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Antibiofilm and Antimicrobial Properties of 1-allyl-3-(2-diisopropylaminoethyl) Benzimidazolium Chloride and its Silver(I)-NHC Complex

Uğur Tutar [1,a,*], Cem Çelik [2,b]

[1] *Department of Pharmaceutical Botany, Faculty of Pharmacy, Sivas Cumhuriyet University, 58140 Sivas, Türkiye*
[2] *Department of Medical Microbiology, Faculty of Medicine, Sivas Cumhuriyet University, 58140 Sivas, Türkiye*
**Corresponding author*

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ABSTRACT

Today, the number of antimicrobials used in treatment has decreased, especially due to drug resistance. We need new antimicrobials. Biofilms are an important cause of antimicrobial resistance. In this study, the antimicrobial and antibiofilm properties of the salt and silver complex of benzimidazolium-based NHC compound, which were previously synthesized and characterized, were evaluated. The antimicrobial properties were tested using the broth microdilution method, while their antibiofilm potential was determined by microtiter plate assay. Salt of the NHC compound (1a) showed antimicrobial activity on microorganisms at concentrations between 31.25-125 µg/mL. The silver complex (2a) of the NHC compound showed higher antimicrobial and antibiofilm activity than the salt compound. This activity was highest on *Candida albicans* yeast (MIC 3.9 µg/mL). Compound 2a reduced the biofilm structure of *C. albicans* yeast by 86.1% compared to the control. In addition, compound 2a showed 76.4-80.6% antibiofilm activity on gram-negative bacteria. NHC compounds are seen as a promising resource for the development of new generation antimicrobials. The NHC compound evaluated in this study was found to have significant antimicrobial and antibiofilm activity. These compounds could be an important resource for the discovery of future biofilm-acting antimicrobials.

Keywords: N-heterocyclic carbene, Silver, Antimicrobial activity, Antibiofilm activity.

[a] *ututar@cumhuriyet.edu.tr* *https://orcid.org/0000-0002-8058-0994* [b] *cemcelik58@gmail.com* *https://orcid.org/0000-0002-7141-5874*

Introduction

Microorganisms are very important to live on our planet. These tiny creatures make the world usable for other life. Microbial decomposing communities prevent our world from turning into a garbage dump of dead organisms. They also make other important contributions to all living things [1]. However, microorganisms also cause one of the most important problems in the world, which can cause infectious diseases, impair people's health and even cause death [2]. Antibiotics are used to prevent these harms of microorganisms. The first antibiotic, salvarsan, was introduced in 1910, and in a little over 100 years, antibiotics have drastically changed modern medicine. During this period, the average human lifespan was extended by 23 years. However, since then, there has been a decrease in the number of antimicrobials used in treatment, mainly due to drug resistance and problems in the discovery of new antimicrobials [3].

Antimicrobial resistance is a global public health crisis that prevents us from successfully treating bacterial infections. Many infectious agents that can be successfully treated with one of several drug classes have acquired resistance to almost all of these drugs in some cases [4]. An important reason for the spread of antimicrobial resistance is biofilms. Biofilms are environments where microorganisms live as a community by clinging to a surface and surrounding them with a protected material. Biofilms play an important role in multidrug resistance and cause high morbidity and mortality in infections [5]. New treatment strategies and new antimicrobials are needed to overcome the threats posed by this increase in antimicrobial resistance. Currently, no drug that specifically targets bacterial biofilms is in clinical use. Moreover, the development of new antimicrobial agents is currently declining for different reasons [4,6,7].

N-heterocyclic carbene (NHC) compounds and their silver complexes have antimicrobial activities on microorganisms. Therefore, NHC compounds are seen as a promising reservoir for the development of new generation antimicrobials [8]. In this study, antimicrobial and antibiofilm activities of salt and silver complex of NHC compound, which were previously synthesized and characterized, were evaluated. It is thought that this study will contribute to the literature, especially today, where sufficient new antimicrobials cannot be developed against increasing antimicrobial resistance and there are no antibiotics that will affect biofilms.

Materials and Methods

Compounds

In this study, previously synthesized and characterized 1-Allyl-3-(2-diisopropylaminoethyl)benzimidazolium chloride (1a), Chloro[1-allyl-3-(2-diisopropylaminoethyl) benzimidazol-2-ylidene]silver(I) (2a), [9] were evaluated in terms of antimicrobial and antibiofilm properties. The

Minimal Inhibition Concentration (MIC) test was used to determine the antimicrobial activities of the compounds, and the Biofilm Inhibition Concentration (BIC) test was used to determine the antibiofilm activities.

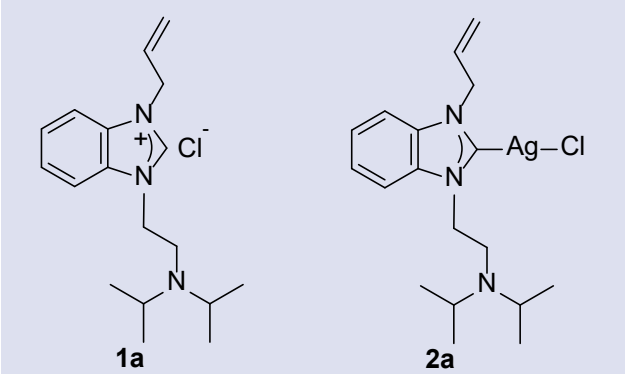

Figure 1. Structure of compounds (1a, 2a)

Microorganisms

In this study, Gram negative bacteria; *Escherichia coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC 17978), Gram positive bacteria; *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), and yeast strain; *Candida albicans* (ATCC 10231) obtained from the standard strains of the American Type Culture Collection were used. Standard microorganisms were stored at -80 °C and revived at room temperature before the study.

Antimicrobial activity

Microdilution method was used to determine the antimicrobial activities of the compounds according to the CLSI [10] recommendations. The minimal inhibitory concentration (MIC) values of the compounds were investigated for the studied strains using the microwell dilution method. Bacterial and fungus cultures were adjusted to 0.5 McFarland standard turbidity ($1x10^8$ CFU/mL microorganisms) to prepare suspensions of microorganisms.

2 mg of the compound was weighed and dissolved in 4 mL of MHB to prepare a stock solution. For the test, the compounds were dissolved in Mueller-Hinton broth (MHB) containing 10% (v/v) DMSO. Serial twofold dilutions were prepared in 96-well plates with MHB at a concentration ranging from 1.9 to 250 µg /ml.

100 microliters of the prepared solutions were added to each well and all concentrations were distributed in the wells. Then, the microorganisms used in the study (100 microliters) were added to each well to be tested. In the study, ciprofloxacin antibiotic was used for bacteria and Fluconazole was used for fungus as positive control. As negative control, wells containing strains of microorganisms prepared by dispensing 200 microliters without any compound were evaluated. The microplate reader was used to measure the optical density (OD) at 620 nm, and it was measured again following the incubation at 37 °C for 24 h. MIC was determined as the lowest concentration of 1a and 2a that inhibits the growth of test microorganisms.

Reduction in biofilm formation

Microtiter plate assay was used to test the anti-biofilm activity of compounds against microorganisms. One aliquot (100 µL) of culture (1×10^8 CFU/mL) containing 2% (w/v) glucose diluted with Tryptic Soy Broth (TSB) dispensed into a test well. Then, 100 µL of decreasing concentrations of compounds (1.9 to 250 µg/ml) were dispensed into the wells. TSB was used as negative control, and compound-free microorganism cultures alone were used as positive control. After 24 hours of incubation at 37 °C, the supernatants of the wells were decanted and each well was gently rinsed three times with 300 µL of sterile distilled water. After drying the plates for 30 minutes in air, they were stained with 0.1% (w/v) crystal violet for 30 minutes at room temperature. It was washed 3 times with distilled water and dried. The crystal violet was then dissolved in 95% ethanol and absorbance was read in a microplate reader at 570 nm. The biofilm inhibition value at SubMIC was calculated with Eq. [11] Biofilm inhibition (%) = { (Ac − As) / Ac } 100 where As and Ac are the absorbance of sample and control, respectively. This test was performed in triplicate and the mean (n = 3) was taken.

Results and Discussion

The resistance of pathogenic microorganisms to antimicrobial treatments is increasing at an alarming rate. As a result, there are difficulties in the fight against infections. One of these difficulties is the current shortage of effective drugs. Infections caused by resistant microorganisms do not respond to conventional treatments. New antimicrobials and strategies are needed for the treatment of these infections. On the other hand, studies on finding new antimicrobials effective on resistant microorganisms have decreased considerably in recent years. For this reason, very few new antimicrobial agents have been used in the period [5]. But the spread of antibiotic resistance threatens the successful treatment of infections. New drugs need to be discovered to prevent a pre-antibiotic era from returning [12].

NHCs are typically organic compounds that mimic the chemical properties of phosphines. Since they are easy to prepare and process, they have found wide use and made a significant impact in the field of organometallic chemistry [13]. Historically, silver and its compounds have been used for medicinal applications. Silver compounds have been used especially for wound care antiseptics and for infections. Although its use has been limited by the discovery of new antibiotics, it has gained importance again with the problem of antimicrobial resistance. In recent years, NHCs and their silver complexes have been synthesized for pharmaceutical applications and are widely used as metal-based drug candidates for medical applications [13].

In our study, antimicrobial and antibiofilm activities of benzimidazolium-derived NHC compound salt and silver complex were evaluated. Salt of benzimidazolium-derived NHC compound (1a) showed antimicrobial activity on

microorganisms at concentrations between 31.25-125 µg/mL. Compound 1a showed the highest antimicrobial activity on *C.albicans*, while the activity on *S.aureus* was lower (Table 1). The silver complex of the NHC compound (2a) showed higher antimicrobial activity on the microorganisms in the study than the salt compound this activity reached the highest activity on *C. albicans* yeast (MIC 3.9 µg/mL). The activity of compound 2a appears to be good compared to the control antifungal Fluconazole. Compound 2a also showed very strong antimicrobial activity on gram-negative bacteria at a concentration of 7.8 µg/mL. The antimicrobial activity of compound 2a on gram-positive bacteria was also found to be strong with a MIC concentration of 15.6 µg/mL (Table 1)

Table 1. Minimum Inhibitory Concentrations (MICs) of Compounds (µg/mL)

Compounds	Microorganism				
	Staphylococcus aureus (ATCC 29213)	Enterococcus faecalis (ATCC 29212)	Escherichia coli (ATCC 25922)	Acinetobacter baumannii (ATCC 17978)	Candida albicans (ATCC 10231)
1a	125	62.5	62.5	62.5	31,25
2a	15.6	15.6	7.8	7.8	3.9
Ciprofloxacin	<1.9	<1.9	<1.9	<1.9	
Fluconazole					<1.9

There are studies in the literature on the antimicrobial properties of silver-bound NHC compounds. Kaloğlu et al. [14] reported that NHC compounds showed antimicrobial activity at concentrations between 6.25 and 100 µg/mL in their study with gram-positive, gram-negative bacteria and yeast strains. When the study results were compared, it was seen that the silver complex we evaluated in our study had higher antimicrobial activity. Mnasri et al. [15] reported that the Ag(I) NHC complexes they synthesized showed strong antimicrobial activity against bacterial and fungal agents at 0.24 and 62.5 µg/ml concentrations. Sarı et al. [16] reported that the benzimidazolium-based Ag(I)-NHC compounds they synthesized showed antimicrobial activity at different concentrations on gram-positive, gram-negative bacteria, and fungus strains**.** Asekunowo et al. [17] stated that Ag(I) NHC complexes showed antibacterial activity on *Staphylococcus aureus* and *Escherichia coli* at concentrations of 12.5-100 µg/mL. When the studies are evaluated, it is understood that the Ag(I)-NHC compound, whose antimicrobial activity was evaluated in our study, has a very strong antimicrobial activity. For this reason, it would be appropriate to carry out further studies in terms of this compound being one of the new antimicrobials at the present time when new antimicrobials are needed.

Biofilm is a stable collection of microorganisms that settle on any surface in the matrix they produce. Many microorganisms in a living or non-living environment can form a biofilm. Biofilms make an important contribution to the development of resistance of microorganisms to antimicrobial drugs. This leads to the presence of bacteria in biofilms that can cause very important diseases. Today, there are no antibiotics that are effective on biofilms and aim to destroy biofilms. Accordingly, the number of resistant microorganisms is increasing, and the number of drugs that can destroy these microorganisms is gradually decreasing. Therefore, it seems very important to design new biofilm inhibitors as antimicrobial agents targeting the inhibition of biofilm formation [18].

Table 2. Reduction in Biofilm Formation on ½ MIC Value of Compounds (Concentration (µg/mL)/ Biofilm Inhibition (%))

Compounds	Microorganism				
	Staphylococcus aureus (ATCC 29213)	Enterococcus faecalis (ATCC 29212)	Escherichia coli (ATCC 25922)	Acinetobacter baumannii (ATCC 17978)	Candida albicans (ATCC 10231)
1a	62.5/37.7±1.1	31.25/38.6±0.9	31.25/70.5±0.5	31.25/50.8±0.3	15.6/50.8±0.2
2a	7.8/64.6±0.3	7.8/68.1±0.3	3.9/76.4±0.2	3.9/80.6±0.3	1.9/86.1±0.1

In this study, the antibiofilm activities of the salt and metal complex of the Benzimidazolium derivative NHC compound were investigated at ½ MIC concentrations. Salt of the NHC compound (1a) showed 37.7-70.5% antibiofilm activity on two gram-positive and two gram-negative bacteria and one yeast strain used in the study. Compound 1a reduced the biofilm structure formed by *E.coli* bacteria by 70.5% compared to the control. This is a remarkable result. Compound 1a also reduced the biofilm structure of *C. albicans* yeast by more than half compared to the control. Antibiofilm activity of compound 1a on gram-positive bacteria was found to be around 38%. The silver complex of NHC compound (2a) inhibited microorganism biofilms at higher rates than the salt compound (1a). Compound 2a showed very high antibiofilm activity, especially by reducing the biofilm structure of *C. albicans* yeast by 86.1% compared to the control. In addition, compound 2a showed 76.4-80.6% antibiofilm activity on gram-negative bacteria and 64.6-68.1% on gram-positive bacteria. It was observed that compound 2a had very strong antibiofilm activity on all microorganisms used in the study (Table 2).

Üstün et al. [11] showed the antibiofilm activities of the Ag(I)-NHC compounds they synthesized on gram-positive and negative bacteria and fungal strains. Celik et al. [19] In their study, they reported that Ag(I)-NHC compounds inhibited the biofilm formation of gram-positive and gram-negative bacteria and yeast strains by

32-84% while degrading mature biofilms by 14-66%. Bernardi et al. [20] reported that they found the most effective results in inhibiting biofilms in silver and copper-linked NHC complexes. Şahin et al. [21] reported that the Ag(I) NHC compounds they synthesized found different concentrations of antibiofilm activity on two gram-positive and two gram-negative bacteria and one yeast strain. Tutar et al. [22] They reported that they found strong antibiofilm activity in their study with Ag(I) NHC complexes. Researchers have found up to 90% antibiofilm activity on gram-positive and gram-negative bacteria and yeast strains. It is very important that the newly discovered antimicrobials have antibiofilm properties. In the literature, there are limited studies on the biofilm activities of Ag(I) NHC compounds. Ag(I) NHC compound, whose antibiofilm properties we examined in our study, showed significant antibiofilm activity. Therefore, this compound is considered to have the potential for new antimicrobials.

Conclusion

Rapidly developing resistance to antimicrobials has reduced the number of drugs used for treatment, but not enough new antimicrobials have been discovered. In addition to the antimicrobial activities of the new drugs to be produced, it is very important that they also have antibiofilm properties. New substances need to be discovered for antimicrobial drugs with these properties. In this study, antimicrobial and antibiofilm activities of the salt and silver-based complex of benzimidazolium-derived NHC compound were evaluated. Significant antimicrobial and antibiofilm activity were found in these compounds on two gram-positive and two gram-negative bacteria and one yeast strain. There are publications in the literature on the antimicrobial activities of NHC compounds. However, the number of publications on the antibiofilm activities of these compounds is not high. These compounds could be an important resource in the process of producing antimicrobials of the future. Therefore, further in vitro and in vivo studies would be beneficial.

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Conflicts of interest

The authors declared that they have no conflict of interest.

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