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High-Throughput Screening of Metal-*N*-Heterocyclic Carbene Complexes against Biofilm Formation by Pathogenic Bacteria

Thierry Bernardi,*[a] Stéphanie Badel,[a] Pascal Mayer,[a] Jérome Groelly,[a] Pierre de Frémont,[b] Béatrice Jacques,[b] Pierre Braunstein,[b] Marie-Laure Teyssot,[c] Christelle Gaulier,[c] Federico Cisnetti,[c] Arnaud Gautier,*[c] and Sylvain Roland*[d]

A set of molecules including a majority of metal-*N*-heterocyclic carbene (NHC) complexes (metal = Ag, Cu, and Au) and azolium salts were evaluated by high-throughput screening of their activity against biofilm formation associated with pathogenic bacteria. The anti-planktonic effects were compared in parallel. Representative biofilm-forming strains of various genera were selected (*Listeria*, *Pseudomonas*, *Staphylococcus*, and *Escherichia*). All the compounds were tested at 1 mg L^{-1} by using the BioFilm Ring Test. An information score (IS, sum of the activities) and an activity score (AS, difference between anti-biofilm and anti-planktonic activity) were determined from normalized experimental values to classify the most active molecules against the panel of bacterial strains. With this method we identified lipophilic Ag^{I} and Cu^{I} complexes possessing aromatic groups on the NHC ligand as the most efficient at inhibiting biofilm formation.

The treatment of certain bacterial infections has become a major public health concern due to the increasing emergence of pathogenic bacterial strains that are resistant to most available antibiotics. This phenomenon involves all major microbial pathogens.[1] The increasing inefficiency of conventional antibiotic drugs used in clinical protocols renders antibacterial therapy more and more problematic. For instance, bacteremia (bloodstream infections) associated with *S. aureus* (one of the most frequently occurring bacteria found in clinical isolates) and *E. coli* lead to significant mortality and morbidity among patients, due to methicillin-resistant *S. aureus* (MRSA) and cephalosporin-resistant *E. coli* strains.[2] Resistant *P. aeruginosa* or MRSA are also responsible for respiratory tract infections, which are difficult to control, resulting in high mortality worldwide.[3] Bacterial resistance is induced by various cellular mechanisms involving efflux pumps, target modification, or drug inactivation by enzymes.[4] In addition, drug resistance in refractory infections is closely connected with the presence of bacterial biofilms,[5–7] which are a major cause of failure in the therapy of infections. Antibiotics have been shown to be 1000- to 1500-fold less active against bacteria that are confined within a biofilm than against individual free-form planktonic bacteria.[8] Strategies alternative to those involving conventional antibiotic treatments are needed to control bacterial resistance and tackle refractory infections. Among these, the development of new drugs able to prevent pathogenic bacteria from forming biofilms is essential.[9]

The combination of metal ions with organic molecules offers the opportunity to develop alternative drugs with new modes of action.[10] Although organometallic compounds are commonly used for the chemotherapy of some diseases such as cancer, the development of new metal-based drugs is of particular interest in the context of increasing chemoresistance to conventional therapeutics. This could open new possibilities for the treatment of pathogenic bacteria.[11] In the last decade, metal-*N*-heterocyclic carbene (NHC) complexes with various metal ions have been investigated for their anticancer and antibacterial properties.[12,13] Metal–NHCs are well adapted for drug design, owing to their synthetic flexibility and to the strength of the M–NHC bond that makes them good candidates as carriers for a wide range of metal ions in biological media.[14,15] In the antibacterial field, the most significant results have been obtained with Ag–NHCs,[16] which are capable of inhibiting the growth of various bacterial strains, including highly pathogenic strains, at very low silver concentrations.[12a,e,17] Metals are known as anti-biofilm agents. Metal ions can be incorporated into the matrix of biofilms and interfere with their formation.[18] To date, few metal–NHCs have been evaluated for their potential against bacterial biofilms.[19] A silver(I) imidazole cyclophane *gem*-diol and two xanthine-derived silver(I) complexes were shown to be effective against biofilms of *B. anthracis* and MRSA at low silver concentrations. Two pyr-

[a] *Dr. T. Bernardi, S. Badel, Dr. P. Mayer, J. Groelly*
Biofilm Control SAS, Biopôle Clermont-Limagne
63360 Saint-Beauzire (France)
E-mail: thierry.bernardi@biofilmcontrol.com

[b] *Dr. P. de Frémont, Dr. B. Jacques, Dr. P. Braunstein*
Laboratoire de Chimie de Coordination, UMR CNRS 8232
Université de Strasbourg
4, rue Blaise Pascal, CS 90032, 67081 Strasbourg (France)

[c] *Dr. M.-L. Teyssot, C. Gaulier, Dr. F. Cisnetti, Dr. A. Gautier*
Clermont-Université – Universite Blaise Pascal and ENSCCF
Institut de Chimie de Clermont-Ferrand, 63177 Aubière (France)
E-mail: arnaud.gautier@univ-bpclermont.fr

[d] *Dr. S. Roland*
Sorbonne Universités, UPMC Univ Paris 6
Institut Parisien de Chimie Moléculaire, UMR CNRS 8232
4, place Jussieu, 75005 Paris (France)
Fax: (+33) 1-44273056
E-mail: sylvain.roland@upmc.fr

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azine-functionalized Ag^{I} and Au^{I} complexes were also found to affect *S. mutans* and *E. coli* biofilms.

Recently, we gathered a significant library of metal–NHCs and azolium salts for biological application. This prompted us to evaluate their anti-biofilm activity against representative pathogenic bacteria. The automatized BioFilm Ring Test, a high-throughput screening tool with high reproducibility was used to detect molecules with high test scores. The most relevant results for 89 compounds are presented herein.

For this study we selected various NHC complexes with group 11 metals (Cu^{I}, Ag^{I}, and Au^{I}), with a majority being Ag–NHCs (Figures S1 and S2 in the Supporting Information). Most of the complexes are heteroleptic neutral complexes of the general formula [(NHC)MX] (M = Ag, Au, Cu) and cationic [$(NHC)_2M^+$, X^-] complexes (M = Ag, Cu). To evaluate the influence of the NHC ligand on anti-biofilm activity, various NHC ligand types derived from imidazolium or imidazolinium salts were tested, in which the nitrogen atoms are substituted with aromatic, heterocyclic (triazolyl, pyridyl), aliphatic, or functionalized groups. The activities of representative metal-complexed *N*,*N*′-diaryl-NHCs substituted with additional polar side chains (ammonium groups, alcohols) were also assessed. A set of imidazol(in)ium salts previously prepared by our research groups was also tested in parallel (Supporting Information Figure S3). All compounds were tested at a final concentration of 1 $\mu g\,mL^{-1}$.

The anti-biofilm activity was evaluated on the following bacterial strains: $Gram^+$ *Listeria monocytogenes* EGDe (food pathogen),[20] *Staphylococcus aureus* (ATCC25923, CIP7625), *Staphylococcus epidermis* (CIP105777), and $Gram^-$ *Pseudomonas aeruginosa* (CIP104116, CIP76110) and *Escherichia coli* DH5α. The results were collected after incubation times of 4, 6, and 8 h. This corresponds to 21 experiments per molecule.

The standard crystal violet method has been widely used to evaluate the propensity of a given compound to inhibit biofilm formation. However, this method is not well adapted for rapid screening of large libraries, mainly due to accumulation of non-standardized manipulations (washing, staining, de-staining, and drying steps), which can lead to high result variations for the same compound.[21] In addition, the test requires at least 24–48 h for completion. In the present study, we preferred the automatized BioFilm Ring Test (BRT), which is much less time-consuming owing to fewer manipulations after the initial bacterial inoculation (no washing and staining steps). Moreover, this method was shown to be highly reproducible. In short, the BRT is based on the immobilization of well-dispersed super-paramagnetic beads by forming bacterial aggregates (biofilm) with sufficient strength to overcome displacement when a magnetic field is applied.[22] In the absence of a biofilm, aggregation of the magnetic beads into a single point is easily detectable after magnet contact at the center bottom of the wells (Figure 1). In contrast, in the presence of an established biofilm, the well-dispersed beads remain in place, and no aggregation is observed upon magnetization. Bead mobility is indicative of the status of biofilm formation, which can be determined by comparison of the images obtained in the presence or absence of a given test compound.

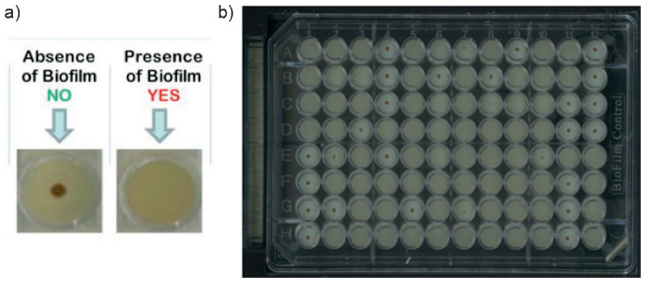

Figure 1. a) Principle of the BioFilm Ring Test: images of the wells containing super-paramagnetic beads after magnetization in the absence and presence of biofilm. b) Example of a 96-well plate (*S. aureus aureus* CIP7625) after magnetization, in which active compounds are visualized by the presence of the brown spots.

The activity of metal–NHCs on planktonic growth and biofilm formation was quantified with the BioFilm Control Elements software tools (BioFilm Control, France). The turbidity of the solution, estimated from an image acquired before magnetization and contrasting liquid addition, gives an indicator of planktonic growth, coined *TURB*. In parallel, the variation of the information content between pre- and post-magnetization images, which is a result of sub-pixel-aligned image analysis algorithms,[23] gives an indicator of biofilm formation coined *BFI* (BioFilm Index). These indices were normalized respectively as $TURB_{norm}$ and BFI_{norm}, by applying the normalization transform $[Val_{sample}-Val_{control}]/[Val_{sample}+Val_{control}]$. Both normalized values range from +1 to −1, i.e., from activation to inhibition, respectively, of biofilm formation and planktonic growth.

To get a general view of the ability of the compounds to inhibit biofilm formation, we determined an information score (IS), which indicates the general level of activity of a given compound against the panel of bacterial strains at different incubation times, regardless of its activity type (anti-biofilm or bactericidal), and an activity score (AS), which indicates the specificity of the molecule as an anti-biofilm agent. The IS and AS values were derived from a global anti-planktonic score ($TURB_{score}$) and a global anti-biofilm score (BFI_{score}). For each compound, these values, calculated as $TURB_{score} = [\{Count(TURB_{norm} > 0.4)\} - \{Count(TURB_{norm} < -0.4)\}]/21$ and $BFI_{score} = [\{Count(BFI_{norm} > 0.4)\} - \{Count(BFI_{norm} < -0.4)\}]/21$, are the average differences across all 21 experiments between the count of the number of normalized values > 0.4 and that of the number of normalized values less than −0.4. The arbitrary limit at 0.4 introduced in this calculation enabled the detection of compounds with the most significant activities. An information score was then expressed as $IS = |BFI_{score}| + |TURB_{score}|$, and an activity score as $AS = BFI_{score} + TURB_{score}$. Because $TURB_{score}$ is negative for compounds with significant antibacterial activity, a negative AS indicates a more pronounced antibacterial activity, whereas a positive AS demonstrates a general anti-biofilm specificity for the compound in question.

From IS and AS values, the set of all molecules could be rapidly classified according to their general activity (IS) and anti-biofilm specificity (AS) (Supporting Information Figures S4 and S5). An initial observation of the results showed that a large majority of the compounds have visible anti-biofilm or anti-

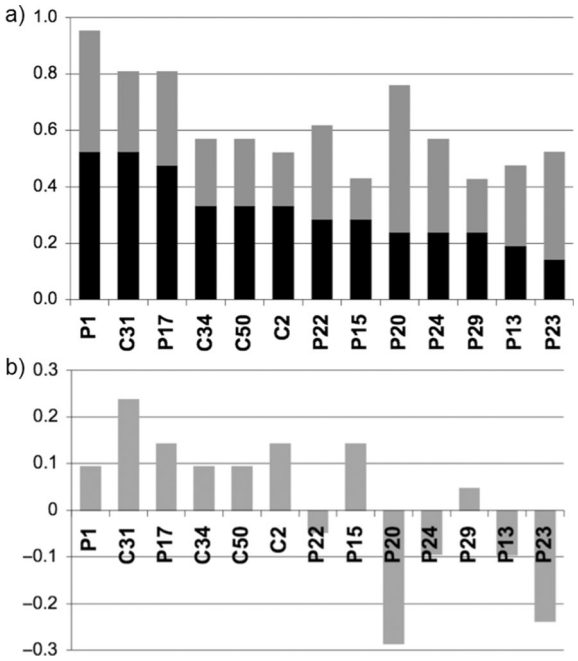

Figure 2. Information score (IS) and activity scores (AS) of the 13 most active molecules selected for IS > 0.4 (see text). a) IS: sum of the absolute values of the global anti-biofilm score (BFI_{score}, black bars) and the global anti-planktonic score ($TURB_{score}$, grey bars). b) AS = BFI_{score} + $TURB_{score}$: positive values indicate more pronounced anti-biofilm activity.

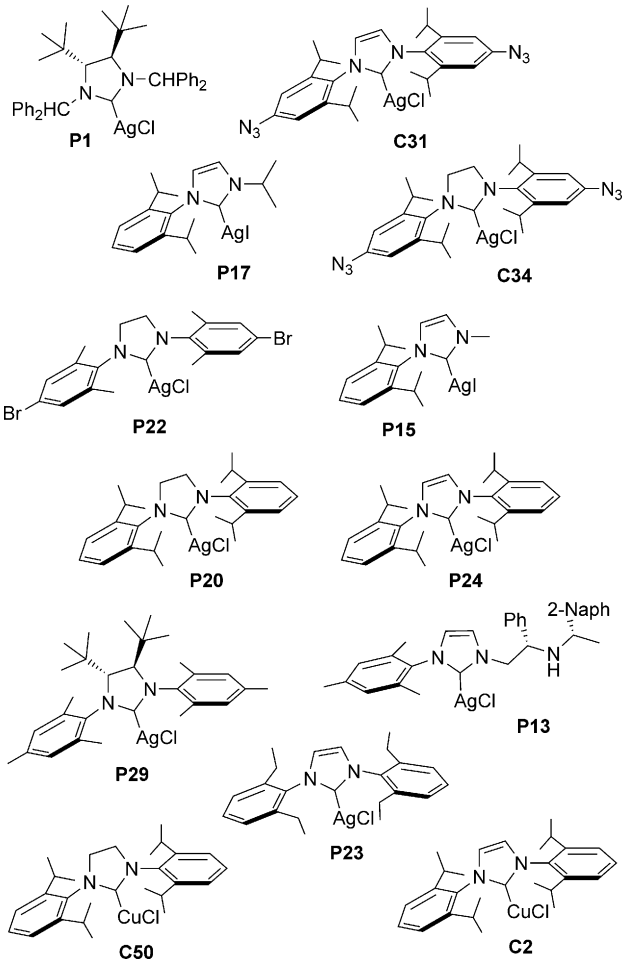

Figure 3. Structures of the 13 most active complexes selected for IS > 0.4.

planktonic activities. In a more precise analysis, we observed that 13 metal–NHCs exhibited IS > 0.4, suggesting high anti-biofilm and anti-planktonic activities against several of the bacterial strains (Figure 2a). As depicted in Figure 3, this family of active complexes is largely characterized by the presence of lipophilic NHC ligands with bulky aromatic groups on the nitrogen atoms. It is exclusively composed of neutral heteroleptic complexes with predominantly silver as the metal. Silver is also present in the three most active complexes (**P1**, **C31**, and **P17**) for which IS > 0.8, and a BFI_{score} value of ~0.5 was determined. Among these, **C31** exhibits the highest positive AS value, which corresponds to the highest anti-biofilm specificity (Figure 2b). In parallel, two Cu–NHCs (**C50** and **C2**) were also found to display high general antibacterial activity, with a BFI_{score} close to 0.3.

Previous results showed that **P1** and **P17** display different antibacterial activities against *S. aureus* and *E. coli*, suggesting that the NHC ligand and the halide play an important role in the selectivity of the complexes for the bacterial strain. Similar factors could affect the selectivity of metal–NHCs as anti-biofilm agents. Therefore, their activity against the different bacterial strains was examined separately.

To select the best method to analyze BFI_{norm} values, we first compared the BFI_{norm} values at $t = 8$ h with the average of the BFI_{norm} values recorded at $t = 4$, 6, and 8 h. Classification of the molecules according to these values gave similar results for some bacterial strains such as *P. aeruginosa* CIP104116 or *S. aureus* ATCC25923 (Supporting Information Figure S5). With other strains such as *E. coli*, the active compounds are also detected with both values, although they appear in a different order (Figure S5). Consequently, it was possible to compare the compounds according to their BFI_{norm} value at 8 h, which is representative of their general potential as anti-biofilm agents (Table 1).

A general analysis of the data shows first that most of the 13 complexes display high activity against *S. aureus* strains regardless of the metal (Ag or Cu). Secondly, none of the 13 complexes is active against the whole set of bacterial strains. Besides, none of the complexes is significantly active against *S. epidermis* or *P. aeruginosa* CIP76110. The superior scores observed with **P1** and **C31** are clearly associated with high BFI_{norm} values (> 0.4) for four of the strains. Both complexes strongly inhibit biofilm formation of *S. aureus* and display significant activity against *E. coli* and *P. aeruginosa* CIP104116. The highest BFI_{norm} value with *L. monocytogenes* was also observed for **P1**, although the inhibition is moderate (~0.4). Both complexes (**P1** and **C31**) are silver chloride complexes with bulky aromatic substituents on the NHC ligand. The other complexes can be classified in four different categories. The first one includes complexes **C2**, **P29**, **P24** (NHC–AgCl) and **P13** (NHC–CuCl), which exclusively display anti-biofilm activity against *S. aureus*. The second category involves **P20**, **P22**, **P23**, **C34** (NHC–AgCl) and **C50** (NHC–CuCl), and is characterized by activity against

Table 1. BFI_{norm} values at $t = 8$ h for molecules with IS > 0.4.[a]

Compd	Bacterial strain						
	L. monocytogenes EGDe	*P. aeruginosa* CIP104116	*S. epidermis* CIP105777	*E. coli* DH5α	*S. aureus aureus* CIP7625	*P. aeruginosa* CIP76110	*S. aureus* ATCC25923
P1	0.398	0.407	0.045	0.713	0.913	−0.047	0.897
C31	0.290	0.543	0.137	0.476	0.919	−0.012	0.902
P17	0.287	0.651	0.229	0.522	0.229	0.101	0.231
C34	−0.01	−0.110	−0.067	0.472	0.920	−0.093	0.904
C50	−0.084	−0.321	−0.084	0.661	0.916	−0.070	0.899
C2	0.038	−0.025	−0.067	0.144	0.887	−0.055	0.887
P22	0.054	−0.321	0.026	0.458	0.914	0.045	0.892
P15	−0.007	0.641	0.033	−0.031	−0.040	0.001	−0.054
P20	0.278	−0.325	0.006	0.432	0.916	0.001	0.900
P24	0.016	−0.181	0.045	0.236	0.915	−0.033	0.898
P29	−0.264	−0.311	0.038	0.063	0.789	−0.026	0.907
P13	0.193	0.003	0.006	0.343	0.736	−0.070	0.820
P23	−0.142	−0.476	−0.008	0.703	0.916	−0.077	0.899

[a] Values are the result of one experiment. The screening strategy was oriented toward the detection of anti-biofilm molecules with high reliability against a range of representative bacterial strains (multiple targets) rather than on obtaining highly precise results on a single target (a given bacterial strain). Low standard deviations have been routinely obtained with the BioFilm Ring Test in repeated experiments (see ref. [22], $\sigma_{avg} \sim 6\%$).

Table 2. BFI_{norm} values at $t = 8$ h for selected molecules with IS < 0.4.

Compd[a]	Bacterial strain						
	L. monocytogenes EGDe	*P. aeruginosa* CIP104116	*S. epidermis* CIP105777	*E. coli* DH5α	*S. aureus aureus* CIP7625	*P. aeruginosa* CIP76110	*S. aureus* ATCC25923
LR36	0.410	0.256	0.056	0.00	−0.053	−0.041	0.009
P33	0.714	−0.224	0.062	−0.017	−0.079	−0.018	−0.033
LR40	0.270	0.463	−0.057	−0.063	−0.062	0.031	−0.025
P4	0.144	0.626	−0.092	0.050	−0.049	0.020	−0.094
P7	0.085	0.562	−0.037	0.019	−0.022	−0.101	−0.094
P8	−0.127	0.527	0.082	0.150	0.073	−0.077	−0.086
P9	0.181	0.469	0.019	0.093	0.066	0.045	−0.017
P14	−0.077	0.594	−0.092	−0.040	0.051	−0.012	−0.070
P38	−0.042	−0.280	−0.014	0.092	0.366	0.441	0.455
P44	−0.150	−0.227	−0.007	0.041	0.509	0.508	0.536

[a] Compound structures are provided in Supporting Information Figures S1–S3.

both *E. coli* and *S. aureus*. Interestingly, except **P23**, all these complexes possess saturated NHC ligands derived from imidazolinium salts. In contrast, **P15** displays a unique and interesting profile owing to selective inhibition of biofilm formation by *P. aeruginosa* CIP104116. Finally, **P17** exhibits activity similar to that of **P15** against *P. aeruginosa* CIP104116 (~0.65), but was also found to inhibit *E. coli* biofilm formation. Both **P15** and **P17** are silver complexes with iodido ligands. This suggests that iodide could favor selectivity against $Gram^-$ bacteria. Importantly, **P23** is as active as **P1** against *E. coli* and *S. aureus*. However, it also promotes biofilm formation in the case of *P. aeruginosa* CIP104116, with a negative BFI_{norm} value of −0.476.

Next, we carefully analyzed the anti-biofilm activities of all compounds that were not identified for IS > 0.4. This was done to check the validity of the initial selection method and to detect other molecules with significant activities against a particular bacteria. This showed that an significant set of compounds (**C12**, **C17**, **C18**, **C19**, **C21**, **C47**, and **P5**) exhibited a profile similar to that of **C2**, **P29**, **P24**, and **P13** with high activity and selectivity against *S. aureus* biofilm formation (Supporting Information Table S1). Analysis of the results revealed that the lower scores of these compounds are due to their ability to activate biofilm formation in the case of *P. aeruginosa* CIP104116. This property was observed with a dozen molecules that display negative BFI_{norm} values for this strain, ranging from −0.3 to −0.5 (Table S1). Note that **C47** and **C18** are azolium salts (NHC·HCl), precursors of **P24** and **C19**/**C21**, respectively. In the latter case, it is not clear whether the activity is metal- or ligand-based. From the data, we also observed that the cationic homoleptic Ag complex **LR36** and the CuI complex **P33** exhibited high selectivity for *L. monocytogenes* biofilms (Table 2). This particular selectivity is rarely observed across the library of compounds. Another set of molecules (**LR40**, **P4**, **P7**, **P8**, **P9**, and **P14**) was identified for near exclusive selectivity against *P. aeruginosa* CIP104116 (Table 2). **LR40** and **P9** are both cationic complexes with Au^I or Ag^I, respectively, as the metal. More, interestingly, **P4**, **P7**, **P8**, and **P14** are Ag–NHC complexes characterized by the presence of iodido ligands. Their profile is similar to that of the AgI complex **P15**. Finally, the study highlight-

ed two compounds (**P38** and **P44**), which are azolium salts, as having notable activity against *P. aeruginosa* CIP76110.

In conclusion, we have shown that various metal–NHC complexes with group 11 metals, as well as some azolium salts, are capable of inhibiting biofilm formation of representative pathogenic bacteria at low concentrations. We have demonstrated the pertinence of the BioFilm Ring Test as a high-throughput screening method for the discovery of antibacterial metallodrug candidates. The results highlight that Ag complexes possessing lipophilic NHC ligands have the broadest anti-biofilm activity, although some Cu complexes also displayed high scores. In addition, we demonstrated that some metal–NHCs and azolium salts, associated with lower scores, exhibited selective but significant activity against one or two pathogenic bacterial strains. This opens new perspectives for the development of new metal-based drugs for the treatment of refractory bacterial infections associated with biofilms.

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Keywords: antibiotics · biofilms · carbene ligands · group 11 elements · high-throughput screening

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