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Original Research Article

Antimicrobial, anti-biofilm and cytotoxic properties of methallyl functionalized benzimidazolium-derived Ag(I)-N-heterocyclic carbene complexes

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Abstract

Purpose: *To investigate the antimicrobial, antibiofilm, and cytotoxicity properties of methallyl substituted benzimidazolium-based, silver-bound N-heterocyclic carbene (Ag(I)-NHC) complexes, with respect to their potential to act as antimicrobial agents.*
Methods: *The antimicrobial, antibiofilm, and cytotoxicity properties of the four complexes, the synthesis and characterization of which were carried out previously, were investigated. The antimicrobial properties were tested using the broth microdilution method, while their antibiofilm potential were determined by microtiter plate assay. The L-929 cell line was used for cytotoxic studies.*
Results: *Strong antibiofilm and antimicrobial effects were produced by Ag(I)-NHC complexes. Compounds 2 and 3 showed potent activities against E. coli strain, with effects similar to that of positive control antibiotic, while compounds 1 and 4 exhibited antimicrobial activity at a concentration of 31.2 µg/mL. The compounds were effective against biofilms formed at concentrations in the range of 32 – 84 %, and degraded mature biofilms at a concentration range of 14 - 66 %. Compounds 1 and 2 did not significantly affect cell survival ($p > 0.05$), while compounds 3 and 4 significantly reduced cell survival, when compared with untreated cells in the control group ($p < 0.001$).*
Conclusion: *This study may be one of the few studies on benzimidazolium-derived NHCs. The compounds which produced antimicrobial, antibiofilm, and cytotoxic properties in this study may be valuable and novel antimicrobial agents. Therefore, there is need for further in vivo and in vitro studies on these compounds.*

Keywords: *N-Heterocyclic carbene, Silver, Benzimidazolium, Antibiofilm, Antimicrobial activity*

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INTRODUCTION

The use of chemicals for curing contagious disease was first reported by Paul Erhlich in 1909. However, in the 1940s, it was understood that microorganisms have the potential to be resistant to antibiotics. Since then, intense and inappropriate use of antibiotics have resulted in a crisis of microbial resistance which has become a significant public health problem [1].

Biofilms are responsible for resistance of microorganisms to antibiotics. Biofilms are composed of live bacterial flora within an extrapolysaccharide protective structure. These microbial groups are ubiquitous in almost every environment. Biofilm-associated bacterial populations cause drug resistance for a number of reasons. Moreover, biofilms increase the number of virulent strains by facilitating gene transfer among bacteria. Therefore, it is very important to identify novel bioactive compounds that can be used to solve health problems caused by biofilms [2].

Advancements in synthetic chemistry have made important contributions to the development of novel drugs. N-Heterocyclic carbenes (NHC) are currently popular in the development of new pharmacological products. Indeed, NHCs have been extensively employed since they were first developed, due to their important features such as catalytic and biological activities [3]. In recent years, nitrogen-containing heterocyclic carbenes have been used mainly as medicinal compounds due to their medicinal properties [4]. Metal ions can be incorporated into the matrix of biofilms to suppress their formation, since metals act as antibiofilm agents [5]. In the literature, there are limited studies on the effectiveness of NHCs on biofilms [5-7]. In the present study, the antimicrobial, antibiofilm, and cytotoxicity properties of benzimidazolium-based Ag(I)-NHC compounds which were synthesized and characterized in an earlier study, were investigated. This was with a view to determining their potential to act as antimicrobial agents.

EXPERIMENTAL

Compounds

In this study, antimicrobial, antibiofilm, and cytotoxicity properties of four benzimidazolium-based Ag(I)-NHC compounds were investigated. These compounds (**1, 2, 3 and 4;** Figure 1**)** were synthesized and characterized in previous studies [8,9].

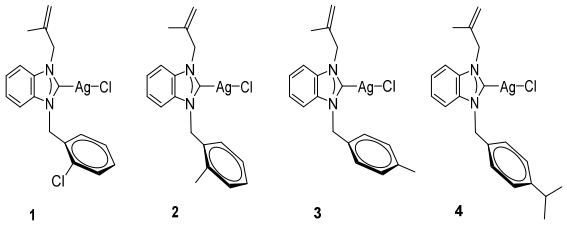

Figure 1: Benzimidazolium-based Ag(I)-NHC complexes (compounds **1-4**)

Microorganisms

The microorganisms employed in this study (25922 *Esherichia coli*, 70063 *Klebsiella pneumoniae*, 29213 *Staphylococcus aureus*, 29212 *Enterococcus faecalis*, and 10231 *Candida albicans*), and multidrug resistance clinical isolate (*Acinetobacter baumannii*) were obtained from standard strains of the American Type Culture Collection (ATCC).

Evaluation of antimicrobial activity and determination of MICs

Using the broth microdilution technique, the antimicrobial activities of the compounds were evaluated, as outlined by Gezegen *et al* [10]. Overnight cultures of microorganisms were used to prepare inoculation cell density of 10^8 CFU/mL. The compounds to be tested were dissolved in broth with 10 % (v/v) dimethyl sulfoxide. Subsequently, serial double dilutions of concentrations 3.9 - 250 µg/mL were prepared in microplates. Ampicillin, ciprofloxacin, and colistin were used as standard antibacterial agents, while flucanozole was used as a standard antifungal agent. Absorbance (A) was read at a wavelength of 620 nm.

Biofilm inhibition concentration (BIC) assay

The microtiter plate method was used in accordance with the method of Şahin *et al* [11]. An overnight culture of microorganisms at 10^8 CFU/mL turbidity containing 1 % (w/v) glucose, was diluted with 100 µL of Tryptic Soy Broth and dispensed into wells of a microplate. Subsequently, 100 µL of specific proportions of compounds **1 - 4** (3.9 to 250 µg/mL) were dispensed into different wells. After 24 h of incubation at 37 °C, the culture medium in each well was discarded, and the wells were washed. The microplates were dried for one hour. Then, 0.1 % (w/v) crystal violet was added to each well, and the wells were left for 30 min, after which they were washed and dried. The dried stain in each well was dissolved with 95 % ethanol, and the absorbance of the resultant solution was measured at 570 nm in a microplate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg/Germany). Biofilm inhibition value at sub-MIC was calculated using Eq 1.

Biofilm inhibition (%) = {(Ac-As)/Ac}100 ..... (1)

where Ac and As are the absorbance of control and sample, respectively. The assay was run in triplicate.

Determination of biofilm eradication concentration (BEC)

The BEC experiment was carried out according to the method of Tutar *et al* [12]. In this assay, 200 µL (10^8 CFU/mL) of microorganisms were inoculated into flat-bottomed, 96-well microplates. The microplates were incubated at room temperature for two days. After the biofilm structure was formed, the wells were washed to remove non-adherent cells. The serial dilutions of compounds **1 - 4** (3.9 to 250 µg/mL) were dispensed into separate wells. The wells were incubated again under the same conditions, after which they were rinsed with distilled water, and the contents were stained with crystal violet. A compound-free biofilm was used as positive control.

The concentration of biofilm settled at the bottom of the wells was taken as BEC. The dried stain in each of the wells was dissolved with 95 % ethanol, and absorbance each solution was read at 550 nm. Biofilm eradication was calculated using Eq 2.

Biofilm eradication (%) = {(Ac-As)/Ac}100 ..... (2)

where Ac and As are the absorbance of control and sample, respectively.

Cell culture

Murine fibroblast L929 cells (ATCC) were used. The cultivation of the cell lines was carried out in Dulbecco's Minimum Eagle Medium supplemented with 10 % fetal calf serum and 1 % penicillin/streptomycin. The cells were incubated at 37 °C in a 5 % carbon dioxide incubator.

Cytotoxicity assay

Murine fibroblast (L929) cells were seeded in microtitre plates at a density of 5×10^3 cells/mL/well. The cytotoxic potential of the Ag(I)-NHC complexes were determined by incubating the L929 cells with graded concentrations of the compounds i.e., 250, 125, 62.5, 31.2, 15.6, and 7.8 µg/mL, for 24 h. After the incubation period, 10 µL of XTT labelling solution was added to each well. After 2 h of the tetrazolium reaction, the absorbance of each well measured at 450 nm in a microplate reader (Thermo Scientific Microplate Photometer, Multiskan FC, USA). The percentage cell viability was calculated by comparing the optical density values of the samples with that of the untreated cells.

Statistical analysis

Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for statistical analysis. Tukey test was used for comparison of the experimental groups. All analyses were done with SPSS version 23.0. Values of $p < 0.05$ were accepted as indicative of statistically significant difference.

RESULTS

Antimicrobial activity

Compounds **2** and **3** showed potent antimicrobial effects against *E. coli* strain, with activities similar to that of the positive control antibiotic (Ampicillin), while compounds **1** and **4** showed antimicrobial activities at concentration of 31.2 µg/mL. The compounds were effective against *S. aureus* and *E. faecalis* strains at concentrations between 7.8 - 62.5 µg/mL. Compound **2** exerted a very strong antimicrobial effect against *K. pneumonia* strain, with MIC value of 7.8 µg/mL. Compounds **2**, **3,** and **4** produced strong activity against the clinical isolate of *A. baumannii*, with MIC value of 15.6 µg/mL, relative to the positive control colistin. The compounds also exhibited antifungal effects against *C. albicans* at concentrations between 7.8 and 31.25 µg/mL (Table 1).

Antibiofilm activity

The inhibitory effects of the compounds on biofilm formations are given in Table 2.

Table 1: Minimum inhibitory concentrations (MICs) of compounds **1 - 4** (µg/mL)

Compound	*E. coli*	*K. pneumoniae*	*S. aureus*	*E. faecalis*	*A. baumannii*	*C. albicans*
1	31.2	31.2	62.5	62.5	62.5	31.2
2	< 3.9	7.8	31.2	31.2	15.6	7.8
3	3.9	15.6	15.6	31.2	15.6	7.8
4	31.2	31.2	7.8	15.6	15.6	15.6
Control	Ampicillin < 3.9	Ciprofloxacin 0.50	Ampicillin < 3.9	Ampicillin < 3.9	Colistin 31.25	Flucanozole < 3.9

Table 2: Biofilm inhibitory concentrations (BICs) at MIC or sub-MIC values {(µg/mL)/(%)}

Compound	*E. coli*	*K. pneumoniae*	*S. aureus*	*E. faecalis*	*A. baumannii*	*C. albicans*
1	15.6/80.6±0.5	15.6/54.0±2.0	31.2/37.0±3.0	31.2/44.6±0.5	31.2/52.6±0.5	15.6/55.0±1.0
2	3.9/84.6±0.5	3.9/56.0±3.0	15.6/43.0±2.0	15.6/32.6±0.5	7.8/53.6±0.5	3.9/64.0±1.0
3	3.9/81.0±2.0	7.8/50.6±0.5	7.8/61.6±0.5	15.6/45.0±1.0	7.8/69.±1.0	3.9/49.0±2.0
4	15.6/67.6±0.5	15.6/48.0±1.0	3.9/54.6±0.5	7.8/54.0±1.0	7.8/49.6±0.5	7.8/52.6±0.5

Table 3: Biofilm eradication concentrations (BECs) at MIC or sub-MIC values {(µg/mL)/(%)}

Compound	*E. coli*	*K. pneumoniae*	*S. aureus*	*E. faecalis*	*A. baumannii*	*C. albicans*
1	15.6/63.0±1.0	15.6/38.0±1.0	31.2/26.6±0.5	31.2/32.6±0.5	31.2/36.0±2.0	15.6/27.0±2.0
2	3.9/66.6±0.5	3.9/42.0±3.0	15.6/25.6±0.5	15.6/14.6±0.5	7.8/40.0±2.0	3.9/30.6±0.5
3	3.9/60.6±0.5	7.8/41.0±3.0	7.8/45.6±0.5	15.6/27.6±0.5	7.8/58.0±1.0	3.9/30.8±0.5
4	15.6/54.6±0.5	15.6/30.0±2.0	3.9/36.6±0.5	7.8/38.0±2.0	7.8/40.0±1.0	7.8/40.6±0.5

The compounds reduced biofilm formation in *E. coli, K. pneumoniae, S. aureus, E. faecalis, A. baumannii* and *C. albicans* by 67 - 84, 48 - 56, 37 - 61, 32 - 54, 49 - 69 and 49 - 64 %, respectively.

The eradication potential of the compounds on mature biofilm formations are given in Table 3. The compounds eradicated mature biofilm formation in *E. coli, K. pneumoniae, S. aureus, E. faecalis, A. baumannii* and *C. albicans* by 54 - 66, 30 - 42, 25 - 45, 14 - 38, 36 - 58 and 27 - 40 %, respectively.

Effect of compounds on survival of L929 cells

In the present study, L929 cells were exposed to decreasing doses of the tested compounds (250, 125, 62.5, 31.2, 15.6, and 7.8 µg/mL) for 24 h, and cell survival was determined using XTT cell proliferation assay. As shown in Figure 2, pre-incubation of the L929 cells with the compounds at doses of 250 and 125 µg/mL for 24 significantly decreased cell survival, when compared with untreated control cells ($p < 0.001$; Figure 2).

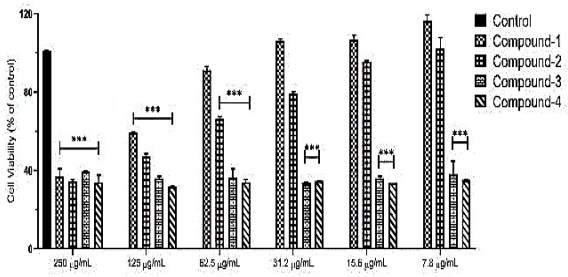

Figure 2: Effects of compounds **1 - 4** at different doses (250, 125, 62.5, 31.2, 15.6, and 7.8 µg/mL) on survival of L929 cells. The data are presented as mean ± SEM. ***$P < 0.001$, compared to control group

However, compound **1** did not affect cell survival at doses of 62.5, 31.2, 15.6, and 7.8 µg/mL, when compared with control-untreated cells ($p > 0.05$; Figure 2). Furthermore, compound **2** had no effect on cell survival at doses of 31.2, 15.6, and 7.8 µg/mL, when compared with untreated control cells ($p > 0.05$; Figure 2). Moreover, compound **3** and compound **4** significantly reduced cell survival at doses of 62.5, 31.2, 15.6, and 7.8 µg/mL, relative to untreated cells in the control group ($p < 0.001$; Figure 2).

DISCUSSION

Nowadays, it is very difficult to identify novel antibiotics. Until recently, herbal resources in traditional medicine were used to produce new medicines. However, novel drug studies have changed drastically in recent times due to high rate of consumption of these natural products as herbal medicines. Novel drug development studies are carried out by synthesizing new chemical molecules [3].

The N-Heterocyclic carbenes are deprotonated cyclic carbenes that have been electrically stabilized as azolium salts [3]. The first report on the antimicrobial activities of Ag(I)-NHC was presented in 2004 [4]. With new studies, it is possible to synthesize specific Ag-NHC complexes that exhibit preferable antimicrobial and antibiofilm effects. In the present study, it was observed that the four methallyl substituted benzimidazolium-derived Ag(I)-NHC compounds exerted antimicrobial and antibiofilm effects on microorganism strains that cause significant infections in humans. Compounds **1** and **2**, which did not show cytotoxic effects on L-929 murine fibroblast cells at MIC concentrations, are particularly promising in terms of their potential as antimicrobial agents. Compound **1** was effective at various concentrations on the microorganisms studied. The antimicrobial activity of compound **2** on *E. coli* was similar to that of ampicillin which is frequently used in treatment of infectious diseases. Compound **2** was more effective on *A. baumannii* (a drug-resistant clinical isolate) than colistin. This compound appears to have a significant potential

for use as an antimicrobial agent in the treatment of *Acinetobacter* infections. Compounds **1** and **2** also exhibited antimicrobial effects on *C. albicans* fungus, with compound **2** producing a higher effect.

There are studies in the literature on the antimicrobial properties of benzimidazolium-derived Ag(I)-NHCs. Several bacteria and fungi isolates were used by Kaloğlu *et al* [13]. to evaluate the antimicrobial activities of the Ag-(I) NHC compounds they synthesized. The researchers reported that all the newly synthesized complexes showed good activity against different microorganisms. When compared with the MIC values of the NHC complexes studied by Kaloğlu *et al*, it was observed that the NHC compounds evaluated in this study exhibited stronger antimicrobial activities on the alike microorganisms. Sarı *et al* [14] used Gram positive and Gram-negative bacteria and fungus to determine the antimicrobial activities of five benzimidazolium-derived silver bound NHC complexes they synthesized. The researchers reported that the compounds synthesized in the study using agar and broth dilution methods exhibited antimicrobial activity on microorganisms, especially a complex that showed a broad-spectrum antimicrobial activity.

However, the antimicrobial activity of the complexes which were tested in this study, were higher than those of the compounds evaluated by Sarı *et al*. The benzimidazolium-derived Ag(I)-NHC compounds synthesized by Gök *et al* exhibited high potency, especially on Gram-positive bacteria and yeast [15]. The compounds evaluated in this study showed high activity, especially on Gram-negative and gram-positive bacterial strains, unlike those in the study carried out by Gök *et al*. In addition to the antimicrobial activities of Ag(I) NHC complexes, there are also studies that tested the cytotoxic properties of these compounds [16].

Most infections are related to biofilms. Therefore, antibiofilm activity is a very important factor in the design of future antibiotics [2]. In this study, it was seen that the four benzimidazolium-derived Ag(I)-NHC complexes exhibited significant antibiofilm activities at MIC and sub-MIC concentrations. In particular, biofilm formation by *E. coli* was inhibited by these compounds up to 85 %, while *E. coli* mature biofilms were eradicated by these compounds up to 67 %. The complexes inhibited *K. pneumoniae* biofilms that cause significant nosocomial infections, by 56 %. The silver complexes showed significant antibiofilm activities on the pandrug-resistant clinical isolate of *A. baumannii* bacteria which causes important nosocomial infections associated with biofilms. In particular, compound **2** inhibited *A. baumannii* biofilm by approximately 70 %, while it eradicated 58 % of the mature biofilm formed. This strong antibiofilm effect on the resistant clinical isolate is very promising. The compounds tested in this study exhibited significant antibiofilm activity on Gram-positive bacteria and *C. albicans*.

Methicillin-resistant *S. aureus* (MRSA) infections are on the increase worldwide. Thus, it is important to discover substances that can inhibit the biofilms formed by this bacterium. Tessier and Schmitzer reported that benzimidazolium salts are potent antibiofilm agents. The researchers demonstrated that the antibiofilm activities of these complexes are due to their ability to destroy the biofilm matrix and bacterial cell membranes, which make them potential drug candidates for the cure of biofilm-related infections [17].

Samanta *et al* [18] reported that methallyl substituted imidazolium-derived N-heterocyclic carbene-metal compounds were effective in eradicating biofilms formed on contact lenses, and that they are promising candidates for the successful treatment of biofilm-associated keratitis infections. Using biofilm ring test, Bernardi *et al* [5] studied the antibiofilm effects of a series of metal-imidazolium and imidazolinium-derived NHC compounds, and reported that the silver metal bound to NHCs yielded the most effective results. This points to the significance of metal NHCs for the development of new metal-based drugs for treating resistant bacterial infections associated with biofilms. In a study, it was reported that Ag(I)-NHC compounds inhibited *Bacillus anthracis* biofilms better than ciprofloxacin and doxycycline which are in clinical use [6]. In another study, it was reported that Au and Ag complexes of synthesized imidazolium derivative silver-bound NHC compounds suppressed biofilm formation in *Streptococcus mutans* MTCC890 and *E. coli* ATCC 25922 standard bacterial strains [7]. The present study also showed strong antibiofilm effects of Ag(I)-NHC compounds on *E. coli* biofilms. Although there are several publications on the anti-biofilm effects of imidazolium and imidazolidinium derivatives of Ag(I)-NHC compounds in the literature, there are no publications on the antibiofilm activities of benzimidazolium derivative Ag(I)-NHC compounds.

CONCLUSION

The four methallyl-based benzimidazolium-

derived Ag(I)-NHC compounds exhibit strong antimicrobial and antibiofilm effects. Although compounds **3** and **4** are cytotoxic to L-929 mouse fibroblast cells at MIC concentrations, compounds **1** and **2** did not exhibit cytotoxic effects. Compounds **1** and **2** appear to be particularly valuable in terms of their potential as new antimicrobial agents. Therefore, there is need for additional *in vivo* and *in vitro* studies on these compounds.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Cem Celik, Ugur Tutar and Neslihan Sahin designed the study and wrote the manuscript. Cem Celik, Ugur Tutar and Aysegül Ozturk performed the experimental work. All the authors reviewed and approved the final manuscript for publication.

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