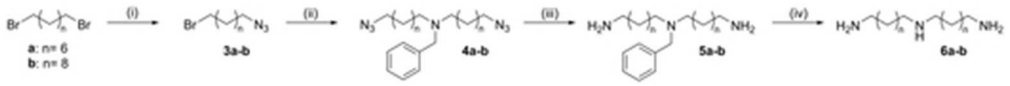

Scheme 1. Synthesis of triamines 6a-b.

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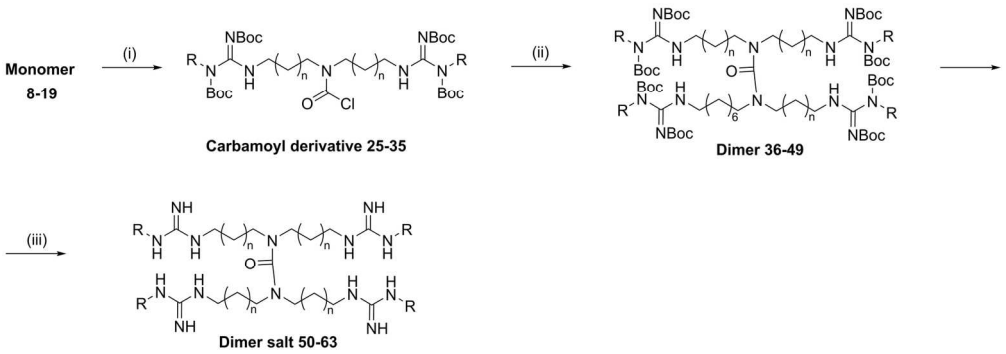

Scheme 3. Synthesis of dimers.

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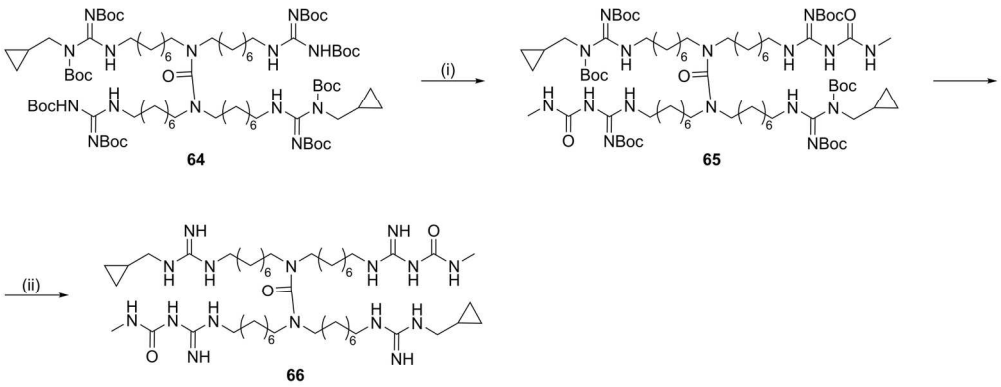

Scheme 4. Synthesis of amidinourea compound 66.

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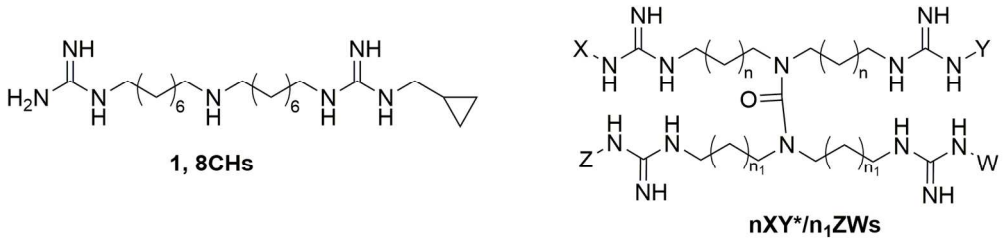

Image for Table 2

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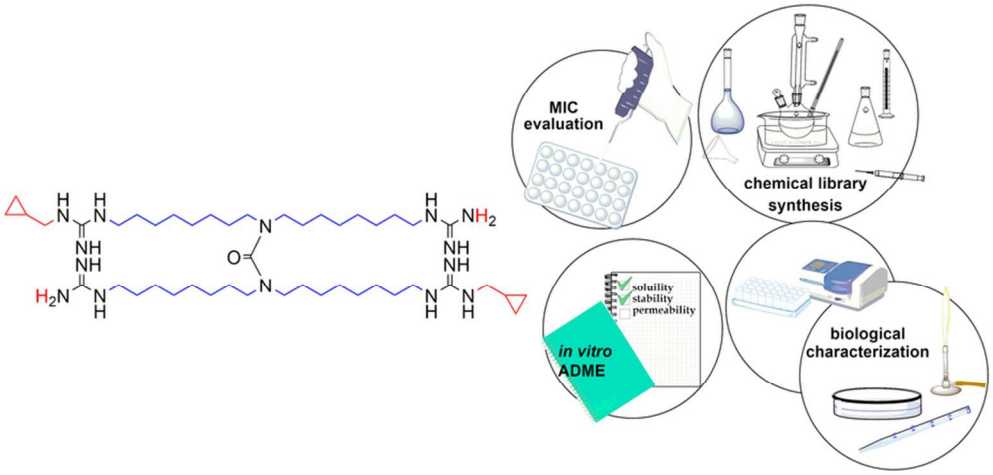

Graphic for Abstract

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