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RESEARCH ARTICLE

Synthesis, Characterization and Biological Studies of Organoselenium *trans*-Palladium(II) Complexes

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Abstract: ***Background*:** Over the years, transition metal complexes have exhibited significant antimicrobial and antitumor activity. It all started with cisplatin discovery, but due to the large number of side effects it shows, there is a growing need to find a new metal-based compound with higher selectivity and activity on more tumors.

***Objectives*:** Two novel *trans*-palladium(II) complexes with organoselenium compounds as ligands, $[Pd(L1)_2Cl_2]$ (L1 = 5-(phenylselanylmethyl)-dihydrofuran-2(3H)-one) and $[Pd(L2)_2Cl_2]$ (L2 = 2-methyl-5-(phenylselanylmethyl)- tetrahydrofuran) were synthesized, in the text referred to as Pd-Se1 and Pd-Se2. Also, a structurally similar *trans*-palladium(II) complex, $[Pd(L3)_2Cl_2]$ (L3= 2,2-dimethyl-3-(phenylselanylmethyl)-tetrahydro-2H-pyran) was synthesized according to an already published work and is referred to as Pd-Se3. The interaction of synthesized complexes with DNA and bovine serum albumin was observed. Also, antimicrobial activity and *in vitro* testing, cell viability, and cytotoxic effects of synthesized ligands and complexes on human epithelial colorectal cancer cell line HCT-116 were studied. Molecular docking simulations were performed to understand better the binding modes of the complexes reported in this paper with DNA and BSA, as well as to comprehend their antimicrobial activity.

***Methods*:** The interactions of the synthesized complexes with DNA and bovine serum albumin were done using UV-Vis and emission spectral studies as well as docking studies. Antimicrobial activity was tested by determining the minimum inhibitory concentrations (MIC) and minimum microbicidal concentration (MMC) using the resazurin microdilution plate method. Cytotoxic activity on cancer cells was studied by MTT test.

***Results*:** The Pd(II) complexes showed a significant binding affinity for calf thymus DNA and bovine serum albumin by UV-Vis and emission spectral studies. The intensity of antimicrobial activity varied with the complexes Pd-Se1 and Pd-Se3, showing significantly higher activity than the corresponding ligand. The most significant activity was shown on *Pseudomonas aeruginosa.* Under standardized laboratory conditions for *in vitro* testing, cell viability and cytotoxic effects of synthesized ligands and complexes were studied on human epithelial colorectal cancer cell line HCT-116, where Pd-Se2 showed some significant cytotoxic effects.

***Conclusion*:** The newly synthesized complexes have the potential to be further investigated as metallodrugs.

Keywords: Palladium, Selenium, DNA, Docking, Antimicrobial activity, Cytotoxicity.

1. INTRODUCTION

Selenium and its compounds have long been known as extremely toxic substances for the human organism. [1] The first research, in the middle of the last century, showed that selenium is necessary for the human organism in small quantities.

To date, it has been observed that there is a link between selenium and cancer, namely, the nutrition enriched with selenium and vitamin E reduces the risk of cancer. [1] It has also been observed that Se is both neuroprotective as well as neurotoxic, a cardiovascular health promoter, an antidiabeto-

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genic and prodiabetogenic agent. In addition, selenium is an essential element that plays a very important role in oxidation-reduction reactions. [1-4] In addition to anti-tumor activity, selenium and its compounds also show antimicrobial and anti-oxidant activity.[4]

Cisplatin is still the most used transition-metal-based medicine in chemotherapy. However, many severe side effects and the resistance that its use brings calls for the need to design a drug with better characteristics. [5-25] Initially, it was believed that Pd(II) complexes could not exhibit antitumor activity, due to their exceptional reactivity, however, studies in recent years show that some Pd(II) complexes have greater cytotoxicity than cisplatin. [26,27] In the last couple of years, research has been conducted to introduce a complex that will exhibit cytotoxic activity by introducing different types of ligands into the coordination sphere of the Pd(II) complex. [28] A *trans*-geometry, complex of Pd(II) with a pyridine-containing ligand was synthesized. It has been found that this complex binds to DNA and exhibits cytotoxicity to certain cancer cells. [29]

The discovery of antibiotics and their successful application have marked a turning point in the fight against infectious diseases. However, after mass and uncontrolled use of antibiotics, bacteria developed different defense mechanisms and became resistant to the effects of antibiotics. In recent years, many researchers work on finding new antimicrobial agents that can be applied effectively in the fight against infections. [30] Significant antibacterial and antifungal effects have been shown by numerous complexes of transition metals, including palladium complexes. [31,32] Selenium is an essential element and complexes of palladium and selenium compounds have anti-tumor, anti-microbial and many other pharmacological activities. [1] The resistance of some different *Rhizobium* and *Bradyrhizobium* strains to the action of selenite and selenate is described. [33] Antimicrobial activity of sodium selenite against *Helicobacter pylori (in vitro* and *in vivo*) was studied. [34] The influence of sodium selenite on the growth of G+ and G- bacteria and their sensitivity to antibiotics have been examined. [35]

Our idea was to merge selenium and palladium and synthesize Pd(II) complexes with selenium-ligands (Fig. **1**), and then examine antimicrobial as well as cytotoxic activities. In this paper, the interactions of synthesized complexes with CT-DNA and albumin, as potential targets for complex activity, were also researched.

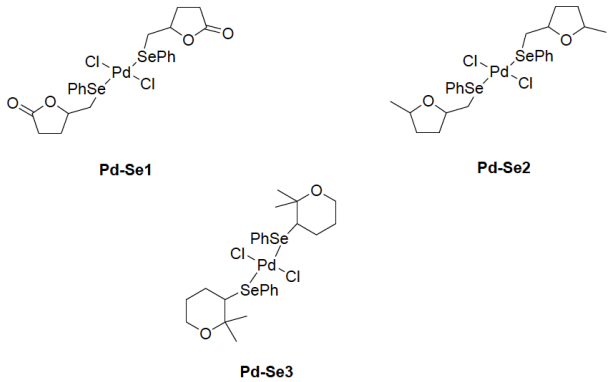


Fig. (1). Structures of the investigated trans-Pd-Se complexes.

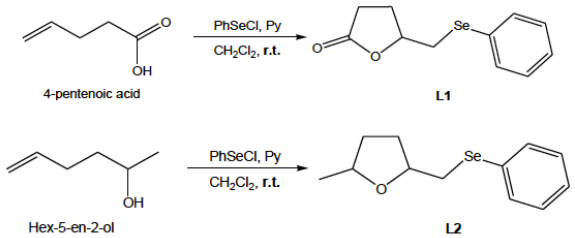
2. MATERIALS AND METHOD

2.1. Chemicals and Solutions

Commercial chemicals were used without prior purification. $PdCl_2$, PBS (phosphate = 0.01 M, c(NaCl) = 0.137, c(KCl) = 0.0027 M, pH 7.4), CT-DNA, ethidium bromide (EB) and BSA were from Sigma Aldrich. CT-DNA was dissolved in triple-distilled deionized water and stored at 4°C for less than a week. The UV absorbance ratio (260nm/280nm) of CT-DNA solutions in PBS was 1.8–1.9, indicating a lack of protein contamination. Nucleophile stock solutions were freshly prepared before use. Double distilled, deionized water was used for all experiments. Preparation of Pd-Se1 and Pd-Se2 complexes was done according to published procedures. [36] Pd-Se3 was prepared as published and characterized by standard analytical methods. [37] Dimethyl sulfoxide (DMSO) was purchased from Acros Organics (New Jersey, USA). Resazurin was obtained from Alfa Aesar GmbH & Co. (KG, Karlsruhe, Germany). DMEM (Dulbecco's Modified Eagle Medium) and trypsin-EDTA were obtained from Sigma Aldrich (St. Louis, MO, USA). Penicillin/streptomycin was from Thermo Fisher Scientific (Waltham, MA, USA). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), FBS (fetal bovine serum) and PBS (phosphate-buffered saline) were obtained from Gibco, Invitrogen, USA.

2.2. Synthesis and Characterization of Pd-Se1 and Pd-Se2

Synthesis of Ligands: Synthesis of Ligands L1 and L2: To the solution of 1 mmol of 4-pentenoic acid (0.1 g) or hex-5-en-2-ol (0.1 g) in 5 cm^3 of dry dichloromethane, an equimolar amount of pyridine (0.079 g) was added. Then 1.1 mmol (0.212g) of solid PhSeCl was added to the mixture and the reaction mixture was stirred at room temperature until the reaction was complete (30 minutes for L1, and instantaneously for L2). The reactions were followed by TLC chromatography and visual discoloration (orange to pale yellow). The resulting solution was rinsed with 2M HCl, saturated aqueous solution of $NaHCO_3$ and saline solution. After drying over Na_2SO_4, the organic layer was concentrated and purified by chromatography on a silica gel column.



Scheme 1. Synthesis of L1and L2 ligands.

The ligands L2 and L3, as well as complex Pd-Se3 were prepared according to the published procedures. [36, 37]

Synthesis and characterization of Pd-Se1 and Pd-Se2 complexes: L1 or L2 ligands (0.3 mmol, 0.077 g) were dis-

solved in 10 cm^3 of EtOH/MeOH (4:1) and stirred rapidly after the addition of $PdCl_2$ (0.33 mmol.0.058 g) in excess. The mixture was stirred at 40°C for 4 days for the Pd-Se1 complex and 5h for the Pd-Se2 complex. After complexation, the color changed from dark brown to yellow (complex Pd-Se1) or dark red (complex Pd-Se2). After filtration, the solution was concentrated by slow evaporation, and the resulting solid was air-dried and recrystallized from ethanol.

Pd-Se1 complex: yellow substance. Yield (0.196 g, 95%). ^{1}H NMR ($CDCl_3$; δ, ppm): 7. 90 (m, 2H, Ph), 7.45 (m, 3H, Ph), 4.72–4.95 (m, 1H, CHO), 3.83-3.98 (m, 1H, CH_aH_bSe)), 3.32-3.45 (m, 1H, CH_aH_bSe), 1.84-2.13 and 2.3-2.64 (m, 4H, $2CH_2$). ^{13}C NMR ($CDCl_3$; δ, ppm): 175.42, 133.49, 130.58, 130.08, 128.82, 84.35, 29.93, 28.71, 28.3. Anal. Calcd. for ($C_{22}H_{24}Cl_2O_4Se_2Pd$) C: 38.42; H: 3.52; O: 9.31. Found: C: 39.05; H: 3.22; O: 9.78; ESI-MS: $[M\text{-}Cl]^+$: Calcd.: 652.9456; Found: 652.9457

Pd-Se2 complex: dark red substance. Yield (0.161 g, 78%). ^{1}H NMR ($CDCl_3$; δ, ppm): 7. 90 (m, 2H, Ph), 7.38 (m, 3H, Ph), 3.85–4.4 (overlapping multiplets, 2H, 2CHO), 3.87–4.01 (m, 1H. CH_aH_bSe), 3.6-3.78 (m, 1H, CH_aH_bSe), 1.42-1.82 and 1.85-2.20 (m, 4H, $2CH_2$), 1.16 and 1.26 (d, J = 5Hz, 6H, CH3 *cis*/*trans* isomers). ^{13}C NMR ($CDCl_3$; δ, ppm): *cis*/*trans i*somers: 133.16 /133.26, 129.06/129.20, 128.82/128.99, 126.54/126.58, 76.64/77.28, 75.21/75.87, 37.31/37.79, 32.77/33.23, 31.25/32.16, 21.04/21.21 Anal. Calcd. for ($C_{24}H_{32}Cl_2O_2Se_2Pd$) C: 41.91; H: 4.69; O: 4.65. Found: C: 41.21; H: 4.28; O: 4.78; ESI-MS: $[M\text{-}Cl]^+$: Calcd.: 652.8728; Found: 652.8719.

2.3. Instrumentation

NMR spectra were recorded on a 200 MHz Varian Gemini-2000. NMR signals were referenced to residual proton or carbon signals of the deuterated solvent (^{1}H- and ^{13}C-NMR) and are reported in ppm relative to TMS. Elemental analyses (C, H, N) were performed by combustion and gas chromatographic analysis with an Elementar Vario MICRO elemental analyzer. pH measurements were done using a Mettler Delta 350 digital pH meter with a resolution ± 0.01 mV and a combination glass electrode calibrated using standard buffer solutions (Sigma) at pH 4, 7, and 9. UV-vis measurements were conducted on a PerkinElmer Lamda 25 and 35 double-beam spectrophotometer with thermostated 1.00 cm quartz Suprasil cells. The temperature was controlled to ± 0.1°C. Fluorescence was measured on a RF-1501 PC spectrofluorometer (Shimadzu, Japan). Mass spectrometry was measured on a Waters Quadrupole-ToF Synapt 2G using electrospray ionization (ESI).

2.4. DNA Interactions

CT-DNA solutions were prepared in PBS, with a UV absorbance ratio A_{260}/A_{280} of ca. 1.8–1.9, indicating negligible protein contamination. Concentrations of CT-DNA solutions were determined at A_{260} with ε = 6600 $M^{-1}cm^{-1}$. [38] Fluorescence spectra were recorded in the range of 550–750 nm with excitation at 527 nm. Excitation and emission bandwidths were both 10 nm.

2.5. UV-vis Absorption Studies

To quantitatively compare the complexes' binding strength, binding constant Kb was determined by monitoring changes in absorption at the MLCT band with an increasing concentration of CT-DNA using the following eq. 1.

$$[DNA]/(\varepsilon_A - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/[K_b(\varepsilon_b - \varepsilon_f)] \quad (1)$$

The ratio of the slope to the y-intercept in plots of $[DNA]/(\varepsilon_A-\varepsilon_f)$ vs. [DNA] gives K_b, where [DNA] is the concentration of DNA in base pairs, $\varepsilon_A = A_{obsd}/[complex]$, ε_f is the extinction coefficient for the unbound complex and ε_b is the extinction coefficient for the complex in the fully bound form.

2.6. Ethidium Bromide (EB) Displacement Studies

The relative binding of complexes to CT-DNA was determined by calculating the quenching constant (K_{sv}) from the slopes of straight lines obtained from the Stern-Volmer equation (eq. 2).

$$I_0/I = 1 + K_{sv}[Q] \quad (2)$$

Where I_0 and I are emission intensities in the absence and presence of quencher (complexes Pd-Se1 and Pd-Se2), respectively, [Q] is the total concentration of quencher, K_{sv} is the Stern-Volmer quenching constant, which was obtained from the slope of the plot of I_0/I vs. [Q].

2.7. Viscosity Measurements

Changes in DNA viscosity were measured in the presence of increasing amounts of complexes Pd-Se1 and Pd-Se2. Flow time was measured with a digital stopwatch. Each sample was measured in triplicate, and the average flow time was calculated. Data are presented as $(\eta/\eta_0)^{1/3}$ against r, where η is the DNA viscosity in the presence of complex and η_0 is the viscosity of DNA in buffer alone. Viscosity values were calculated from the observed flow time of DNA-containing solutions (t) corrected for the flow time of buffer alone (t_0), $\eta = (t - t_0)/t_0$.

2.8. Protein Binding Studies

Protein fluorescence is due to natural fluorophores such as tryptophan, tyrosine, and phenylalanine. Changes in BSA fluorescence were used to monitor interaction with metal complexes. Tryptophan fluorescence quenching experiments were conducted using 2.0 μM BSA in PBS. The emission intensity quenching of BSA tryptophan residues at 363 nm in the presence of increasing concentrations of complexes Pd-Se1 and Pd-Se2 (0–10.0 μM) was monitored. Fluorescence spectra were recorded in the range 300–500 nm with excitation at 295 nm. Excitation and emission bandwidths were both 10 nm.

2.9. *In vitro* Antimicrobial Assay

Test substances, microorganisms, and suspension preparation of the tested compounds were dissolved in DMSO and then diluted into a liquid nutrient medium to achieve a concentration of 10%. An antibiotic, doxycycline (Galenika

A.D., Belgrade), was dissolved in a liquid nutrient medium, a Mueller–Hinton broth (Torlak, Belgrade), while antimycotic, fluconazole (Pfizer Inc., USA) was dissolved in Sabouraud dextrose broth (Torlak, Belgrade). The antimicrobial activity of the ligands and complexes was tested against seventeen microorganisms. The experiment involved nine strains of pathogenic bacteria, including five standard strains and four clinical isolates. Also, the antifungal activity of the complexes with five mold species and three yeast species was tested. The bacterial suspensions were prepared by the direct colony method. The turbidity of the initial suspension was adjusted using a densitometer (DEN-1, BioSan, Latvia). When adjusted to the turbidity of the 0.5 McFarland's standard,[39] the bacteria suspension contained about 108 colony forming units (CFU)/mL, and yeast suspension contained 106 CFU/mL. Ten-fold dilutions of the initial suspension were additionally prepared into sterile 0.85% saline. Bacterial inocula obtained from bacterial cultures were incubated for 24 h at 37°C on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard to approximately 106 CFU/mL. Suspensions of fungal spores were prepared from fresh mature (3-to 7-day-old) cultures that grew at 30°C on a PD (potato dextrose) agar substrate. Spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 106 CFU/mL according to the procedure recommended by NCCLS. [40]

Microdilution method: Antimicrobial activity was tested by determining the minimum inhibitory concentrations (MIC) and minimum microbicidal concentration (MMC) using the microdilution plate method with resazurin. [41] The 96-well plates were prepared by dispensing 100 µL of nutrient broth, Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for fungi in each well. A 100 µL aliquot from the stock solution of the tested compound (with a concentration of 2000 µg/mL) was added into the first row of the plate. Then, two-fold serial dilutions were performed by using a multichannel pipette. The obtained concentration range was from 1000 to 7.8 µg/mL. The method is described in detail in the reported paper.[42] Doxycycline and fluconazole were used as a positive control. 10% DMSO (as solvent control test) was recorded not to inhibit the growth of microorganisms. Each test included growth control and sterility control. All the tests were performed in duplicate, and the MICs were constant. Minimum bactericidal and fungicidal concentrations were determined by plating 10 µL of samples from wells where no indicator color change or no mycelia growth was recorded, on nutrient agar medium. At the end of the incubation period, the lowest concentration with no growth (no colony) was defined as the minimum microbicidal concentration.

The effect of PdSe1, PdSe3, L1 and L3 on formed biofilm of selected bacteria: The effect of PdSe1, PdSe3, L1 and L3 on formed biofilm of S. aureus ATCC 25923, S. aureus and P. aeruginosa was determined according to the method described by O'Toole and Kolter (1998), with some modifications.[43] The tissue culture 96-well microtiter plates (Sarstedt, Germany) were prepared by adding 100 µL of TSB broth and 10 µL of fresh bacterial suspension (1.0 McFarland for Gram-positive and 0.5 for Gram-negative bacteria) to each well. The inoculated microtiter plates were incubated at 37°C for 48 hours. After incubation, the content of each well was gently pulled out. Then, 100 µL of dissolved complexes PdSe1 and PdSe3, and ligands L1 and L3 were added to each well, and the microtiter plates were incubated at 37°C for 24 hours. The concentration of complexes and ligands ranged from 1000-7.8 µl. After incubation, the content of each well was gently removed by tapping the microtiter plates. After that, the experiment was carried out according to the published procedure.[44] Biofilm inhibitory concentration required to reduce biofilm coverage by 50% (BIC50) was defined as the lowest concentration of extract that showed 50% inhibition of biofilm formation.[45]

2.10. Molecular Docking Simulations

The starting structures of studied complexes before docking were optimized at the ωB97XD [46] theory level combined with the def2-TZVP basis set. [47] Gain structures were characterized as minima by preforming and examining the vibrational frequencies at the same theory level. For this type of calculation, the GAUSSIAN suite program was used. [48] The starting three-dimensional (3D) structures of DNA fragments representing a canonical B-DNA (PDB: 1BNA), DNA with an intercalation gap (PDB: 1Z3F), and bovine serum albumin (PDB: 4F5S), as well as structures of *S. aureus* tyrosyl-tRNA synthetase (TyrRS; PDB: 1JIJ) and topoisomerase II DNA gyrase (DNA Gyr; PDB: 2XCT) were obtained from Protein Data Bank (PDB; http://www.rcsb.org). [49] Water molecules, ligands, and heteroatoms were removed if present. In the rigid structure of DNA, BSA, TyrRS, and DNA Gyr flexible, compounds were docked using Molegro Virtual Docker (MVD, version 2013.6.0.1). [50] Grid resolution of the binding side was 0.3 Å. Docking towards BSA was performed by MVD generated cavity, near a Trp-213 residue, for *S. aureus* DNA Gyr around ciprofloxacin, while for *S. aureus* TyrRS around SB-239629 ligand. The docking procedure parameters were: a maximum number of iterations 1500, population size 50, energy threshold 100.00, and a maximum number of steps 300. A maximum population of 100 and a maximum number of iterations of 10 000 were used for each run. The MolDock SE as a search algorithm was used with the number of runs set to 100, and the number of generated poses was 5. The MVD-related scoring functions described the estimation of investigated complexes and DNA/BSA/DNA Gyr/TyrRS interactions: MolDock, Docking, Rerank, and H-bond. [50] Molegro scores were evaluated relatively. The five best poses were retained. Docked poses with DNA fragments were visualized using CHIMERA molecular graphics program (http://www.cgl.ucsf.edu/chimera/).

2.11. *In vitro* Cytotoxic Activity

Cytotoxic activity on cancer cells was studied by MTT test. The HCT-116 cell line was obtained from the American Tissue Culture Collection (Manassas, VA, USA). The stock solutions were prepared in DMSO at a concentration of 100 mM and diluted to final concentrations (0.1, 1, 10, 50, 100 and 500 µM) in DMEM medium for cell treatment (percent of DMSO in the highest treatment concentration was 0.5%, thus not toxic to the cells). The cells were grown in complete medium – DMEM, 10% fetal bovine serum, antibiotics 100

IU/mL penicillin, and 100 µg/mL streptomycin, in T75 culture flasks, on a controlled atmosphere of 5% CO_2 at 37 °C. All experiments were done with cells at 70 to 80% confluence.

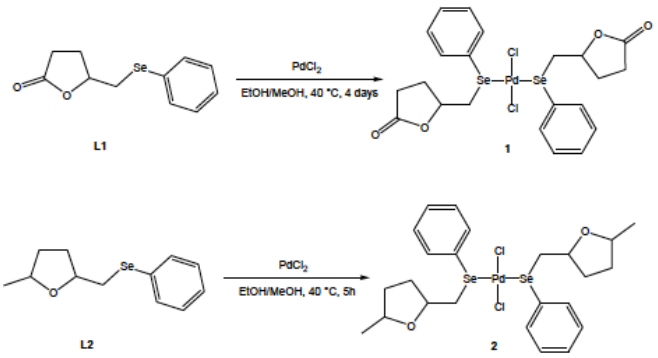
Cells were seeded in a 96-well plate (104 cells per well), and after 24 h of pre-incubation, treated with 100 µL of each concentration of investigated substances. Untreated cells served as a control. At the end of the treatment period (24 and 72 h), 25 µL MTT (concentration 5 mg/mL in PBS) was added to each well and then incubated at 37 °C in 5% CO_2 for 2 h. This test is based on mitochondrial dehydrogenase reaction from living cells with MTT and resulting in colored crystals of formazan. Purple formazan was dissolved in 150 µL DMSO, and the absorbance was measured at 550 nm on Rayto 96-well plate ELISA reader, RT-2100C.

The effects on cell viability were calculated as a ratio of the absorbance of the treated samples divided by the absorbance of control samples (untreated cells), multiplied by 100 to express as a percentage of viable cells. The data are expressed as the means of two independent experiments and were done using an SPSS (Chicago, IL) statistical software package. The cytotoxic effect was expressed by IC_{50} (a dose that reduces 50% of cell viability), calculated from the dose curves by a computer program, CalcuSyn v 2.1.

3. RESULTS AND DISCUSSION

3.1. Preparation and Structure of Pd-Se1 and Pd-Se2

Complexes $[Pd(L1)_2Cl_2]$ (L1 = 5-(phenylselanylmethyl)-dihydrofuran-2(3H)-one) and $[Pd(L_2)_2Cl_2]$ (L2 = 2-methyl-5-(phenylselanylmethyl)-tetrahydrofuran) in text are referred to as Pd-Se1 and Pd-Se2, respectively (Figure 1). These complexes were synthesized by stirring equimolar amounts of $PdCl_2$ and L1 or L2 ligands, Scheme **2**, in EtOH/MeOH (4:1) mixture and stirred rapidly at 40°C for 4 days. The synthesized Pd(II) complexes were characterized by ^1H and ^{13}C NMR spectroscopy, elemental analysis, and ESI-MS mass spectrometry.



Scheme 2. Synthesis of Pd(II) complexes, Pd-Se1, and Pd-Se2.

Elemental analyses on these complexes were in very good agreement with a complex composition of $[Pd(L1)_2Cl_2]$ or $[Pd(L_2)_2Cl_2]$. The ^1H NMR, as well as the ^{13}C NMR spectra, of the Pd-Se1 and Pd-Se2 complexes indicated that only this distinct species is formed. The complexes are also characterized by ESI-MS mass spectrometry, in the m/z range of 400−700 including main peaks at m/z = 652.9456 (1+), and 652.8728 (1+), representing fragments of Pd-Se1 and Pd-Se2 complexes, respectively, formed by losing one chloride (Figure S1 and S2).

3.2. DNA-Binding Studies

Transition metal complexes react with nucleic acids, and the model of interaction depends on the structure of the complex, charge, and the type of inert ligand. It has been shown that the geometry of both the inert ligand and the complex affects the interaction between the complexes and the DNA molecule.[51] Transition metal complexes can interact with the DNA through non-covalent interactions (intercalation, "groove binding", "minor binding", *etc*.), as well as covalent interactions, *i.e*., the substitution of one labile ligand with nitrogen from a guanine molecule of DNA. [52-54] In this paper, we were researching the interactions of the Pd-Se complexes, Figure 1, with the DNA molecule as a potential target for the binding of the metal complexes. These interactions were examined using Uv-vis spectroscopy, fluorescence spectroscopic, and viscosity measurement.

3.2.1. Electronic Absorption Method

The addition of the metal complex solutions to a CT-DNA solution can lead to spectral changes, which can indicate an interaction between the examined complexes and the DNA molecule. [55-58] The spectral changes shown in Fig. (**2**) were obtained by recording the UV-Vis spectra of the complexes Pd-Se1 and Pd-Se2 (constant concentrations, 8 µM; phosphate buffer) in the absence and presence of different concentrations of the CT-DNA solution (0-40 µM).

The investigated complexes Pd-Se1 and Pd-Se2 give similar spectral changes and absorb in the same part of the spectrum, Fig. **2**. A hyperchromic effect was observed after adding the complex solution to the DNA solution at a wavelength of about 260 nm. This confirms that there is an interaction between the complexes and the DNA molecule. In addition, a peak shift of couple nm was observed, indicating a stabilization of the DNA duplex. The observed hyperchromic effect, as well as the shift of the maximum, can indicate intercalation, *i.e*., interactions between the aromatic chromophores of the complex and the aromatic chromophores of the DNA chain. [59,60] However, the exact type of interaction between the complex and the DNA chain cannot be determined solely based on UV spectroscopy.

The binding constant, K_b, is determined by monitoring the absorbance changes at the appropriate wavelength after adding the growing concentration solution of the DNA based on the equation (1).

Table **1** shows the obtained values for the K_b constant for the investigated Pd-Se complexes and the structurally similar complexes. The binding affinity of the studied complexes to DNA is decreasing in the sequence Pd-Se2 > Pd-Se1, although the difference is very small. The investigated complexes interact well with the DNA molecule namely, ethidium bromide (EB), a classical intercalator for DNA, which has a lower value of the constant K_b compared to the investigated complexes $K_b = (1.23 \pm 0.07) \times 10^5\ M^{-1}$. [61] Table 1 shows that the formerly studied complexes 1, 2, and Pd-Se3 have about two times smaller K_b constant compared to the

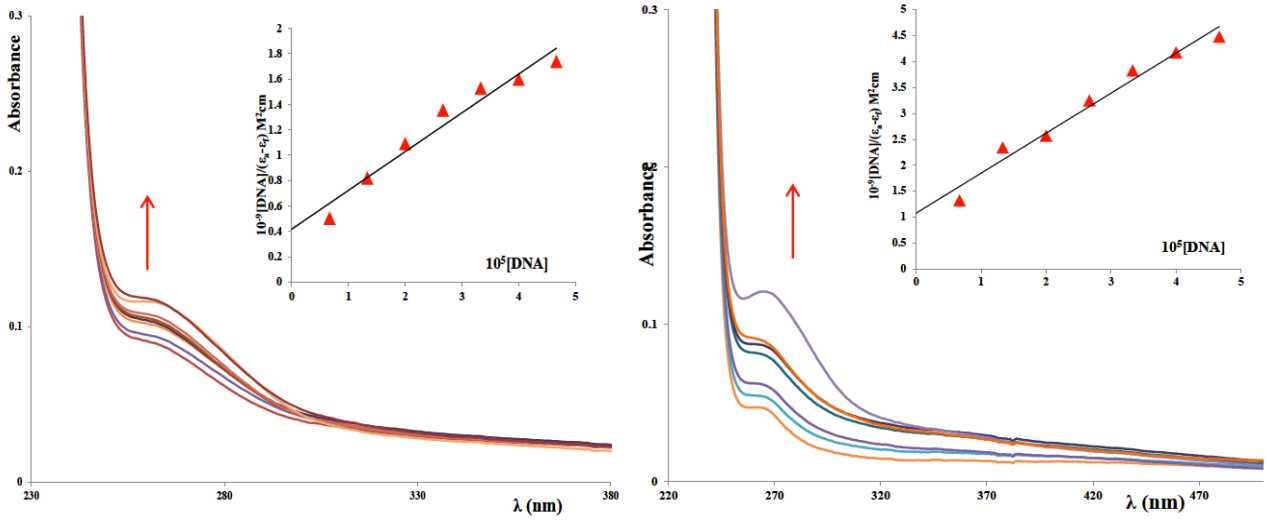


Fig. (2). Absorption spectra of the complexes Pd-Se1 and Pd-Se2 in 0.01 M phosphate buffer after addition of CT-DNA solution. [complex] = 8 μM, [CT-DNA] = 0-40 μM. The arrow indicates a change in the absorbance with an increase in DNA concentration. Inserted graph: plots of $[DNA]/(\varepsilon_a - \varepsilon_f)$] vs. [DNA].

Table 1. DNA binding constants and Stern-Volmer constants for different Pd-Se complexes.

	DNA				
	K_b [M^{-1}]	K_{sv}[M^{-1}]	K_{bin}[M^{-1}]	*n*	Ref.
Pd-Se1	$(7.5 \pm 0.1) \times 10^5$	$(1.3 \pm 0.1) \times 10^5$	$(5.0 \pm 0.1) \times 10^7$	1.6	This paper
Pd-Se2	$(8.0 \pm 0.1) \times 10^5$	$(9.6 \pm 0.1) \times 10^5$	$(1.1 \pm 0.1) \times 10^7$	1.5	This paper
1	$(5.2 \pm 0.1) \times 10^5$	$(1.6 \pm 0.1) \times 10^5$	/	/	37
2	$(4.4 \pm 0.1) \times 10^5$	$(1.1 \pm 0.1) \times 10^5$	/	/	37
Pd-Se3	$(3.9 \pm 0.1) \times 10^5$	$(0.9 \pm 0.1) \times 10^5$	/	/	37

complexes Pd-Se2 and Pd-Se1. [37] For the difference in K_b's values, the nature of the ligand's ring is probably responsible, *i.e.*, five-membered rings have higher values of K_b. [37].

3.2.2. Fluorescence Spectroscopic Methods

Based on spectroscopic absorption measurements, it has been found that the investigated complexes interact efficiently with the DNA molecule, but only absorption measurements are insufficient to confirm the way of interaction between the complexes and the DNA chain. Fluorescence measurements were subsequently made to confirm the interaction between the newly examined complexes and the DNA. Ethidium bromide (EB) is a molecule that shows a small intensity of fluorescence as a free molecule. However, EB is a classical intercalator showing a significant intensity of fluorescence emissions at about 600 nm when intercalated between the DNA strands. [61] When the EB molecule from the EB-DNA pair is replaced by another molecule, the fluorescent emission decreases. Changes that occur in the EB-DNA spectra after the addition of different complex concentrations are used to examine complex and DNA interactions namely, if the complex can intercalate into the DNA chain stronger than EB, it will replace the EB molecule in the DNA chain, which would lead to a reduction in the fluorescent emission of EB-DNA compounds. [62] In Figure 3, the dependence of the intensity of EB-bound emission related to DNA in the absence and presence of the complex in the wavelength function for the Pd-Se1 and Pd-Se2 complex is presented. The addition of solutions of the Pd-Se1 and Pd-Se2 complexes of increasing concentrations leads to a significant reduction in the emission intensity to about 610 nm, indicating competition between the complex and the EB in binding to the DNA (Fig. **3**). [63,64] Reduce in the DNA-EB complex's fluorescence intensity indicates that the tested complexes can suppress EB from the EB-DNA compound, *i.e.*, that the Pd-Se1 and

Pd-Se2 complexes exhibit affinity for binding to the DNA molecule, which agrees with the results obtained by UV-Vis spectroscopy.

The Stern-Volmer constants (K_{sv}) are calculated using the Stern-Volner equation (2). The investigated complexes show high values for the K_{sv} constants of the same order of magnitude (10^5 M^{-1}) (Table 1), indicating the possibility that the complexes replace EB and interact with the DNA molecule,

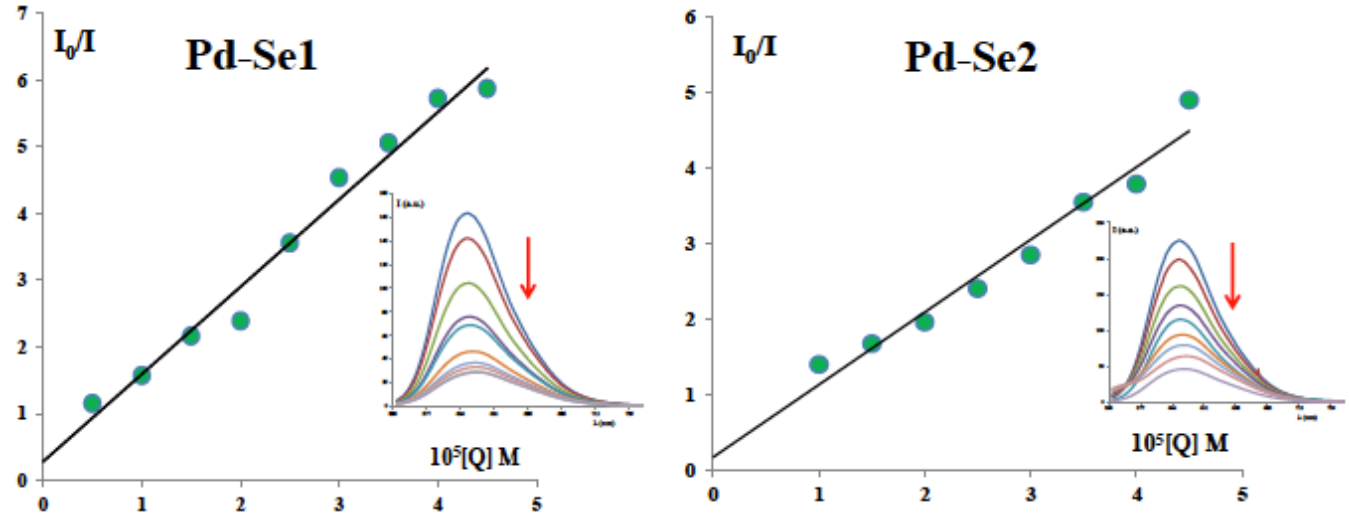


Fig. (3). Dependence of I_0/I from the concentration of [Q] (Q = complex), where the experimentally obtained points are represented with (●), and full lines represent linear dependence. Embedded graph: Emission spectra of EB-DNA in the presence of the complexes Pd-Se1 and Pd-Se2. [EB] = 10 μM, [DNA] = 10 μM, [complex] = 0-10 μM; λ_{ex} = 527 nm. Arrows show changes in intensity after adding solutions of a growing concentration complex.

which is in agreement with the values of the binding constants K_b obtained by UV-Vis spectroscopy. From the results presented in Table 1. the complex Pd-Se2 shows a slightly higher affinity for competition with the EB molecule than the Pd-Se1 complex. The investigated complexes also show a higher affinity for intercalation with the DNA molecule than previously examined structurally similar complexes(Table **1**).

The obtained data based on the fluorescence measurement can also be used to determine the number of binding sites for the complex to the DNA molecule, n, and the equilibrium binding constant, K_{bin}, based on the Scatchard [65] equation S1 (see supporting information). The obtained values are shown in Table 1 and Figure S3. For n, the values of 1.6 and 1.5 indicate one binding site for the complex on the DNA molecule. Based on the obtained values of constants (Table 1), we can assume that the most probable way of interaction between the investigated complexes and the CT-DNA molecule is intercalation.

3.3. Viscosity

In order to further confirm that the synthesized complexes interact with the DNA molecule, the viscosity of the DNA solution was measured in the presence and absence of the increasing concentrations of the Pd-Se1 and Pd-Se2 complexes (Figure S4). Adding rising concentrations (up to r = 1.0) of the Pd-Se1 and Pd-Se2 complexes to the CT-DNA solution (0.01 mM) led to an increase in the relative viscosity of the DNA, which was most pronounced after the addition of the Pd-Se2 complex. In classical intercalation, the compound is inserted between the pairs of DNA bases, which lead to an increase in the length of the DNA chain and, therefore, the viscosity of the DNA solution. The intercalation strength is usually proportional to the increase in the viscosity of the DNA. Accordingly, the observed viscosity increase for the complexes Pd-Se1 and Pd-Se2 is another evidence of intercalation with the DNA molecule, most likely between the phenyl group of the complex and the purine and pyrimidine bases of DNA.

3.4. Protein Binding Studies

Serum albumin is the most common protein in the blood plasma. Its role in the organism is multiple, for instance, to transport and storage exogenous substances. For this reason, it is important to examine the interaction of serum albumin with metal ion complex. Bovine serum albumin (BSA) is a suitable model of the molecule for testing because it is structurally like human serum albumin (HSA). [65,66] The bovine serum albumin solution showed an intense fluorescent emission at λ_{em}, max = 352 nm when excited at 295 nm. The addition of the Pd-Se1 and Pd-Se2 complexes to the serum albumin solution showed a reduction in fluorescence to about λ = 363 nm, which is shown in Figure 4. The resulting reduction in fluorescence can be attributed to changes in the tertiary structure of proteins resulting from changes in the tryptophan environment in the serum albumin due to the protein-complex binding. [67]

The values of the Stern-Volmer constant (K_{sv}) for the interactions of the complexes Pd-Se1 and Pd-Se2 with serum albumin were determined using the Stern-Volmer equation (2), where I_0 is the initial intensity of fluorescence of tryptophan in the albumin, I is the intensity of fluorescence of tryptophan in the albumin after the addition of the complex, and [Q] is the concentration of the complex. K_{sv} can be calculated from the linear dependence of I_0/I to [Q] (Figure 4). The values of the K_{sv} constant for the interaction of the studied complexes with serum albumin are given in Table 2. Based on the Scatchard [65] equations S1 (see supporting information), the number of binding sites, n, and the equilibrium binding constant, K_{bin}, were determined and are shown in Table **2**.

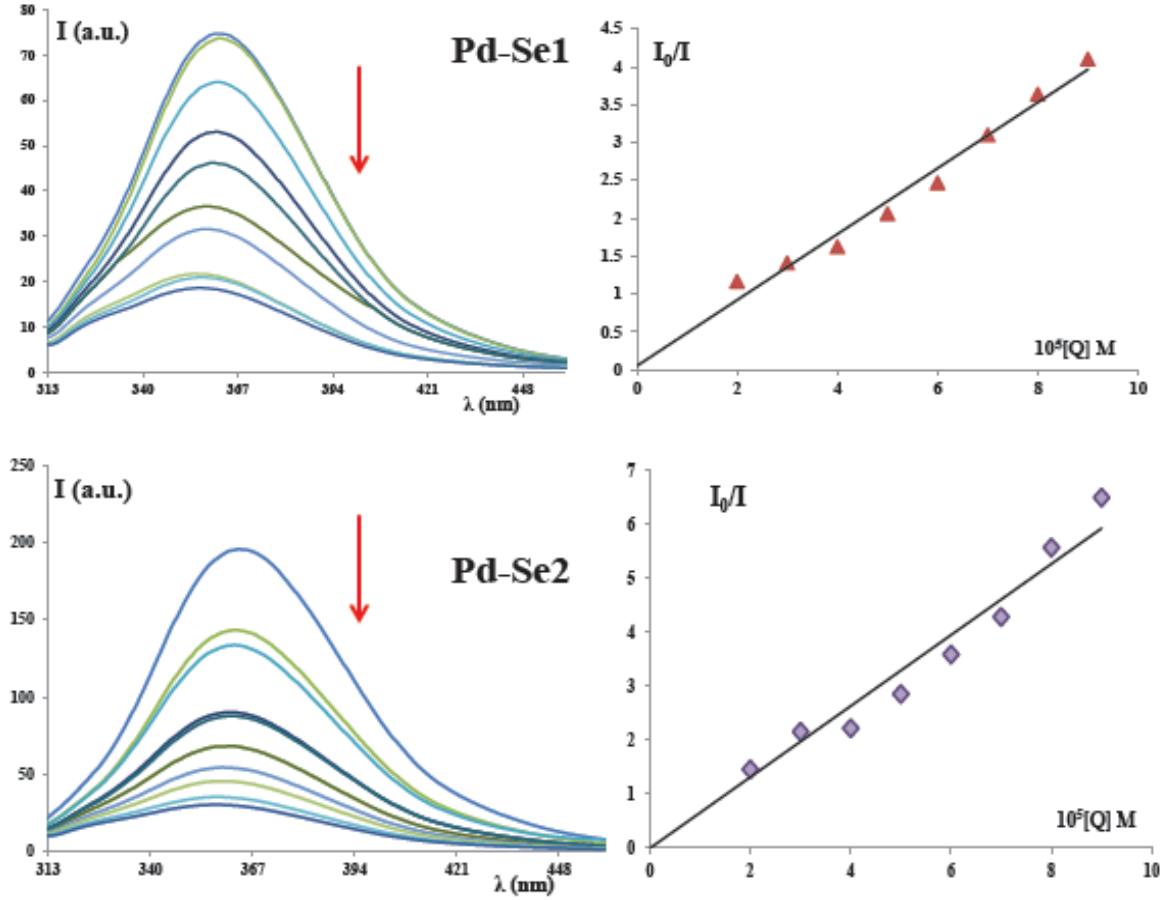


Fig. (4). Emission spectra of serum albumin in the presence of the Pd-Se1 and Pd-Se2 complexes [serum albumin] = 2 μM, [complex] = 0-10 μM, λ_{ex} = 295 nm. Arrows show changes in intensity after adding complex solutions of the growing concentration; The dependence of I_0/I on the concentration [Q] (Q = complex), where the experimental points are presented by (●), and the full lines represent the linear dependence.

Table 2. BSA binding constants for investigated Pd-Se complexes.

	BSA		
	K_{sv}[M^{-1}]	K_{bin}[M^{-1}]	*n*
Pd-Se1	$(4,4 \pm 0.1) \times 10^6$	$(3,6 \pm 0,1) \times 10^{12}$	2.4
Pd-Se2	$(6,6 \pm 0.1) \times 10^6$	$(4,8 \pm 0,1) \times 10^8$	1.6

It can be noted that the investigated Pd-Se complexes interact very well and exhibit high affinity with binding to serum albumin, Table 2. A graphical representation of the obtained values for K_{sv} constants for all investigated complexes with DNA and serum albumin is shown in Fig. (**5**). The obtained data indicate that the investigated complexes show a significant binding affinity for DNA and serum albumin. Complexes Pd-Se1 and Pd-Se2 have a higher binding affinity for serum albumin compared to DNA, Tables **1** and **2**, Fig. **5**. The most likely mechanism for the interaction of DNA complexes examined based on the obtained data is intercalation. A slightly higher affinity for DNA molecules and serum albumin shows the Pd-Se2 complex compared to the Pd-Se1 complex.

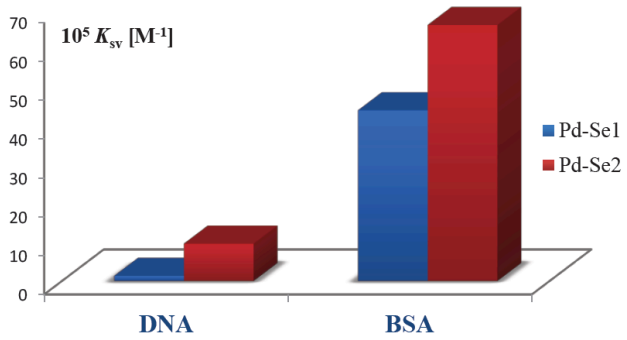


Fig. (5). The obtained K_{sv} values for investigated complexes with BSA or CT-DNA.

3.4. *In vitro* Antimicrobial Activity

The results of *in vitro* antimicrobial activity of studied compounds against 17 strains of bacteria and fungi, with control results, were determined by the microdilution method. The results are presented in Table S1. Inhibitory effects of 10% DMSO on microorganism's growth were not observed. Generally, the compounds showed different degrees of antimicrobial activity.

The intensity of antimicrobial activity varied depending on the type of substances. According to the previous research, complexes Pd-Se1 and Pd-Se3 have significantly higher activity than the corresponding ligands. [42,68,69] MICs and MMCs values were in the range from 15.63 to >1000 μg/mL, which agrees with previous research. There is no difference in the antimicrobial activity of the tested compounds between Gram-negative and Gram-positive bacteria. Complexes Pd-Se1 and Pd-Se3 showed the most significant activity on Pseudomonas aeruginosa. Activities of Pd-Se1 and Pd-Se3 were better than that of the positive control. The Pd-Se3 complex also had significant activity on *P. aeruginosa* standard and *Staphylococcus aureus* standard and isolate. The Pd-Se1 complex has significant activity on filamentous fungi (*Trichoderma viridae* ATCC 13233 and *genus Aspergillus*), and that activity was either in the range of the positive control or even better. These results are in accordance with previously done research. [36] The values of MIC and MBC observed in this study agree with those reported for a similar set of microorganisms. They were treated with Pd(II) complexes with sulfur and nitrogen donor ligands, Schiff bases ligands. [70] Significantly lower antifungal activity of palladium(II) complexes was demonstrated. [71,72] Only a study in 2012 showed that palladium(II) complexes with a thiosalicylic acid derivative as a ligand have significantly higher antifungal activity against the genus species Aspergillus than the positive control, fluconazole.[70] Antimicrobial testing of selenite was rare and sporadic. It was found that sodium selenite has an inhibitory effect on Helicobacter pylori.[73] No antibacterial effect of sodium selenite was shown on the species Bacillus subtilis, Bacillus mycoides, Escherichia coli, and Pseudomonas sp. Sodium selenite may affect the inhibitory effect of antibiotics on bacterial growth. That influence is due to the dynamics of growth through a lower population density and a lower amount of extracellular proteins. In the presence of sodium selenite, the inhibitory effect on the bacterial growth of ampicillin, and streptomycin increases. [35]

3.5. The Effect of PdSe1, PdSe3, L1, and L3 on Formed Biofilm of Selected Bacteria

The effect of complexes Pd-Se1 and Pd-Se3 and ligands L1 and L3 on formed biofilm of *S. aureus* ATCC 25923, S. aureus and *P. aeruginosa* was determined, and the results are presented in Table **3**.

In general, tested complexes showed a lower effect on the formed biofilm of tested bacteria than the tested ligands (Table **3**). Complex Pd-Se1 showed a better effect on tested biofilm of *S. aureus* (BIC50 at 500 μg/ml), while complex Pd-Se3 showed a significant effect on tested biofilm of *P. aeruginosa* (BIC50 at 125 μg/ml). BIC50 for ligand L3 was in the range of 780-1000 μg/ml, while for ligand L1, was in

Table 3. The effect of PdSe1, PdSe3, L1 and L3 on formed biofilm of selected bacteria. Values are given as BIC50.

Species / Tested compounds (μg/mL)	L1	PdSe1	L3	PdSe3
Staphylococcus aureus ATCC 25923	˃1000	˃1000	780	˃1000
Staphylococcus aureus	768.27	500	1000	˃1000
Pseudomonas aeruginosa	690.8	˃1000	1000	125

Table 4. Score values for DNA docking with investigated complex PdSe1-3.

DNA Docking				
PDB ID of DNA	**Complex**	**MolDock**	**Rerank**	**Docking**
1BNA - canonical gap	PdSe1	-156.86	-68.35	-132.39
	PdSe2	-175.17	-92.33	-171.83
	PdSe3	-135.31	-68.35	-132.39
1Z3F - intercalation gap	PdSe1	-167.58	-82.58	-160.72
	PdSe2	-169.99	-88.18	-167.72
	PdSe3	-129.05	-71.65	-123.81

the range of 690.8-768.27 μg/ml. Only *S. aureus* ATCC 25923 showed resistance to ligand L1. The antibiofilm effect of 1,2,3-triazole and Pd nanoparticles was confirmed on Pseudomonas aeruginosa [74], and palladium(II) complex with terpyridine as a ligand.[75]

3.6 Molecular Docking

Molecular docking simulations are powerful tools to determine the structural compatibility between the investigated complexes and the target molecules (in our case, DNA, BSA, DNA Gyr, and TyrRS), their interaction mode and surrounding. These results can be compared with experimental findings and further put light on the structural activity of docked compounds towards the targeted biomolecules.

Investigated complexes PdSe1-3, were docked in two different DNA fragments, one representing canonical B-DNA (PDB 1BNA) and the second DNA fragment with an intercalation gap (PDB 1Z3F). 1BNA is the crystal structure of a synthetic DNA dodecamer, while 1Z3F is the crystal structure of a 6 bp DNA fragment in complex with an intercalating anticancer drug, ellipticine. Comparing these results, we are opposing the favorable interaction ability of investigated complexes. The best-docked poses of complexes with DNA are displayed in Figs. (**6**) and (**7**) with the top-ranked poses according to used scoring functions displayed in Table **4**.

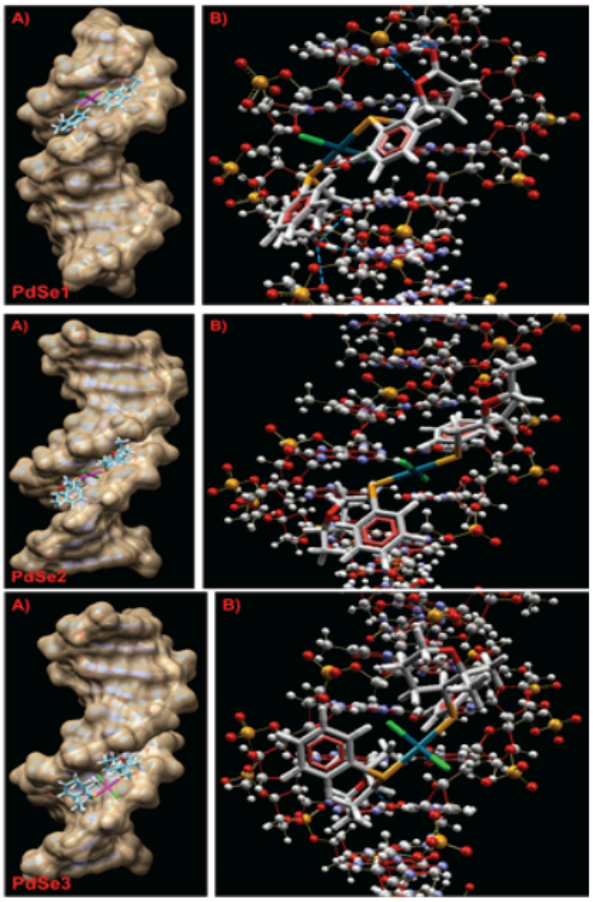


Fig. (6). A) Computational docking model illustrating interactions between investigated complexes PdSe1-3 and DNA with the canonical gap; B) Possibilities of formation a hydrogen bonds (blue dotted lines) between investigated complexes and DNA fragments.

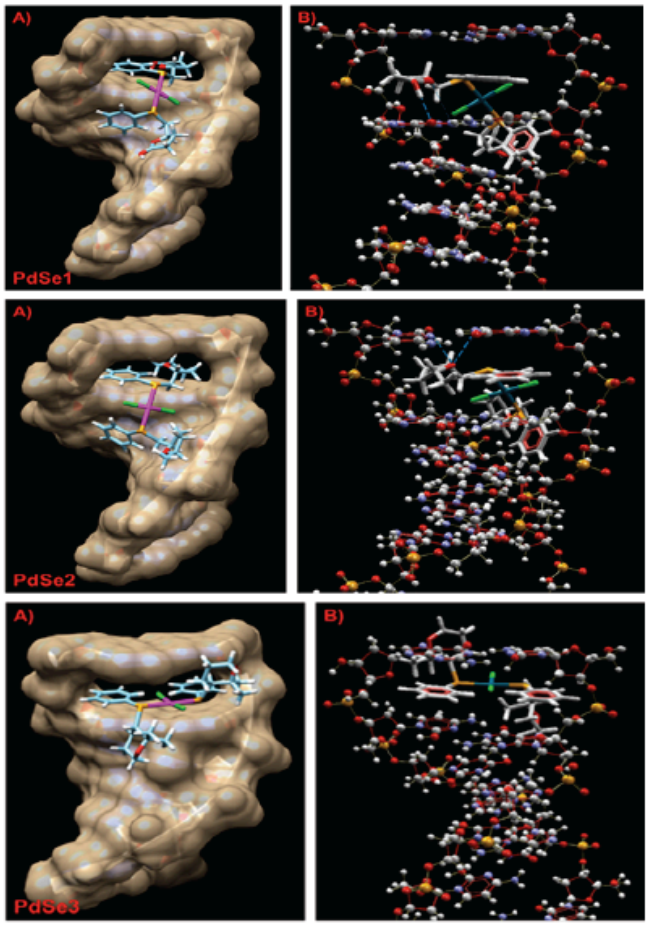


Fig. (7). A) Computational docking model illustrating interactions between investigated complexes PdSe1-3 and DNA with the intercalation gap; B) Possibilities of formation a hydrogen bonds (blue dotted lines) between investigated complexes and DNA fragments.

Based on molecular docking scores of investigated complexes, PdSe1-3 exhibited a very similar ability to interact with both DNA fragments, where PdSe1 is a better intercalator, while PdSe2 and PdSe3 have proven to be better minor groove binders. In general, binding to both DNA fragments follows the order PdSe2 > PdSe1 > PdSe3 which aligns with the experimental DNA binding studies.

Docking simulation with a BSA molecule reveals that the complexes PdSe1-3 are bound to the subdomain IIA (the site I) of BSA proteins which is consistent with the experimental data by which with the increasing amount of complex, a fluorescence quenching was observed due to the interaction between the complexes and Trp-213 residue. Interaction results between investigated complexes and BSA protein are illustrated in Fig. (**8**), while top-ranked poses according to the used scoring functions are presented in Table **5**.

From gain docking score values, all investigated complexes are well accommodated into the binding pocked located at the site I of BSA protein. Most prominent interaction is formed with the amino acid residues Ser-453(H), Tyr-340, Gln-220(H), Val-343(H), Asp-450, Arg-194, Leu-197, Arg-198, Trp-213, Ser-201, Leu-480(H), Ser-343 with

Table 5. Top-score values for investigated complex PdSe1-3 with BSA proteins.

BSA Docking					
PDB ID	**Complex**	**MolDock**	**Rerank**	**Hbond**	**Docking**
4F5S – bovin serum albumine	PdSe1[a]	-149.37	-112.44	-6.07	-157.99
	PdSe1[b]	-143.49	-48.57	-8.48	-143.86
	PdSe2[a]	-145.58	-99.29	-4.57	-143.17
	PdSe2[b]	-145.37	-93.12	-5.19	-144.71
	PdSe3[a]	-116.35	-86.91	-2.24	-116.42
	PdSe3[b]	-110.85	-78.77	-5.59	-109.11

[a]Best complex pose according to MolDock, Docking, and Rerank scoring functions.
[b]Best complex pose according to Hbond scoring function.

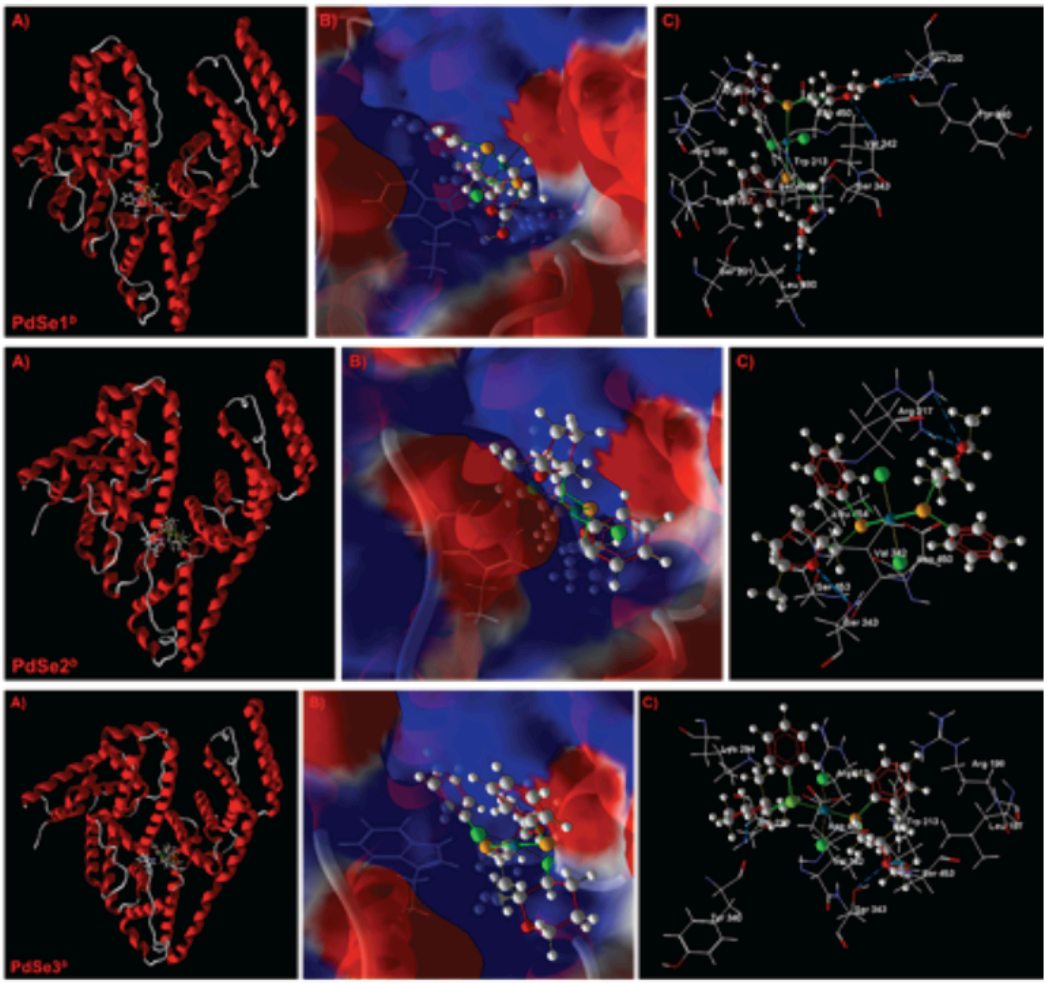


Fig. (8). Best poses with BSA for complexes PdSe1-3 [a]MolDock, Docking, and Rerank scoring functions, and [b]H-bond values: A) molecular docking results illustrated regarding the BSA protein's backbone; B) complex embedded inside the active site of BSA proteins in the electrostatic view; C) binding site of investigated complexes on BSA protein and selected amino acid residues represented by stick models. Hydrogen bonds are shown in blue dotted lines.

complex PdSe1, Arg-217(H), Val-342, Leu-454, Ser-343(H), Asp-450, Ser-453 with complex PdSe2 and Leu-197, Val-342, Ser-343(H), Ser-453(H), Trp-213, Arg-198, Asp-450, Lys-294, Tyr-340, Gln-220(H), and Arg-217 in the case of complex PdSe3 ((H) indicating a hydrogen bond formation (Fig. **8**). This kind of hydrogen bond contributes to the additional stabilization of the drug/BSA-complex and is considered one of the major factors for the successful transporta-

Table 4. IC_{50} values (μM) of the investigated substances.

IC_{50} [μM]		
Compound	**HCT-116**	
	24 h	**72 h**
L1	>500	>500
L2	>500	238.35
L3	>500	>500
Pd-Se1	>500	218.78
Pd-Se2	>500	81.85
Pd-Se3	>500	>500

Inhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate experiment.

tion of the drug through the body. Results obtained by performing the molecular docking simulations are in good agreement with the result obtain in experimental albumin binding studies and follow the order PdSe2 > PdSe1 ≥ PdSe3 by regarding an H-bond score function.

Topoisomerase II DNA gyrase [76] and tyrosyl-tRNA synthetase [77] represent an attractive target enzyme for finding a new antibacterial agent. DNA topoisomerases catalyze changes in the DNA topology and are essential for cell survival. DNA gyrase is a type II topoisomerase that can introduce negative supercoils into DNA by ATP consumption. It is present in all bacteria but is absent in higher eukaryotes, making it an attractive target for antimicrobial agents. [78] TyrRS belongs to the aminoacyl-tRNA synthetases (aaRSs) and is responsible for catalyzing the covalent binding of amino acids to their respective tRNA to form charged tRNA. Thus, inhibition of aaRSs affects cell growth due to their crucial role in the protein biosynthesis process. Molecular docking results between investigated complexes and DNA Gyr/TyrRS are illustrated in Figures S6 and S7, while top-ranked poses according to the used scoring functions are presented in Tables S2 and S3, respectively.

Docking results against DNA Gyr and TyrRS indicated a well-conserved binding region. For both enzymes, the interaction trend follows the order of complexes PdSe2 > PdSe1 > PdSe3, where the observed trend in binding is consistent with the experimental inhibition trend for the complexes. Summarizing the results, it can be concluded that the driving force behind a prominent activity of a complex PdSe2 is the ability to better adapt to the binding cavities of the proteins and form more stable hydrogen bonds with the amino acid residues. In the case of DNA Gyr, noticeable interactions are formed with amino acid residues Trp-592(D), Asp-589(D), Ser-449(D)(H), Asp-448(D)(H), Ser-445(D), Leu-1298(B), Ala-588(D), Gly-1111(B), Ser-1112(B)(H) with complex PdSe1, Leu-1298(B)(H), Ser-445(D), Arg-447(D), Ser-449(D)(H) with complex PdSe2 and Thr-1296(B), Gly-441(D), Ser-445(D), Asp-589(D), Ala-588(D)(H), and Ser-1112(B) in the case of complex PdSe3 ((H) indicating a hydrogen bond formation and (D) or (B) protein chain (Figure S6). For the TyrRS protein, amino acids involved in the interactions with studied complexes that should be pointed out are Gly-49(H), Thr-42, Val-191, Gln-190, Cys-37, Ala-39, Asn-124(H), Gly-72, Asp-40(H), Thr-75(H), Gln-174, Gly-38 for PdSe1 complex, Gln-190, Cys-37(H), Gly-38(H), Gly-193, Gly-49 in the case of PdSe2 complex and Asp-40, Val-191, Tyr-36, Gln-190, Gly-38, Pro-53, and His-50 in the case of PdSe3 complex. ((H) indicating a hydrogen bond, Figure S7).

3.7. *In vitro* cytotoxic activity

The cytotoxic activity of investigated complexes and corresponding ligands was evaluated in human colorectal cancer cell line HCT-116, by MTT assay after 24 and 72 hours of treatment. [79] Results are presented as a percentage of viable cells (Figure S5), while cytotoxic effects are expressed as IC_{50} values (Table **4**). Tested substances reduce cell viability in a dose- and time-dependent manner and showed moderate cytotoxic activity against HCT-116 cells. According to the obtained results, the effects on the cancer cells indicated IC50 higher than 500 μM after 24 h of treatment, while some treatments showed significant cytotoxic activity after 72 h. It is noticed that complex PdSe2 has the most potent effect on investigated cell lines among all our treatments, with IC50 lower than 100 μM.

CONCLUSION

New *trans*-palladium(II) complexes with organoselenium ligands named Pd-Se1 and Pd-Se2 were synthesized and characterized. Also, an already known structurally similar trans-palladium(II) complex named Pd-Se3 was studied.

The obtained data indicated that the investigated complexes show a significant binding affinity for both DNA and serum albumin, with a higher binding affinity for serum albumin compared to DNA. The most likely mechanism for the interaction of DNA with the examined complexes based on the obtained data is intercalation. The intensity of antimicrobial activity varied, with the complexes Pd-Se1 and Pd-Se3 having higher activity than the corresponding ligands. There is no difference in the antimicrobial activity of tested compounds between Gram-negative and Gram-positive bacteria. Complexes Pd-Se1 and Pd-Se3 showed the most significant activity on *Pseudomonas aeruginosa*, with the activ-

ities of Pd-Se1 and Pd-Se3 being better than that of the positive control. Complex Pd-Se1 showed a better effect on tested biofilm of *S. aureus*, while complex Pd-Se3 showed a significant effect on tested biofilm of *P. aeruginosa*. The fact that Pd(II) complexes exerted better activity against HCT-116 cells in comparison to ligands is not surprising because of the effect of palladium. Complex Pd-Se2 shows the most significant effect and could be considered for detailed investigations on HCT-116 and other cancer cell lines.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used in this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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