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Angélique Mottais[a], Mathieu Berchel[b], Tony Le Gall[a], Yann Sibiril[a], Frédérique d'Arbonneau[a], Véronique Laurent[a], Paul-Alain Jaffrès[b], and Tristan Montier[a,*]

[a] *Univ Brest, INSERM, groupe "Transfert de gènes et thérapie génique", UMR 1078, CHRU de Brest, 22 avenue Camille Desmoulins, F-29238 Brest, France*

[b] *Univ Brest, CNRS, CEMCA, UMR CNRS 6521, 6 Avenue Victor Le Gorgeu, F-29238 Brest, France*

Corresponding author* : UFR Médecine et Sciences de la Santé, CHRU Brest, 22 avenue Camille Desmoulins, 29238 Brest (France); Tel: (+33) 2 98 01 80 80; Fax: (+33) 2 98 46 79 10; E-mail address: tristan.montier@univ-brest.fr (T. Montier).

Abbreviations

Ag, silver; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; NHC, N-heterocyclic carbene complexe ; MIC, minimum inhibitory concentration; MRSA, methicillin resistant *Staphylococcus aureus ;* Sa, *Staphylococcus aureus* ; Pa, *Pseudomonas aeruginosa*

Abstract

The development of new antibacterial molecules is essential in view of the emergence of pathogenic strains resistant to multiple antibiotics. Among the infectious pathologies, pulmonary infections are particularly difficult to treat due to the complexity of lung anatomy and the presence of natural barriers such as mucus. At present, the aerosol delivery of antibacterial compounds is still poorly employed. Furthermore, the presence of bacteria in lungs negatively affects aerosolized Cystic Fibrosis gene therapy efficiency. A multi-functional formulation (antibacterial and transfection activities) could increase the therapeutic effect. This work reports the synthesis of new *N*-heterocyclic carbene silver complexes (Ag-NHC) featuring a lipid chain and the evaluation of their antibacterial potency, especially when delivered following aerosolization. When formulated alone in water, these Ag-NHC displayed remarkable antibacterial activities against some *Staphyloccocus aureus* strains and *Pseudomonas aeruginosa* clinical strains. Moreover, combined with cationic lipid and DNA (ternary combination), they could be used to deliver therapeutic genes *via* aerosolization in infected lungs. Altogether, the data reported herein support n-alkyl chain Ag-NHC as a possible alternative to conventional antibiotics for treating respiratory infections and to combat the emergence of multi-resistant bacteria.

Keywords

silver *N*-heterocyclic carbene; lung therapy; antibacterial activity; aerosolization; synthetic vector

1. Introduction

The World Health Organization has identified the research of new antibacterial drugs as a pressing priority particularly for infectious respiratory context, due to the emergence of extremely-drug resistant strains (Jansen et al., 2018; Tacconelli et al., 2018). Nowadays, lung infections and other disorders with infectious complications are more and more difficult to treat with classical antibiotics such as beta-lactam class delivered *per os* (Ciofu et al., 2015). Considering the pneumonitis, new antibacterial molecules are required but also an efficient and well-tolerated administration method such as aerosol delivery (Mottais et al., 2017). In this sense, Cystic Fibrosis (CF) may be considered as a challenging model. The absence/malfunction of the CFTR (cystic fibrosis transmembrane conductance regulator), a chloride channel located at the apical membrane of epithelial cells, is due to some mutations in the *CFTR* gene (Riordan et al., 1989). It leads to a hyper-viscous mucus obstructing the respiratory tract. This environment favors the development of microbial infections (with bacteria, fungi and viruses) and their presence stimulates the immune system which damages the pulmonary parenchyma (Cookson et al., 2018; Frayman et al., 2017; Pihet et al., 2009). The infections caused by *Pseudomonas aeruginosa*, a Gram-negative bacillus, are chronic in adult CF patients and their treatment require recurrent intravenous injections of antibiotics. Although such treatment is still quite efficient, it also causes some major side effects such as hepatic cytolysis and renal failure. In addition, repeated injections of antibiotics can promote the emergence of multi-resistant strains that are now frequently reported (Stefani et al., 2017). A similar scenario is also documented with methicillin-resistant *Staphylococcus aureus* (MRSA) implicated in numerous nosocomial infections (Cohen et al., 2017). Thus, the development of new treatments that could be administrated in a safe way and that would limit the emergence of resistance represents a major healthcare challenge.

The antibacterial properties of silver are now well-established (Barillo and Marx, 2014; Barras et al., 2018; Chernousova and Epple, 2013). Silver derivatives (*N*-heterocyclic carbenes (NHC) (Liang et al., 2018; Teyssot et al., 2009), hybrid materials (Rueff et al., 2015), and polymers (Radheshkumar and Münstedt, 2006)) offer promising perspectives as bactericidal agents due to their likely multi-target mode of action. Furthermore, little emergence of resistance has been reported so far with the use of compounds incorporating silver (Youngs et al., 2012). However, its action mechanism is still not

completely understood; Ag^+ ions may interact with the plasma membrane and some key enzymes (Youngs et al., 2012). Silver has been used clinically as a standalone compound (e.g. $AgNO_3$) or in combination with an antibiotic (e.g. with sulfamides in sulfadiazine), mainly for the treatment of burn wound infections (Jodar et al., 2015). Ag^+ ions have also been shown to be effective to treat a CF patient infected with a bacterial strain resistant to multiple antibiotics (Baral et al., 2008). Other metallic ions are under investigation in CF, like gallium, for their antibacterial properties (Goss et al., 2018).

N-Heterocyclic carbene (NHC) complexes were first described in the 1960s by Wanzlick (Wanzlick, 1962). They can be associated with metals to form stable metallo-carbene complexes (Teyssot et al., 2009; Garrison and Youngs, 2005). NHC complexes were mainly studied for their catalytic properties (Ren et al., 2017). In recent years, bioactive properties have been identified for these compounds (anticancer, antibacterial...) suggesting new biomedical perspectives (Hindi et al., 2009a, 2009b; Hopkinson et al., 2014; Iqbal et al., 2015; Medvetz et al., 2008; Patil et al., 2015; Saturnino et al., 2016). Thus, the biological potential of NHC complexes is an important research area in the field of organometallic chemistry. The main bioactive NHCs incorporate gold (Au), silver (Ag), copper (Cu), palladium (Pd) or platinum (Pt). Silver *N*-heterocyclic carbene complexes (Ag-NHC) are thus used for their bactericidal properties (Hindi et al., 2009a; Kascatan-Nebioglu et al., 2007; Roland et al., 2011; Leid et al., 2012; Lim et al., 2015). Ag-NHC 5 ((4,5-dichloro-1,3-dimethyl-2,3-dihydro-1H-imidazol-2-yl) silver acetate) (**Fig. 1**) has shown antibacterial activities against a panel of Gram-positive and Gram-negative bacteria such as *P. aeruginosa* (Berchel et al., 2011; Hindi et al., 2008). Ag-NHC 12 (**Fig. 1**), a structural analog of Ag-NHC **5** incorporating a polycyclic aromatic hydrocarbon (naphthalene), was shown to be active against two Gram-negative bacteria which are involved in respiratory tract infections (*Burkholderia pseudomallei* and *Burkholderia mallei*) (Panzner et al., 2009).

Besides our interest in Ag-NHC compounds, we have previously reported that some cationic amphiphiles can exhibit remarkable bactericidal activities against Gram-positive bacteria (Le Gall et al., 2013). We showed that the structure of the lipid domain deeply influenced the antibacterial activity of these compounds. In another report, we demonstrated that Ag-NHC 5 can be combined with a

cationic lipid in view to enlarge the spectrum of its antibacterial activity (Mottais et al., 2018). Nevertheless, it was not determined whether Ag-NHC 5 remained as a standalone compound or if it effectively assembled with cationic lipids. In the present report, we wondered whether the incorporation of a long alkyl chain within the structure of Ag-NHC compound would further improve their bactericidal effects while preserving the transfection capacity of the formulations. After their synthesis and formulations, we first evaluated the antibacterial potency of each new Ag-NHC compound in comparison with benchmarks compounds, (i) under standard conditions (by microdilution evaluations in liquid culture) and (ii) under particular condition, highly relevant with a treatment of respiratory tract (i.e. by means of nebulization). Secondly, the cytotoxicity of the new Ag-NHCs was evaluated by direct deposition or post-nebulization on bronchial epithelial cells. Lastly, the transfection efficiency following nebulization was determined considering human-derived epithelial cells' cultures.

2. Material and methods

2.1. Materials

Compounds 1a and 1b. 4,5-dichloroimidazole (2.0 g, 14.60 mmol) and KOH (901 mg, 16.06 mmol) were combined in anhydrous acetonitrile (ACN) (50 mL) at room temperature. The reaction was allowed to stir for 3 hours before 1-bromotetradecane (1a, 4.5 g, 11.05 mmol) or 1-Bromooctadecane (1b, 5.4 g, 16.04 mmol) was added and the reaction was heated to reflux for 18 h. After being cooled at room temperature, the precipitated salts were separated by filtration and dried *in vacuo*.

1a. (3.4 g, 99 % Yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (t, 3H, J= 6.4 Hz), 1.22-1.38 (m, 22H), 1.74 (m, 2H), 3.87 (t, 2H, 6.4 Hz), 7.33 (s, 1H). ^{13}C NMR (162 MHz, $CDCl_3$): δ 14.1, 22.6, 26.4, 28.9-31.9, 35.9, 49.4, 118.9, 119.9, 137.0.

1b. (3.0 g, 87 % Yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, 3H, J= 6.4 Hz), 1.24-1.35 (m, 30H), 1.74 (m, 2H), 3.87 (t, 2H, 6.4 Hz), 7.35 (s, 1H). ^{13}C NMR (162 MHz, $CDCl_3$): δ 14.1, 22.6, 26.4, 28.9-31.9, 35.9, 49.4, 118.9, 119.9, 137.0.

Compounds 2a-2d. Methyl iodide (1.0 equiv. for 2a and 2b) or 2-bromomethylnaphthalene (1.0 equiv. for 2c and 2d) was added to compounds 1a (500 mg, 1.50 mmol) or 1b (500 mg, 1.30 mmol) in anhydrous ACN (20 mL) and the reaction was allowed to stir at reflux for 18 hours. After being cooled to room temperature, the precipitated salts were separated by filtration and dried *in vacuo*.

2a. (391 mg, 67% Yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (t, 3H, *J*= 6.4 Hz), 1.22-1.40 (m, 22H), 1.96 (m, 2H), 4.07 (s, 3H), 4.31 (t, 2H, 6.4 Hz), 10.78 (s, 1H). ^{13}C NMR (162 MHz, $CDCl_3$): δ 14.1, 22.6, 26.4, 28.9-31.9, 35.9, 49.4, 118.9, 119.9, 137.0.

2b. (601 mg, 87% Yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (t, 3H, J= 6.4 Hz), 1.22-1.40 (m, 24H), 1.95 (m, 2H), 4.34 (t, 2H, 6.4 Hz), 5.85 (s, 2H), 7.50 (m, 2H), 7.65 (m, 1H), 7.80-7.83 (m, 3H), 7.85 (s, 1H), 10.78. ^{13}C NMR (162 MHz, $CDCl_3$): δ 14.1, 22.7, 26.2, 28.9-31.9, 35.8, 42.3, 49.5,137.1.

2c. (693 mg, 87% Yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (t, 3H, J= 6.4 Hz), 1.21-1.31 (m, 24H), 1.72 (m, 2H), 3.85 (t, 2H, 6.4 Hz), 4.65 (s, 2H), 7.43-7.50 (m, 2H), 7.79-7.83 (m, 1H), 7.80-7.85 (m, 3H), 8.08 (s, 1H), 11.66 (s, 1H). ^{13}C NMR (162 MHz, $CDCl_3$): δ 14.1, 22.7, 26.2, 28.9-29.7, 31.9, 49.8, 52.7, 113.5, 125.5-133.1, 133.4, 137.8.

2d. (690 mg, 87% Yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (t, 3H, J= 6.4 Hz), 1.21-1.38 (m, 24H), 1.95 (m, 2H), 4.34 (t, 2H, 6.4 Hz), 5.84 (s, 2H), 7.50 (m, 2H), 7.65 (m, 1H), 7.80-7.90 (m, 3H), 8.07 (s, 1H), 11.60 (s, 1H). ^{13}C NMR (162 MHz, $CDCl_3$): δ 14.1, 22.7, 26.2, 28.9-29.7, 31.9, 49.8, 52.7, 113.5, 125.5-133.1, 133.4, 137.8.

Compounds 3a-d. Imidazolium salts (2a-2d, 1.0 equiv.) and silver acetate (2.0 equiv.) were combined in anhydrous dichloromethane (DCM) (10 mL). The flask was covered with foil and the reaction was stirred in darkness for two days. The final solution was filtered over Celite, rinsed with DCM and the filtrate was concentrated. The oily residue was diluted with a minimum amount of DCM and excess diethyl ether was added. The reaction was cooled to -20°C for 72 h. White crystals were filtered, dried *in vacuo* and stored in the dark at -20°C.

3a. (520 mg, 70% Yield). ^{1}H NMR (500 MHz, CD_2Cl_2): δ 0.86-0.89 (t, 3H, J= 6.0 Hz), 1.26-1.33 (m, 22H), 1.79 (m, 2H), 1.94 (s, 3H), 3.81 (s, 3H), 4.11-4.13 (t, 2H, J= 6.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.1, 22.6, 26.4, 29.1-31.9, 51.6, 54.9, 117.4, 117.6, 124.9-129.1, 131.6, 133.1, 179.1.

3b. (620 mg, 71% Yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, 3H, J= 6.0 Hz), 1.24-1.35 (m, 30H), 1.77 (m, 2H), 2.08 (s, 3H), 3.08 (s, 3H), 4.10 (t, 4H, J= 6.0 Hz). ^{13}C NMR (162 MHz, $CDCl_3$): δ 14.1, 22.6, 26.4, 29.1-31.9, 51.6, 54.9, 117.4, 117.6, 124.9-129.1, 131.6, 133.1, 179.1.

3c. (500 mg, 62% Yield). ^{1}H NMR (500 MHz, CD_2Cl_2): δ 0.87 (t, 3H, J= 6.0 Hz), 1.24-1.31 (m, 22H), 1.80 (m, 2H), 2.08 (s, 3H), 4.16 (t, 2H, J= 6.0 Hz), 5.48 (s, 2H), 7.43-7.50 (m, 3H), 7.79-7.83 (m, 4H). ^{13}C NMR (125 MHz, CD_2Cl_2): δ 14.2, 22.8, 23.1, 26.7, 29.5-30.1, 32.3, 51.9, 55.1, 117.7, 118.0, 125.4-129.2, 132.5-133.6, 178.5, 180.6.

3d. (350 mg, 41% Yield). ^{1}H NMR (500 MHz, CD_2Cl_2): δ 0.87-0.90 (t, 3H, J= 6.0 Hz), 1.26-1.36 (m, 30H), 1.82-1.85 (m, 2H), 1.95 (s, 3H), 4.17-4.20 (t, 4H, J= 6.0 Hz), 5.53 (s, 2H), 7.47-7.52 (m, 3H), 7.83-7.87 (m, 4H). ^{13}C NMR (125 MHz, CD_2Cl_2): δ 14.2, 22.8, 23.1, 26.7, 29.5-30.1, 32.3, 51.9, 55.1, 117.7, 118.0, 125.4-129.2, 132.5-133.3, 178.5, 180.6.

Formulations: Unlike Ag-NHC 5 diluted in water, the neo-synthesized silver carbenes (3a-d), are much less water soluble, which is likely due to their hydrophobic alkyl chain. The water solutions of compounds 3a-d were prepared by adding an ethanol solution of compounds 3a-d. The final concentration of the stock solutions of compounds 3a-d were 1.5 mM and the content of ethanol in these water solutions was 5%. The formulation of the Ag-NHC 3a-d with the cationic lipid KLN47 in sterile water (formulation with a molar ratio KLN47/3a-d was 1/0.25) was prepared by hydration of a lipid film containing the correct proportion of the two components. KLN47 was synthesized following a reported procedure (**Fig.S1**) (Picquet et al., 2005).

2.2. Methods

2.2.1. Bacterial culture

The bacterial strains used in this work are Gram-positive bacteria (i.e. four *S. aureus* N315, RN4220, Xen31® and Newman) and Gram-negative bacteria (i.e. one *Escherichia coli* MG1655 and three *P aeruginosa* Xen5®, 130709 and 240709). The characteristics of each strain was detailed in

previous studies (Le Gall et al., 2013; Mottais et al., 2018) and again reported in supplementary information.

2.2.2. *Cell line*

The 16HBE$^{14o-\ line}$ (bronchial epithelial cells) was used in this work (Cozens et al., 1994). These non-cancerous cells were seeded in a 96-well plate at a rate of 25,000 cells by well in complete medium (Essential Minimum Eagle medium (EMEM, Lonza) supplemented with 10% of fetal bovine serum, 1% of an antibiotics mixture (penicillin, streptomycin) and 1% of L-glutamine).

2.2.3. *Determination of the minimum inhibitory concentration (MIC)*

For a given compound, it corresponds to the minimal concentration able to inhibit the growth of a bacterial strain. The various chemical compounds were diluted to obtain a concentration range from 5 to 200 µM (5, 10, 20, 30, 40, 50, 60, 70, 80, 100 and 200 µM) in a final volume of 200 µL (medium inoculated with the different bacterial strains). After incubation of the plates for 24 hours at 37°C with stirring (180 rotations per minute), a reading of the optical density was carried out at 650 nm using a microplate reader (Mithras 2 LB943, Berthold).

2.2.4. *Determination of the median inhibitory concentration (IC50) and cytotoxicity evaluation*

In order to determine the dose required to kill 50% of total eukaryotic cell population, a growing range (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM) of different carbene solutions was performed then deposited on bronchial epithelial cells. After 24 or 48 hours of exposure, eukaryotic 16HBE^{14o-} cells were lysed with 75 µL of Passive Lysis buffer (Promega, 0.5X) and the cell viability was evaluated by the bioluminescent measurement of the ATP with the Vialight® kit (Lonza). Untreated cells were used to determine the normal cell viability (set to 100%).

2.2.5. *Aerosol procedure*

The Ag-NHC compound alone (in water solutions) or the lipoplexes [cationic lipids/Ag-NHC complexes, (1.2 mM/0.3 mM) + pDNA (1 mg)] were mixed in sterile water (10 mL final volume). After incubation at room temperature for 30 minutes, the solutions were introduced into the nebulizer reservoir (PariLC plus, Pulmomed). This nebulizer was connected to an exposure box by a pipe on one side, and on other side to an air outlet (the experimental set-up was already published (Mottais et al.,

2018)). Typically, a nebulization lasted about half an hour. It corresponds to the time necessary for the 10 mL drugs total nebulization. At the end point, the fog generated in the exposure box disappeared.

2.2.6. Antibacterial activity post-aerosol

Before the aerosolization, petri dishes were inoculated by the flooding method with one of the bacterial strains tested. After drying, a plug was placed in the center of the petri dish to protect an area from aerosolization. The post-aerosolization antibacterial activity was determined by comparing the growth density between the treated area and the untreated area. Three effects were distinguished. A total bactericidal effect was marked by a lack of growth in the treated area. An intermediate effect showed a lower growth density in the treated area than in the untreated area. Identical densities in both zones indicated the absence of antibacterial activity of the aerosol formulation.

2.2.7. Transfection efficiency post-aerosolization

One mg of plasmid (pGM144 incorporating a luciferase gene cassette, **Fig. S2**., Davies et al., 2012; Hyde et al., 2008) was mixed with the different formulations of cationic lipids/Ag-NHC complexes in 10 mL of sterile water. After 30 min of incubation at room temperature, each formulation was introduced into the nebulizer and the aerosolization was carried out as detailed above. Following this treatment, cells were incubated for 48 h at 37°C. Once the medium was removed, the bronchial epithelial cells were lysed with 75 µL of Passive Lysis Buffer (Promega). The luciferase expression was expressed in relative light unit (RLU) per mg of total protein, as determined using the Luciferase Assay System (Promega) and the BCA protein assay Kit (Interchim).

2.2.8. Statistical analysis

The Student t-test was employed to estimate the significance. A p-value ≤ 0.05 was considered to denote a statistically-significant difference. For each experiment, the N conforms to the number of individual experiments performed and the n conforms to the replicates i.e. the number of samples or wells included in one experiment.

3. Results and Discussion

3.1. Chemistry

Four analogues of Ag-NHC 5 or Ag-NHC 12 incorporating an alkyl chain, namely 3a/3c and 3b/3d respectively, were synthetized (**Fig. 2**). The synthetic routes to these alkyl functionalized silver carbene complexes is outlined in **Fig. 3**. In the first step, 4,5-dichloro-imidazole was mono-alkylated with 1-bromotetradecane or 1-bromooctadecane in the presence of KOH to produce compounds 1a and 1b in nearly quantitative yield (95-99%). Subsequent alkylation was performed either with methyl iodide or 2-bromomethylnaphthalene to lead to four imidazolium salts 2a-d. Their reaction with silver acetate in dichloromethane produced the desired Ag-NHC compounds 3a-d in moderate yields (41-71%). These yields are likely ascribed to the purification step which was achieved by recrystallization in DCM/Et_2O mixture at -20°C for three days.

Ag-NHC compounds 3a-d were fully characterized by 1D NMR experiments (^{1}H, ^{13}C). 2D NMR spectra (HMBC) were also recorded when the carbon (C-Ag bond) of the imidazole ring was not visible on the ^{13}C NMR spectra (see Experimental part and supplementary information).

3.2. Structure-antibacterial activity analysis

A range of concentrations (from 5 to 200 μM) of each Ag-NHC compound was evaluated with eight different bacterial strains, either Gram-positive (*S. aureus*) or Gram-negative bacteria (*E. coli* and *P. aeruginosa*). It is worth noticing that *S. aureus* and *P. aeruginosa* bacteria strains are usually found in sputum of CF patients. Many of these strains are multi-resistant to antibiotics (antibiotic resistance profiles were reported in the **Table S1**) (Le Gall et al., 2013; Mottais et al., 2018). Relevant antibiotics (kanamycin and ampicillin) were used as controls. The compounds 3a, 3b, 3c and 3d were prepared as a 5% ethanolic dispersion in water. It is noteworthy here that 5% ethanol in water solution alone does not exhibit any antibacterial effect against the different strains tested (Tables 1 and 2). Ag-NHC 5 was also used as standard, and its bactericidal activities found herein were consistent with those reported in previous studies (Berchel et al., 2011; Hindi et al., 2008; Le Gall et al., 2013; Mottais et al., 2018). The minimum inhibitory concentrations (MIC) are presented in the **Table 1**. Of note, the Ag-NHCs 3a-d exhibited antibacterial activities from micro-molar concentrations, at doses in the same range as referent antibiotics used in clinical settings (**Tables 1** and **2**).

The grafting of a naphthylmethylene moiety and/or long alkyl chain (3a-d) was beneficial for antibacterial activity against Gram-positive bacteria (**Table 1**). In contrast to Ag-NHC 5, silver

carbene complexes incorporating an alkyl chain (($C_{14}H_{29}$ (3a and 3c) or $C_{18}H_{37}$ (3b and 3d)) were active against Gram-positive bacteria (MICs are ranging from 5 to 20 µM). It has previously been described that the addition of alkyl chains (C_6H_{13} named Ag-NHC 10) improved the antibacterial activity (Hindi et al., 2009a). The Ag-NHC incorporating $C_{14}H_{29}$ alkyl (3a and 3c) displayed a lower MIC against *S. aureus* strains (≤ 5 µM) in comparison with the Ag-NHC compound incorporating $C_{18}H_{37}$ alkyl (3b and 3d) (**Table 1**). However, the antibacterial activity against Gram-negative bacteria was variable depending on the strains considered (**Table 2**). It is worth noting that this observation correlates with the information in another study reporting the antibacterial effect of arsonium polar headgroup based cationic amphiphiles in which the structure of the lipid chains also influenced the bactericidal activity. As observed herein, the use of C14:0 lipid chains also yielded the most efficient bactericidal agent (Le Gall et al., 2013). The Ag-NHC featuring a lipid chain (3a-d) are therefore more efficient (lower MIC) than the Ag-NHC 5 or 12 on Gram-positive strains.

3.3. Importance of Ag^+ ions

The antibacterial activity of Ag-NHC compounds is likely due to the release of Ag^+ ions. To confirm this point, the MICs of different imidazolium salts (2a-d) precursors of the Ag-NHC compounds were also determined (**Tables 3** and **4**).

The precursors 2 (*N,N'*-dimethyl-4,5-dichloroimidazolium iodide) proved to be inefficient at least up to 200 µM (**Tables 3** and **4**). The incorporation of a long alkyl chain (2a-d) induced a noticeable bactericidal effect against Gram-positive strains (**Table 3**). A strong bactericidal effect was reported for compounds 2a-b (MIC rate of between ≤ 5 and 10 µM) and a lower antimicrobial efficiency for the compound 2c. In this series of four compounds (2a-d), the less efficient was 2d, which was completely inactive against *S. aureus* RN4220. These results indicate, firstly, that the long alkyl chain is beneficial for the antibacterial activity, and secondly, that the addition of naphthyl moiety negatively impacted the bactericidal effect. It must be noted that, unlike Ag-NHC compounds, these precursors are positively charged (**Fig. 3**). The incorporation of a long alkyl chain and the positive charge are reminiscent of the structure of detergents. These compounds were also tested on Gram-negative strains. Nevertheless, as shown in **Table 4**, they were found inactive. We can therefore assume that Ag^+ ions are involved in the antibacterial activity against Gram-negative bacteria; as for Gram-positive

strains, the antibacterial effect may imply a dual effect since the degradation of Ag-NHC compounds produces Ag^+ ions and imidazolium salts, both expressing activity against these strains.

3.4. Cytotoxicity evaluation on bronchial epithelial cells

The cytotoxicity of these compounds was also evaluated on human bronchial epithelial cells ($16HBE^{14o-}$) following direct deposition in the liquid culture medium (Cozens et al., 1994). We determined the median inhibitory concentration (IC50), i.e. the concentration of compound for which half of the cell population is still viable (**Fig. 4**).

A control consisting of a 5% alcoholic solution was deposited in parallel with the different Ag-NHC compounds. Ag-NHC 5 exhibited no cytotoxic effect towards the bronchial epithelial cells. This was reflected by an IC50 greater than 100 µM. The silver carbene complexes incorporating a naphthalene (Ag-NHC 12) has an IC50 between 80 and 100 µM, meaning that the incorporation of the naphthalene moiety induced only a slight cytotoxicity. However, the presence of a long alkyl chain led to a high cytotoxicity with an IC50 between 2 to 16 µM, depending on the Ag-NHC considered. Of note, no cytotoxic data has been reported before for Ag-NHC compound incorporating a C_6H_{13} alkyl chain (Ag-NHC 10) (Hindi et al., 2009a; Li et al., 2010; Panzner et al., 2009). Thus, the alkyl chain length plays a major role in the cytotoxicity. The single fatty chain could destabilize the plasma membrane of eukaryotic cells and induce the cell death.

3.5. Antibacterial effects of Ag-NHC compounds after aerosol delivery

Aerosolization is a mode of administration that can alter the expected biological effects due to the pressure applied to the supramolecular assemblies. In a previous report, an Ag-NHC derived from caffeine was aerosolized in mice infected with a strain of *P. aeruginosa* (Cannon et al., 2009). A decrease in bacterial density was observed post-nebulization, indicating that aerosolization did not affect the antibacterial potential of this family of compounds. Herein, the neo-synthesized Ag-NHC compounds were aerosolized using a solution of 0.3 mM in 10 mL introduced into the nebulizer reservoir. It should be noted that the concentration deposited during the aerosolization i.e. reaching the samples hosted in the exposure box was much lower due to the important and inherent losses with this kind of procedure (Mottais et al., 2018). From our own experience conducted with a colorimetric solution assay, only 20% of the quantity introduced first in the nebulizer reservoir actually deposit at

the bottom of the exposure box (data not shown). For each bacterial strain evaluated, an agar plate was inoculated and then the central area was covered to prevent its treatment during nebulization. Following nebulization and an overnight incubation at 37°C, the difference in bacterial growth density between the treated *versus* protected areas allowed to distinguish between three antibacterial effects i.e. total, intermediate or no effect (**Fig. 5A**).

The results obtained are summarized in **Fig. 5B**. In contrast to a previous study from our group where Ag-NHC 5 was used at 1.2 mM, Ag-NHC 5 was evaluated here at a lower concentration i.e. 0.3 mM. In this context, Ag-NHC 5 showed an intermediate effect only against Gram-negative strains of *P. aeruginosa* (130709 and 240709) (Mottais et al., 2018). 3a aerosolized allowed an intermediate effect against most Gram-positive bacteria and some Gram-negative strains (*E. coli* MG1655 and *P. aeruginosa* 240709). Considering 3b, it showed a very good antibacterial activity against all Gram-positive bacteria and had an intermediate effect on clinical strains of *P. aeruginosa*. Thus, the grafting of an alkyl chain on Ag-NHC 5 allowed the broadening of the antibacterial spectrum post-aerosolization. Regarding the naphthalene derivatives, an intermediate antibacterial activity against Gram-negative bacteria as *E. coli* MG1655 and *P. aeruginosa* 130709 was found. When a $C_{14}H_{29}$ alkyl chain was engrafted (3a and 3c), the antibacterial activity against Gram-positive bacteria was also detected. Nevertheless, this effect was not observed for 3d incorporating a $C_{18}H_{37}$ alkyl chain.

3.6. Combinations of cationic lipids and Ag-NHC compounds

Previous works have reported that the encapsulation of Ag-NHC compounds in nanoparticles promotes accumulation in the lungs (Leid et al., 2012; Lim et al., 2015; Ornelas-Megiatto et al., 2012). This accumulation enhances the release of a high concentration of Ag^+ ions at the site of infection. Another Ag-NHC compound featuring two naphthyl moieties (Hindi et al., 2008; Leid et al., 2012; Panzner et al., 2009) was loaded into dextran. Post-aerosolization, nanoparticles showed no change in their structures (Ornelas-Megiatto et al., 2012). Our research groups have developed gene transfer agents mainly based on the use of cationic lipids (Mottais et al., 2018; Montier et al., 2008; Lindberg et al., 2012, 2015; Berchel et al., 2016). We seek to develop formulations that are able to cross the different lung extracellular barriers and that conserve their transfection ability towards the target cells (Mottais et al., 2017; Resnier et al., 2016). Cationic lipids allow the formation of nanostructures called

lipoplexes when combined with nucleic acid constructs. The gene transfer agent selected herein is the cationic lipid KLN47, an arsonium-containing lipophosphoramide possessing an antibacterial activity against Gram-positive bacteria when directly deposited in a liquid medium (Le Gall et al., 2013). The neo-synthesized Ag-NHC compounds being hydrophobic, were formulated with a cationic lipid like KLN47 by hydration of the lipid film (Picquet et al., 2005). For these experiments, we used the derivatives of Ag-NHC 5 (3a and 3b). All of the cationic lipid/Ag-NHC complexes were formulated in sterile water at the initial concentrations of 1.2 mM and 0.3 mM respectively with 1 mg of a luciferase encoding pDNA (pGM144) (Davies et al., 2012). In parallel, the aerosolization of cationic lipids/Ag-NHC complexes was performed simultaneously on agar plates of different bacteria and on bronchial epithelial cells seeded in 96-well plate (experimental system previously reported) (Mottais et al., 2018). Furthermore, the aerosolization procedure did not affect the physicochemical characteristics (size and zeta potential measurements) of nanoparticles (**Table S2**).

Regarding the antibacterial effect post-nebulization, the formulation associating cationic lipid (KLN47)/pGM144 did not have any antibacterial activity, as reported earlier (Mottais et al., 2018). However, once aerosolized, the related combinations (pDNA/KLN47/3a and pDNA/KLN47/3b) exhibited an antibacterial activity against the different strains of *S. aureus* (Gram-positive bacteria) and a variable activity against Gram-negative bacteria (**Fig. 6A** and **S3**). The combination did not alter the antibacterial potency observed. It even seems to enhance the activity against Gram-negative bacteria.

Regarding the post-aerosolization cytotoxicity by these compounds, the eukaryotic cells viability observed was about 70-80% (**Fig. 6B**). The formulation with 3b was less toxic than with 3a compound ($p<0.05$). In view of IC_{50} previously determined by direct deposition, this cytotoxicity was acceptable.

Regarding the transfection efficiency, the presence of Ag-NHC compound incorporating an alkyl chain did not modify the gene transfer activity of the cationic lipid KLN47 (**Fig. 6B**). The transfection activity was indeed roughly the same as the results observed with the cationic lipid formulated alone (Mottais et al., 2018).

4. Conclusions

The new Ag-NHC compounds reported in this study were prepared in three steps from commercial compounds. These compounds 3a-d exhibited increased bactericidal effect on both Gram-positive and Gram-negative strains when compared to the parent compound Ag-NHC 5. These results indicated that the incorporation of a long alkyl chain in the structure of Ag-NHC compounds enhanced the antibacterial activity. However, this incorporation increased also the toxicity towards eukaryotic cells. The nebulization of compounds 3a-d on bacteria preserved the bactericidal activity on Gram-positive strains whereas a lower activity was found on Gram-negative strains. Finally, derived Ag-NHC/cationic lipids complexes are efficient formulations against Gram-positive strains and at a lesser extent, against Gram-negative strains, while possessing the capacity to carry nucleic acids in eukaryotic cells. Due to the structural analogy of compounds 2a-d with those of detergents that could be ascribed to their cytotoxicity, it can be suggested to incorporate the Ag-NHC fragment within a molecular structure possessing two lipid chains. To improve the transfection efficiency for *in vivo* application, the formulations could incorporate mucus-penetrating particles like polyethylene glycol. In order to decrease this cytotoxicity, the quantity of Ag-NHC compounds could be reduced. A balance between the cytotoxic concentrations and those needed for the antibacterial activity must be established. This cytotoxicity could also be decreased by modulation of the chemical structure of Ag-NHC compounds. In addition to their antibacterial potency, Ag-NHC compounds may also present some anticancer properties. These compounds could therefore be used in combination with cationic lipids in order to slow down the progression of pulmonary metastases.

Author Contributions

Mathieu Berchel and Paul-Alain Jaffrès performed the synthesis of compounds and their characterization. Angélique Mottais, Tony Le Gall, Yann Sibiril and Véronique Laurent performed the evaluation of the different formulations in term of antibacterial activities, cytotoxicity assays and transfection procedures. Tristan Montier and Frédérique d'Arbonneau planned and organized the experimental procedures.

All authors contributed to the writing of the manuscript and all of them have given approval to its final version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at…

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Figure captions:

Fig. 1. Ag-NHC compounds derived from 4,5-dichloroimidazole reported in literature and used as control compounds in this study.

Fig. 2. Structures of the lipophilic Ag-NHC compounds newly reported in this study.

Fig. 3. Synthesis of the Ag-NHC compounds from 4,5-dichloroimidazole.

Fig. 4. Median inhibitory concentration (IC50) of the different Ag-NHC compounds observed on bronchial epithelial cells (16HBE^{14o-} cell line) (N=1, n=3).

Fig. 5. Antibacterial effects of Ag-NHC following aerosolization. To define the antibacterial effect 24 h after treatment, bacterial growth density was compared between an area not exposed and an area treated. Three antibacterial effects were defined (A): total effect, no bacteria in the area treated; intermediate effect, some bacteria in the area treated; and no effect, same density in both areas. This experiment was repeated 3 times (N=3, n=1).
[a] Sa: *Staphylococcus aureus*. [b] Pa: *Pseudomonas aeruginosa.*

Fig. 6. Antibacterial effects, cytotoxicity and transfection efficiency post-nebulization of the formulations prepared with the arsonium-containing lipophosphoramidate KLN47 used alone or in mixture with Ag-NHC 3a or 3b. Untreated bronchial epithelial cells are used as negative control (CTRL) for cytotoxicity and transfection efficiency evaluations. In the first chart, the dashed line represents 100% of viability. In the second chart, it represents the baseline of transfection efficiency. Of note, Ag-NHC compounds are devoid of any gene transfer activity (data not shown). This experiment was repeated twice (N=2, n=24). The asterisk denotes a statically significant difference as determined using the Student t-test (p-value ≤ 0.05), and non-significant results are notified by ns.
[a] Sa: *Staphylococcus aureus*. [b] Ec: *Escherichia coli*. [c] Pa: *Pseudomonas aeruginosa.*

Table captions:

Table 1
Minimum inhibitory concentration (in µM) of Ag-NHC compounds newly synthetized, as determined by liquid broth micro-dilution method, on four Gram-positive bacteria.

Table 2
Minimum inhibitory concentration (µM) of Ag-NHC compounds newly synthetized, as determined by liquid broth micro-dilution method, on three Gram-negative bacteria.

Table 3
Minimum inhibitory concentration (µM) of Ag-NHC precursors, as determined by liquid broth micro-dilution method, on four Gram-positive bacteria.

Table 4
Minimum inhibitory concentration (µM) of Ag-NHC precursors, as determined by liquid broth micro-dilution method, on three Gram-negative bacteria.

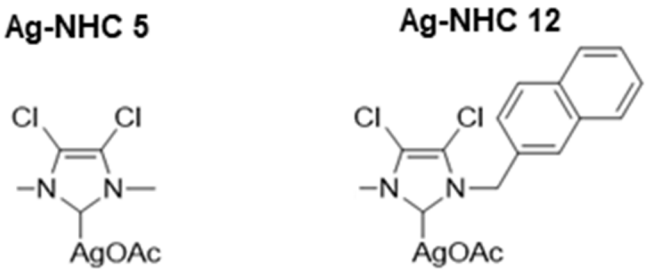


Fig. 1. Ag-NHC compounds derived from 4,5-dichloroimidazole reported in literature and used as control compounds in this study (Hindi et al., 2009a; Medvetz et al., 2008; Panzner et al., 2009).

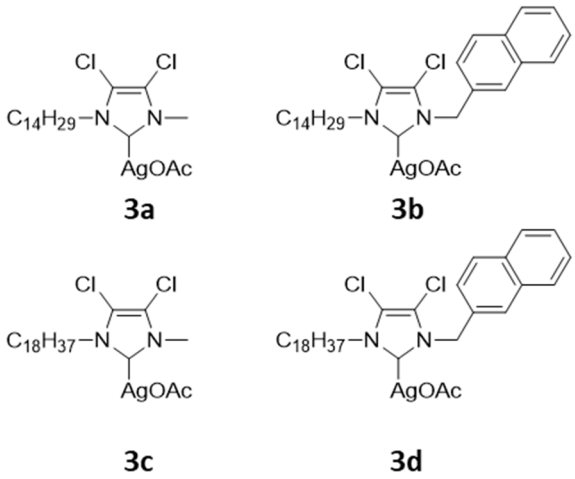


Fig. 2. Structures of the lipophilic Ag-NHC compounds newly reported in this study.

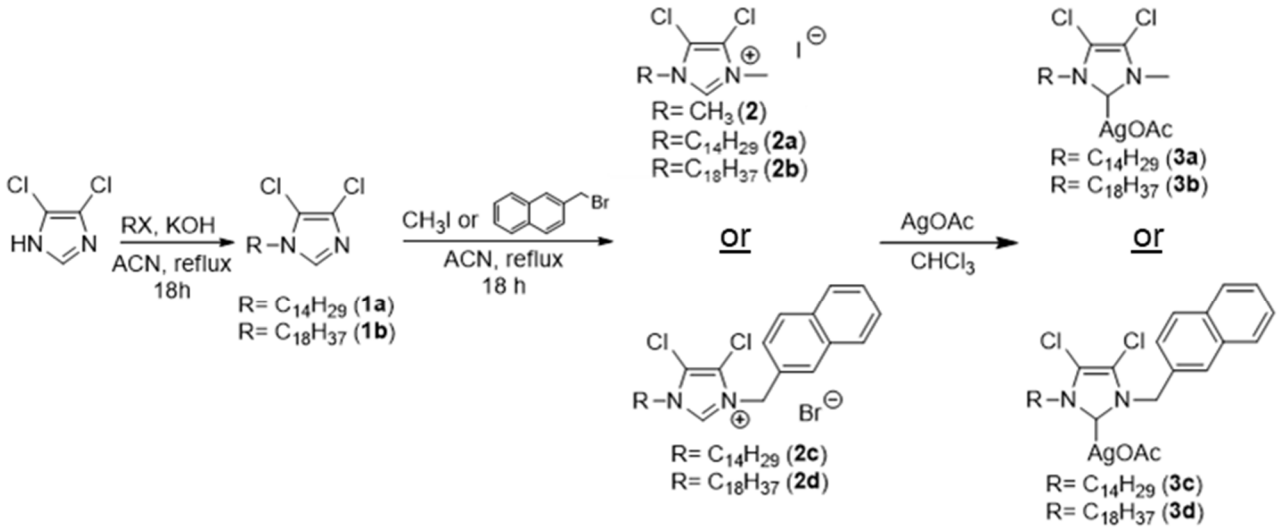


Fig. 3. Synthesis of the Ag-NHC compounds from 4,5-dichloroimidazole.

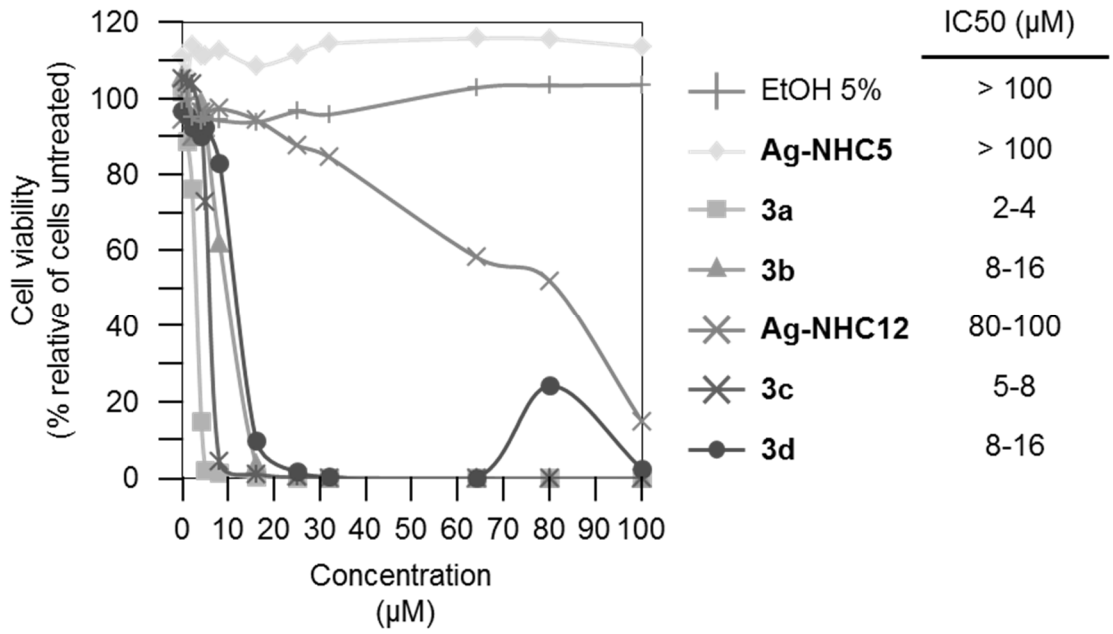
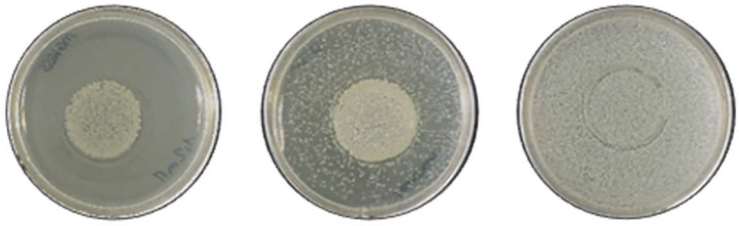


Fig. 4. Median inhibitory concentration (IC50) of the different Ag-NHC compounds observed on bronchial epithelial cells (16HBE^{14o-} cell line) (N=1, n=3).

A **Typical antibacterial effects**



Total effect (+) Intermediate effect (+/-) No effect (-)

B **Results**

Gram (+) strains	Sa[a] N3115	Sa RN4220	Sa Newman	Sa Xen31
Ag-NHC5	-	-	-	-
3a	+	+/-	+/-	+/-
3b	+	+	+	+/-
Ag-NHC12	-	-	-	-
3c	+	+	+	+/-
3d	-	-	-	-

Gram (-) strains	Pa[b] Xen5	Pa 240709	Pa 130709
Ag-NHC5	-	+/-	+/-
3a	-	+/-	-
3b	-	+/-	+/-
Ag-NHC12	-	-	+/-
3c	-	+/-	-
3d	-	+/-	-

Fig. 5. Antibacterial effects of Ag-NHC following aerosolization. To define the antibacterial effect 24 h after treatment, bacterial growth density was compared between an area not exposed and an area treated. Three antibacterial effects were defined (A): total effect, no bacteria in the area treated; intermediate effect, some bacteria in the area treated; and no effect, same density in both areas. This experiment was repeated 3 times (N=3, n=1).
[a] Sa: *Staphylococcus aureus*. [b] Pa: *Pseudomonas aeruginosa.*

A **Antibacterial effects post-nebulization**

Gram (+) strains	Sa[a] N3115	Sa RN4220	Sa Newman	Sa Xen31
pDNA / KLN47	-	-	-	-
pDNA / KLN47 / **3a**	+	+	+	+
pDNA / KLN47 / **3b**	+	+	+	+/-

Gram (-) strains	Pa[b] Xen5	Pa 240709	Pa 130709
pDNA / KLN47	-	-	-
pDNA / KLN47 / **3a**	-	+/-	+/-
pDNA / KLN47 / **3b**	-	+/-	+

+ : total effect ; +/- : intermediate effect; - : no effect

B **Cytotoxicity and transfection efficiency post-nebulization**

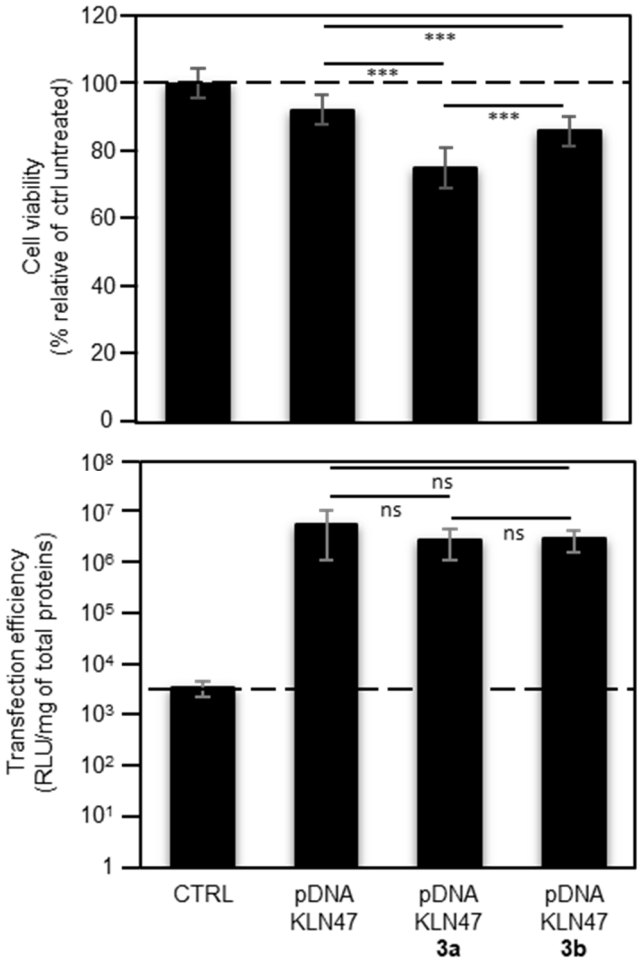


Fig. 6. Antibacterial effects, cytotoxicity and transfection efficiency post-nebulization of the formulations prepared with the arsonium-containing lipophosphoramidate KLN47 used alone or in mixture with Ag-NHC 3a or 3b. Untreated bronchial epithelial cells are used as negative control (CTRL) for cytotoxicity and transfection efficiency evaluations. In the first chart, the dashed line represents 100% of viability. In the second chart, it represents the baseline of transfection efficiency. Of note, Ag-NHC compounds are devoid of any gene transfer activity (data not shown). This experiment was repeated 2 times (N=2, n=24). The asterisk denotes a statically significant difference as determined using the Student t-test (p-value ≤ 0.05), and non-significant results are notified by ns.
[a] Sa: *Staphylococcus aureus*. [b] Ec: *Escherichia coli*. [c] Pa: *Pseudomonas aeruginosa.*

Table 1
Minimum inhibitory concentration (in µM) of Ag-NHC compounds newly synthetized, as determined by liquid broth micro-dilution method, on four Gram-positive bacteria.

Strain	Sa[a] N315	Sa RN4220	Sa Newman	Sa Xen31
Ag-NHC 5	60	200	100	100
3a	≤ 5	≤ 5	≤ 5	≤ 5
3b	≤ 5	10	10	10
Ag-NHC 12	60	40	50	50
3c	≤ 5	10	≤ 5	≤ 5
3d	20	20	20	20
EtOH 5%	> 200	> 200	> 200	> 200
Ampicillin	> 200	> 200	≤ 5	≤ 5
Kanamycin	> 200	> 200	10	10

The values reported in this table are representative of 3 independent experiments conducted with different solution preparations (N=3, n=1).
[a] Sa: *Staphylococcus aureus.*

Table 2
Minimum inhibitory concentration (μM) of Ag-NHC compounds newly synthetized, as determined by liquid broth micro-dilution method, on three Gram-negative bacteria.

Strain	Pa[a] Xen5	Pa 240709	Pa 130709
Ag-NHC 5	70	20	20
3a	20	30	20
3b	30	30	20
Ag-NHC 12	30	20	20
3c	30	20	20
3d	60	30	40
EtOH 5%	> 200	> 200	> 200
Ampicillin	> 200	60	> 200
Kanamycin	60	> 200	50

The values reported in this table are representative of 3 independent experiments conducted with different solution preparations (N=3, n=1).
[a] Pa: *Pseudomonas aeruginosa.*

Table 3
Minimum inhibitory concentration (μM) of Ag-NHC precursors, as determined by liquid broth micro-dilution method, on four Gram-positive bacteria.

Strain	Sa[a] N315	Sa RN4220	Sa Newman	Sa Xen31
2	> 200	> 200	> 200	> 200
2a	≤ 5	10	10	10
2b	≤ 5	≤ 5	ND[b]	ND
2c	20	50	50	40
2d	80	> 200	ND	ND

(N=2, n=3).
[a] Sa: *Staphylococcus aureus*. [b] ND: Data not determined.

Table 4
Minimum inhibitory concentration (μM) of Ag-NHC precursors, as determined by liquid broth micro-dilution method, on three Gram-negative bacteria.

Strain	Pa[a] Xen5	Pa 240709	Pa 130709
2	> 200	> 200	> 200
2a	> 200	> 200	> 200
2b	ND[b]	> 200	> 200
2c	> 200	> 200	> 200
2d	ND	> 200	> 200

(N=2, n=3).
[a] Pa: *Pseudomonas aeruginosa.* [b] ND: Data not determined.

Graphical abstract