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 <7.81 µg/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 GAAFAR, 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 heksahloridoplatinata (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-hexachloroplatinum(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.

Scheme 1. Synthesis of the platinum complexes (IV) with S-alkyl derivatives of thiosalicylic acid.

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 (OD_{630 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 synthesized (RADIĆ *et al.*, 2012) it can be concluded that these complexes had better antibacterial activity than the ligands. HEGAZY and GAAFAR (2012) tested synthesized 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.

Species	C1		C2		C3		C4		C5		Doxycycline	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Lactobacillus plantarum</i>	500	1000	500	1000	500	1000	500	1000	500	1000	0.45	7.81
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i>	62.5	125	125	250	125	250	250	250	250	250	31.25	62.5
<i>Bacillus subtilis</i> IP 5832	500	500	500	>1000	1000	1000	1000	1000	1000	1000	1.95	15.63
<i>B. subtilis</i>	62.5	500	250	500	31.25	500	62.5	500	62.5	500	0.11	1.95
<i>B. subtilis</i> ATCC 6633	1000	1000	1000	1000	1000	1000	1000	>1000	1000	>1000	1.95	31.25
<i>Staphylococcus aureus</i>	62.5	125	62.5	500	250	500	250	500	250	500	0.45	7.81
<i>S. aureus</i> ATCC 25923	<7.81	62.5	125	250	125	500	125	250	125	250	0.22	3.75
<i>Enterococcus faecalis</i>	1000	1000	500	500	100	1000	1000	1000	1000	1000	7.81	62.5
<i>Escherichia coli</i>	1000	1000	500	500	500	1000	500	1000	500	1000	7.81	15.63
<i>E. coli</i> ATCC 25922	250	500	250	500	250	500	250	500	250	500	15.63	31.25
<i>Proteus mirabilis</i>	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	250	500
<i>P. mirabilis</i> ATCC 12453	250	500	500	500	500	500	1000	1000	1000	1000	15.63	62.5
<i>Pseudomonas aeruginosa</i>	500	>1000	250	1000	250	1000	500	>1000	500	>1000	250	1000
<i>P. aeruginosa</i> ATCC 27853	1000	1000	1000	1000	1000	>1000	1000	>1000	1000	>1000	62.5	125
<i>Salmonella enterica</i>	1000	1000	500	500	500	1000	500	1000	500	1000	15.63	31.25
<i>Salmonella typhimurium</i>	1000	1000	500	500	500	1000	500	1000	500	1000	15.63	125

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 ($\mu\text{g/ml}$) – biofilm inhibitory concentration, nt – not tested.

Species	C1	C2	C3	C4	C5	Doxycycline
	BIC					
<i>Staphylococcus aureus</i>	250	62.5	125	500	125	250
<i>S. aureus</i> ATCC 25923	500	62.5	1000	250	1000	250
<i>Proteus mirabilis</i> ATCC 12453	1000	>1000	1000	nt	1000	nt
<i>Pseudomonas aeruginosa</i>	1000	1000	1000	1000	1000	2000

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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