Biological Evaluation of Mechlorethamine-Pt(II) Complex, Part II: Antimicrobial Screening and Lox Study of the Complex and its Ligand

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Abstract: The reaction of K_2PtCl_4 with anticancer-alkylating agent mechlorethamine hydrochloride ($CH_3NH(C_2H_4Cl)_2$ = HN2×HCl), in the molar ratio 1 : 2, affords the complex $[H2N2]_2[PtCl_4]$. *In vitro* antimicrobial and lipoxygenase inhibitory activities of the complex and its precursor were evaluated. Antimicrobial activity of the HN2×HCl and $[H2N2]_2[PtCl_4]$ complex was investigated against 29 species of microorganisms. Testing is performed by microdilution method. Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) have been determined. The difference between antimicrobial activity of precursor and corresponding platinum(II) complex is noticed and the activity of the precursor was higher. Tested compounds demonstrated the high and significant antifungal activity and low to moderate antibacterial activity. It was shown that the gram-positive bacteria were more sensitive than the gram-negative. UV absorbance-based enzyme assays were performed with HN2×HCl and $[H2N2]_2[PtCl_4]$ complex, in order to evaluate their *in vitro* inhibitory activity of soybean lipoxygenase (LOX), also. Assay with LOX showed significantly greater inhibitory activity of the complex, than the precursor.

Keywords: Antibacterial activity, Antifungal activity, Mechlorethamine hydrochloride, Platinum(II) complex, Soybean lipoxygenase inhibition

INTRODUCTION

Alkylating agents are highly reactive compounds. Their action is based on the causing of various changes in the DNA molecule and it is correlated with cytotoxicity [1, 2]. Mechlorethamine and its analogues are bifunctional alkylating agents. They react with nucleophilic centers of cellular molecules DNA, RNA, and proteins [3]. Mode of action of mechlorethamine on the DNA is the primary mechanism responsible for its antitumor effect [4]. Mechlorethamine, also known as HN2, mustine, chlormethine, or as Mustargen (brand name) is one of the first clinical anti-tumor drugs [5 - 8]. Experimental studies of reactions of nitrogen mustards with nucleic acids and proteins have demonstrated that mechlorethamine reacts faster than its aromatic analogues and forms greater amounts of crosslink adducts [9, 10]. Biological effects of mechlorethamine and other compounds from the family of nitrogen mustard were studied on different microorganisms in different test systems *in vivo* and *in vitro* [11 - 17].

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Platinum(II) complexes have many medicinal, chemical and industrial applications. Interaction of platinum(II) with biological molecules is of great medical interest, mostly because some of these complexes are anticancer drugs [18].

Platinum compounds exhibited antitumor activity, but have relatively high cytotoxicities. These compounds behave as non-classical alkylating agents, regarding their principle function of bonding to the N7 position of the imidazole ring of the purine bases of DNA [19].

The effect of platinum complexes on proteins and various enzymes was investigated [20 - 24]. It was found that enzymes containing reactive sulfhydryl groups are particularly sensitive to inhibition by platinum complexes, while other histidine containing enzymes gave a slight protection against inhibition [25].

Although the activity and the use of cisplatin in the treatment of tumors is known [18, 26] activity of the platinum and other metal of the platinum group are interesting as antibacterial and antifungal agents. Antimicrobial activity of the Pt(II) complexes and different precursors were investigated in literature [27 - 35].

In our previous study we have elaborated that the novel $[H2N2]_2[PtCl_4]$ complex can act as an artificial metallopeptidase [36]. The aim of this study was to continue with in-

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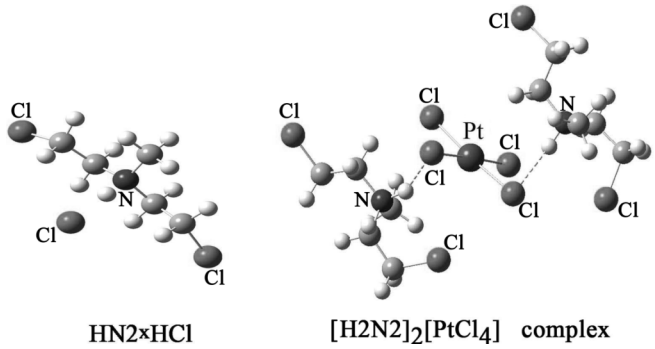

Fig. (1). The Optimized Structures of Pt(II) Complex and its Precursor.

vestigation of the biological activity of this complex. Now we investigate *in vitro* antimicrobial and lipoxygenase inhibitiory activity of the complex and its precursor.

MATERIALS AND METHODS

Chemical

The compounds K_2PtCl_4 and caffeic acid were obtained from Aldrich Chemical Co. All common chemicals were of reagent grade. Mechlorethamine hydrochloride (HN2×HCl), soybean lipoxygenase and linoleic acid sodium salt were obtained from Sigma Chemical Co. Nutrient liquid medium, a Mueller–Hinton broth was from Liofilchem, Italy, while a Sabouraud dextrose broth was from Torlak, Belgrade. An antibiotic, doxycycline, was purchased from Galenika A.D., Belgrade, and antimycotic, fluconazole, was from Pfizer Inc., USA. The IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer using the KBr pellet technique. Elemental microanalyses for carbon, hydrogen, and nitrogen were performed at the Faculty of Chemistry, Belgrade University.

Computational Method

Calculations was conducted using Gaussian09 [37] with the M06 hybrid functional [38]. The triple split valence basis set 6-311++G(d,p) was used for C, H, O, N, and Cl [39]. Geometrical parameters were optimized in water, using the CPCM model (Polarizable Conductor Calculation Model, ε = 78.36). Vibrational analysis was performed. Calculated structure was verified to be local minima (all positive eigenvalues) for ground state structures by frequency calculations.

Structure Examination of Mechlorethamine Ligand and the [H2N2]$_2$[PtCl$_4$] Complex

The optimized structure of HN2×HCl is presented in Fig. (**1**). It is worth pointing out that NBO analysis reveals the N–H bond, implying that HN2×HCl consists of chloride anion and $CH_3NH(C_2H_4Cl)_2$ cation. Also, the N-H bond distance is 1.065 Å, while the distance between Cl anion and H is 1.95 Å. The $[H2N2]_2[PtCl_4]$ complex synthesis, as well as its optimized structure are reported in [36].

Soybean Lipoxygenase Inhibition Study

In vitro study was evaluated as reported previously [40]. The tested compounds were dissolved in DMSO were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 cm^3 of enzyme solution (1/9×10-4 w/v in saline). The conversion of sodium linoleate to 13-hydroperoxy-linoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor.

In vitro Antimicrobial Assay

Test Microorganisms

Antimicrobial activity of the precursor and corresponding platinum(II) complex was tested against 29 microorganisms. The experiment was involved 16 strains of pathogenic bacteria, including 7 standard strains (*Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923; *Sarcina lutea* ATCC 9341; *Bacillus subtilis* ATCC 6633; *Proteus mirabilis* ATCC12453) and 9 clinical isolates (*Escherichia coli*; *Enterococcus faecalis*; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; *Sarcina lutea*; *Bacillus subtilis*; *Proteus mirabilis*; *Salmonella enterica* and *Salmonella typhymirium*). Also, four species of probiotic bacteria (*Lactobacillus plantarium* PMFKG-P31, *Bacillus subtilis* IP 5832 PMFKG-P32, *Bifidobacterium animalis subsp. lactis* PMFKG-P33; and *Lactobacillus rhamnosus* PMFKG-P35) five species of pathogenic fungi (*Aspergillus fumigatus* PMFKG-F23; *Aspergillus flavus* PMFKG-F24; *Aspergillus restrictus* PMFKG-F25; *Aspergillus niger* PMFKG-F26 and standard strain *Aspergillus niger* ATCC 16404); and four yeast species (*Candida albicans* (clinical isolate), *Candida albicans* ATCC 10231, *Rhodotorula* sp. PMFKG-F27 and *Saccharomyces boulardii* PMFKG-P34) were tested. All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from a collection held by the Microbiology Laboratory Faculty of Science, University of Kragujevac.

Suspension Preparation

Bacterial suspensions and yeast suspension were prepared by the direct colony method. The colonies were taken directly from the plate and were suspended in 5 mL of sterile 0.85% saline. The turbidity of initial suspension was adjusted by comparing with 0.5 McFarland's standard (0.5 mL 1.17% w/v $BaCl_2{\times}2H_2O$ + 99.5 mL 1% w/v H_2SO_4) [41]. When adjusted to the turbidity of the 0.5 McFarland's standard, bacteria suspension contains about 10^8 colony forming unites (CFU)/mL and suspension of yeast contains 10^6 CFU/mL. Ten-fold dilutions of initial suspension were additionally prepared into sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spore from slopes with growing aspergilli. The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

Microdilution Method

Antimicrobial activity was tested by determining the minimum inhibitory concentrations (MIC) and minimum microbicidal concentration (MMC) by using microdilution plate method with resazurin [42]. The 96-well plates were prepared by dispensing 100 µL of nutrient broth, Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi and yeasts, into each well. A 100 µL from the stock solution of tested compound (concentration of 2000 µg/mL) was added into the first row of the plate. Then, twofold, serial dilutions were performed by using a multichannel pi-

Table 1. Antibacterial Activity of the HN2×HCl and Corresponding Platinum(II) Complex

Species	HN2×HCl		Pt - H2N2		Doxycycline	
	MIC*	MMC**	MIC	MMC	MIC	MMC
Sarcina lutea ATCC 9341	125	250	125	125	< 0.448	7.81
Sarcina lutea	125	125	125	250	< 0.448	3.75
Enter. faecalis ATCC 29212	500	500	500	500	7.81	62.5
Enter. faecalis	1000	1000	1000	1000	7.81	62.5
Bacillus subtilis ATCC 6633	250	250	250	250	1.953	31.25
Bacillus subtilis	125	250	250	250	0.112	1.953
Staphylococcus aureus ATCC 25923	500	500	500	1000	0.224	3.75
Staphylococcus aureus	250	500	500	500	0.448	7.81
Escherichia coli ATCC 25922	1000	>1000	1000	>1000	15.625	31.25
Escherichia coli	1000	1000	1000	1000	7.81	15.625
Pseud. aeruginosa ATCC 27853	1000	1000	1000	1000	62.5	125
Pseud. aeruginosa	125	1000	250	1000	250	> 250
Proteus mirabilis ATCC12453	500	1000	1000	1000	15.625	62.5
Proteus mirabilis	1000	1000	1000	1000	250	> 250
Salmonella enterica	1000	>1000	1000	>1000	15.625	31.25
Salmonella typhymirium	1000	>1000	1000	>1000	15.625	125
Lactobacillus rhamnosus	31.25	500	500	1000	7.81	31.25
Lactobacillus plantarum	500	500	500	1000	0.448	7.81
Bifidobacterium animalis subsp. lactis	500	500	1000	1000	31.25	62.5
Bacillus subtilis IP 5832	500	500	500	1000	1.953	15.625

*MIC values (μg/mL) – means inhibitory activity.
**MMC values (μg/mL) – means microbicidal activity.

pette. The obtained concentration range was from 1000 to 0.49 μg/mL. A 10 μL of diluted bacterial, yeast suspension and suspension of spores was added to each well to give a final concentration of 5 x 10^5 CFU/mL for bacteria and 5 x 10^3 CFU/mL for fungi and yeast. Finally, 10 μL resazurin solution was added to each well inoculated with bacteria and yeast. Resazurin is an oxidation–reduction indicator used for the evaluation of microbial growth. It 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 for bacteria, 28 °C for 48 h for the yeast and 28 °C for 72 h for fungi. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink. For fungi, MIC values of the tested substance were determined as the lowest concentration that visibly inhibited mycelia growth.

Doxycycline and fluconazole were used as a positive control. Solvent control test was performed to study an effect of 10% DMSO on the growth of microorganism. It was observed that 10% DMSO did not inhibit the growth of microorganism. Also, in the experiment, the concentration of DMSO was additionally decreased because of the twofold serial dilution assay (the working concentration was 5% and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant. Minimum bactericidal and fungicidal 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 microbicidal concentration.

Statistical Analysis

All statistical analyses were performed using SPSS package. Mean differences were established by Student's t-test. Data were analyzed using one-way analysis of variance (ANOVA). In all cases P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Microbiological Screening

The results of *in vitro* testing of antimicrobial activities of the precursor and corresponding platinum(II) complex are shown in Tables **1** and **2**. For comparison, MIC and MMC values of doxycycline and fluconazole are also listed in Tables **1** and **2**. The tested substances showed broad range an-

Table 2. Antifungal activity of the HN2×HCl and Corresponding Platinum(II) Complex

Species	HN2×HCl		Pt - H2N2		Fluconazol	
	MIC*	MMC**	MIC	MMC	MIC	MMC
Candida albicans ATCC 10231	62.5	250	125	250	31.25	1000
Candida albicans	62.5	125	125	250	62.5	1000
Rhodotorula sp.	15.625	31.25	31.25	62.5	62.5	1000
Saccharomyces boulardii	125	125	125	250	31.25	1000
Aspergillus niger ATCC 16404	15.625	15.625	62.5	62.5	62.5	62.5
Aspergillus niger	15.625	15.625	62.5	62.5	500	1000
Aspergillus restrictus	3.91	15.625	7.81	31.25	500	2000
Aspergillus fumigatus	3.91	62.5	7.81	62.5	500	1000
Aspergillus flavus	3.91	7.81	7.81	15.625	1000	1000

*MIC values (μg/mL) – means inhibitory activity.
**MMC values (μg/mL) – means microbicidal activity.

timicrobial activity. The precoursor and corresponding platinum(II) complex showed different degree of antimicrobial activity in relation to the groups of microorganisms (bacteria or fungi). In general, the activity of precursor was higher than the corresponding platinum(II) complex ($p < 0.05$). Also, the precoursor and corresponding platinum(II) complex demonstrated more potent inhibitory effects on the growth of fungi than bacteria ($p < 0.05$).

The tested precoursor and corresponding platinum(II) complex showed significant antifungal activity. MICs were from 3.91 μg/mL to 62.5 μg/mL while MMCs were from 7.81 μg/mL to 62.5 μg/mL, depending on the species of fungi. The precoursor and corresponding platinum(II) complex inhibited the growth of *Aspergillus* species at low concentrations and no statistically significant difference was in their action. Their activity was stronger than the control fluconazole ($p < 0.05$).

MIC values for yeasts were in range from 15.625 μg/mL to 125 μg/mL, while MMCs were from 31.25μg/mL to 250 μg/mL. Values for MIC and MMC were lower in for the precursors but with no significant differences in action between them and Pt(II) complex.

The precoursor and corresponding platinum(II) complex demonstrated low to moderate antibacterial activity. MIC values were in range from 125μg/mL to 1000μg/mL, and MMC values from 125μg/mL to >1000μg/mL depending on the strain of bacteria. The gram-positive bacteria were more sensitive than the gram-negative bacteria. Among G+ bacteria the best results were observed against *Sarcina lutea* and *Bacillus subtilis* (clinical isolates and standard strains). The tested compounds did not affect the growth of clinical isolates and standard strains of G- bacteria or their activities were very low (except *Pseudomonas aeruginosa* MIC=125μg/mL to 250μg/mL). Also, probiotic bacteria showed high resistance to the effects of tested compounds. MICs and MMCs were at 500 μg/mL and 1000 μg/mL (except in the case of *Lactobacillus rhamnosus* when precursor realized the MIC at 31.25 mg /mL).

Soybean Lipoxygenase Inhibition Study In vitro [40]

In continuation of our study on biological significant derivatives of histidine [43] and biological activities of Pd(II)-diethanolamine and Pd(II)-mechlorethamine complexes, [44, 45] this part of our work is devoted to the study of *in vitro* inhibition of soybean lipooxygenase (LOX) with novel platinum(II)-mechlorethamine complex and its precursor. UV absorbance-based enzyme assays with these compounds were done in order to evaluate their inhibitory activity of soybean LOX. Availability and stability of mammalian lipoxygenases is limited, and therefore research on lipoxygenases was done with readily obtainable enzyme from soybean seeds. The active site in soybean LOX is non-heme Fe(III) atom coordinated by three histidines, isoleucine, asparagine and a hydroxide group [46] (Fig. **2**).

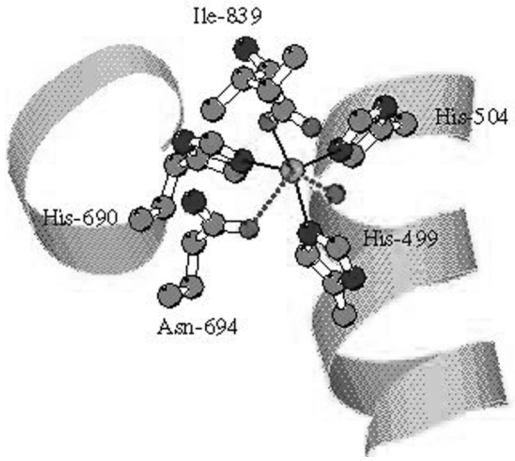

Fig. (2). The active site in Soybean LOX.

Most of the LOX inhibitors are antioxidants or free radical scavengers, since lipoxygenation occurs *via* radicals. Considering the radical mechanism of inhibition of LOX and the fact that platinum(II) ion is "soft" Lewis acid, and electrophyle, we assumed that platinum(II) complex can be free

radical scavenger in LOX-catalyzed reaction of dioxigenation of fatty acids.

Studies of inhibitors on soybean LOX points to several possible mechanisms, e.g., binding to allosteric sites around the active site of the enzyme molecule [47], preventing the formation of the active Fe(III) form of LOX50 or trapping the free radicals formed during the lipoxygenase-catalyzed oxygenation of polyunsaturated fatty acids [48]. In our cases it is reasonable to expect that platinum(II)-chlorido moiety of investigated complex, as Lewis acid and free radical scavenger cause the blocking of the catalytic cycle. Also, taking into account great affinity of platinum(II) ion of the complex for nitrogen of histidine imidazole ring [49, 50, 51, 52] it is reasonable to expect that competitive coordination reaction of Pt(II) ion to the imidazole nitrogen occurs, also [53].

Perusal of % inhibition values, or IC50 values, shows that HN2×HCl as a complex precursor has lower inhibition than platinum(II) complex (Table 3). Higher inhibitory activity of platinum(II) complex, relative to HN2×HCl, clearly shows that platinum(II)-chlorido moiety of the complex is meritorious for inhibitory activity. Complex is better inhibitor of soybean LOX than the reference compound caffeic acid. Low inhibitory activity of mechlorethamine hydrochloride can be assigned to the fact that enzyme assay was done in the presence of the tris buffer (pH = 9.00). Under this assay condition hydrochloric acid from HN_2×HCl, as electrofile and potent inhibitor, is being neutralized.

Table 3. Inhibitory Activity of the HN2×HCl and Corresponding Platinum(II) Complex

Compound	LOX IC_{50} (μM)
Complex	50 μM
HN2×HCl	2 % (0.1 mM), 18,4 % (0.5 mM)
CA	600 μM

CA Caffeic Acid; HN2×HCl Mechlorethamine; Each value Represents the mean of two Independent Experiments

CONCLUSIONS

Results of this evaluation show that the $[H2N2]_2[PtCl_4]$ complex and mechlorethamine hydrochloride as its precoursor demonstrated more potent inhibitory effects on the growth of fungi than bacteria. Antifungal activity is higher on the Aspergillus species related to the tested yeast species. It was shown that the gram-positive bacteria (*Sarcina lutea* and *Bacillus subtilis*) were more sensitive than the gram-negative and probiotic bacteria. The activity of precursor was higher than the corresponding platinum(II) complex. Soybean lipoxygenase inhibition assay with LOX showed higher inhibitory activity of the complex then mechlorethamine hydrochloride. Complex is better inhibitor of soybean LOX than the reference compound caffeic acid.

$[H2N2]_2[PtCl_4]$ complex, with high LOX inhibitory effect and significant antifungal activity, deserves attention as a potentionally multiple useful compound and, can therefore be candidate for further stages of screening *in vitro* and/or *in vivo*.

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