N, N’-Olefin Functionalized Bis-Imidazolium Gold(I) Salt Is an Efficient Candidate to Control Keratitis-Associated Eye Infection

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Abstract

Keratitis treatment has become more complicated due to the emergence of bacterial or fungal pathogens with enhanced antibiotic resistance. The pharmaceutical applications of N-heterocyclic carbene complexes have received remarkable attention due to their antimicrobial properties. In this paper, the new precursor, 3,3’-(p-phenylenedimethylene) bis[(2-methyl-allyl)imidazolium] bromide (1a) and its analogous PF₆ salt (1b) were synthesized. Furthermore, silver(I) and gold(I)-N-heterocyclic carbene (NHC) complexes [Ag₂LBr₂/Au₂LBr₂; 2a/3a], [(Ag₂L₂)(PF₆)₂/(Au₂L₂)(PF₆)₂; 2b/3b] were developed from their corresponding ligands. All compounds were screened for their antimicrobial activities against multiple keratitis-associated human eye pathogens, including bacteria and fungi. Complexes 2a and 3a showed highest activity, and the effectiveness of 3a was also studied, focusing eradication of pathogen biofilm. Furthermore, the structures of 1a, 2a and 3a were determined using single crystal X-ray analysis, 2b and 3a were optimized theoretically. The mechanism of action of 3a was evaluated by scanning electron microscopy and docking experiments, suggesting that its target is the cell membrane. In summary, 3a may be helpful in developing antimicrobial therapies in patients suffering from keratitis-associated eye infections caused by multidrug-resistant pathogens.

Introduction

Keratitis is a common corneal infection in tropical areas of the world. This kind of infection is quite dangerous, and in many cases can lead to permanent blindness, especially if not diagnosed promptly and treated effectively. Keratitis can develop from fungal (Candida, Aspergillus, Fusarium etc.) and bacterial (Staphylococcus aureus, Pseudomonas aeruginosa etc.) infections, especially those caused by the use of contact lenses or by eye injuries [1,2]. The emergence of bacterial and fungal pathogens with enhanced antibiotic resistance has arisen due to a number of reasons, such as mutations, gene transfer, biofilm formation and inappropriate use of traditional antibiotics [3,4]. The association of antibiotic-resistant pathogens with keratitis infection is a matter of great concern, since many infections have had no effective treatment yet.

Since antibiotic resistance seems to be inevitable, strenuous efforts have been made to develop new antimicrobial agents. The pharmaceutical application of N-heterocyclic carbene (NHC) and their metal complexes have gained enormous attention due to their antimicrobial properties. Recently, pyrazine functionalized-NHC complexes with clear deleterious effects against multidrug-resistant pathogens were seen to show an unusual mechanism of action [5]. Among them, gold (I) and silver (I)-NHCs seem to be remarkable candidates for antibiotic development, due to their higher activity and relatively low toxicity to mammalian cells when compared to other metals, such as Ru(II), Ru(I), Cu(I) and Pd(II)-NHC complexes. Hindi et al. [6] summarized the antimicrobial activities of several NHCs showing clear benefits, but demonstrated the need for higher amounts in order to obtain effective pathogen control. However, NHCs are in a versatile ligand class which has the advantage of easy manipulation. Furthermore, they also have the ability to bind simply to metals and can be readily functionalized [7]. Ag(I) and Au(I)–NHC complexes have also been found in many medicinal applications including antimicrobials [8]. Gold in vivo biochemistry still remains enigmatic, mainly due to a scarcity of adequate models and an incomplete understanding of gold reactivity. Imidazolium salts react with silver oxide and produce an NHC silver complex, which serves as a useful trans-metallating reagent [9] which has been widely studied. Finally, an interesting aspect of silver (I)-NHC complexes is the aggregation of silver (I) centers by argentophilic attraction with Ag-Ag separations [10] shorter than the sum of their van der Waals radii.

Research in this area is made easier and more interesting by the fact that steric and electronic properties as well the reactivity of
metal-NHC complexes can be studied by varying the N-substituents of imidazole [11]. Many NHC complexes have been reported to include NHCs with simple N-alkyl or aryl substituents. Various functional groups [5] such as pyridyl, phosphinyl, pyrazolyl and thiazole have also attracted considerable attention due to their use in constructing N-functionalized NHCs. In this paper, we look at this reaction, and report the synthesis of a new type of silver-NHC complex. The hemi-labile nature of the allylic [12] system changes the metal-organic coordination mode during the formation of the complex, which encouraged us to design Ag and Au-complexes of the allylic ligand.

Data reported here show the antimicrobial activity of N, N'-olefin functionalized bis-imidazolium gold (I) and silver (I) salts against several resistant human fungal and bacterial pathogens, which can cause the serious eye infection keratitis. Their synthesis, crystal structures, characterization, and mode of action were analyzed and ex vivo bioassays were carried out.

Materials and Methods

Reagents and Chemicals

All the reagents - imidazole, α,α'- dibromo p-xylene, 3-chloro-2-methyl propene, Ag$_2$O and NH$_4$PF$_6$ - were purchased from Sigma-Aldrich, UK, and were used without further purification. All manipulations were carried out in an open atmosphere unless otherwise stated. Au(SMe$_2$)Cl was prepared according to the reported procedure [5]. Solvents EtOH, MeOH and CHCl$_3$ of analytical-grade type were distilled and dried over appropriate drying agents and dinitrogen prior to use. All the selected antibiotics and antifungals tested in this study were purchased from HIMEDIA, India. $^1$HNMR spectra were recorded on a Bruker ARX 400 in (CD$_3$)$_2$SO$_2$ as the solvent and $^{13}$CNMR data were recorded on 75 MHz instrument respectively and referenced to residual protons and $^{13}$C signals of deuterium solvents. MALDI MS spectra were recorded on an Applied Biosystem, Voyager time-of-flight mass spectrometer (Text S4).

Synthesis of Compounds

A solution of (680 mg, 10 mmol) imidazole and (400 mg, 10 mmol) NaOH was prepared in THF, 3-Chloro-2-methyl propene (905 mg, 10 mmol) was added to the solution, and the mixture was refluxed for 15 h at 65°C. The reaction mixture was cooled at room temperature and the solvent was removed. After removal of the solvent, a brown, oily liquid was isolated and characterized as 1-(2-methyl allyl) imidazolide. Then 1-(2-methylallyl)imidazolide (244 mg, 2 mmol) and α,α’ - dibromo p-xylene (264 mg, 1 mmol) was added to the reaction mixture (neat reaction), heated for 6 h producing a colorless solid. The solid mass was washed with Pet-ether and recrystallized in MeOH. 3,3’-(p-phenylendimethylene) bis[1-(2-methyl-allyl)imidazolide] hexafluorophosphate salt (1b) was synthesized using the aqueous solution of ammonium hexafluorophosphate (NH$_4$PF$_6$) in complex (1a) and produces the colorless, water-insoluble hexafluorophosphate salt (1b).

Furthermore, 3,3’-(p-phenylendimethylene) bis[1-(2-methylallyl)imidazolide] silver bromide (2a) salt was synthesized after a reaction of complex 1a (102 mg, 0.2 mmol) and Ag$_2$O (47 mg, 0.2 mmol) in dichloromethane, and the mixture was stirred at room temperature for 12 h with exclusion of light, then filtered to remove a small amount of unreacted Ag$_2$O. The filtrate was collected and solvent was removed producing a white solid mass (2a). Furthermore, 144 mg (0.2 mmole) of solid silver bromide complex (2a) was taken in a round-bottomed flask and dissolved in 20 mL dichloromethane solvent. Au(SMe$_2$)Cl (59 mg, 0.2 mmol) was added to it in N$_2$ medium and stirred for 1 h, and precipitation of AgCl was observed. It was then filtered and the filtrate was collected; after removal of the solvent, the colorless solid gold complex produced 3,3’-(p-phenylendimethylene) bis[1-(2-methylallyl)imidazolide]gold(I) bromide (3a) salt.

3,3’-(p-phenylendimethylene) bis[1-(2-methyl-allyl)imidazolide]silver hexafluorophosphate (2b) was synthesized by the reaction of complex (1b) (64 mg, 0.15 mmol) with excess Ag$_2$O (27 mg) in acetonitrile (CH$_3$CN). The mixture was stirred at room temperature for 8 h with exclusion of light and then filtered to remove a small amount of unreacted Ag$_2$O. The filtrate was collected and solvent was removed to produce a white solid mass of complex (2b). 90 mg (0.15 mmole) of solid silver hexafluorophosphate complex (2b) was put into a round-bottomed flask and dissolved in 10 mL of acetonitrile solvent. Au(SMe$_2$)Cl (45 mg, 0.15 mmol) was added to (2b) in N$_2$ medium and stirred for 1 h. Precipitation of AgCl was observed, which was then filtered. Filtrate was collected and, after removal of solvent, produced the colorless solid 3,3’-(p-phenylendimethylene) bis[1-(2-methylallyl)imidazolide]silver hexafluorophosphate complex (3b). The chemical shift (1HNMR and 13CNMR) and mass (m/z) data of all synthesized compounds were described in the supplementary file.

X-Ray Structure Determinations

Crystals of 1a, 2a and 3b were coated in paraffin oil, mounted on a kryo loop, and placed on a goniometer at a temperature of 296 K in an open atmosphere. X-ray data sets were collected using a Bruker Apex CCD diffractometer with graphite monochromated MoK$_\alpha$ X-ray radiation ($\lambda = 0.71073 \, \AA$). The unit cell determination was achieved by using reflections from three different orientations. An empirical absorption and crystal refinement corrections were performed by using multiscan SADABS. Structure solution, refinement and modeling were accomplished by using the SHELXL-97 package [13]. The structure was obtained by full matrix least square refinement of $F^2$ and selection of appropriate atoms from the generated difference map. Additional data collection and refinement parameters are summarized in Table S1 and S2.

Structure Optimization

In order to understand the structure of synthesized complexes 2b and 3a, the structures were drawn according to the NMR and mass data found, and also further generated the structures of complexes 2a and 3b. Geometries at the B3LYP/LANL2DZ level of theory using the Gaussian G-03-E01 [14] program were optimized. The number of imaginary frequencies of all the molecules turns out to be zero, implying that they correspond to minimum energy structures on the potential energy surface.

Microbial Strains and Growth Conditions

The Aspergillus fumigatus JMJ3 (filamentous fungus), Candida albicans SJ11 (unicellular fungus) Pseudomonas aeruginosa (Gram-negative bacteria), and Staphylococcus aureus (Gram-positive bacteria) used in this study were obtained from Priyamvada Birla Aravind Eye Hospital in Kolkata, India. The phenotypic characteristics of clinically isolated fungal strains and antibiotic sensitivity profiling of bacterial strains were described in the supplementary section. All the strains were isolated from patients associated with keratitis, and fungal strains were previously characterized [2,15]. Fungal species were collected from a mature solid medium culture plate (Sabouraud dextrose agar) and mixed with liquid RPMI 1640 (Himedia, India), and were incubated for 24 h at 30°C to get the relevant turbidity of 0.5 ×10$^5$ CFU/mL. Bacterial cultures were
maintained in Mueller-Hinton Broth (MHB) and a total inoculum load of ca. 10^6 cells per well-maintained.

**Antimicrobial Assays**

Minimum inhibitory concentration (MIC) values of all the synthesized compounds and antifungal/antibacterial antibiotics were determined according to CLSI guidelines [16]. The concentration of each compound used for the assay ranged from 0.97 μM to 1 mM. MIC values were determined where no visible growth was observed. The characteristic features of clinical isolates with their antimicrobial sensitivity were described in [Text S3]. The culture conditions and bacterial growth were monitored as described earlier by Roymahapatra et al. [5]. Likewise, fungal growth and culture conditions were monitored as described earlier, following Sengupta et al. [2]. Two wells, where no compounds were added, were used as positive controls, and compounds with the absence of microorganisms were used as negative controls in order to maintain the experimental sterility, and all independent experiments were repeated four times.

To examine the bacterial growth or killing kinetics in the presence of compound 3a, bacterial cells were grown in 100 mL of Mueller–Hinton Broth (MHB) supplemented with different doses of complex (0.25 to 4.0 μM), at 37°C with continuous agitation at 180 rpm. Growth or killing rates and bacterial concentrations were determined according to the OD600 and using the conversion factor of 3.1861 milliliters of mock-inoculated Sabouraud dextrose broth served as a negative control. The in vitro growth kinetics of fungal strains were determined following the protocol described by Mitchell et al. [17]. In brief, fungal strains (10^3 CFU) were inoculated into 2 mL Sabouraud dextrose broth supplemented with different doses of complex 3a (0.25 to 4.0 μM), and incubated at 30°C with continuous rocking at 180 rpm. Two milliliters of mock-inoculated Sabouraud dextrose broth served as a negative control. The in vitro growth rates were measured at OD600 and using the conversion factor of 3.1861 CFU/mL per 1 U OD600 [17]. All independent experiments were repeated four times.

**Scanning Electron Microscopy (SEM)**

*Pseudomonas aeruginosa* and *Candida albicans* cells were harvested from the log phase of their respective growth medium. The cells were then washed three times with 1X PBS buffer and resuspended in the same saline buffer. The cells were treated with gold (I) complex (3a) with their individual MIC concentration for 30 min. Cells were repeatedly washed with saline water and 5–10 μl of resuspended solution containing bacteria/fungus was placed on the lysine-coated glass cover slip as a drop-caste method. The fixed cells were dried and kept on desiccators until use. The samples were then fixed onto a graphite stub and kept in an auto sputter coater (E5200, Bio-Rad) under low vacuum for gold sputter coater (E5200, Bio-Rad) under low vacuum for gold coating up to 120 seconds. Surface morphology was studied by using a scanning electron microscope (JEOL, JSM5800) with an accelerated voltage between 5–20 kV.

**Eradication of Biofilm on Contact Lenses**

The formation of *Aspergillus fumigatus* and *Candida albicans* biofilm on contact lenses was carried out according to Sengupta et al. [2]. Soft contact lenses were separately submerged in 6 wells of flat-bottomed polystyrene plates containing 2 mL of RPMI 1640 medium, inoculated with the respective fungal strain with an inoculum dose of 3.5 x 10^6 CFU/mL and incubated for 72 h at 30°C. After 72 h, the lenses were removed and the planktonic cells were washed gently and repeatedly with 1X PBS buffer. To determine the effect of gold (I) complex (3a) on biofilm eradication of the contact lenses, complexes were added to the biofilm in serial double-diluted concentrations (0.97 μM to 1 mM), and the final volume of 2 mL with RPMI medium, which was then incubated further for 24 h at 30°C. A series of complex-free wells and biofilm-free wells was also included to serve as positive and negative controls, respectively. After incubation, the remaining biofilm metabolic activity was quantified by the XTT-reduction assay as described earlier [13]. For imaging, the lenses were flipped, and stained biofilms were visualized by fluorescence microscopy to compare their gross morphologies. Live-dead analysis was performed with BacLight Live/Dead dye (Invitrogen Inc. USA). The images were captured from the stained biofilms under fluorescence microscope (OLYMPUS IX 51, fitted with Evolution VF CCD Camera).

Biofilm formation by bacterial clinical isolates of *P. aeruginosa* and *S. aureus* on contact lenses were determined by using a polystyrene crystal violet adherence assay, as described previously by Croes et al. [18]. In brief, soft contact lenses were separately submerged in 6 wells of flat-bottomed polystyrene plates containing 2 mL of Trypticase Soy Broth (TSB) with 0.5% glucose, inoculated with the respective bacterial strain with an inoculum dose of 10^6 CFU/mL and incubated for 48 h at 37°C. The biofilm that had grown over contact lenses was used for minimum biofilm eradication concentrations (MBEC) assay following Roymahapatra et al. [5] and complex concentrations were used in the same way as in the previous fungal assay. The crystal violet retained within the biofilm was extracted by adding 100 μl of 70% (vol/vol) ethanol with 10% isopropyl alcohol (vol/vol) and measured at 590 nm (A590). All independent assays were performed four times. Subsequently, the biofilm formations in different concentrations were visualized under fluorescence microscope (OLYMPUS IX 51, fitted with Evolution VF CCD Camera) by staining with BacLight Live/Dead dye (Invitrogen Inc. USA).

**Cytoplasmic Material Release Study**

Cytoplasmic material release study was carried out according to Sahu et al. [19], with minor modifications. To summarize, 1 mL of overnight bacterial (*P. aeruginosa* and *S. aureus*) and fungal (*C. albicans*) culture (relevant turbidity of 0.5 x 10^8 CFU/mL) were centrifuged and the pellet was washed with PBS (1X) buffer. Next, the pellet was suspended in 1 mL of PBS (1X) buffer and divided into five aliquots of 200 μL each. Different aliquots of cell suspensions were treated with different concentration of 3a (0.5 to 4 μM) and incubated at 30°C for 2 h. The samples were filtered to remove bacteria, and OD values of supernatants were recorded at 260 nm. The percentage of cytoplasmatic material release was measured considering the OD values obtained with TntonX-100 (0.1%) treatment with 100% release. The experiment was repeated three times.

**Molecular Docking**

Two membrane models were built through the CHARMM-GUI server [20] for simulating fungal and bacterial membranes, both with 50 Å^2. For fungal membrane, a proportion of 1:1:1:1:1 of POPA (palmitoyloleoylphosphatidylamine), POP3 (palmitoyloleoylphosphatidylserine), POPE (palmitoyloleoylphosphatidylethanolamine), DOPC (dioleoylphosphatidylcholine), DPPC (dipalmitoylphosphatidylcholine) and cholesterol was used according to Jo et al. [20]. For the bacterial membrane, a 3:1 proportion of of POPE and POPG (phosphatidylglycerol) was used according to Murzyn et al. [21].

Molecular docking studies were performed by AutoDock Vina and AutoDock Tools as previously described [5,22–24]. Grid
boxes were set to the center of the membrane models and then
discholocated to cover one layer of membrane models, while for other
receptors the grid boxes were kept in the center of the receptor.
The grid box sizes for membrane models were set to 20, 20 and 30
for X, Y and Z axes, respectively. The 3a structure was set as a
flexible ligand by using the default parameters of the AutoDock
Tools. Final models were analyzed on PyMol (The PyMOL
Molecular Graphics System, Version 1.4.1, Schrodinger, LLC).

MTT Assay
Non-carcinoma mouse embryo fibroblast cell (3T3) and human
breast carcinoma cell (MDA-MB-231) lines were used in this
study. The cells were cultured as monolayers in Dulbecco’s
Modified Eagle Medium (DMEM) supplemented with 10% (v/v)
heat-inactivated fetal bovine serum and antibiotics. After over-
night incubation, the medium was aspirated off, washed with PBS
(1X) buffer and 200 mL of complex 2a, and 3a (0–100 mM
concentration) medium were added separately in triplicates. The
MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-
con] assay was performed following the method described by
Mandal et al. [25].

Compatibility Test
Ophthalmologists generally have a large repertoire of antibac-
terial and antifungal antibiotics in the form of eye drops,
ointments, tablets and parenteral applications to control eye
infections. Complex 3a was used in an eye drop and tested for
48 h, six times a day on goat (Capra aegagrus hircus) eyes, and the
concentration was 5-fold of that used in in vitro experiments. The
eye drops were collected from a butcher’s shop at the local market.
In general, enucleation of the entire globe of the eyes was done
after death. The cornea was excised from globe under an aseptic
condition in a biosafety cabinet. Next, the excised cornea was kept
in a McCarey-Kaufman medium for preservation without any
antibiotics. Experiments were repeated with three eyes. Each eye
was kept at 4°C in a petri dish and drops were applied at 4 hour
intervals. After 48 hours of treatment, the blubber conjunctiva,
retina and lens were evaluated for any damage, cracking or
dilution by visual observation.

Statistical Analysis
The results are presented as the mean ± SD. The statistical
significance of the experimental results was determined by one-
way Student’s t-test or one-way ANOVA followed by Dunnett’s
test. Values of p<0.05 were considered statistically significant.
Prism version 5.0 was used for all statistical analyses.

Results
Synthesis of the Complexes
The ligand 1a was synthesized by the addition of imidazole and
NaOH in dry THF under stirring conditions at room temperature
and a subsequent addition of 3-chloro-2-methylpropene. The
mixture was refluxed at 65°C for 15 h and allowed to cool at room
temperature, and the solvent was removed. A brown oily liquid
form of 1-(2-methyl allylimidazole was obtained. Then 1-(2-
methyl-allylimidazole and α,β – dibromo- p-xylene were mixed
and the reaction mixture was heated for 6 hours to obtain a
colorless solid 1a (Yield: 490 mg, 0.96 mmol, 96%). Quantitative
conversion of PF6 salt (1b) of the prodigian was achieved by the
addition of aqueous solution of ammonium hexafluorophosphate in
solution 1a. The presence of NCHN proton in 1H NMR spectra
confirmed the formation of ligands 1a ( δ = 9.47 ppm) and 1b
(δ = 9.25 ppm). Elemental analysis, 13CNMR and MALDI mass
spectra support the formulation of proligands 1a and 1b. Complex 2a was prepared (Yield of 2a: 115 mg, 0.15 mmol,
81%) by the complexation of proligand- 1a and Ag2O in
dichloromethane with exclusion of light (Fig. 1) as reported in
the general procedure [3]. Complex 3a was prepared by the silver
carbene transmetallation [5] method (Yield of 3a: 135 mg, 0.17
mMol, 83%). Afterward, complex 2a and Ag(SMe2)2Cl were
combined in DCM under nitrogen atmosphere at room temper-
ature. Complex 2b was prepared by mixing prodigian 1b with
Ag2O in acetonitrile under stirring (4 h) at room temperature
(Yield of 2b: 52 mg, 0.085 mmol, 86%). 3b was also prepared by a
transmetallation method using 2b and Au(SMe2)2Cl in acetonitr-
ele (Yield of 3b: 94 mg, 0.14 mmol, 92%). Formation of the
complexes 2a, 2b, 3a and 3b was confirmed by the absence of
imidazolium protons (Che) and a downfield shift of aromatic
protons. In the case of 2a, the carbene signal appeared at 178.2 ppm
in the 13CNMR spectra, and a downfield shift of 38.9 ppm in comparison to free imidazolium salt was observed
after complexation. A significant 37–39 ppm shift of carbenic
and Au(SMe2)2Cl in acetoni-
carbon in the 13CNMR spectrum confirmed the formation of complexes 2b, 3a and 3b. This was further supported by the mass
spectrometric data. The single crystal X-ray structures of 1a, 2a
and 3b were determined (see supplementary file). 1HNMR, 13CNMR and MALDI mass spectra of all synthesized complexes
were supplied in the supplementary file and their corresponding
structures were determined (Fig. S1 to S5, Text S1 and S2).

Antimicrobial Activity
The antibacterial and antifungal activities of all synthesized
compounds were tested against both Gram-positive and -negative
bacteria as well as against unicellular and filamentous fungus. The
antibacterial activities of complexes 2a and 3a were higher than
2b and 3b (Tables 1 and 2). Complex 3a was more active
against P. aeruginosa and A. fumigatus than complex 2a and other
tested antibiotics. The eradication of pathogen-formed biofilm is
an important criterion for the successful development of new
generation antimicrobials. All the pathogenic strains were used to
develop their biofilm over contact lenses and the biofilm eradication
ability after 24 h of treatment (Fig. 2). The killing activity of
complex 3a for P. aeruginosa was different from S. aureus (Fig. S6).
The killing activity was concentration-dependent and no cell
growth was seen even after 0.5 and 2.5 μM concentration of
complex 3a for P. aeruginosa and S. aureus, respectively. Similarly,
no cell growth was observed after 2.5 and 0.5 μM concentration
of complex 3a for C. albicans and A. fumigatus, respectively (Fig. S7).

Insights into Mechanism of Action
The interactions between microbial cell membrane and
complex 3a were observed by using a scanning electron
microscope (SEM). SEM pictures clearly indicated a morpholog-
ical change after 1 h of treatment with complex 3a (Fig. 3). The
morphological change indicates that the target should be found on
the microorganism surface such as lipid bilayer or cell walls. In
order to determine the morphological changes observed in SEM
images were due to the membrane damage or not, the leakage of
cytoplasmic materials was monitored at 260 nm wavelength. The
leakage of cytoplasmic materials is considered a characteristic

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indication of damage to the cytoplasmic membrane [19]. Different concentrations of complex 3a were incubated with bacterial and fungal cell suspensions for 2 h. Cytoplasmic material leakage was observed after increasing the complex concentration, and maximum leakage was found in P. aeruginosa in comparison to S. aureus and C. albicans (Fig. 4), which indicates that the main target might be lipid bilayer.

Therefore, docking experiments were performed in order to verify the binding affinities of 3a compound to microbial cell membranes (Fig. 5). Docking data shows the complexes formed between 3a compounds with bacterium- and fungus-like membranes. The 3a molecule is clearly more buried into bacterium-like membrane in comparison to fungus-like lipid bilayer. Moreover, docking results suggested that 3a affinity to bacterium-like membranes is stronger than fungus-like membranes since the affinities were $2.7.4 \text{ kcal.mol}^{-1}$ and $2.6.1 \text{ kcal.mol}^{-1}$ respectively. Furthermore none polar interactions were observed in both cases, being complexation mainly driven by van der Walls forces.

**Compatibility Test**

Both complexes 2a and 3a inhibited cell proliferation of MDA-MB-231 and 3T3 cells in a dose-dependent manner. Cell inhibition was prominent mainly at concentrations after 25 μM for both the complexes, as confirmed by MTT assay (Fig. S8). The IC$_{50}$ values of complex 3a against MDA-MB-231 and 3T3 cancer cells were 29.21±2.38 μM and 23.44±2.01 μM, and the values of 2a against MDA-MB-231 and 3T3 cells were 32.94±2.72 μM and 26.21±1.88 μM, respectively. In addition,
complex 3a was used as an eye drop at five times the concentration observed in the in vitro experiment and applied onto the normal eye that had been excised from a goat. After 48 h of treatment with complex 3a, which was applied 6 times daily, the complex was revealed to have low toxicity to the eye (Fig. 6). The pigment and lens were neither diluted nor fractured after 48 h of treatment with complex 3a, which suggests its low toxicity and future application in keratitis control.

**Discussion**

N,N'-olefin functionalized allylic ligands, 3,3'-p-phenylenedimethylenes-bis[1-2-methyl-allylimidazolium] bromide(1a) and 3,3'-p-phenylenedimethylenes-bis[1-2-methylallylimidazolium] hexafluorophosphate(1b) were used to synthesize four bis-imidazolium silver(I) and gold(I) salts as well as 2a, 2b and 3a, 3b. Syntheses of these compounds were confirmed by using NMR (1H and 13C) and MS analysis. Single-crystal X-ray diffraction data were collected for compounds 1a, 2a and 3b. The molecules (2a, 2b and 3a, 3b) possessed N-C carbene bond distances N-Carbene-N angles in imidazole moiety were comparable with other imidazolium systems. The carbene-Ag-carbene bond angle in 2a was 170.83(15)' and C-Ag-Br bond angle was about 170.6 - 171.6 '. i.e. linear, whereas Ag-Ag-Br bond angle was nearly 90', which indicates the Ag-Ag bond was perpendicular to C-Ag-Br in a 'T' shape. It revealed that the complex was of the bis-carbene type with its olifinic system confirmed by its dinuclear crystallinity Ag(2)(1a-H)(2) motif. The same structural geometry was found in the optimized structure of complex 3a. For 2a, Ag-Ag shortest bond distance was found to be 3.084(3) Å (Ag(4)-Ag(1)) [Fig. S2] and that of Au-Au for 3a was 3.08415(5) Å [Au(110-Au(112)] [Fig. S3]. These metal-metal bond distances are shorter than the reported olefin systems and even shorter than the sum of their van der Waals radii [26]. The single crystal structure of complex 3b was determined by XRD study and that of 2b through theoretical optimization. 2b and 3b looked like a ‘chair’ with two ligands bonding by metal center from the opposite side.

The synthesized complexes 2a, 2b, 3a and 3b were evaluated for their antimicrobial properties against human pathogens causing keratitis infection. Among them, complexes 2a and 3a showed the strongest antimicrobial activities against resistant pathogenic strains. The bacterial strains were resistant to the commonly used antibiotics cefazidime, vancomycin, and piperacillin. The fungal strains were also resistant to amphotericin B, fluconazole, and voriconazole. Several resistant mechanisms such as enzymatic inactivation, chemical modification, alteration of the antibiotic target site and efflux pumps are well reported [27]. The most promising feature of this research was that all resistant strains evaluated here were highly susceptible to complexes 2a and 3a. This leads us to suggest that complexes 2a and 3a follow different mechanisms in order to inhibit microorganism growth.

Inhibition of biofilm formation or eradication of grown biofilm is the most important feature of these complexes. Complex 3a was able to eradicate the biofilm formed by bacterial and fungal strains both significantly and successfully. Biofilm is a complex matrix of exopolymeric substances of the organisms that prevent drugs from direct penetration. Hence, it is also believed that the ability of complex 3a to penetrate the biofilm matrix at very low doses might be related to the presence of a dinuclear [Au2(1a-H)(2)]2+ olifinic motif. Indeed, gold complexes showed good and selective activity against both Gram-positive and -negative bacteria, as well toward fungi. It is apparent that the functionalization of the nitrogen atoms of the NHC ligands and the complexation with Au(I) at the C2 site might be the cause of higher antimicrobial activity. The difference in activities depends on several factors since these complexes are known to interact freely with cellular components when they are in a flexible co-planar [28] configuration and have rotational freedom [29]. The relative energy as a function of the torsional angle [5] is also a relative descriptor for the co-planarity and free rotation which endorse easy binding of the metal center of the complex with the active site. In the present study, among the four complexes, the metal centers of 2a and 3a are bonded with a single ligand (other side attached to Br atom), having scope for free rotation. This free rotation promotes a co-planar configuration within the reaction medium for the easy formation of complexes with cellular components. On the other hand, ligand dissociation is prohibited in the case of cationic tetrahedral Au(I) phosphine complexes as they gain stability through chelating. This is due to the high stability of the metal-phosphorus bond [30], metal-F bond and probably formation of H-bond with ‘F’ of PF6 -, as is the case for 2b and 3b. Since metal centers are connected with two ligands, free rotation is prohibited and they are more stabilized through chelating with PF6 -, so their reactivity is lower than 2a and 3a. Our results are also in agreement with an earlier report by Ozdemir et al. [31] that imidazolium salt bearing Cl and Br salts is very active; but the same derivative completely lost its activity when the anion was changed to PF6 or BF4. The probable mechanism of action consists in the bacterial and fungal membranes destabilization, reducing the structural integrity and leading to clear microorganism shape modification. The membrane’s destabilizing effect is probably given by insertions of

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**Table 2. Minimum biofilm eradication concentration of the synthesized compounds, antibacterial and antifungal antibiotics against keratitis-associated eye pathogens.**

| Compounds   | C. albicans | A. fumigatus | P. aeruginosa | S. aureus |
|-------------|-------------|--------------|--------------|-----------|
| 2a          | 15.62       | 7.81         | 7.81         | 7.81      |
| 2b          | 250         | 125          | 125          | 125       |
| 3a          | 3.90        | 1.95         | 1.95         | 3.90      |
| 3b          | 125         | 125          | 125          | 125       |
| Voriconazole| 125         | nt           | nt           | nt        |
| Amphotericin B| 31.25      | 15.62        | nt           | nt        |
| Fluconazole  | 500         | 500          | nt           | nt        |
| Cefazidime   | nt          | nt           | 62.5         | 62.5      |
| Vancomycin   | nt          | nt           | 31.25        | 31.25     |
| Piperacillin | nt          | nt           | 250          | 250       |

Data are representing as μM concentration. nt means “not tested”.

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Figure 3. SEM micrographs of bacteria and fungi treated with compound 3a. Cells were treated with compound 3a (2 μg.mL⁻¹) and control group was treated with 0.1% DMSO. Control P. aeruginosa cells (a) and treated with 3a (b); Control C. albicans cells (c) and treated with 3a (d).

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Figure 4. Effect of complex 3a on the release of cytoplasmic material at 260 nm. P. aeruginosa (blue line), S. aureus (red line) and C. albicans (green line) were incubated with different concentrations (0.5 to 5.0 μM) of complex 3a at 30°C for 2 h. Release of cytoplasmic material was monitored at 260 nm wavelength and percent values were calculated in relation to triton treatment (positive control). The treatment with triton X-100 (0.1%) was considered as 100% of cytoplasmic material release. Data points represent mean and standard deviation of three experiments.

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multiple molecules of 3a, intercalating the lipids and leading to membrane disruption (Figs. 2 and 3). However, 3a was more active against bacteria than fungi. This difference could be related to two elements, the cell walls thickness and the membrane affinities. According to the docking affinities, the binding of 3a is more favorable to bacterium-like membranes than fungus-like ones. For bacterium-like membranes, a binding affinity of $-7.4$ kcal.mol$^{-1}$ was observed whereas the affinity for fungus-like ones was $-6.1$ kcal.mol$^{-1}$. In addition, the 3a activity is influenced by the composition of the cell wall, the compound must cross the cell wall before acting in the lipid bilayer. The differences between the amounts of leakage materials are due to the variations of their cell wall compositions. P. aeruginosa, is a Gram-negative bacterium with a thinner peptidoglycan layer which makes cytoplasmic leakage more likely compared to the thicker peptidoglycan layer of Gram-positive bacterium, S. aureus. Furthermore, in C. albicans, the main cell wall composition is chitin, which makes a rigid layer surrounding the membrane. The cytoplasmic material leakage is more prominent and more rapid in P. aeruginosa followed by S. aureus. The leakage was nominal in C. albicans (Fig. 4). In addition, a small mammalian cell toxicity degree was observed in their corresponding MIC values between 0.5 to 5.0 $\mu$M through the MTT assay.

However, a question arises: why was 3a more active than 2a? The most critical difference between the two compounds is in their stability. The covalent bond between gold and NHC is more stable than the covalent bond constructed between silver and NHC. Silver seems to be worse than gold for NHC $\leftrightarrow$ metal $\pi$-back donation [3]. This fact makes 2a more reactive to free compounds, yielding more interactions with other molecules in the environment than 3a complexes. Therefore, to compensate these interactions with non-target molecules, a larger number of 2a complexes are needed, while 3a complexes seem to go straight to the membranes.

Finally, the results obtained from the MTT assay are found to be in agreement with the low toxicity at concentrations of their MIC and MBEC values between 0.5 to 5.0 $\mu$M, and preliminary ex vivo toxicity studies demonstrated very low toxicity on the eye when applied externally. Thus, it may prove useful as an external eye drop therapy in patients suffering with keratitis. Similar studies are in progress with some determining the molecular level of interaction with binding modes to bacterial and fungal membrane lipids while others are exploring the synthesis of other gold complexes with different halide groups to enable higher water solubility and lower toxicity. Nevertheless, data reported here are extremely promising, and the reported salts could probably be
Figure 6. Compatibility test of complex 3a on goat (*Capra aegagrus hircus*) eyes, as an eye drop. Eye just excised from goat (a); after 48 h treated with compound 3a (b), conjunctiva is not damaged or cracked after 48 h treatment with complex 3a applied 6 times daily (c); and lens is neither diluted nor fractured after 48 h treatment (d). Images were captured at a magnification of 5X.
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used in the near future as an important tool to control keratitis-associated eye infection causing multi drug resistant pathogens.

Supporting Information

Figure S1 Ortep View (40% probability, H removed for clarity) of single crystal X-ray structure of ligand 3,3,3”- (p-phenylene-)dienyl methane bis[1-(2-methylallyl)imidazolium] bromide (1a). Pertinent bond lengths (\( \AA \)) and angles (\( ^\circ \)): N1-C7 = 1.320(9), N2-C7 = 1.325(9), bond angles (\( ^\circ \)): N1-C7-N2 = 108.5(6).

Figure S2 Ortep View (30% probability, H removed for clarity) of single crystal X-ray crystallographic structure of complex (2a). Pertinent bond lengths (\( \AA \)) and angles (\( ^\circ \)): N3-C29 = 1.33(3), N5-C29 = 1.36(3), C29-Ag3 = 2.08(2), Ag3-Br2 = 2.439(2), N7-C40 = 1.30(3), N5-C40 = 1.34(3), C40-Ag4 = 2.09(2), Ag4-Br4 = 2.409(4), Ag1-Ag105 = 3.084(3), bond angles (\( ^\circ \)): N5-C29-N6 = 104.0(2), C29-Ag3-Br2 = 171.7(7), C40-Ag4-Br4 = 172.2(7), C40-Ag4-Ag1 = 97.4(6), Br4-Ag4-Ag = 87.11(12).

Figure S3 ORTEP View (H atoms have been removed for clarity) of optimized structure of complex (3a). Pertinent bond lengths (\( \AA \)) and angles (\( ^\circ \)): N35-C55 = 1.31818, N54-C53 = 1.30648, C58-Au11 = 2.06414, Au11-Br107 = 2.43997, N35-C39 = 1.33557, N34-C39 = 1.32853, C39-Au110 = 2.10169, Au110-Br2 = 2.41073, Au110-Au112 = 3.08413, bond angles (\( ^\circ \)): N55-C58-N35 = 102.87193, C58-Au111-Br107 = 171.45808, N34-C39-N53 = 107.52845, C39-Au110-Br2 = 171.45603, C39-Au110-Au112 = 97.47505, Br2-Au110-Au112 = 87.03457.

Figure S4 Ortep View of optimized structure of complex (2b). Pertinent bond lengths (\( \AA \)) and angles (\( ^\circ \)): N55-C54 = 1.37564, N56-C54 = 1.38058, C54-Ag105 = 2.12572, N6-C1 = 1.38045, N5-C1 = 1.37665, C1-Ag105 = 2.12643, bond angles (\( ^\circ \)): N55-C54-N56 = 104.25472, N5-C1-N6 = 104.27794, C1-Ag105-C55 = 179.81391.

Figure S5 ORTEP View (40% probability, H and PF6 removed for clarity) of single crystal X-ray crystallographic structure of complex (3b). Pertinent bond lengths (\( \AA \)) and angles (\( ^\circ \)): C18-Au1 = 2.016(7), C7-Au1 = 2.12645, N3-C18 = 1.353(10), N4-C18 = 1.349(10), N2-C7 = 1.331(9), N1-C7 = 1.357(9), bond angles (\( ^\circ \)): N3-C18-N4 = 103.9(7), N2-C7-N1 = 105.7(6), C7-Au1-C18 = 179.1(3).

Figure S6 Growth kinetics of bacterial strains with 10³ CFU/mL in the presence of different concentrations of compound 3a.

Figure S7 Growth kinetics of fungal strains with 10⁵ CFU/mL in the presence of different concentrations of compound 3a.

Figure S8 Dose-dependent cytotoxic activity of compound 3a and 2a. Human breast carcinoma cell (MDA-MB-231) (red and violet lines) and non-carcinoma mouse embryo fibroblast cell (3T3) (blue and black lines) were grown in vitro in 96-well plates and treated with different concentrations (0.0 to 100 μM) of complex 3a (blue and violet color) and 2a (black and red color). The mean of the percentage of inhibition of cell proliferation compare to control (without complex) along with standard deviation of triplicate results are indicated.

Table S1 Crystal and X-ray diffraction data table of synthesized compounds.

Table S2 Important bond parameters for synthesized compounds.

Text S1 Chemical shift (\( ^{1}HNMR \) and \( ^{13}CNMR \)) and Mass (m/z) data of all synthesized compounds.

Text S2 X-ray crystallographic analysis.

Text S3 Characteristic features of clinical isolates.

Text S4 Procedure for MALDI MS analysis.

Author Contributions

Conceived and designed the experiments: SMM. Performed the experiments: SMM, JD, TS, GR, S. Collected data: S. Saha, JD, OLF, SMM. Contributed reagents/materials/analysis tools: JD OLF SMM. Wrote the paper: JD OLF SMM.

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