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The effect of non-specific binding of Pd(II) complexes with *N*-heteroaromatic hydrazone ligands on the protein structure

NEMANJA D. MIJIN[1#], JELICA MILOŠEVIĆ[1], NENAD R. FILIPOVIĆ[2#], DRAGANA MITIĆ[3#], KATARINA ANĐELKOVIĆ[1#], NATALIJA Đ. POLOVIĆ[1#] and TAMARA R. TODOROVIĆ[1*#]

[1]University of Belgrade – Faculty of Chemistry, Studentski trg 12–16, 11000 Belgrade, Serbia, [2]University of Belgrade – Faculty of Agriculture, Nemanjina 6, 11000 Belgrade, Serbia and [3]Innovation Centre of University of Belgrade – Faculty of Chemistry, Studentski trg 12–16, 11000 Belgrade, Serbia

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Abstract: Previously, the cytotoxic actions of five Pd(II) complexes with bidentate *N*-heteroaromatic chelators (complexes **1**–**5**) on a palette of several cancer cell lines were investigated. However, the results of the cytotoxic activity did not correlate with the hydrophobic character of the complexes. To gain further insight into the structure–activity relationship, essential for the design of novel potential drugs, other factors, such as non-specific interactions with cellular proteins, have to be taken into account. To explore the potential non-specific influence of the complexes on protein structures, ovalbumin (OVA) was chosen as a model system to mimic cellular non-specific crowding environments with high protein concentrations. A Fourier-transform infrared spectroscopy study implied that the binding of **3** and **4** led to only moderate alternations in the secondary structures of the protein, without the possibility to penetrate into hydrophobic core of the protein and disruption of protein native fold. Contrary, the effect of complex **5** on OVA secondary structures was concentration-dependent. While the lower concentration of complex **5** had no effect on OVA structure, a doubled concentration of complex **5** led to complete disruption of the content native-like secondary structures. The concentration-dependent effect of complex **5** on the changes in secondary structures and considerable increase in the exposure of OVA hydrophobic surfaces to water may be related to a potential crosslinking that leads to OVA aggregation.

Keywords: ovalbumin model system; protein aggregation; DMSO effect; ligand hydrophobicity.

* Corresponding author. E-mail: tamarat@chem.bg.ac.rs
# Serbian Chemical Society member.
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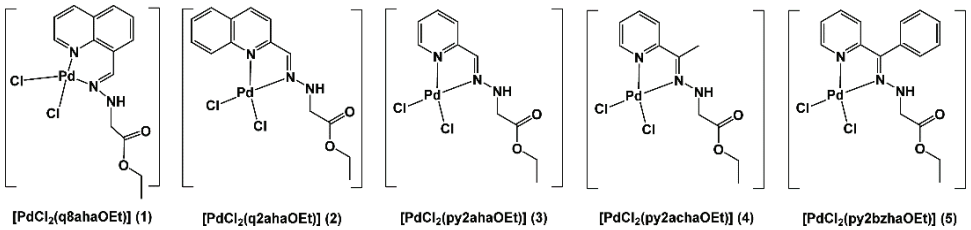
INTRODUCTION

The coordination compound cis-diamminedichloroplatinum(II), known as cisplatin or CDDP, is a blockbuster anticancer drug that entered the market a half-century ago as the first metal-based chemotherapeutic (MBC).[1] Cisplatin is used to treat various types of solid tumors[2] and proved to be especially active against testicular cancer, with a 90% cure rate.[3] For all so called 'platins' – platinum(II)-based chemotherapeutics, including cisplatin, that entered the market, DNA was identified as a primary target. Their mechanism of action includes covalent binding of platinum to DNA bases, with formation of mainly intrastrand cross links, which eventually leads to apoptosis.[4] In order to overcome the side effects of cisplatin, such as nephrotoxicity and development of tumor resistance, novel MBCs based on platinum and other metals were developed.[1,4,5] Among them, palladium(II) complexes have been studied due to significant similarity of coordination chemistry of Pt(II) and Pd(II).[6,7] The advantage of Pd(II) complexes over analogues Pt(II) complexes is firstly the better solubility of the former.[8] In the terms of anticancer activity, Pd(II) complexes have shown comparable or even better activities than approved platinum-based chemotherapeutics, with reduced cross-resistance and decreased toxicity.[6,7,9] On the other hand, due to high reactivity, Pd-based complexes are often unable to reach their biological targets. Moreover, isomerization of active cis into trans isomers results most frequently in decreased activity of Pd(II) complexes. Preparation of Pd(II) complexes with *N*,*N*′ chelating ligands was proven to be an effective strategy for the prevention of isomerization.[8]

In previous studies, the cytotoxic action of several Pd(II) complexes with bidentate *N*-heteroaromatic chelators (complexes **1**–**5**, Scheme 1), which are the condensation products of ethyl hydrazinoacetate hydrochloride and quinoline-8--carboxaldehyde, quinoline-2-carboxaldehyde, 2-formylpyridine, 2-acetylpyridine and 2-benzoylpyridine, were investigated. The antiproliferative activity of the complexes was evaluated on a palette of several cancer cell lines.[9,10] Complex **1** with an q8a-based ligand (Scheme 1) appeared to be the most effective with an activity comparable to that of cisplatin in all the investigated cell lines. The observed antiproliferative effect was predominantly mediated through the induction of apoptotic cell death.



Scheme 1. Structures of Pd(II) complexes **1**–**5**.

Generally, the antiproliferative activity of a compound could be related, in part, to its log *P* value, as a measure of lipophilic character. Since **1**–**5** all possess a $PdCl_2$ core, the liphophilicity should vary only as a function of the log *P* value of the ligands. The calculated log *P* values for the ligands in **1**–**5** are: 2.12, 2.24, 1.16, 1.48 and 2.48, respectively.[9,11,12] Based only on the log *P* values, it would be expected that **5** possesses the most pronounced antiproliferative effect, since the resulting hydrophobicity may contribute to an increased uptake of the compound by the cells.[13] However, despite a favorable log *P* value, **5** showed no anticancer activity against acute monocytic leukemia (THP-1) and mammary adenocarcinoma (MCF-7) cell lines, while complex **3**, with the lowest log *P* value, induced apoptosis in the investigated cell lines.[10] Thus, it was hypothesize that reduction of the cytotoxic action within this group of similar complexes could be related to non-specific interactions with cellular proteins. Thus, complexes **3**–**5** were selected for further studies. In order to explore the potential non-specific influence of **3**–**5** on protein structure, ovalbumin (*Gallus gallus*) was chosen as a model system. Ovalbumin (OVA) is a 45 kDa reserve protein expressed in chicken egg whites. Based on its homology, it belongs to the serpin family of proteins, which includes certain inhibitors of serine proteases.[14] OVA accounts for about 50 % of hen egg white proteins, which makes it an easily available model system for studying protein behavior, including protein conformational changes.[15–17] Even though OVA is an extensively studied protein, there are no reports about its natural ligands. Another common protein model system, human serum albumin (HSA), is a transport protein that has many binding sites for various natural ligands and, hence, it is likely to bind structurally related molecules including potential therapeutics under investigation. The low affinity of binding of diverse molecules makes OVA a better model system for studying the effect of different compounds on a protein structure. Due to its low specificity, OVA is even used to mimic cellular non-specific crowding environments with protein concentrations up to above 300 g L^{-1}.[18,19]

EXPERIMENTAL

Materials and methods

Ethyl hydrazinoacetate hydrochloride (98 %), 2-formylpyridine (98 %), 2-acetylpyridine (97 %) and 2-benzoylpyridine (98 %) were obtained from Acros Organics (BVBA, Geel, Belgium), while potassium tetrachloropalladate(II) (98%) was obtained from Aldrich. All solvents (reagent grade) were obtained from commercial suppliers and used without further purification.

Elemental analyses (C, H, N) were performed by standard micromethods using a Elementar Vario ELIII C,H,N,S/O analyser, and their results were found to be in good agreement (±0.4 %) with the calculated values. NMR spectra were obtained on a Bruker Avance 500 instument equipped with a broad-band direct probe. All spectra were measured at 298 K in $CDCl_3$ or DMSO-d_6. Chemical shifts are given on δ scale (ppm) relative to tetramethylsilane (TMS) as an internal standard for ^1H and ^{13}C.

*Synthesis of ethyl (*E*)-((pyridin-2-ylmethylene)amino)glycinate-*N,N*-dichloridopalladium(II) [PdCl$_2$py2ahaOEt] (**3**), ethyl (*E*)-((1-(pyridin-2-yl)ethylidene)amino)glycinate-*N,N*-dichloridopalladium(II) [PdCl$_2$py2achaOEt] (**4**) and ethyl (*E*)-((phenyl(pyridin-2-yl)methylene)amino)glycinate-*N,N*-dichloridopalladium(II) [PdCl$_2$py2bzhaOEt] (**5**)*

The complexes **3**–**5** were synthesized as described previously[9,10] by the template reaction of ethyl hydrazinoacetate hydrochloride, the corresponding carbonyl compound and potassium tetrachloropalladate(II) (mole ratio 1:1:1). The purity of the complexes was checked by elemental analysis and NMR spectroscopy (Supplementary material to this paper).

Calculation of absorption, distribution, metabolism, and excretion (ADME) parameters and pan assay interference compounds (PAINS) evaluation

Physicochemical properties, lipophilicity, water solubility, pharmacokinetics, druglikeness and medicinal chemistry parameters were determined using the free SwissADME tools available at the website of the Swiss Institute of Bioinformatics (http://www.swissadme.ch/).[11] The structures of the complexes obtained by X-ray diffraction studies were converted to Sybyl Mol2 format using the Mercury 2022.1.0 program.[20] Input SMILES formats of the complexes were constructed using the Open Babel Package v3.1.1.[21]

Protein purification

OVA was extracted from hen egg whites by a two-step method according to a previously published protocol.[22] The globulins were first precipitated using a 50 % saturation solution of ammonium sulfate, after which they were removed *via* 30 min of centrifugation at 3000*g*. Thereafter, the OVA was precipitated from the received supernatant by adjusting the pH to its isoelectric point of 4.6 through titration with 2 M acetic acid. The precipitate was separated by centrifugation (30 min at 3000*g*) and then resuspended in 100 mM Tris-HCl buffer (pH 7.4). The obtained OVA solution was then dialysed against 100 mM Tris-HCl buffer (pH 7.4) for 16 h in order to remove excess salts. The OVA was then stored at –20 °C. The OVA concentration was determined *via* the Bradford assay, using bovine serum albumin (BSA) as the standard.

8-Anilinonaphthalene-1-sulphonic acid (ANS) fluorescence measurements

The binding of ANS fluorescent dye was measured using a FluoroMax-4 Jobin Yvon spectrofluorimeter. The dye was dissolved in 100 mM Tris-HCl buffer (pH 7.4) with a concentration of 8 mM. Mixtures containing 200 μL of ANS, 1900 μL of the 100 mM Tris-HCl buffer (pH 7.4), and 100 μL of the samples (containing the protein and each of the complex in equimolar ratio) were prepared. The concentrations used were based on already published IC_{50} values of the selected complexes and were 50 μM for complex **3**, and 100 μM for complexes **4** and **5**. Complex **5** was also tested in a concentration of 50 μM. Emission spectra (wavelength range 440–550 nm) were obtained, with the excitation wavelength set to 390 nm.[23]

Fourier transform infrared spectroscopy (FTIR)

Infrared spectra of the OVA-Pd complex samples (in 100 mM Tris-HCl buffer, pH 7.4 and DMSO) were collected using a Thermo Fisher Scientific Nicolet Summit FTIR Spectrometer in the ATR mode. Aliquots of 1.0 μL were applied to the diamond crystal, and their solvents were evaporated *via* a mild stream of argon. Thereafter, composite spectra in the mid-IR region (400–4000 cm^{-1}) were collected in 32 scans using a DTGS KBr detector, with a scanning resolution of 2 cm^{-1}. The received spectra were automatically corrected for background absorption. FTIR corrections (automatic ATR correction and automatic baseline cor-

rection) were performed using OMNIC32 software. The amide I region was deconvoluted to its constituents corresponding to certain secondary structures using the same software, essentially as described previously.[22]

RESULTS AND DISCUSSION

ADME parameters and PAINS evaluation

The *in-silico* ADME profiles of complexes **1**–**5** were assessed through the robust SwissADME program and the results are presented in Table I. All compounds showed the desirable Lipinski rule principles such as MW ≤ 500, number

TABLE I. Pharmacological profiles, medicinal chemistry principles and lead-likeness properties of compounds **1**–**5**; ++: high; +: activity; –: no activity

Parameter	Compound				
	1	**2**	**3**	**4**	**5**
Physicochemical properties					
Molecular weight	434.61	434.61	384.56	398.58	460.56
#Heavy atoms	22	22	18	19	24
#Aromatic heavy atoms	10	10	6	6	12
Fraction Csp^3	0.21	0.21	0.30	0.36	0.19
#Rotatable bonds	5	5	5	5	6
#H-bond acceptors	2	2	2	2	2
#H-bond donors	1	1	1	1	1
Molar Refractivity	88.74	88.74	71.23	76.04	95.72
Topological polar surface area (TPSA), $Å^2$	55.62	55.62	55.62	55.62	55.62
log $P_{o/w}$	1.55	1.61	0.69	0.98	1.94
Pharmacokinetics					
GI absorption	++	++	++	++	++
BBB permeant	+	+	+	+	+
P-gp substrate	–	–	–	–	–
CYP1A2 inhibitor	+	+	–	–	+
CYP2C19 inhibitor	+	+	+	+	+
CYP2C9 inhibitor	–	–	–	–	–
CYP2D6 inhibitor	+	+	–	–	+
CYP3A4 inhibitor	–	–	–	–	+
log (K_p / cm s^{-1})	–6.00	–5.77	–6.41	–6.27	–5.47
Druglikeness					
Lipinski #violations	0	0	0	0	0
Ghose #violations	0	0	0	0	0
Veber #violations	0	0	0	0	0
Egan #violations	0	0	0	0	0
Muegge #violations	0	0	0	0	0
Bioavailability score	0.55	0.55	0.55	0.55	0.55
Medicinal chemistry					
PAINS #alerts	0	0	0	0	0
Leadlikeness (1 violation: $MW > 350$)	No	No	No	No	No
Synthetic accessibility	3.72	3.77	3.64	3.69	3.92

of atoms that act as hydrogen bond acceptors ≤ 10 and number of hydrogen bond donors ≤ 5.[24] Other physicochemical properties, such as number of rotatable bonds (≤ 10), molar refractivity (from 40 to 130) and topological polar surface area (TPSA ≤ 140 Å^2), were also found within the acceptable range. All compounds were predicted to be highly absorbed by the gastrointestinal (GI) system after oral administration, while some of them are likely to inhibit cytochrome P450 gene isoforms (*i.e.*, CYP1A2, CYP219). Relevant strategies for selection of molecules with preferred drug-like profiles examined by SwissADME indicate that the all complexes represent drug candidates since they possess important functional groups and bioavailability. Finally, according to a recently published editorial,[25] in order to remove suspicion of artificial activity, in addition to SwissADME, the compounds were evaluated by the ZINC PAINS pattern identifier.[26] The applied algorithms did not report the compounds as potential PAINS or covalent inhibitors, although due to *MW* > 350 none of the compounds represent potential lead compound, which is usually the case for MBCs with nontrivial organic ligands.

However, one of the most important drug-likeness descriptors is related to the lipophilic character of the drug candidate. This property is usually measured by 1-octanol/water partition coefficient (log $P_{o/w}$). The calculated log $P_{o/w}$ for the complexes given in Table I represent the average value of five computational models.[12,24,27–30] The order of the log $P_{o/w}$ values for the complexes is the same as for the parent ligands, where complex **5** possesses the highest hydrophobic character and lipo-solubility. However, the results of the cytotoxic activity do not correlate with the hydrophobic character of the complexes. In order to gain insight into the structure–activity relationship, other factors, such as non-specific interactions with cellular proteins, have to be taken into account. Thus, for the further studies, complex **3**, which showed to be more potent apoptosis inducer than cisplatin in THP-1 and MCF-7 cell lines, and complexes **4** and **5**, which showed no activity, were chosen.

The effect of DMSO on OVA secondary structures

In exploring the biological effect of potential medicinal therapeutics on biological systems including proteins, nucleic acids and cells, usually the presence of co-solvent is needed to enable dissolution of the potential therapeutics in simulated physiological conditions (aqueous buffers). For this purpose, DMSO is commonly used in final concentrations up to 5 %.[10] FTIR spectroscopy was employed to monitor the changes in the secondary structures of OVA in the absence and presence of different concentration of DMSO (1, 2 and 4 %) in order to explore whether any change in DMSO concentration could influence the protein structure in a non-specific way. The obtained spectra are shown in Fig. 1.

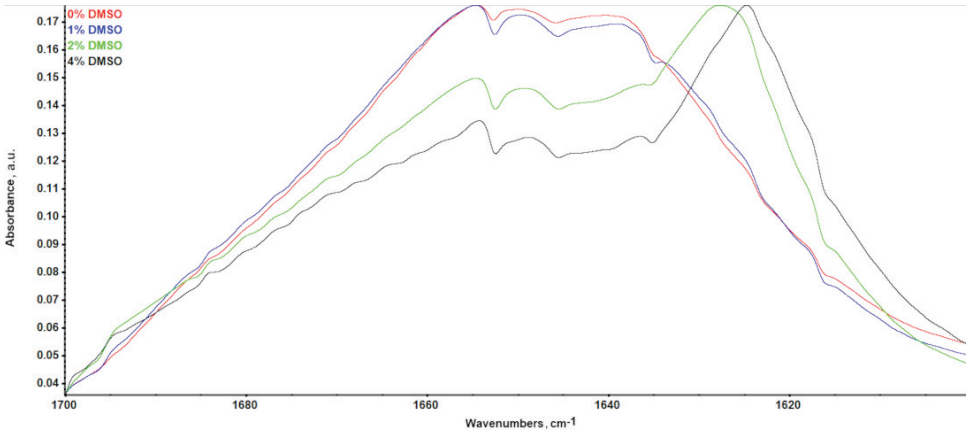


Fig. 1. FTIR spectra of OVA in the presence of 0–4% DMSO.

The most commonly used spectral region within an IR spectrum is the amide I region. The associated absorption bands, which are almost entirely the result of C=O stretching vibrations of the peptide bonds, make this region the most sensitive for the analysis of the secondary structure of proteins.[31] Bands attributed to the most abundant native-like secondary structures were identified according to previously published data: native-like β-sheets (1636 cm^{-1}),[22,32] random coils (1646 cm^{-1}),[22] α-helices (1653 cm^{-1}),[22] unordered structures (1662–1675 cm^{-1})[33] and β-turns (1683 cm^{-1}).[34] Additional bands, which were barely visible in the spectrum of the starting OVA sample, were also identified as side-chain peaks (1611 cm^{-1}), and low-frequency and high-frequency aggregation specific β-sheets (1625 and 1695 cm^{-1}, respectively).[17,35,36]

Qualitative examination of the spectra showed that 1 % DMSO appeared not to affect the OVA structure, while 2 and 4 % DMSO induced concentration-dependent conformational changes in OVA. Peaks attributed to native-like secondary structures are less prominent in favor of low frequency aggregation specific β-sheets (1622–1625 cm^{-1}). For quantitative determination of secondary structures, second order derivatives of the deconvoluted amide I peaks were created and the respective secondary structure percentages are given in Table II.

TABLE II. The effect of DMSO concentration on the content of secondary structure (contribution of polypeptide chain total structure, %) in OVA

OVA	Structure				
	β-Sheets	Unordered	α-Helix	β-Turn	Error
0 % DMSO	37.2	29.5	25.5	7.8	1.0
1 % DMSO	36.7	30.3	25.0	8.0	1.2
2 % DMSO	41.7	27.3	23.6	7.4	0.9
4 % DMSO	50.3	21.2	19.4	9.1	0.7
X-Ray structure	37.9	28.1	26.3	7.7	–

While the control of OVA and OVA treated with 1 % DMSO showed a content of secondary structures similar to that of the crystal structure of the protein, higher DMSO concentrations led to significant structural rearrangements. Specifically, the total β-sheet content was elevated by about 13 % at the expense of other secondary structures in 4 % DMSO, such as α-helices and unordered structures.

It was noted in several studies that DMSO has a potential influence on various protein properties at higher DMSO concentrations[37–40] by inducing changes in the binding and biochemical properties of proteins[41] through the indirect disruption of protein–solvent interactions, thus causing subsequent protein denaturation.[37,39,42] However, it has also been shown that DMSO may have such an effect on several protein systems at considerably lower concentrations (0.1–3 vol. % DMSO).[41] Despite the similarly noticeable trend of α-helix to β-sheet conversion in these studies,[43] based on our findings and research it is believe that, in the present *in vitro* system, DMSO concentrations of up to 1% are adequate, as they do not display such a denaturing influence on considerably lower protein concentrations.

*The effect of the complexes **3**–**5** on OVA secondary structures*

Even a simple inspection of the protein IR spectra (Fig. 2) implied that the binding of **3** and **4** led to only moderate alternations in the protein secondary structures. This conclusion was fortified by insignificant changes in the well-described content of protein secondary structures (Table III).

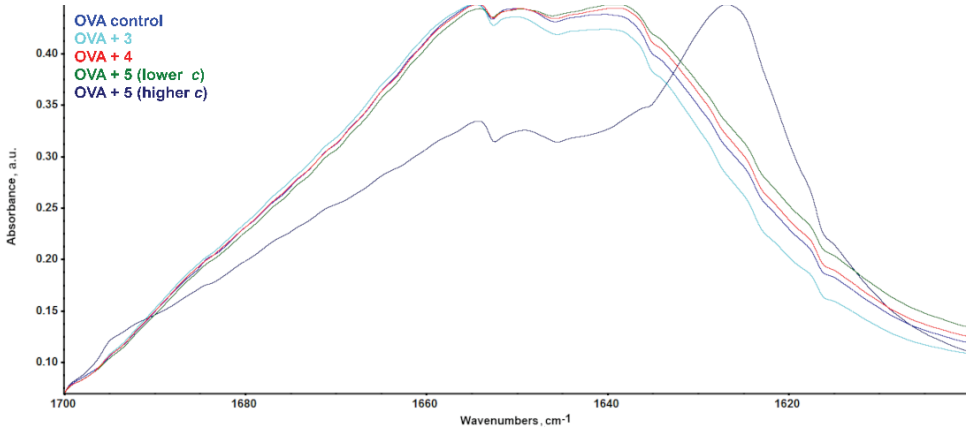


Fig. 2. FTIR spectra of OVA in the presence of the complexes **3**–**5** in 1 % DMSO.

On the other hand, the effect of complex **5** on OVA secondary structures was concentration-dependent. While lower concentration of complex **5** did not have any effect on the OVA structure, even though present in equimolar ratio, doubled

concentration of complex **5** led to complete disruption of native like secondary structures. In this higher concentration, complex **5** induced a shift toward *β*-fold at the expense of native-like secondary structures, such as *α*-helices and unordered chain (Table III). Elevated intensity of peaks at frequencies of 1625 and 1695 cm^{-1} suggested denaturation and aggregation of OVA (Fig. 2).[22,23]

TABLE III. The effect of the complexes **3**–**5** on the content of OVA secondary structures (Contribution of polypeptide chain total structure, %)

Sample	Structure				
	β-Sheets	Unordered	*α*-Helix	*β*-Turn	Error
OVA control	36.7	30.3	25.0	8.0	1.2
OVA + **3**	34.8	31.5	25.3	8.4	1.5
OVA + **4**	34.2	30.2	27.0	8.6	1.4
OVA + **5** (lower *c*)	37.5	29.5	24.1	8.9	0.9
OVA + **5** (higher *c*)	51.8	19.9	20.1	8.2	1.7

*The effect of complexes **3**–**5** on OVA water exposed hydrophobic surface*

To address such a wide range effects of complexes **3**–**5** on the native structure of OVA, from none to denaturation, the potential effect on their preferential binding to the protein surface was explored by monitoring the water exposed hydrophobic surface of the protein in the absence and the presence of the complexes. Due to the effect of the present palladium complexes on the secondary structure of OVA, conformational changes in the level of exposure of hydrophobic amino acid residues to water were noted. These changes were established *via* the binding of ANS to water-exposed hydrophobic surfaces and are depicted in Fig. 3.

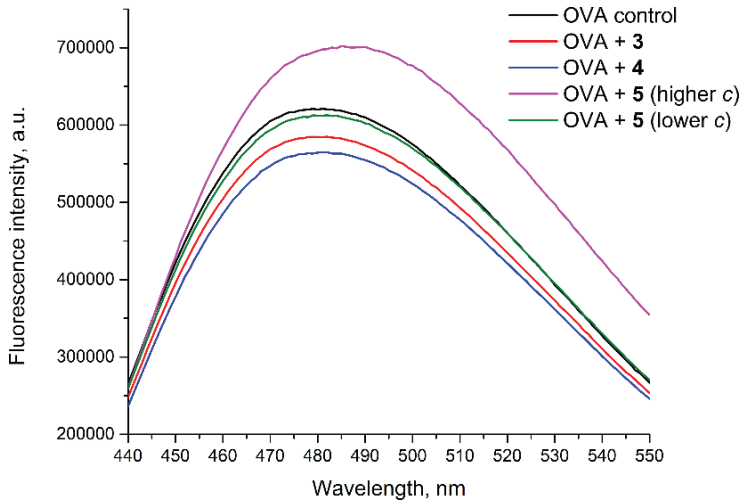


Fig. 3. The effect of complexes **3**–**5** on ANS fluorescence.

A small but notable decrease of water-exposed hydrophobic protein surfaces may be observed in the cases of the complexes **3** and **4**. The observed change in water exposed protein surface is in line with non-specific, reversible binding of ligands to a protein surface.[44,45] In this sense, complexes **3** and **4** bind to water exposed hydrophobic patches on the OVA surface by weak hydrophobic interactions, thus lowering ANS binding. Bearing in mind their insignificant effect on the presence of protein structural motives (Table III), their binding suggested non-specificity and reversibility, without the possibility of penetration into hydrophobic core of the protein and disruption of the protein native fold. Beside secondary structures changes, binding of the complex **5** to OVA had a concentration-dependent controversial effect on the water exposed hydrophobic surface protein, as well. In the case of the sample with the lower concentration of **5**, only negligible changes in water exposed hydrophobic OVA surface could be observed (Fig. 3). This result is in line with insignificant changes in the content of protein secondary structures and implies that complex **5** does not bind to water exposed hydrophobic patches on protein surface, thus its presence does not interfere with ANS binding. Doubled concentration of both complex **5** and protein, however, induced a considerable increase in the exposure of hydrophobic surfaces to water, as may be observed in the case of sample with the higher concentration of **5**, hinting towards notable conformational changes, including exposure of parts of the hydrophobic core of the protein.

It is well documented that non-specific ligand binding can influence the protein native fold. Induction of protein conformational changes was proven to be in line with induced changes in protein flexibility and the hydrophobic nature of ligands.[46] Since complex **5** is the most hydrophobic among the tested complexes, the obtained results fit well. However, the concentration-dependent effect of complex **5** on secondary structures changes and considerable increase in the exposure of hydrophobic surfaces of OVA to water may be related to a potential crosslinking that leads to OVA aggregation. Exposure of at least parts of hydrophobic core is well documented because that leads to changes in the secondary structures and the consequential aggregation of the denatured proteins.[22,23,31]

CONCLUSIONS

The calculated pharmacological profiles, medicinal chemistry principles and lead-likeness properties of investigated Pd(II) complexes with bidentate *N*-heteroaromatic chelators **1**–**5** indicated that the all complexes represent drug candidates since they possess important functional groups and bioavailability. However, the previously obtained results of the cytotoxic activity do not correlate with the hydrophobic character of the complexes. To gain more insight into structure–activity relationship, essential for design of novel potential drugs, the potential non-specific influence of the complexes on the OVA structure, as a

model system used to mimic cellular non-specific crowding environments with high protein concentrations were explored. FTIR study implied that the binding of **3** and **4** led to only moderate alternations in OVA secondary structures, without a possibility to penetrate into hydrophobic core of the protein and disruption of the protein native fold. On the contrary, the effect of complex **5** on OVA secondary structures was concentration-dependent. While a lower concentration of complex **5** did not have any effect on OVA structure, doubled the concentration of complex **5** led to complete disruption of the contents of native like secondary structures. Concentration-dependent effect of complex **5** on secondary structures changes and considerable increase in the exposure of OVA hydrophobic surfaces to water may be related to a potential crosslinking which leads to OVA aggregation. It has been demonstrated that a non-specific influence of a compound on a protein structure could be successfully assessed using OVA as a model system. In addition to ADME parameter calculation, this experimental test run at concentrations close to the anticipated or determined IC_{50} values could serve as experimental pre-evaluation of a potential drug candidate and could aid in the selection of a lead compound.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/11863, or from the corresponding author on request.

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ИЗВОД

ЕФЕКАТ НЕСПЕЦИФИЧНОГ ВЕЗИВАЊА КОМПЛЕКСА ПАЛАДИЈУМА(II) СА *N*-ХЕТЕРОАРОМАТИЧНИМ ХИДРАЗОНСКИМ ЛИГАНДИМА НА СТРУКТУРУ ПРОТЕИНА

НЕМАЊА Д. МИЈИН[1], ЈЕЛИЦА МИЛОШЕВИЋ[1], НЕНАД Р. ФИЛИПОВИЋ[2], ДРАГАНА МИТИЋ[3], КАТАРИНА АНЂЕЛКОВИЋ[1], НАТАЛИЈА Ђ. ПОЛОВИЋ[1] и ТАМАРА Р. ТОДОРОВИЋ[1]

[1]Универзитет у Београду – Хемијски факултет, Студентски трг 12–16, 11000 Београд, [2]Универзитет у Београду – Пољопривредни факултет, Немањина 6, 11000 Београд и [3]Иновациони центар Универзитета у Београду – Хемијског факултета, Студентски трг 12–16, 11000 Београд

Наша претходна истраживања су била усмерена ка испитивању цитотоксичног дејства пет Pd(II) комплекса са бидентатним *N*-хетероароматичним хелаторима (комплекси **1**–**5**) на палети од неколико туморских ћелијских линија. Међутим, резултати цитотоксичне активности нису били у корелацији са хидрофобним карактером комплекса. Како би се стекао бољи увид у однос између структуре и активности, који је од суштинског значаја за дизајн нових потенцијалних лекова, потребно је узети у обзир и друге факторе, као што су неспецифичне интеракције са ћелијским протеинима. У циљу проучавања потенцијалног неспецифичног утицаја комплекса на структуру протеина, овалбумин (OVA) је изабран као модел систем који опонаша ћелијско неспецифично окружење са високим концентрацијама протеина. Резултати инфрацрвене спектро-

скопија са Фуријe трансформацијом су указали на то да је везивање **3** и **4** довело до умерених промена у секундарним структурама протеина, без могућности продирања у хидрофобно језгро протеина и поремећаја нативног типа увијања протеина. Насупрот томе, ефекат комплекса **5** на секундарне структуре OVA био је зависан од концентрације. Комплекс **5** при мањој концентрацији није имао утицај на структуру OVA, али је при двоструко већој концентрацији довео до потпуног поремећаја садржаја нативних секундарних структура. Овакав концентрационо-зависан ефекат комплекса **5** на промене секундарних структура и значајно повећање изложености хидрофобних површина OVA протеина молекулима воде може бити повезан са потенцијалним умрежавањем које доводи до агрегације OVA.

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