ANTIBACTERIAL AND ANTIBIOFILM SCREENING OF NEW PLATINUM(IV) COMPLEXES WITH SOME S-ALKYL DERIVATIVES OF THIOSALICYLIC ACID

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ABSTRACT. The influence of 5 new Platinum(IV) (Pt(IV)) complexes with S-alkyl derivatives of thiosalicylic acid (C1-benzyl, C2-methyl, C3-ethyl, C4-propyl and C5-butyl) was studied on 16 strains of bacteria. Antibacterial activity was tested using microdilution method with resazurin while antibiofilm activity was observed by tissue culture plate method, using doxycycline as a positive control. The results were expressed as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and biofilm inhibitory concentration (BIC). The best result on Gram positive bacteria exhibited C1 and MIC was ≤7.81µg/ml against Staphylococcus aureus ATCC 25923. Bifidobacterium animalis subsp. lactis (probiotic) was sensitive to C2 (MIC at 15.625 µg/ml). The highest sensitivity of Gram negative bacteria was observed in Escherichia coli ATCC 25922 treated with C1, C2, C3 and C4, in Proteus mirabilis ATCC 12453 treated with C1, and in Pseudomonas aeruginosa treated with C2, C3 and C5 (all MICs at 250 µg/ml). The C2 complex were more efficient as antibiofilm agents and the best results were obtained with C2 acting against S. aureus and S. aureus ATCC 25923 biofilms. In conclusion, we noticed that the tested compounds exhibited promising properties as antibacterial and antibiofilm agents.

Key words: platinum(IV) complex, antibacterial activity, antibiofilm.

INTRODUCTION

The interest in determining the influence of new metal complexes on microorganisms is increasing due to the growing resistance of pathogenic bacteria. Studies on antimicrobial activity of platinum (Pt(IV)) complexes have been conducted, showing wide influence on microorganisms but being more or less effective.

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Staphylococcus aureus, as Gram positive bacteria and Shigella flexneri, as Gram negative were a part of AL-HASANI (2007) investigation of the antibacterial activity for the ligand and their metal complexes, which were bimetallic. Square planar Pd (II) and octahedral Pt (IV) complexes with novel spherical aramides nanoparticles containing flexible linkages ligands were tested for antimicrobial activity (ELHUSSEINY and HASSAN, 2013). In this investigation, Pt complexes as polymeric nanoparticles showed high potency as antitumor and antimicrobial agents. Different polymers with Pt(IV) (NARTOP et al., 2013) exhibited a moderate activity against selected microorganisms. Pt(IV) complexes with unsymmetrical tetradentate schiff bases (HEGAZY and GAFFAR, 2012) have been tested on Bacillus subtilis, S. aureus, Escherichia coli, Salmonella typhi, also yeast and fungi. They have proven to act as antimicrobials. Other studies of platinum (Pt(IV)) complexes include Pt(IV) chelate (HEGAZY, 2012), Pt(IV) dithiocarbamate complexes (MANAV et al., 2006), thiodiamines with Pt(IV) (MISHRA and KAUSHIK, 2007), etc.

The goal of this study was in vitro testing of new synthesized Pt(IV) complexes (labeled as: C1 for Pt(S-bz-thiosal)₃, C2 for Pt(S-met-thiosal)₃, C3 for Pt(S-et-thiosal)₃, C4 for Pt(S-pr-thiosal)₃ and C5 for Pt(S-bu-thiosal)₃) in order to obtain information on their antimicrobial activity and for the first time the antibiofilm activity of any of Pt(IV) complexes.

MATERIALS AND METHODS

Chemicals

Ammonium sulphate was purchased from Zorka Pharma (Šabac, Serbia), magnesium sulphate from Merck-Alkaloid (Skopje, FYRM), while potassium dihydrogen phosphate and sodium dihydrogen citrate were purchased from Kemika (Zagreb, Croatia). Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (New Jersey, USA). Resazurin was obtained from Alfa Aesar GmbH & Co. (KG, Karlsruhe, Germany). Crystal violet stain was obtained from Fluka AG (Buchs SG, Switzerland), nutrient liquid medium, a Mueller–Hinton broth was purchased from Torlak (Belgrade, Serbia), an antibiotic, doxycycline, from Galenika A.D. (Belgrade, Serbia).

The synthesis of complexes

The platinum(IV) complexes with an S-alkyl derivatives of thiosalicylic acid [PtCl₂(S-R-thiosal)₂] were obtained by reacting potassium heksahlorido platinita (IV) and S-alkyl derivatives of thiosalicylic acid (R= benzyl, methyl-, ethyl-, propyl- or butyl) in a molar ratio of 1:2 with the addition of aqueous lithium hydroxide (Scheme 1). S-alkyl derivatives of thiosalicylic acid were synthesized according to the procedure described by SMITH et al., (2011).

S-alkyl derivatives of thiosalicylic acid (for C1-benzyl, C2-methyl, C3-ethyl, C4-propyl and C5-butyl) in amount of 0.6 mmol was slowly added to the solution of 0.2 mmol (0.1 g) potassium-hexachloro platinum(IV) with 10 ml of distilled water. The reaction mixture was heated in a water bath with stirring for 3 h. During this period, small portions from a solution of LiOH (0.6 mmol with 10 ml of distilled water) were added. The precipitate of the complex was separated by filtration, rinsed with distilled water and dried in air.
Determination of antibacterial and antibiofilm activity

Test microorganisms

The antibacterial activity was tested against 16 strains of bacteria (Table 1) and antibiofilm activity against 4 bacterial strains (Table 2). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac, Serbia. The ATCC strains were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Suspension preparation

The suspensions were prepared by direct colony method. The turbidity of the initial suspension was adjusted using 0.5 McFarland densitometer (DEN-1, BioSan, Latvia). The initial suspensions were additionally diluted in 1:100 ratio in sterile 0.85% saline.

Microdilution method

Antibacterial activity was tested by determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) using microdilution method with resazurin (Sarker et al., 2007). The tested compounds were first dissolved in dimethyl sulfoxide (DMSO) (10% of total volume) and then into nutrient liquid medium (up to 100% of total volume). The stock concentrations of tested compounds were 2000 µg/ml. Next, serial twofold dilutions were made in a concentration range from 1000 µg/ml to 7.81 µg/ml in sterile 96-well microtiter plates containing nutrient broth. After that, 10 µl of diluted suspensions were added to appropriate wells. Finally, 10 µl resazurin solution, as an indicator of microbial growth, was added to each well. Resazurin is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink. Minimum bactericidal concentration was determined by plating 10 µl of samples from wells, where no indicator color change was recorded, on nutrient agar medium. At the end of the incubation period the lowest concentration with no growth (no colony) was defined as minimum bactericidal concentration (MBC).

Doxycycline, dissolved in nutrient liquid medium, was used as a positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganisms. Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.
**Tissue culture plate method (TCP)**

The TCP assay described by CHRISTENSEN et al. (1985) is the most widely used test for detection of biofilm formation. We screened all strains for their ability to form biofilm by TCP method with some modifications. Each test included biofilm formation control. Bacterial biofilm formation properties were well described by O’TOOLE et al. (2000).

The tissue culture 96-well plates (Sarstedt AG & Co., Germany) were prepared by dispensing 100 µl of nutrient broth, Mueller–Hinton broth for bacteria, into each well. A 10 µl of fresh bacterial suspension was added to each well. The inoculated plates were incubated at 37 °C for 24 h for Gram negative bacteria and 48 h for Gram positive bacteria. A 100 µl from the stock solution of tested complexes (concentration of 2000 µg/ml) was added into the first row of the plate. Then, twofold, serial dilutions were made for each next row using a multichannel pipette. After 24 h, the incubation content of each well was gently removed by tapping the plates. The wells were washed with 200 µl of saline buffer (0.15 M ammonium sulfate, 0.1 M potassium dihydrogen phosphate, 0.034 M sodium dihydrogen citrate and 0.001 M magnesium sulphate) to remove free-floating bacteria. Biofilms formed by adherent cells in plate were stained with crystal violet (0.1% w/v) and incubated at the room temperature for 20 minutes. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of ethanol-acetone solution (4:1). Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA plate reader at wavelength of 630 nm (OD630 nm). Biofilm inhibitory concentration (BIC) was defined as the lowest concentration of each complex where the biofilms were dispersed. Only broth or broth with dissolved complexes served as control to check sterility and non-specific binding of the media. All tests were performed in duplicate.

**RESULTS AND DISCUSSION**

**Antibacterial activity**

The test results of in vitro antibacterial activity of Pt(IV) complexes are presented in Table 1. The detected values were in range from less than 7.81 up to more than 1000 µg/ml. For comparison, MIC and MBC values of doxycycline are also listed. Gram positive bacteria showed higher sensitivity than Gram negative bacteria.

Significant sensitivity of Gram positive bacteria in the presence of Pt(IV) complexes, was observed in *Bifidobacterium animalis* subsp. *lactis*, *B. subtilis*, *S. aureus* and *S. aureus* ATCC 25923. The best result was obtained with C1 with MIC on *S. aureus* ATCC 25923 was <7.81 µg/ml. *B. animalis* subsp. *lactis* (probiotic) showed high sensitivity in relation to C1 (MIC at 62.5 µg/ml) and even higher sensitivity in relation to C2 (MIC at 15.625 µg/ml). MIC for the Gram-negative bacteria was in the range from 250 to >1000 µg/ml. The highest sensitivity was observed in *E. coli* ATCC 25922 in relation to C1, C2, C3 and C4, *Proteus mirabilis* ATCC 12453 in relation to C1 and *Pseudomonas aeruginosa* in relation to C2, C3 and C5 (all MICs at 250 µg/ml).

Comparing the results obtained for the Pt(IV) complexes with the results of corresponding ligands from which they were synthetized (RADIĆ et al., 2012) it can be concluded that these complexes had better antibacterial activity than the ligands. HEGAZY and GAAFFAR (2012) tested synthetized Pt(IV) complex on 10 pathogenic bacteria and had high efficiency against all the strains, including *Salmonella sp.*, *S. aureus* and *B. subtilis*, while the Pt(IV) dithiocarbamate complexes investigated by MANAV et al. (2006) were less active against *E. coli*, *B. subtilis* and *P. aeruginosa*. 

Table 1. Antibacterial activity of Pt(IV) complexes C1 to C5 and positive control (doxycycline), MIC values (µg/ml) – mean inhibitory activity, MBC values (µg/ml) – mean bactericidal activity.

<table>
<thead>
<tr>
<th>Species</th>
<th>C1 MIC</th>
<th>C1 MBC</th>
<th>C2 MIC</th>
<th>C2 MBC</th>
<th>C3 MIC</th>
<th>C3 MBC</th>
<th>C4 MIC</th>
<th>C4 MBC</th>
<th>C5 MIC</th>
<th>C5 MBC</th>
<th>Doxy MIC</th>
<th>Doxy MBC</th>
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<td>250</td>
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<td>&gt;1000</td>
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</table>
**Antibiofilm activity**

Biofilm appears when microbial cells get attached to the surface and get embedded in the extracellular polymeric substances (EPS) (Donlan, 2002). Biofilms are making various problems in food industry, medicine and everyday life. Antibiofilm examinations are a step forward in preclinical testing in microbiology. Bacteria which are in biofilm structure are different than planktonic cells and are generally more resistant to antimicrobial agents (Lewis, 2001), so we decided to perform the test on 4 strains of bacteria to obtain the *in vitro* antibiofilm activity of Pt(IV) complexes. The results are presented in Table 2. The best results were obtained with C2 acting against *S. aureus* and *S. aureus* ATCC 25923 biofilm and it was noticed that obtained values were lower than antibiotic values which is important to notice because this is the first antibiofilm testing of this kind of complexes.

Table 2. Antibiofilm activity of Pt(IV) complexes C1 to C5 and positive control (doxycycline), BIC values (µg/ml) – biofilm inhibitory concentration, nt – not tested.

<table>
<thead>
<tr>
<th>Species</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>Doxycycline</th>
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<tr>
<td></td>
<td>BIC</td>
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**CONCLUSIONS**

The Pt(IV) complexes showed a significant activity against the tested bacteria. The strongest antibacterial and antibiofilm activity was observed against *S. aureus* and *S. aureus* ATCC 25923. Since these bacteria can cause different medical problems, the potential use of Pt(IV) complexes should be the subject of future studies.

**Acknowledgments**

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**References:**


to plastic tissue culture plates: a quantitative model for the adherence of Staphylococci to medical devices. *Journal of Clinical Microbiology.* **22:** 996-1006.


