Joksimović, N., et al. (2024). Anticancer potential of novel palladium(II) complexes with acyl pyruvates as ligands: DNA and BSA interactions and molecular docking study. *STED Journal, 6*(1), 1-14.

ANTICANCER POTENTIAL OF NOVEL PALLADIUM(II) COMPLEXES WITH ACYL PYRUVATES AS LIGANDS: DNA AND BSA INTERACTIONS AND MOLECULAR DOCKING STUDY

Nenad Joksimović[1*], Jelena Petronijević[1], Marina Serafinović[1], Nenad Janković[2], Dejan Baskic[3]

[1]University of Kragujevac, Faculty of Science, Department of Chemistry, Radoja Domanovića 12, 34000 Kragujevac, Serbia, nenad.joksimovic@pmf.kg.ac.rs
[2]University of Kragujevac, Institute for Information Technologies Kragujevac, Department of Sciences, Jovana Cvijića bb, 34000 Kragujevac, Serbia
[3]University of Kragujevac, Faculty of Medical Sciences, Centre for Molecular Medicine and Stem Cell Research, Svetozara Markovica 69, 34000 Kragujevac, Serbia

ORIGINAL SCIENTIFIC PAPER

ISSN 2637-2150
e-ISSN 2637-2614
UDC 616-006.04-084:543.2446:5
DOI 10.7251/STED2401001J
COBISS.RS-ID 140644097

Paper Submited: 28.04.2024.
Paper Accepted: 16.05.2024.
Paper Published: 30.05.2024.
http://stedj-univerzitetpim.com

Corresponding Author:
Nenad Joksimović, University of Kragujevac, Faculty of Science, Department of Chemistry, Radoja Domanovića 12, 34 000 Kragujevac, Serbia. nenad.joksimovic@pmf.kg.ac.rs;

ABSTRACT

Bearing in mind that some palladium complexes showed good antitumor potential while exhibiting less kidney toxicity comparing to cisplatin, to discover a new agents for chemotherapy with improved properties two novel palladium(II) complexes [Pd $(L)_2$] (**3A** and **3B**) with acyl pyruvates (*O,O* bidentate ligands) were synthesized and characterized by spectral (UV-Vis, IR, NMR, ESI-MS) and elemental analysis. The novel palladium(II) complexes were analyzed for their cytotoxic potential on human cancer cell lines (HeLa and MDA-MB 231) and normal fibroblasts (MRC-5). Results showed that complex **3A** displayed very good cytotoxic activity, while complex **3B** had moderate activity on the tested tumor cell lines. After 48h incubation with complex **3A**, his IC_{50} values were similar to the IC_{50} values of cisPt. Notably, all IC_{50} of complex **3A** on human fetal lung fibroblasts (MRC-5) were higher than 100 μM, indicating good selectivity. In addition, complex **3A** induced apoptotic type of cell death, cell cycle arrest in G0/G1 phase in both HeLa and MDA-MB 231 cell lines. In addition, we revealed that **3A** can be useful as adjuvants in cancer therapy by reducing the dose of cisplatin and in this manner its' side effects. For the investigations of interactions between novel palladium(II) complex **3A** and CT-DNA or bovine serum albumin (BSA) fluorometric titrations method was used. The obtained results implied that **3A** has great affinity to displace ethidium bromide (EB) from the EB-DNA complex through intercalation, suggesting strong competition with EB. Results in fluorescence titration of BSA with complex **3A** showed that the fluorescence quenching of BSA happens because of the formation of the **3A**-BSA complex. Obtained K_a value is in the optimal range signifying that appropriate amount of **3A** can be transported and distributed through the cells. In order to better understand the binding of newly synthesized complex **3A** to BSA or DNA, molecular docking study was further performed.

Keywords: Palladium(II) complexes, acyl pyruvates, anticancer activity, protein binding study, DNA interactions, molecular docking.

INTRODUCTION

Since finding the cisplatin metal-based compounds have gained great importance in medicinal science (Rosenberg, Van Camp, & Krigas, 1965; Desoize, & Madoulet, 2002). Although platinum-based drugs have been used extensively as anticancer drugs they are showing many shortcomings such as acute drug resistance on some tumors, nephrotoxicity, and neurotoxicity (Ott, & Gust, 2007; Hartmann, & Lipp, 2003; Sastry, & Kellie, 2005). Therefore, the examination for novel anticancer agents with enhanced properties has been focused on the discovery of some other metal-based drugs. The similar coordination behavior of platinum(II) and palladium(II) was one of the greatest interests for the development of the antitumor palladium based drugs (González, et al., 1997; Ali, et al., 2002). Investigations showed that palladium complexes hold better, or at least comparable antitumor activity compared to cisplatin (Lee, et al., 2015; Mazumder, Beale, Chan, Yu, & Huq, 2012). It has been also reported that numerous Pd complexes displayed less kidney toxicity comparing to cisplatin and other Pt complexes (Trevisan, et al., 2002). In addition, palladium complexes with organic ligands showed very good cytotoxic activity on various cancer cell lines compared to cisplatin (Chiririwa, Moss, Hendricks, Meijboom, & Muller, 2013; Krogul, et al., 2012; Abu-Surrah, et al., 2010; Ajloo, et al., 2015).

Organic ligands such as acyl pyruvates have been broadly used as starting substrates in medicinal chemistry to obtain novel potential drugs (Guo, et al., 2016; Shehab, & El-Bassyouni, 2018; Stepanova, Dmitriev, & Maslivets, 2019; Kulakov, et al., 2017). This structural fragment can be found in many biologically active compounds and natural products. Their crucial feature in acyl pyruvate molecule is the presence of keto-enol tautomerism, where the equilibrium of the ketone and the enol forms in is strongly shifted towards the enol form due to the formation of the distinct resonance structure as a six-membered ring (Zalesov, Kataev, Pulina, & Kovylyaeva, 2002). The most important, the capacity to form stable complexes with most metals is a direct consequence of the occurrence of such compounds in the enol form. Also, the combination of highly electrophilic character and oxygen-rich coordination sites combined with the capacity to form stable complexes with most metals is making acyl pyruvates excellent candidates for synthesis of novel metal-based compounds (Prokop, Gelbrich, Sieler, Richter, & Beyer, 2001). Therefore, new metal-based agents such as palladium complexes appear to be promising for the development of novel and improved chemotherapeutic drugs.

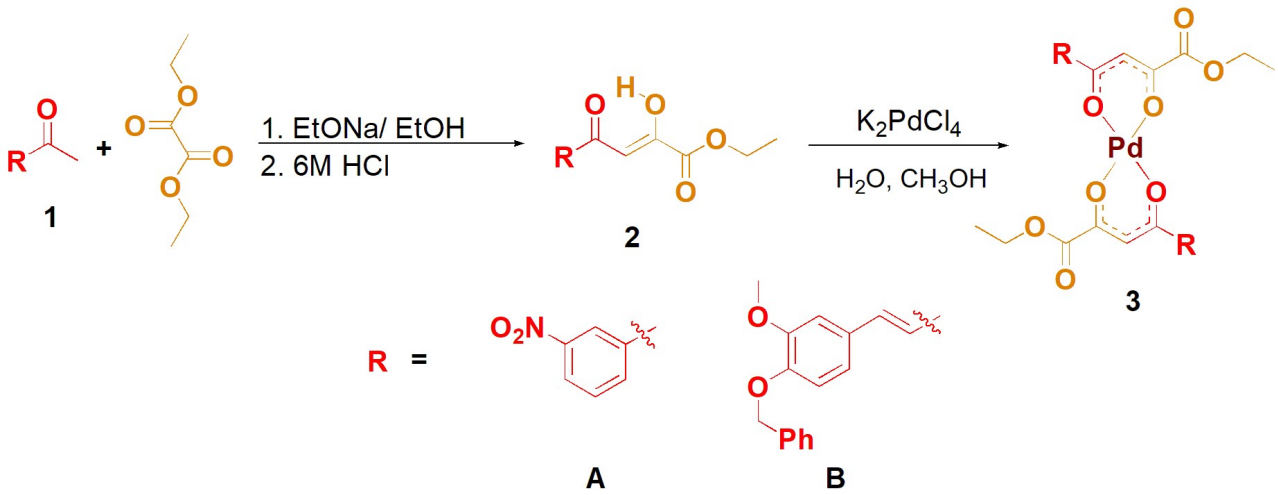
It is very important to study the binding modes with DNA molecule and potential drug in order to examine the probable mechanism of antitumor activity. DNA is major target for the multiplicity of drugs and small molecules (Lazić, Arsenijević, Puchta, Bugarčić, & Rilak, 2016; Li, et al., 2015; Sánchez, Penas, Vásquez, & Mascareñas, 2014). Conventionally, a small molecular size compound can interact with DNA through covalent or noncovalent interaction (Lauria, et al., 2014; Bork, et al., 2014; Abd Karim, et al., 2014). Investigations of this mode of interactions are very useful in the discovery of a good efficiency drug, their mechanism of action or to examine its toxic properties (Zhang, & Liu, 2011; Shen, Shao, Xu, Li, & Pan, 2011; Hartwig, 2010). For the molecules that showed good antitumor property, it is imperative to investigate the mode of binding to transport proteins, to better verify the potential use of a drug in clinical practice in future. The serum albumins exhibit a significant role in the transport and deposition of the biologically significant molecules or drugs in the circulatory system (Kandagal, et al., 2006). Furthermore, it is very important for a small molecule or a potential drug to bind reversibly to serum albumins (Bertucci, & Domenici, 2002).

RESULTS AND DISCUSSION

Synthesis and characterization

The Claisen condensation reaction between diethyl oxalate and different methyl ketones 1 under basic conditions was used to obtain *O,O*-bidentate ligands **2A** and **2B** (**Scheme 1**). The synthesis of the ligands was performed according to the early published procedure (Andrzejak, et al., 2010). The reaction procedure that we published earlier, between $K_2[PdCl_4]$ and acyl pyruvates ligands in 1 : 1 water/methanol solution, was used for obtaining two novel palladium(II) complexes **3A** and **3B** (**Scheme 1**) (Joksimovic, et al., 2020). In a short reaction time (~10 min) at

room temperature this salt yielded complexes **3A** and **3B** with corresponding ligands. The obtained yields for complexes **3A** and **3B** were 86% and 83%, respectively.



Scheme 1. Synthesis of acyl pyruvate ligands **2A** and **2B** and their palladium(II) complexes **3A** and **3B**.

Both new complexes were characterized *via* spectral (UV-Vis, IR, NMR, ESI-MS) and elemental analysis. As in the previously published palladium complexes with similar ligands (Joksimovic, et al., 2020), IR spectra of complexes **3A** and **3B** showed bands approximately at 1570 and 1510 cm^{-1} that are assigned to ν(C=C) coupled with ν(C=O) and ν(C=O) coupled with ν(C=C) respectively. The IR spectra of all complexes compared to corresponding spectra of ligands showed that ν(γ-C=O) is approximately 15 cm^{-1} negative shifted. The absence of intense bands near 1270 cm^{-1} in the spectra of complexes, that appears in the spectrum of corresponding ligands as the consequence of bending O–H vibrations in the plane (dos Santos, & Cavalheiroll, 2014; Zolezzi, Decinti, & Spodine, 1999) is caused by the coordination of the ligand to palladium(II) which is followed by the deprotonation of the O–H group.

Anticancer potential

The antitumor potential and selectivity of two novel palladium(II) complexes **3A** and **3B** was investigated on two tumor cell lines (HeLa and MDA-MB 231) and one normal MRC-5 cell after 24 and 48h treatment with a range of concentrations. Based on the achieved results, IC_{50} values were calculated and presented in **Table 1**.

As shown in **Table 1**, complex **3A** showed very good cytotoxic activity, while complex **3B** showed moderate activity on the tested tumor cell lines. After 48h incubation with complex **3A**, his IC_{50} values were similar to the IC_{50} values of cisPt. Tested cells, MDA-MB 231 and HeLa, showed diverse sensitivity on tested complexes. Obtained IC_{50} values were lower in HeLa cells, thus indicating a higher sensitivity of these cells compared to MDA-MB 231.

Based on obtained results, we carefully chose complex **3A** for further investigation. To determine its selectivity, the cytotoxic effects of complex **3A** were examined on human fetal lung fibroblasts. MRC-5 cells were treated with the same concentrations of complex **3A** for 24 and 48 hours. The determined IC_{50} values are given in **Table 1**. Notably, all IC_{50} values were higher than 100 μM, indicating good selectivity of complex **3A**. This is crucial since good selectivity is one of the main factors for a new antitumor drug candidate.

Table 1. In vitro inhibitory activity (IC_{50}) (μM) of novel palladium(II) complexes **3A** and **3B** on human cancer cell lines (HeLa and MDA-MB 231) and human fibroblasts (MRC-5) after 24 and 48 h of treatment. Cisplatin was used as a positive control. (n.d. - not determined).

	MDA MB 231		HeLa		MRC-5	
	24h	**48h**	**24h**	**48h**	**24h**	**48h**
3A	158.6±45.4	95.8±52.5	58.2±7.0	5.9±3.2	>100	>100
3B	183.0 ± 43.4	94.5 ± 20.4	99.7 ± 11.0	43.8 ± 0.3	n.d.	n.d.
cisPt	101.2±6.7	53.8±2.8	25.7 ± 2.9	8.7 ± 1.3	183.7 ± 16.2	43.1 ± 8.8

Palladium(II) complex 3A induced apoptosis and cell cycle arrest in HeLa and MDA-MB 231 cells

The obtained results showed, when annexin V-FITC/7-AAD staining of cells were treated with IC_{50} concentration of **3A,** that complex induced apoptotic type of cell death in both HeLa and MDA-MB 231 cell lines (**Figure 1 (A)**). The investigations were performed by the procedure we previously published (Joksimovic, et al., 2020). Treated cells were primarily early or late apoptotic, and a slight percentage of cells were necrotic. Necrosis is type of cell death that induce inflammation and injury of surrounding tissue, opposite to apoptosis that is restricted to the individual cells. Therefore, induction of apoptosis in cancer cells and their elimination without induction of undesirable inflammatory response is the aim of anticancer therapy.

In addition, it is important to emphasize that the cell cycle progression is well-controlled process controlled by conserved mechanisms. Regulatory pathways monitoring proper progression through the cell cycle are referred to as cell cycle checkpoints. To maintain tissue homeostasis, cell proliferation and cell death are equally important. Based on those facts it can be concluded that apoptosis and cell cycle share certain regulatory molecules. In case of harm or cellular stress these regulatory molecules can arrest cell cycle until the damage is repaired or stress signals eliminated, while in contrary apoptotic death program is activated. Thus, apoptosis can be activated by the agents that induce cell cycle arrest. The investigations of cell cycle arrest were performed by the procedure we previously published (Joksimovic, et al., 2020). Obtained results in cell cycle analysis displayed that complex **3A** induces cell cycle arrest on both tested cell lines, and its influence on cell cycle is presented on **Figure 1 (B)**. As can be noticed, in both HeLa and MDA-MB 231cells, complex **3A** induced cell cycle arrest in G0/G1 phase. These results suggest that the cell cycle arrest is probably linked with apoptosis induction.

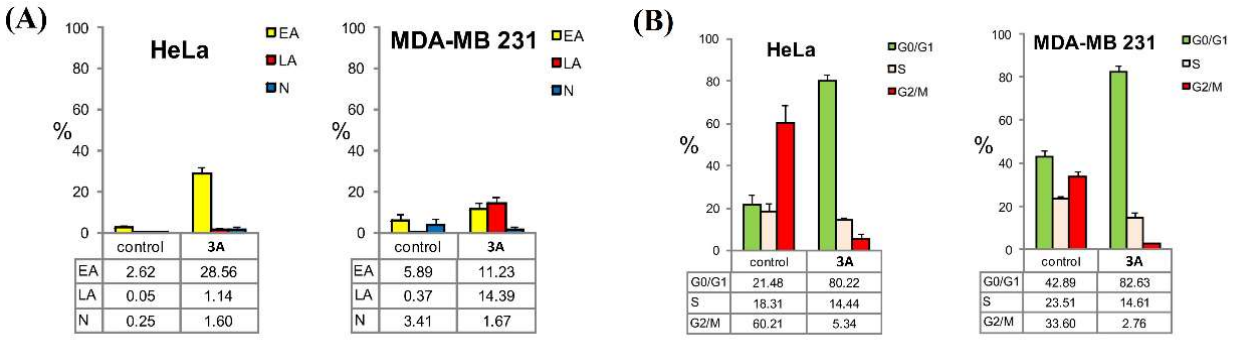


Figure 1. (A) Flow cytometric analysis of Annexin V-FITC/7-AAD staining. Graphs showing percent of early apoptotic (EA), late apoptotic (LA) and necrotic cells (N) in untreated (control) and treated HeLa and MDA-MB 231 cells with complex **3A**. Results are presented as an average of two independent experiments; **(B)** Cell cycle analysis**.** Graphs showing cell cycle distribution in untreated (control) and treated HeLa and MDA-MB 231 cells with complex **3A**. Results are presented as an average of three independent experiments.

The interactions of cisplatin and palladium(II) complex 3A

The cytotoxic effect of cisplatin and complex **3A** separately and different concentrations of **3A** on HeLa cells (**Figure 2**) were used for determining combination index (CI). The investigations were performed by the procedure we previously published (Joksimovic, et al., 2020). F_a indicates fraction of cell viability affected (**Figure 2**). F_a=0.05-0.97 corresponds to 5-97% toxicity. The results indicated that combinations of the lowest concentrations of both cisplatin and complex **3A** were most effective, considering that CI value showed strong (the lowest concentrations of **3A**) to moderate synergism (the lowest concentrations of cisplatin and higher concentrations of **3A**). These results imply that **3A** can be useful as adjuvants in cancer therapy by reducing the dose of cisplatin and in this manner its' side effects.

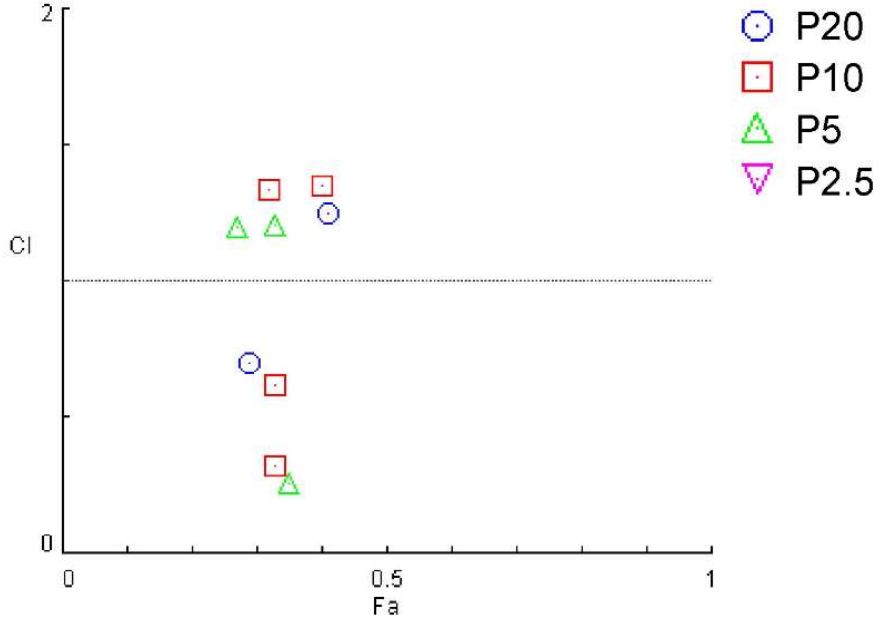


Figure 2. Combination Index Plots presenting the combined effects of palladium(II) complex **3A** and cisplatin on HeLa cells. Various concentrations of **3A** were combined with cisplatin concentrations: 20μM (Pt20), 10μM (Pt10), 5μM (Pt5) and 2,5μM (Pt2,5).

Interactions of palladium(II) complex 3A with DNA

Ethidium bromide (EB) is a chemical with a favorably planar structure. Because of its structural characteristics, there is an opportunity of intercalating into molecule of DNA. These interactions lead to an increase in fluorescence intensity. Fluorescence emission comes as a result of effective intercalation between the base pairs of DNA and EB. Emission of EB-DNA complex that is formed can be quenched by adding some potential drug. If a drug or small molecule intercalates into DNA molecule the binding positions of DNA accessible for EB decreases, resulting in quenching of the fluorescence of the EB–DNA system (Bertucci, & Domenici, 2002). Therefore, we tested the competitive binding mode of **3A** with EB-DNA complex using the procedure described earlier (Joksimovic, et al., 2020). Fluorescence quenching spectra of titration EB–DNA with **3A** (**Figure 3**), were recorded in the range of 550–750 nm. These spectra at 610 nm pointed a decreasing trend with the increasing concentration of **3A**, implying that EB was replaced by the compounds **3A**.

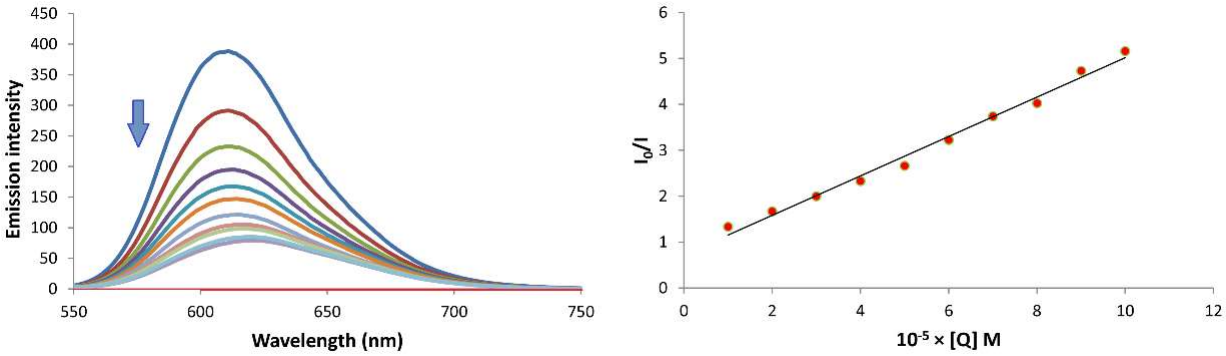


Figure 3. Left: emission spectra of EB-DNA in the absence (blue lines) and presence of compounds 3A. The red lines denote solutions: buffer + quencher. [EB] = 100 μM, [DNA] = 100 μM; [**3A**] = 0–100 μM; pH = 7.4; λ_{ex} = 500 nm. Right: plots of I_0/I versus [Q].

A quenching constant (K_q) was determined by examining the dependency of I_0/I on [Q] (**Figure 3**) using Sterne-Volmer equation **(1)** (Lakowicz, & Weber, 1973). These investigations were performed in order to better understand the binding strength of compound **3A** to CT-DNA. The quenching constants for **3A** that are given in **Table 2** specify that **3A** has the competence to displace EB from the EB–DNA complex by binding to DNA through intercalation (Petronijević, et al., 2018).

$$I_0/I = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \qquad \textbf{(1)}$$

Table 2 The bimolecular quenching rate constant (k_q), Stern–Volmer constant (K_{sv}), and correlation coefficient (R) for compound **3A**.

Compound	k_q [M^{-1} s^{-1}]	K_{sv} [M^{-1}]	R
3A	$(4.3 \pm 0.2) \times 10^{13}$	$(4.3 \pm 0.2) \times 10^{5}$	0.994

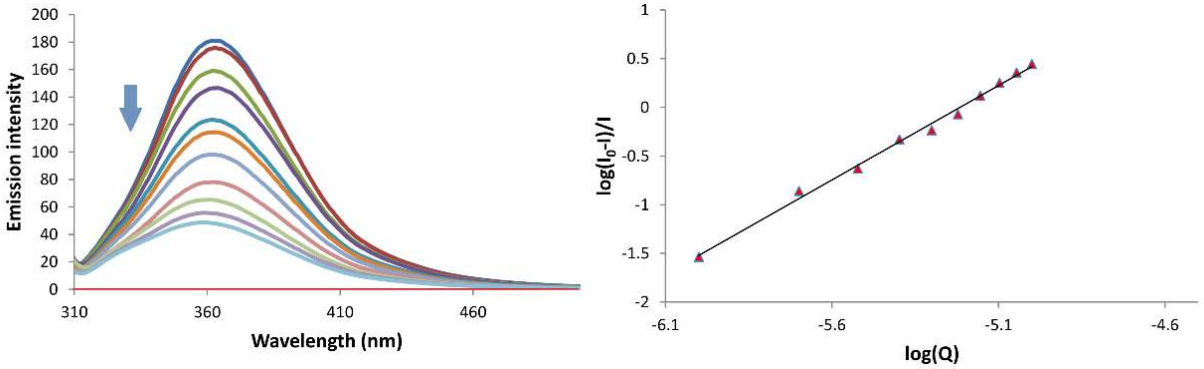
Interactions of palladium(II) complex 3A with bovine serum albumin

Bovine serum albumin (BSA) which has very similar structural properties to human serum albumin (HSA) is frequently used in studies of the interactions with drugs or small molecules and proteins, doe to it is responsible for the transport of drugs in biological systems. Since the efficiency of potential drugs depends on their capacity to bind to transport proteins, we investigated the binding mode of **3A** with BSA using the procedure we described previously (Joksimovic, et al., 2020). The fluorescence spectroscopy titration method was used to investigate the binding of different compounds to serum albumins, giving binding results mechanism, binding method, and binding sites in the protein. The fluorescence emission spectra were obtained in the wavelength range of 310–500 nm (**Figure 4**).

The binding constant (K_a) and a number of binding sites for each BSA molecule (n) were determined using the equation **(2)** (Strekowski, & Wilson, 2007):

$$\log(I_0\text{-}I/I) = \log K_a + n \log[Q] \qquad \textbf{(2)}$$

I_0 is the emission intensity in the absence of **3A**, I is the emission intensity for the complexes **3A**–BSA. [Q] is the concentration of **3A**. The K_a values and n are determined by investigating the dependence of $\log[(I_0-I)/I]$ versus log[Q] (**Figure 4**). The obtained K_a values, given in **Table 3,** indicate that **3A** has a strong binding affinity to BSA. The number of binding site for **3A** (n≈2, **Table 3**) shows that **3A** binds to BSA in the molar ratio 2 : 1



.

Figure 4. Left: emission spectra of BSA in the absence (blue lines) and presence of compound **3A**. The red lines denote solutions: buffer + quencher. [BSA] = 10.0 μM; [**3A**] = 0–10 μM; pH = 7.4; λ_{ex} = 280 nm. Right: the dependence of log(I_0-I/I) on log[Q].

Table 3. Binding parameters (K_a and n) and the correlation coefficient (R) for interactions of **3A** with BSA.

Compound	K_a [M^{-1}]	n	R
3A	(2.4 ± 0.2) × 10^5	1.94	0.996

Docking study of palladium(II) complex 3A with DNA and BSA

Simulations of binding of complex **3A** to DNA or BSA were performed using molecular docking tools in order to further confirm results obtained from study of interactions with DNA and BSA and to examine possible modes of binding. Results of the docking experiments are given in Table **4**. In case of complex **3A** with BSA the obtained value of ΔG was -7.98 kcal·mol^{-1}, while with DNA was is -6.93 kcal·mol^{-1}. Thus, represented data (**Table 4**) predicts strong binding to DNA and **3A** complex system. The best docking poses are presented in **Figure 5** (between **3A** and BSA) and **Figure 6** (between **3A** and DNA).

As far as interaction is concerned, in case of complex **3A** with BSA protein primary comes from van der Waals force and conventional hydrogen bonds (**Figure 7**). In the case of the same complex with DNA molecule contributions are also given by conventional hydrogen bonds and by van der Waals force (**Figure 8**).

Table 4. Overview of molecular docking calculations for tested complex **3A** with BSA and DNA.

Label	Complex	ΔG[a]
3A	**3A** with BSA	-7.98
3A*	**3A** with DNA	-6.93

[a]kcal·mol^{-1}.

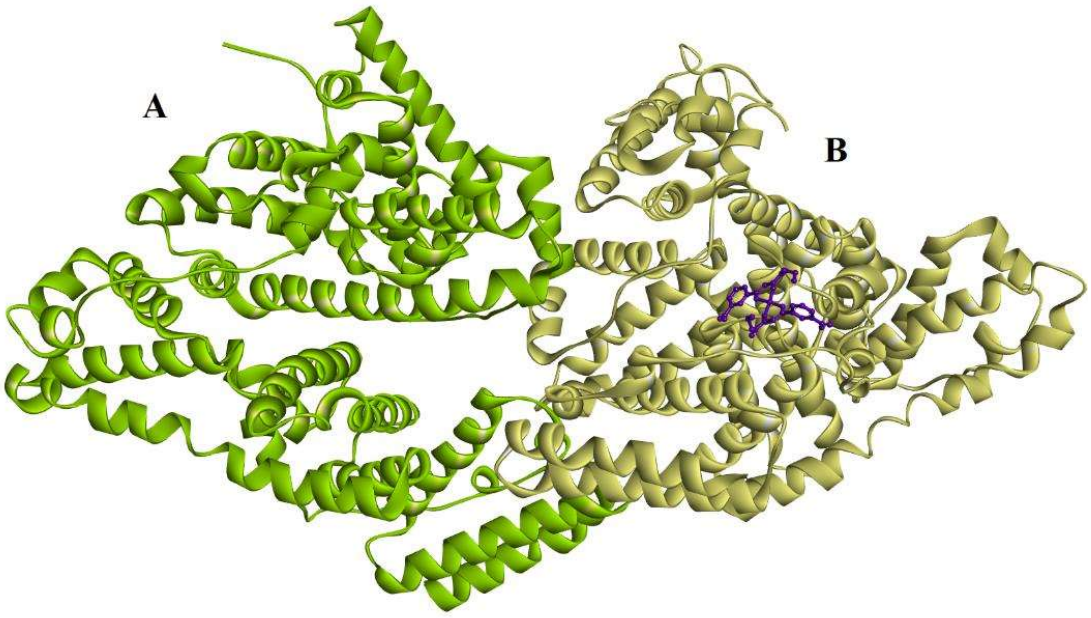


Figure 5. Best docking pose of complex **3A** (purple colored) with BSA protein (with domains A (green colored) and B (yellow colored).

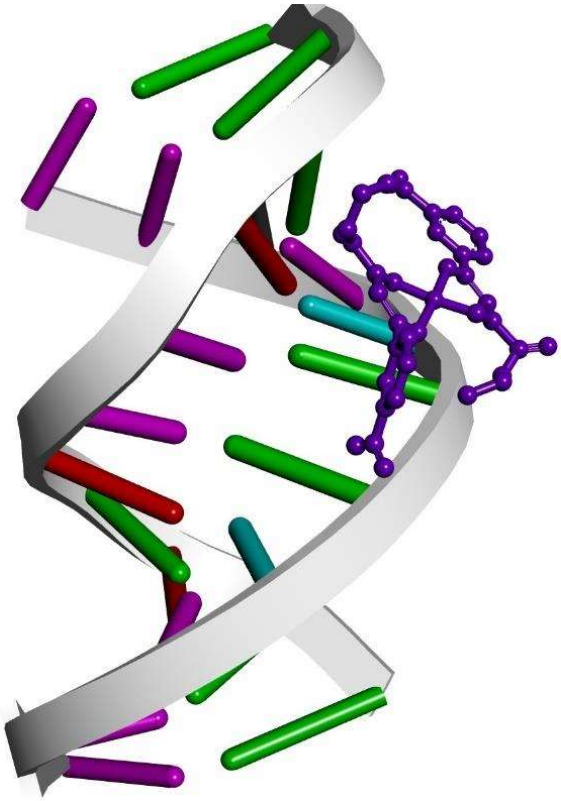


Figure 6. Best docking pose of complex **3A** (purple colored) with DNA molecule

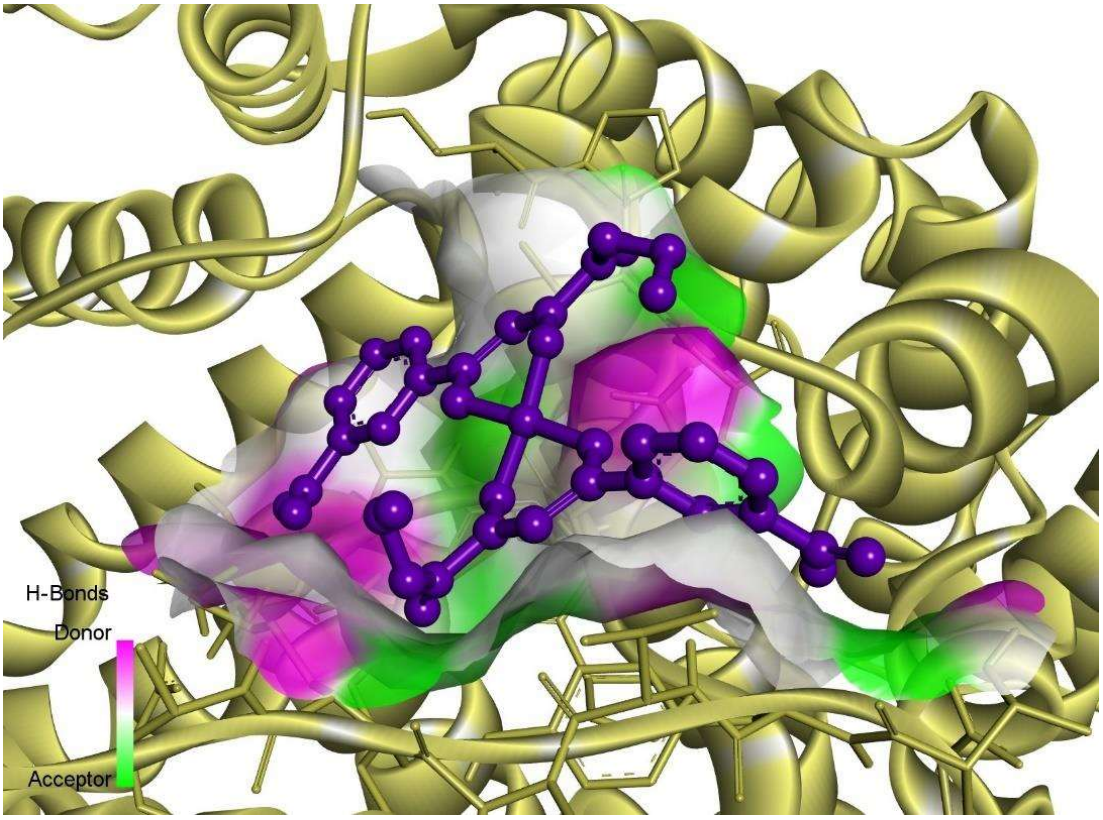


Figure 7. Interaction contributions for the complex **3A** with BSA protein.

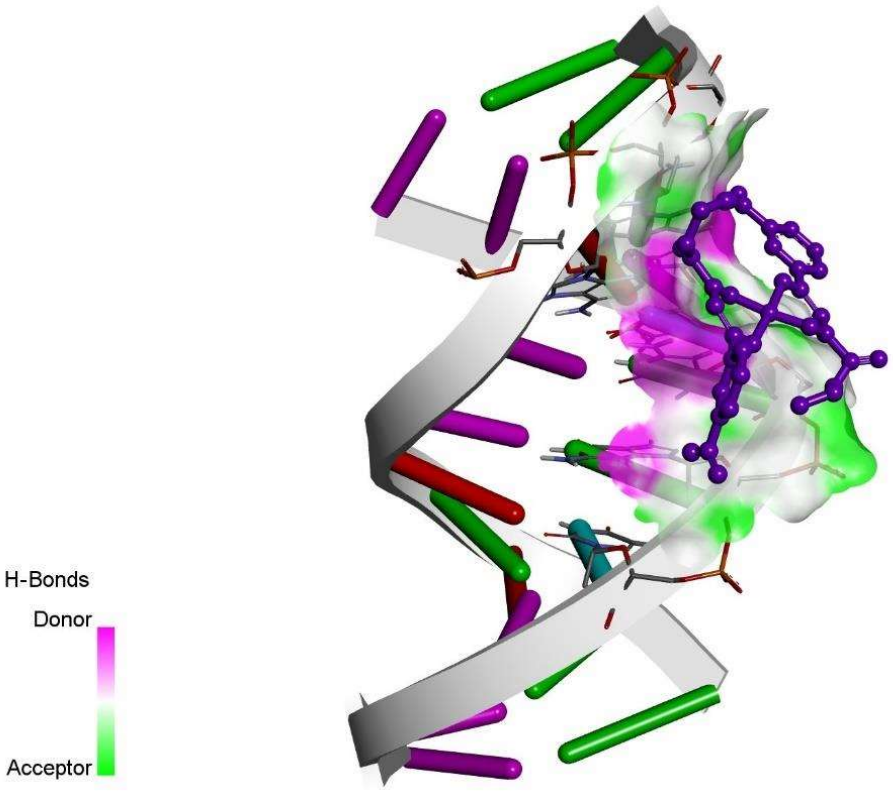


Figure 8. Interaction contributions for the complex **3A** with DNA molecule.

EXPERIMENTAL

All solvents and substrates were purchased from Sigma. Phosphate buffered saline (PBS) tablets were purchased from Fisher BioReagents. In 10 mM PBS buffer at pH = 7.4 a fresh solution of CT-DNA and EB in doubly distilled water was prepared. The DNA solution gave a ratio of UV absorbance at 260 nm and 280 nm (A_{260}/A_{280}) of ca. 1.8–1.9, indicating that the DNA was sufficiently free of proteins. CT-DNA concentration was measured by the UV absorbance at 260 nm (ε = 6600 M^{-1} cm^{-1}) (Meadows, Liu, Sou, Hudson, & McMillin, 1993).

Synthesis of palladium(II) complexes 3A and 3B and corresponding ligands 2A and 2B

The synthesis of the ligands was performed according to the early published procedure (Andrzejak, et al., 2010). The ligands were characterized using NMR spectroscopy. The synthesis of the novel palladium(II) complexes **3A** and **3B** was performed according to the early published procedure (Joksimovic, et al., 2020). Both new complexes were characterized *via* spectral [UV-Vis, IR, NMR, ESI-MS] and elemental analysis.

2A (ethyl 2-hydroxy-4-(3'-nitrophenyl)-4-oxo-2-butenoate). ^{1}H NMR ($CDCl_3$, 200 MHz) δ (ppm) : 1.40-1.48 (t, *J* = 7.1 Hz, 3H, CH_3), 4.38-4.49 (q, *J* = 7.1 Hz, 2H, CH_2), 7.13 (s, 1H, CH=CO), 7.75-7.79 (m, 1H, CH_{Ar}), 8.31-8.36 (m, 1H, CH_{Ar}), 8.44-8.50 (m, 1H, CH_{Ar}), 8.81-8.83 (m, 1H, CH_{Ar}) and 14.66 (br. s., 1H, OH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 14.0, 62.9, 97.8, 122.6, 127.7, 130.1, 133.2, 136.4, 148.6, 161.6, 171.3 and 187.5.

2B (ethyl 2-hydroxy-4-[(*E*)-2-(3'-metoxy-4'-benzyloxyphenylvinyl)]-4-oxo-2-butenoate). ^{1}H NMR (DMSO-d_6, 200 MHz) δ (ppm): δ 7.86 – 6.98 (m, 11H, CH_{Ar} + CH=), 6.58 (d, *J* = 7.7 Hz, 1H, CH=), 5.15 (s, 2H, CH_2), 4.28 (q, *J* = 7.1 Hz, 2H, CH_2), 3.83 (s, 3H, CH_3), 1.29 (t, *J* = 7.1 Hz, 3H, CH_3).^{13}C NMR (DMSO-d_6, 50 MHz) δ (ppm): 14.0, 55.9, 62.2, 70.1, 111.1, 113.4, 121.6, 128.0, 128.2, 128.6, 136.8, 149.6, 150.8, 161.8 and 185.9.

3A [Pd(**2A**)$_2$]. Yellow crystal; yield: 86%; mp = 287 °C; UV-Vis (PBS, λ_{max}/nm, (log(ε/M^{-1} cm^{-1})): 339(4.11); IR (KBr, cm−1): ν 3451, 3084, 2988, 1725, 1587, 1556, 1520, 1441, 1350, 1298, 1078, 1017, 711; ^{1}H NMR (DMSO-d_6, 400 MHz) δ : δ 1.16-1.36 (m, 6H, 2×CH_3), 4.25-4.36 (m, 4H, 2×CH_2), 7.71-7.90 (m, 2H, 2×CH=), 8.14 (s, 2H, 2×CH_{Ar}), 8.31-8.70 (m, 6H, 2×CH_{Ar}) ppm; ESI-MS (m/z): [M+] = 634; Calcd for $C_{24}H_{20}N_2O_{12}Pd$ (%): C 45.41, H 3.18, N 4.41; found: C 45.50, H 3.20, N 4.39.

3B [Pd(**2B**)$_2$]. Orange powder; yield: 83%; mp = 205 °C; UV-Vis (PBS, λ_{max}/nm, (log(ε/M^{-1} cm^{-1})): 310(4.49); IR (KBr, cm−1): ν 3507, 2933, 1725, 1558, 1508, 1417, 1260, 1164, 1137, 1019, 843, 747; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.15-1.31 (m, 6H, 2×CH_3), 3.74-3.85 (m, 6H, 2×OCH_3), 4.19-4.32 (m, 4H, 2×CH_2), 5.11-5.16 (m, 4H, 2×OCH_2), 7.03-7.76 (m, 22H, CH= + CH_{Ar}) ppm; ESI-MS (m/z): [M+] = 870; Calcd for $C_{44}H_{42}O_{12}Pd$ (%): C 60.80, H 4.87; found: C 60.88, H 4.88.

Cell lines

Human cervix adenocarcinoma cells (HeLa), breast tumor cell line (MDA-MB 231) and human fetal lung fibroblasts (MRC-5) were obtained from the American Type Culture Collection (ATCC). Cells were maintained in DMEM (Dulbecco's modified Eagle's medium) medium suppemented with 10% heath-inactivated FBS (Fetal Bovine Serum), L-glutamine (2mM), non-essencial amino acids (0,1mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL)(Sigma, Germany). Cells were cultivated at 37°C in an 5% CO_2 atmosphere, and absolute humidity.

MTT assay

Cytotoxicity of palladium(II) complexes **3A** and **3B** against human cell lines was performed by tetrazolium colorimetric MTT assay (Sigma, Germany). The assay is based on the transformation of the tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to formazan by mitochondrial dehydrogenase in viable cells. Briefly, cells were harvested and plated in 96-well microtiter plates (Thermo Fisher Scientific, United States) at an optimal seeding density of 5x10^3 cells per well and incubated overnight for adherence. After overnight incubation, the medium was replaced with medium containing a range concentration of tested compounds (100, 30, 10, 3, 1 and 0.3µM) or with fresh medium as a control. Cisplatin was used as reference compound.

Cells were incubated at 37°C in a 5% CO_2 atmosphere and absolute humidity for 24 and 48 hours. After incubation, media was removed and 100μL of MTT (0.5 mg/mL PBS) was added to each well. After 4h incubation under culture conditions MTT solution was removed and 150μL of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 550 nm with a multiplate reader (Zenith 3100, Anthos Labtec Instruments GmbH, Austria). Experiments were performed in triplicates and repeated in three independent series. Cytotoxicity was calculated according to the formula: $[(A_{CONTROL}-A_{TEST})/A_{CONTROL}]x100$. The IC_{50} value (50% inhibitory concentration) was calculated using ED50plus v1.0 software.

Docking studies

The X-ray crystal structureS of B-DNA (PDB ID: 1BNA) and BSA (PDB ID: 4F5S) were acquired from the Protein Data Bank (PDB) (RCSB PDB: Homepage). Docking processes were carried out using Autodock 4.2 (Frisch, et al., 2013) software equipped with the graphical user interface (GUI) Auto-DockTools (ADT 1.5.6rc3) (Morris, et al., 2009). Then the polar hydrogen atoms were added, and ADT was used to remove crystal water, Geisteiger charges were added to each atom, and merge non-polar hydrogen atoms to the DNA structure. The structures were then saved in PDBQT file format, for further studies in ADT. For the visualization of the docking results, a free version of the Discovery Studio Visualizer 3.5.0 Accelrys Software Inc. (Ju, Ding, Sun, & Chen, 2015) software has been used (Sanner, 1999).

CONCLUSION

In order to investigate the anticancer potential, two novel palladium(II) complexes with acyl pyruvates as ligand were tested on MDA-MB-231 and HeLa cancer cell lines. Complex **3A** showed excellent cytotoxic activity, while complex **3B** showed moderate activity on the tested tumor cell lines. In addition, IC_{50} values for complex **3A** were similar to the IC_{50} values of cisplatin. To determine the selectivity, the cytotoxic effects of complex **3A** were examined on human fetal lung fibroblasts (MRC-5), showing that all IC_{50} values were higher than 100 μM, indicating good selectivity which is crucial since good selectivity is one of the main factors for a new antitumor drug candidate. Investigations of mechanism of anticancer activity reviled that complex **3A** induced apoptotic type of cell death in both HeLa and MDA-MB 231 cell lines. The results of cell cycle analysis showed that **3A** induced cell cycle arrest in G0/G1 phase. In addition, the investigations of interactions with cisplatin revealed that **3A** can be useful as adjuvants in cancer therapy by reducing the dose of cisplatin and in this manner its' side effects. Further investigations of interactions with biomacromolecules such as DNA and a transport protein were performed. Calculated K_{sv} values for the interactions of complex **3A** with DNA implied that our compound interacts with the DNA molecule through intercalation. By examining the binding mode of complex **3A** to a transport protein, we calculated the binding constant K_a, indicating that is able to be transported and distributed to cells in an adequate manner. A molecular docking study was performed to investigate in more detail the mode of binding to DNA and serum albumin molecules. Finally, all results indicate the great potential for future application of novel palladium(II) complex **3A** in clinical practice in the future.

ACKNOWLEDGMENTS

The authors thank the Ministry of Education, Science and Technological Development of the Republic of Serbia (Agreements No. 451-03-66/2024-03/ 200122 and 451-03-9/2021-14/200378).

DECLARATION OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Abd Karim, N.H., Mendoza, O., Shivalingam, A., Thompson, A.J., Ghosh, S., Kuimova, M.K., & Vilar, R. (2014). Salphen metal complexes as tunable G-quadruplex binders and optical probes. *RSC advances*, *4*(7), 3355-3363.

Abu-Surrah, A.S., Safieh, K.A.A., Ahmad, I.M., Abdalla, M.Y., Ayoub, M.T.,

Qaroush, A.K., & Abu-Mahtheieh, A.M. (2010). New palladium (II) complexes bearing pyrazole-based Schiff base ligands: Synthesis, characterization and cytotoxicity. *European journal of medicinal chemistry*, *45*(2), 471-475.

Ajloo, D., Moghadam, E.M., Ghadimi, K., Ghadamgahi, M., Saboury, A.A., Divsalar, A., Sheikh Mohammadi, M., & Yousefi, K. (2015). Synthesis, characterization, spectroscopy, cytotoxic activity and molecular dynamic study on the interaction of three palladium complexes of phenanthroline and glycine derivatives with calf thymus DNA. *Inorganica Chimica Acta*, *430*, 144-160.

Ali, M.A., Mirza, A.H., Butcher, R.J., Tarafder, M.T.H., Keat, T.B., & Ali, A.M. (2002). Biological activity of palladium (II) and platinum (II) complexes of the acetone Schiff bases of S-methyl-and S-benzyldithiocarbazate and the X-ray crystal structure of the [Pd (asme) 2](asme= anionic form of the acetone Schiff base of S-methyldithiocarbazate) complex. *Journal of Inorganic Biochemistry*, *92*(3-4), 141-148.

Andrzejak V., Millet R., El Bakali J., Guelzim A., Gluszok S., Chavatte P., Bonte P.J., Vaccher C., Lipka E. (2010). Synthesis of 2, 3 and 4, 5-dihydro-hydroxy-isoxazoles and isoxazoles under different pH conditions. *Letters in Organic Chemistry*, *7*(1), 32.

Bertucci, C., & Domenici, E. (2002). Reversible and covalent binding of drugs to human serum albumin: methodological approaches and physiological relevance. *Current medicinal chemistry*, *9*(15), 1463-1481.

Bork, M.A., Gianopoulos, C.G., Zhang, H., Fanwick, P.E., Choi, J.H., & McMillin, D.R. (2014). Accessibility and external versus intercalative binding to DNA as assessed by oxygen-induced quenching of the palladium (II)-containing cationic porphyrins Pd (T4) and Pd (t D4). *Biochemistry*, *53*(4), 714-724.

Chiririwa, H., Moss, J.R., Hendricks, D., Meijboom, R., & Muller, A. (2013). Synthesis, characterisation and in vitro evaluation of palladium (II) iminophosphine complexes for anticancer activity. *Transition Metal Chemistry*, *38*, 165-172.

dos Santos, S.X., & Cavalheiro, É.T. (2014). Using of a graphite-polyurethane composite electrode modified with a Schiff base as a bio-inspired sensor in the dopamine determination. *Journal of the Brazilian Chemical Society*, *25*, 1071-1077.

Desoize, B., & Madoulet, C. (2002). Particular aspects of platinum compounds used at present in cancer treatment. *Critical reviews in oncology/hematology*, *42*(3), 317-325.

Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Scalmani, G., Barone, V., Mennucci, B., Petersson, G.A., Nakatsuji, H., Caricato M., Li, X., Hratchian, H.P., Izmaylov, A.F., Bloino, J., Zheng, G., Sonnenberg, J.L., Hada, M., Ehara, M., Toyota, K., Fukuda, R., Hasegawa, J., Ishida, M., Nakajima, T., Honda, Y., Kitao, O., Nakai, H., Vreven, T., Montgomery J.A. Jr., Peralta, J.E., Ogliaro, F., Bearpark, M., Heyd, J.J., Brothers, E., Kudin, K.N., Staroverov, V.N., Kobayashi, R., Normand, J., Raghavachari, K., Rendell, A., Burant, J.C., Iyengar, S.S., Tomasi, J., Cossi, M., Rega, N., Millam, J.M., Klene, M., Knox, J.E., Cross, J.B., Bakken, V., Adamo, C., Jaramillo, J., Gomperts, R., Stratmann, R.E., Yazyev, O., Austin, A.J., Cammi, R., Pomelli, C., Ochterski, J.W., Martin, R.L., Morokuma, K., Zakrzewski, V.G., Voth, G.A., Salvador, P., Dannenberg, J.J., Dapprich, S., Daniels, A.D., Farkas, O., Foresman, J.B., Ortiz, J.V., Cioslowski, J. and Fox, D.J. (2013). Gaussian 09, Revision D.01, Gaussian, Inc., Wallingford CT.

González, L.M., Tercero, M.J., Matilla, A., Niclós-Gutiérrez, J., Fernández, T.M., López, C. M., Alonso, C., González, S. (1997). Cis-dichloro (α, ω-diamino carboxylate ethyl ester) palladium (II) as palladium (II) versus platinum (II) model anticancer drugs: synthesis, solution equilibria of their aqua, hydroxo, and/or chloro species, and in vitro/in vivo DNA-binding properties. *Inorganic chemistry*, *36*(9), 1806-1812.

Joksimović, N., et al. (2024). Anticancer potential of novel palladium(II) complexes with acyl pyruvates as ligands: DNA and BSA interactions and molecular docking study. *STED Journal, 6*(1), 1-14.

Guo, G., Liu, J., Wang, G., Zhang, D., Lu, J., & Zhao, G. (2016). Synthesis and biological evaluation of 3-(4-fluorophenyl)-1H-pyrazole derivatives as androgen receptor antagonists. *Anti-Cancer Drugs*, *27*(4), 278-285.

Hartmann, J.T., & Lipp, H.P. (2003). Toxicity of platinum compounds. *Expert opinion on pharmacotherapy*, *4*(6), 889-901.

Hartwig, A. (2010). The role of DNA repair in benzene-induced carcinogenesis. *Chemico-Biological Interactions*, *184*(1-2), 269-272.

Joksimovic, N., Jankovic, N., Petronijevic, J., Baskic, D., Popovic, S., Todorovic, D., Zaric, M., Klisuric, O., Vranes, M., Tot, A., Bugarcic, Z., (2020). Synthesis, anticancer evaluation and synergistic effects with cisplatin of novel palladium complexes: DNA, BSA interactions and molecular docking study. *Medicinal Chemistry*, *16*(1), 78-92.

Ju, H., Ding, L., Sun, C., Chen, J. (2015). Quantum Chemical Study on the Corrosion Inhibition of Some Oxadiazoles. *Adv. Mater. Sci. Eng*., 2015, 1-5.

Kandagal, P.B., Ashoka, S., Seetharamappa, J., Shaikh, S.M.T., Jadegoud, Y., & Ijare, O.B. (2006). Study of the interaction of an anticancer drug with human and bovine serum albumin: spectroscopic approach. *Journal of pharmaceutical and biomedical analysis*, *41*(2), 393-399.

Krogul, A., Cedrowski, J., Wiktorska, K., Ozimiński, W.P., Skupińska, J., & Litwinienko, G. (2012). Crystal structure, electronic properties and cytotoxic activity of palladium chloride complexes with monosubstituted pyridines. *Dalton Transactions*, *41*(2), 658-666.

Kulakov, I.V., Karbainova, A.A., Shulgau, Z.T., Seilkhanov, T.M., Gatilov, Y.V., & Fisyuk, A.S. (2017). Synthesis and Analgesic Activity of bis (3, 4-dihydroquinoxalin-2 (1 H)-one) and bis (3, 4-dihydro-2 H-1, 4-benzoxazin-2-one) Derivatives. *Chemistry of Heterocyclic Compounds*, *53*, 1094-1097.

Lakowicz, J. R., & Weber, G. (1973). Quenching of fluorescence by oxygen. Probe for structural fluctuations in macromolecules. *Biochemistry*, *12*(21), 4161-4170.

Lauria, A., Bonsignore, R., Terenzi, A., Spinello, A., Giannici, F., Longo, A., Almerico, A.M., Barone, G. (2014). Nickel(II), copper(II) and zinc(II) metallo-intercalators: structural details of the DNA-binding by a combined experimental and computational investigation. *Dalton Transactions*, *43*(16), 6108-6119.

Lazić, D., Arsenijević, A., Puchta, R., Bugarčić, Ž.D., & Rilak, A. (2016). DNA binding properties, histidine interaction and cytotoxicity studies of water-soluble ruthenium (II) terpyridine complexes. *Dalton Transactions*, *45*(11), 4633-4646.

Lee, J.Y., Lee, J.Y., Chang, Y.Y., Hu, C.H., Wang, N.M., & Lee, H.M. (2015). Palladium complexes with tridentate N-heterocyclic carbene ligands: Selective “normal” and “abnormal” bindings and their anticancer activities. *Organometallics*, *34*(17), 4359-4368.

Li, Y., Cheng, M., Hao, J., Wang, C., Jia, G., & Li, C. (2015). Terpyridine–Cu (II) targeting human telomeric DNA to produce highly stereospecific G-quadruplex DNA metalloenzyme. *Chemical Science*, *6*(10), 5578-5585.

Mazumder, M.E.H., Beale, P., Chan, C., Yu, J.Q., & Huq, F. (2012). Synthesis and Cytotoxicity of Three trans-Palladium Complexes Containing Planaramine Ligands in Human Ovarian Tumor Models. *ChemMedChem*, *7*(10), 1840-1846.

Meadows, K.A., Liu, F., Sou, J., Hudson, B.P., & McMillin, D.R. (1993). Spectroscopic and photophysical studies of the binding interactions between copper phenanthroline complexes and RNA. *Inorganic Chemistry*, *32*(13), 2919-2923.

Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., & Olson, A.J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of computational chemistry*, *30*(16), 2785-2791.

Ott, I., & Gust, R. (2007). Non platinum metal complexes as anti-cancer drugs. *Archiv der Pharmazie: An International Journal*

Pharmaceutical and Medicinal Chemistry, *340*(3), 117-126.

Petronijević, J., Janković, N., Stanojković, T.P., Joksimović, N., Grozdanić, N.Đ., Vraneš, M., Tot, A., Bugarčić, Z. (2018). Biological evaluation of selected 3,4-dihydro-2(1H)-quinoxalinones and 3,4-dihydro-1,4-benzoxazin-2-ones: molecular docking study. *Arch. Pharm. Chem. Life Sci. 351*(5), 1700308. doi: 10.1002/ardp.201700308.

Prokop, P., Gelbrich, T., Sieler, J., Richter, R., & Beyer, L. (2001). Kristallstrukturen ferrocenhaltiger 1,3-Diketon-und Enaminoketon-Derivate. *Zeitschrift für anorganische und allgemeine Chemie*, *627*(5), 965-972.

Rosenberg, B., Van Camp, L., & Krigas, T. (1965). Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode. *Nature*, *205*(4972), 698-699.

Sánchez, M.I., Penas, C., Vázquez, M.E., & Mascareñas, J.L. (2014). Metal-catalyzed uncaging of DNA-binding agents in living cells. *Chemical science*, *5*(5), 1901-1907.

Sanner, M.F. (1999). Python: a programming language for software integration and development. *J Mol Graph Model*, *17*(1), 57-61.

Sastry, J., & Kellie, S.J. (2005). Severe neurotoxicity, ototoxicity and nephrotoxicity following high-dose cisplatin and amifostine. *Pediatric hematology and oncology*, *22*(5), 441-445.

Shehab, W.S., & El-Bassyouni, G.T. (2018). Synthesis and cyclization of β-keto-enol derivatives tethered indole and pyrazole as potential antimicrobial and anticancer activity. *Journal of the Iranian Chemical Society*, *15*, 1639-1645.

Shen, H.Y., Shao, X.L., Xu, H., Jia, L.I., & Pan, S.D. (2011). In vitro study of DNA interaction with trichlorobenzenes by spectroscopic and voltametric techniques. *International Journal of Electrochemical Science*, *6*(3), 532-547.

Stepanova, E.E., Dmitriev, M.V., & Maslivets, A. N. (2019). Reaction of Acylpyruvic Acids and Their Esters with N-(2-Aminophenyl) acetamide. *Russian Journal of Organic Chemistry*, *55*, 402-405.

Strekowski, L., & Wilson, B. (2007). Noncovalent interactions with DNA: an overview. *Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis*, *623*(1-2), 3-13.

Trevisan, A., Marzano, C., Cristofori, P., Borella Venturini, M., Giovagnini, L., & Fregona, D. (2002). Synthesis of a palladium (II)-dithiocarbamate complex: biological assay and nephrotoxicity in rats. *Archives of toxicology*, *76*, 262-268.

Zalesov, V.V., Kataev, S.S., Pulina, N.A., & Kovylyaeva, N.V. (2002). 4-Aryl-2, 4-dioxobutanoic acids and their derivatives in reactions with diazoalkanes. *Russian journal of organic chemistry*, *38*, 840-844.

Zhang, K.J., & Liu, W.Y. (2011). Investigation of the electrochemical interaction behavior of DNA with 5-fluorouracil derivatives. *International Journal of Electrochemical Science*, *6*(5), 1669-1678.

Zolezzi, S., Decinti, A., & Spodine, E. (1999). Syntheses and characterization of copper (II) complexes with Schiff-base ligands derived from ethylenediamine, diphenylethylenediamine and nitro, bromo and methoxy salicylaldehyde. *Polyhedron*, *18*(6), 897-904.