Communication

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Synthesis and activity of biomimetic biofilm disruptors.

Thomas Böttcher[#], Ilana Kolodkin-Gal[§], Roberto Kolter[+], Richard Losick[§], Jon Clardy[#,*]

[#]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115, USA.

[+]Department of Microbiology and Immunobiology, Harvard Medical School, 77 Ave. Louis Pasteur, Boston, MA 02115, USA

[§]Department of Molecular and Cell Biology, Harvard University, 16 Divinity Ave., Cambridge, MA 02138, USA.

*Email: jon_clardy@hms.harvard.edu

Supporting Information Placeholder

ABSTRACT: Biofilms are often associated with human bacterial infections, and the natural tolerance of biofilms to antibiotics challenges treatment. Compounds with anti-biofilm activity could become useful adjuncts to antibiotic therapy. We used norspermidine, a natural trigger for biofilm disassembly in the developmental cycle of *Bacillus subtilis*, to develop guanidine and biguanide compounds with up to 20x increased potency in preventing biofilm formation and breaking down existing biofilms. Compounds also were active against pathogenic *Staphylococcus aureus*. Using an integrated approach involving structure-activity relationships, protonation constants and crystal structure data on a focused synthetic library, revealed that precise spacing of positively charged groups and total charge at physiological pH distinguished potent biofilm inhibitors.

Most bacteria form biofilms – multicellular microbial communities embedded in a self-produced exopolymeric substance (EPS) largely composed of a protein anchor and different extracellular polymers. Bacteria within a mature biofilm community exist in an altered metabolic state and different physical environment than their free-floating, or planktonic, relatives. Biofilm bacteria generally tolerate antibiotic treatment[1,2], and antibiotics can induce biofilm formation.[3,4] Consequently, biofilm inhibitors can be applied to decrease antibiotic tolerance of bacteria.[5] Biofilms play a major role in many bacterial infections.[2] In humans, the antibiotic tolerance of biofilm communities frustrates the treatment of persistent bacterial infections such as those associated with cystic fibrosis, endocarditis, joint prostheses, heart catheters, or replacement heart valves.[6,7]

In response to this challenge, high-throughput assays have been developed to identify small molecules with the ability to prevent biofilm formation or disrupt existing biofilms.[8] We recently explored an alternative strategy that exploits the normal developmental cycle of bacteria. Biofilms form when planktonic bacteria in the aqueous phase aggregate on a solid surface or at an air-liquid interface. The biofilm colony grows both by recruitment and cell division to form a mature colony. Mature colonies eventually disintegrate, and the dispersed bacteria resume a planktonic lifestyle (Fig. 1). Bacterially produced small molecules orchestrate the creation and disintegration of biofilms, and identifying these molecular signals could lead to therapeutically useful templates.

We previously identified D-amino acids as potent biofilm disruptors due to their ability to release the protein component of EPS from the bacterial cell wall.[9] Recently we identified norspermidine as a key disruptor of the polymeric component of EPS.[10] Mixtures of norspermidine with D-amino acids were highly synergistic, single digit nanomolar, in disrupting biofilms (Fig. 1).[10] Here we report synthetic mimics of norspermidine with increased potency and a structure-based rationale for their activity.

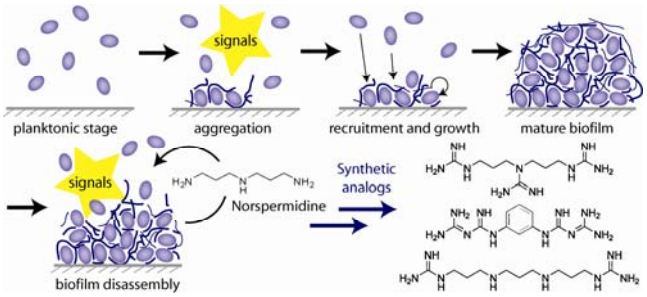
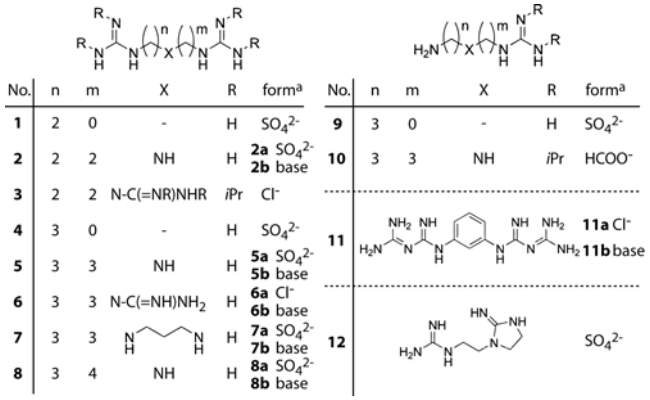


Figure 1. Stages in the developmental cycle of biofilm formation and disruption. Norspermidine both prevented the formation of new biofilms and collapsed the structure of existing biofilms.

Norspermidine appears to disrupt biofilms by targeting the extracellular component of EPS in *Bacillus subtilis*, and it seemed likely that it did so by binding to negatively charged or possibly neutral groups using Coulombic attraction and hydrogen bonding as important features.[10] We tested a set of commercially available polyamines. Norspermidine was most active in inhibiting biofilms for *B. subtilis* and *S. aureus*, followed by norspermine which has an additional aminopropyl unit in its structure (Figure S1, Table S2). Perhaps surprisingly, spermidine with one longer aminobutyl residue in place of an aminopropyl and diethylenetriamine with two shorter aminoethyl groups were inactive in both species. This sharp length dependence indicated that matching the NH to NH distance (4.9 Å) of the (poly)propyleneamine motif of norspermidine and norspermine to the pitch (4.6-5.3 Å) of various helical EPS structures determined or modeled for potential exopolymers (Table S1) formed a key feature. Binding of these polyamines to negatively charged secondary structures would neutralize the charge and collapse the aqueous

meshwork characteristic of mature biofilms.[10] This simple model – three or four positively charged groups separated by propyl units – could be tested against biofilm formation of *B. subtilis* with synthetic mimics, and guanidines and biguanides emerged as particularly appealing substitutes for polyamines because of their potentially increased overall charge at physiological pH values.



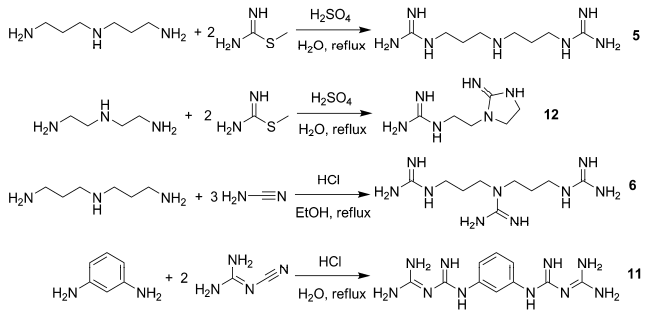
No.	n	m	X	R	form[a]
1	2	0	-	H	SO_4^{2-}
2	2	2	NH	H	**2a** SO_4^{2-} **2b** base
3	2	2	N-C(=NR)NHR	*i*Pr	Cl^-
4	3	0	-	H	SO_4^{2-}
5	3	3	NH	H	**5a** SO_4^{2-} **5b** base
6	3	3	N-C(=NH)NH_2	H	**6a** Cl^- **6b** base
7	3	3		H	**7a** SO_4^{2-} **7b** base
8	3	4	NH	H	**8a** SO_4^{2-} **8b** base

No.	n	m	X	R	form[a]
9	3	0	-	H	SO_4^{2-}
10	3	3	NH	*i*Pr	$HCOO^-$
11					**11a** Cl^- **11b** base
12					SO_4^{2-}

Figure 2: Library of synthetic guanidinylated or biguanidylated polyamine analogs. [a]Counter ion of salt or free base, for stoichiometry see Supporting Information.

We used three different synthetic strategies to generate a small library of compounds (Figure 2) with guanidinyl or biguanidyl groups as chloride or sulfate salts (Scheme 1). Guanidines can be conveniently prepared from amines with *S*-methylisothiourea (Scheme 1),[11] and that reagent led to terminal guanidines (**1**, **2**, **4**, **5**, **7**-**9**, **12**) from commercially available primary amines.

Scheme 1



Alternatively, cyanamide (or the alkylated form of its carbodiimide tautomer), which reacts with secondary amines (Scheme 1),[12] was used to prepare triguanidinylated compounds (**3**, **6**) and alkylated guanidines (**3**, **10**). Finally, a biguanide (**11**) was synthesized from *m*-phenylenediamine and dicyandiamide (Scheme 1) according to Cohn.[13] While aromatic amines are known to readily react with dicyandiamide,[13] our attempts to extend the reaction to primary aliphatic amines were unsuccessful.

Compounds were tested with inhibiting biofilm formation in *B. subtilis*, the model organism that led to norspermidine[10] and *Staphylococcus aureus*, as a related pathogenic species with high clinical relevance. The minimum biofilm inhibitory concentrations (MBICs) of all compounds are given in Table S2. Some of the compounds exhibited remarkable activity for the inhibition of biofilms with 5x to 20x increased activity with *B. subtilis* and more than 8x increased activity with *S. aureus* compared to norspermidine (Figure 3A, Tables 1 and S2). In addition to preventing biofilm formation the most potent compound was also able to disrupt existing biofilms (Figure 3B)

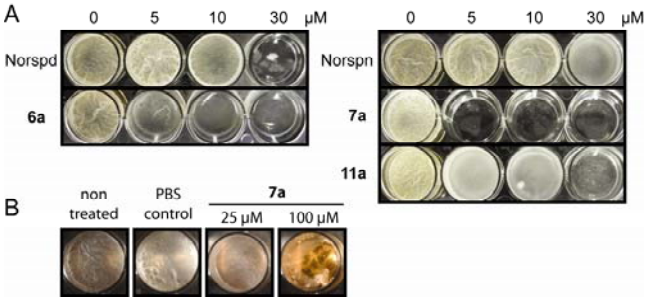


Figure 3: Enhanced activity of synthetic compounds **6a**, **7a** and **11a** against *B. subtilis*. A) Inhibition of biofilm formation compared to corresponding polyamines and B) breakdown of pre-existing biofilms within 12 h. Norspd: norspermidine; Norspn: norspermine; PBS: buffer.

Early on it became clear that the counter ion of the amines and guanidines had a significant effect on activity. For instance, the free base of norspermidine was 3x more active than the chloride salt, which in turn was 3x more active than the sulfate salt in the *B. subtilis* assay (Table S2). Therefore, we generated the free bases of selected compounds and compared them with the corresponding salts. For *B. subtilis* a free base's activity for biofilm inhibition was greater than or equal to the salt, while for *S. aureus* there was no clear trend. Solubility products (K_s) showed no correlation with the activity of the compounds, and bioavailablitiy and delivery into the biofilm matrix are probably critical parameters (Table S3). None of the compounds significantly inhibited bacterial growth at or close to its corresponding MBIC value, which rules out biofilm inhibition being an artifact of reduced viability (Supporting Information). Only compound **7** started to affect growth at concentrations above 200 μM in *B. subtilis* which corresponds to 40x its MBIC.

In *B. subtilis* the most active compounds were **6**, **7**, **8**, **10**, and **11** (as salt and base, Tables 1 and S2) with MBICs between 2 μM (**7b**) and 30 μM (**10**, **11a**). Active compounds exhibited the proposed binding motif[10] of three to four amino or guanidine groups formally separated by propyl chains (**5**-**7**, **10**, **11**, Tables 1 and S2). Additionally, the shorter compound (**9**) with only a single propyl chain displayed activity only at 100 μM. The activity pattern for *S. aureus* was slightly different as the minimal motif required for activity was two guanidine or one amino and one guanidine groups separated by a propyl chain (**4**-**11**). Compounds with ethyl chains instead of propyl (**1**-**3**, **12**) were inactive (≥1 mM) for *B. subtilis* and only weakly active (750 μM) or inactive for *S. aureus*. Biguanide itself was inactive for both species (Tables 1 and S2).

Table 1. Activity of selected compounds

Compound		MBIC (μM) at pH 7.4	
		B. subtilis	*S. aureus*
4		>1000	50
5	**a**	500	75
	b	375±125	400
6	**a**	10[a]	>1000 (500[a])
	b	10	50

7	**a**	5	55±15
	b	2	250
9		100	500
10		30	20±10
11	**a**	30	300
	b	7±3	750±250

[a]incomplete inhibition

The most active inhibitors of biofilm formation of *S. aureus* were **4**, **5a**, **6b**, **7a**, and **10** with MBICs in the range of 10-75 μM. The activity of the best biofilm inhibitors in this initial library is comparable to the lower range of what has been reported in the literature for biofilm inhibitory compounds that do not adversely affect bacterial growth.[8]

Table 2. pK_a values of selected compounds

Compound	pK_a values			
	1	2	3	4
Norspermidine	11.1±0.1	9.4±0.2	7.1±0.0	-
Norspermine	10.6±0.3	10.5±0.3	8.7±0.2	6.7±0.1
Spermidine	11.1±0.1	9.8±0.1	7.8±0.1	-
DET[a]	11.6±0.1	9.0±0.3	3.9±0.1	-
1	13.6±0.1	12.6±0.2	-	-
2	13.5±0.0	12.8±0.2	6.3±0.0	-
4	13.7±0.1	13.0±0.3	-	-
5	13.4±0.1	12.0±0.3	8.4±0.1	-
6	13.8±0.2	13.2±0.3	12.7±0.5	-
7	13.5±0.1	12.0±0.3	9.8±0.1	7.6±0.1
8	13.6±0.0	12.2±0.1	9.2±0.1	-
9	13.6±0.1	9.4±0.1	-	-
11	12.1±0.1	10.6±0.1	-	-

[a]diethylenetriamine

Our results support a model in which the binding of polyamine-based inhibitors to the exopolymer depends on the correct spacing of multiple amino or guanidine groups. The structure-activity relationship in this library further indicates that though there is a common motif in both species, the composition and structure of biofilms of *S. aureus* and *B. subtilis* are different and allow customized inhibition of biofilm formation.

In addition to the structural properties described above, the charge of the compounds could be an important contributor to their inhibitory activity.[10] To investigate this possibility, we determined the pK_a values of selected compounds at 25 °C at 25 mM. Cumulative association constants were calculated by HypNMR[14,15] (Figures S2-S11) and values for pK_a (D_2O) were finally converted to pK_a (H_2O).[16] The pK_a values for the compounds are given in Table 2. For comparison, similar pK_a values have been reported before for spermidine with pK_1 = 10.90, pK_2 = 9.71, and pK_3 = 8.25.[17]

Speciation data derived from pK_a values were generated using the program HySS (Supporting Information, Figures S12-S14) and average charge of the molecules was plotted against pH (Figure 4). For convenience protonation states will be given by a digit string with (1) for a protonated site and (0) for a non-protonated site. In this notation, fully protonated norspermidine is (111). Diethylenetriamine (DET) has one extremely low protonation constant of 3.9 that results in one uncharged amino group – (101) – in the wide pH range of 5-8 (Figure 4A). Although guanidine groups in the related structure **2** significantly increase the third pK_a value to 6.3, the central amino group remains unprotonated (101) at physiological pH values, which was confirmed by X-ray structure analysis (Supporting Figure S17).

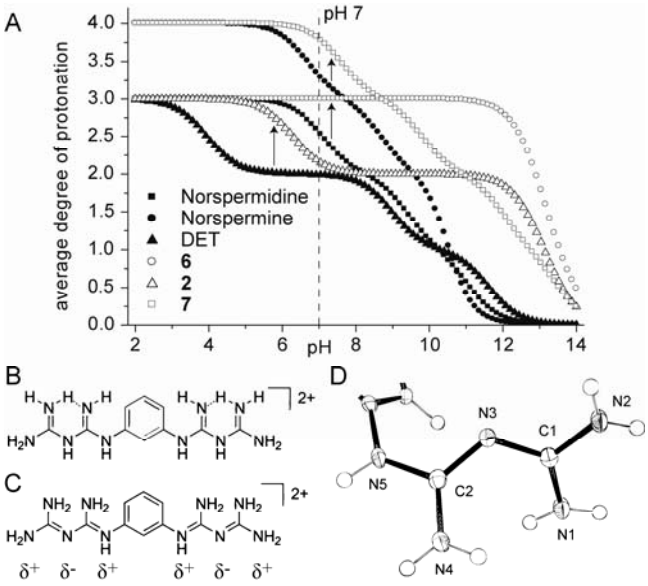


Figure 4: Protonation and charge states. A) Average protonation of polyamines and corresponding di- or triguanidines as a function of pH. B) Classical, incorrect representation of protonated biguanides, C) correct protonation state for **11**. D) Cropped biguanide moiety from crystal structure of **11**.

Its lack of activity at pH 7 reflects both structural and charge liabilities. Not surprisingly, guanidine groups on the scaffold of norspermidine or norspermine increased all individual pK_a values compared to the corresponding polyamines (Figure 4A) causing the average degree of protonation to rise, which is in line with the increased activity of **6** over norspermidine and **7** over norspermine. However, while **5** was active it did not display increased activity over norspermidine, despite its higher degree of protonation (Supporting Figure S16). On average, norspermine carries 3.3 positive charges at pH 7 corresponding to about 30% fully charged (1111) molecules and the rest triply charged (1110 or 1101) with the protonation micro-state (1101) with one non-charged secondary amine as the likely predominant species.[15,17,18] The (1101) species does not comply with the triply charged motif (111) and may contribute to the higher activity of norspermidine over norspermine.

The active compound **11** exists at maximum in a doubly protonated form due to its high pK_a values, and this form is virtually the only relevant species until pH 8.5 (Supporting Figure S16). In the literature, the structure and protonation of biguanides is frequently misrepresented as reported by Bharatam et al.,[19] whose computational studies indicated that the central nitrogen of biguanides is not bonded to a hydrogen both in neutral and charged states (Figure 4B and C). This N atom is partially negatively charged while the positive charge is delocalized between the terminal nitrogen atoms of a biguanide. The crystal structure of protonated **11** confirmed these results (Figure 4D and S19), making **11** analogous to the (1111) motif of fully charged compound **7** which explains its activity.

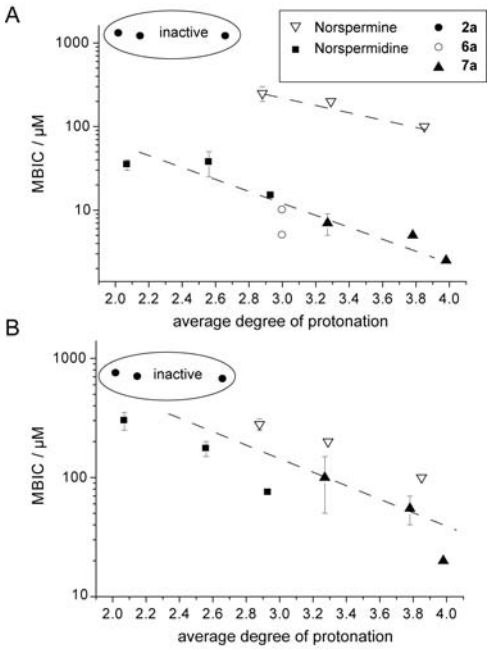


Figure 5: Inhibition of biofilm formation (MBICs) is dependent on the average degree of protonation of polyamines and guanidine compounds for A) *B. subtilis* and B) *S. aureus* show a clear trend. Error bars: SD of MBIC values.

Finally, to confirm the importance of charge for biological activity, we determined the biofilm inhibition in a pH-dependent assay that should directly affect the average charge state of assayed compounds. We plotted MBIC values in *B. subtilis* and *S. aureus* assays against the calculated degree of protonation (Figure 5). Although biofilm morphology and physiology as well as bioavailability of the compounds are expected to change with pH, the potency of active compounds correlated well with the degree of protonation. For all active compounds, activity generally increased (lower MBICs) in both species at higher protonation states while the inactive compound **2** did not respond to changes in protonation. Absolute activities of different compounds, however, did not coincide with the degree of protonation, suggesting that a combination of structure and charge determine biological activity.

In conclusion, chemical synthesis generated a focused library of guanidine and biguanide compounds that mimic norspermidine structurally and in some cases functionally with an ability to inhibit biofilm formation in *B. subtilis* and *S. aureus*. The best compound also mimicked norspermidine's ability to disrupt a mature biofilm. A detailed investigation of structure-activity relationships involving protonation constants and crystal structure data provided insights into the ways that charge and spacing between positively charged groups affect biological activity.

ASSOCIATED CONTENT

Supporting Information. Syntheses and characterization, biofilm inhibition data, pK_a measurements, speciation data and crystal structures. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* Email: jon_clardy@hms.harvard.edu (J. C.)

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

EPS exopolymeric substance, MBIC minimal biofilm inhibitory concentration, PBS phosphate buffered saline.

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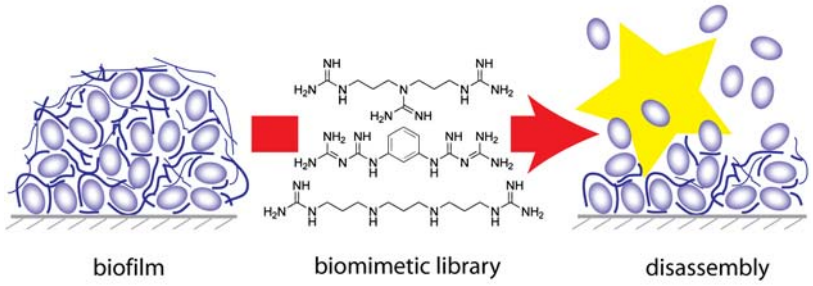
biofilm
biomimetic library
disassembly