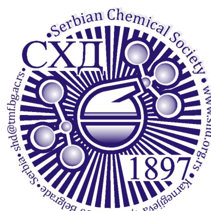

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DNA/BSA interactions and cytotoxic studies of tetradentate *N*,*N*,*O*,*O*-Schiff base copper(II) complexes

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Abstract: Three Schiff base Cu(II) complexes, (*N*,*N'*-*bis*(acetylacetone)propylenediimine)copper(II) complex, [Cu(acac$_2$pn)] (**1**), (*N*,*N'*-*bis*-(benzoylacetone)propylenediimine)copper(II) complex, [Cu(phacac$_2$pn)] (**2**) and (*N*,*N'*-*bis*-(trifluoroacetylacetone)propylenediimine)copper(II) complex, [Cu(tfacac$_2$pn)] (**3**), were used to investigate the interactions with calf thymus DNA (ct-DNA) and bovine serum albumin (BSA) using the electronic absorption and spectroscopic fluorescence methods. UV–Vis absorption studies showed that studied complexes interact with DNA molecule and exhibit moderate binding affinity. Fluorescence studies of complexes **1**–**3** also showed a possibility for DNA intercalation as well as a relatively high binding ability toward BSA. Among the tested complexes, the highest affinity for DNA and BSA molecules was shown by complex **1**. Cytotoxic analyses, performed on human colorectal carcinoma HCT-116 and healthy lung fibroblast MRC-5 cell lines, showed that complex **2** exhibited activity on both cell lines, while complexes **1** and **3** did not show any activity.

Keywords: antitumor; mechanism; cell line; biomolecule; affinity; drug design.

INTRODUCTION

Inorganic medicinal chemistry is an extensive interdisciplinary field in which main research areas is focused on the design and synthesis of transition metal ion complexes as new anticancer agents. Within the transition metals,

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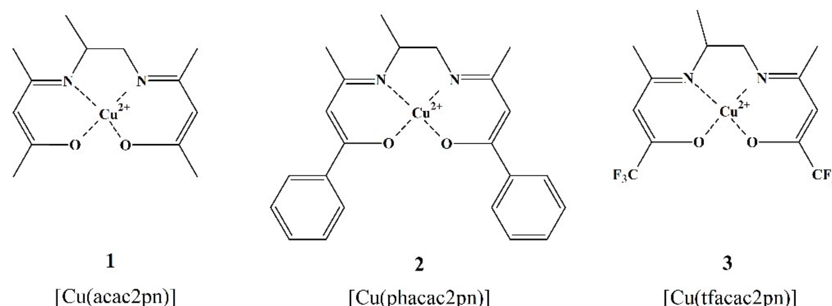
redox-active copper is of particular interest because it is endogenous to humans and has a strong affinity for nucleobases. Copper(II) complexes have shown remarkable potential due to their antimicrobial, anti-inflammatory and cytotoxic activities.[1–4]

Schiff bases are good chelating agents that are popular among the researchers because of their simple and inexpensive synthesis. Some fine-tuning of molecular electronic properties makes Schiff bases extremely attractive for the development of pharmacological agents.[5,6] Besides the fact that many transition metal ion complexes containing Schiff bases have shown excellent anticancer activity, the anticancer activity of some Schiff bases increases when they are used as ligands.[5–10] Certain Schiff base Cu(II) complexes showed potent cytotoxic activity against human colorectal and breast cancer cells.[11–14]

The primal intracellular target for most anticancer drugs is the DNA molecule.[15,16] Therefore, when developing new anticancer drugs, it is crucial to determine the compounds interactions with DNA under physiological conditions. Transition metal ion complexes interact with DNA *via* covalent or non-covalent interactions (intercalation, groove binding, and electrostatic interactions). In addition to DNA, transition metal complexes can also interact with some proteins. Serum albumin proteins are essential for the transport and delivery of pharmaceutical agents to tissues and cells.[17,18] Considering this, the study of the interactions of anticancer agents with albumin provides valuable information about the structural features of the agents and their pharmacological response. Originally, it was believed that only DNA-binding properties were relevant for determination of the anticancer activity of agents. However, protein binding properties are essential for the pharmacokinetics and pharmacodynamics of drugs.[18] Generally, noncovalent binding is the most important binding mode for anticancer drug-protein interactions.

This work aimed to study the DNA/BSA interactions of three structurally similar Cu(II) complexes (Fig. 1) in which copper(II) ion is coordinated to two nitrogen and two oxygen donor atoms.[19–21] The influence of different substi-



1 [Cu(acac2pn)] 2 [Cu(phacac2pn)] 3 [Cu(tfacac2pn)]

Fig. 1. Structures of the studied Cu(II) complexes.

tuents on the activity of complexes was determined. These studies were carried out using calf thymus DNA (ct-DNA) and bovine serum albumin (BSA). The work also shows the results of the *in vitro* cytotoxic assay against human colorectal carcinoma cells HCT-116, which are compared with the effects on healthy human MRC-5 fibroblast cells of the human pleura.

EXPERIMENTAL

Materials and methods

The complexes, (*N*,*N'*-bis(acetylacetone)propylenediimine)copper(II) complex, [Cu(acac2pn)] (**1**), (*N*,*N'*-bis-(benzoylacetone)propylenediimine)copper(II) complex, [Cu(phacac$_2$pn)] (**2**) and (*N*,*N'*-bis-(trifluoroacetylacetone)propylenediimine)copper(II) complex, [Cu(tfacac$_2$pn)] (**3**), were synthesized according to the previously published procedures.[19-21] The obtained complexes were characterized by UV–Vis, IR and mass spectrometry (LTQ Orbitrap XL), Figs. S-1–S-5 of the Supplementary material to this paper. Acetylacetone was obtained from Merck. Phosphate buffer (PBS) were obtained from Fluka. Ethanol absolute anhydrous, chloroform and dichloromethane were obtained from Carlo Erba. Copper(II) acetate hydrate was obtained from Kemika, Zagreb. Bovine serum albumin (BSA), calf thymus DNA (ct-DNA), ethidium bromide (EB) and 1,2-diaminoethane (en) were purchased from Sigma–Aldrich and were used as received without further purification. For *in vitro* experiments all reagents were of the molecular biology grade. Dulbecco's modified eagle medium (DMEM) and phosphate-buffered saline (PBS) were obtained from GIBCO, Invitrogen, USA. Fetal bovine serum (FBS) and trypsin-EDTA were from PAA (The Cell Culture Company, Pasching, Austria). Water was of Millipore quality and it was autoclaved before use in any experiments. All other chemicals were of the highest purity, commercially available, and were used without further purification.

Instrumentation

Perkin-Elmer Lambda 25 double-beam spectrophotometer with a 3.0 mL quartz cuvette and 1.0 cm path length was used for the recording of UV–Vis spectra. ct-DNA solutions were prepared by dissolving the commercially obtained ct-DNA in PBS, with a ratio of the absorbances at 260 and 280 nm between 1.8–1.9, indicating that ct-DNA was adequately free of protein.

Concentration of the ct-DNA solution was determined using A_{260} with $\varepsilon = 6600\ M^{-1}\ cm^{-1}$.[22,23] RF-1501 PC spectrofluorometer was used to record fluorescence spectra in the range 550–750 nm. Fluorescence intensity was measured at an excitation wavelength of 527 nm and emission wavelength of 612 nm. For all experiments the slit widths for excitation and emission (10 nm each) and scan rate were kept constant. Spectrophotometric analyses of cell cytotoxicity were conducted on Multiskan SkyHigh UV/Vis spectrophotometer (ThermoFisher Scientific).

DNA-binding studies

UV–Vis spectroscopy studies. The interactions of studied complexes with ct-DNA were examined using UV–Vis spectroscopy in order to determine the possible binding modes. The intrinsic equilibrium binding constants, K_b, were determined using Eq. (S-1), Supplementary material. DNA binding experiments were performed at room temperature and all absorbance measurements were made in a buffered solution (0.01 M PBS, pH 7.4). The series of solutions containing studied complexes and DNA were prepared by mixing a constant concentration of complex solutions (8 μM) with an increase of DNA concentration from 0 to 40 μM.

Fluorescence quenching studies. Fluorescence spectroscopy was also used to study the interactions of studied complexes with ct-DNA. For fluorescence determination the complex–DNA solutions were prepared by mixing constant DNA–EtBr solutions (DNA EtBr ratio was 1:1) with different concentrations of Cu(II) complex. Concentration of DNA was 25 μM, ethidium bromide (EtBr) was 25 μM while complex concentrations varied from 0 to 50 μM. The system was shaken and incubated at room temperature for 5 minutes before measurement. Stern–Volmer equation (Eq. (S-2)) was used to calculate the fluorescence quenching constant K_{sv}.

BSA-binding studies. Solutions of complexes **1**–**3** and BSA (2 μM) were prepared in 0.01 M phosphate buffer solution. During the fluorescence quenching experiments, the concentration of BSA was kept constant. The quenching of the emission intensity of the tryptophan residues in the BSA at 352 nm was followed using complexes **1**–**3** as quenchers with increasing concentration in the range of 0–30 μM. The fluorescence spectra were recorded in the range of 300–500 nm at an excitation wavelength of 295 nm. The data of the binding of Cu(II) complexes were analyzed using the Stern–Volmer equation. Observed diagrams were used to confirm the interactions of the complexes with albumin and to calculate the corresponding constants.

In vitro *cell cytotoxicity analyses*

The HCT-116, colorectal carcinoma cell line, and MRC-5, healthy lung fibroblast cell line, were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin. For *in vitro* cytotoxicity analyses, 10^4 cells per well in 96-well microtiter plates were seeded overnight and treated with complexes for 24 and 72 h. After treatment incubation, the cell viability was estimated by standard MTT assay, briefly described in our previous studies.[24,25]

RESULTS AND DISCUSSION

DNA-binding studies

The DNA molecule has long been recognized as a main binding target in the human body for the transition metal ion complexes exhibiting anticancer activity. Consequently, the first step when examining the mechanism of action of different transition metal ion complexes is usually the investigation of their affinity for DNA binding.[26–28] Electronic absorption spectroscopy is a commonly used method for verifying the extent and nature of the binding between the metal complexes and the DNA molecule. The absorption intensity of the complexes can decrease (hypochromism) or increase (hyperchromism) upon addition of the DNA, with a slight shift in absorption wavelength, indicating an interaction between complexes and DNA.[28] Within this work, the interactions of complexes **1**–**3** with ct-DNA were examined using UV–Vis titrations. During the titration, the concentrations of complex solutions were kept constant (8 μM; PBS) while the concentrations of ct-DNA solution were increasing (0–40 μM). The equilibrium binding constants, K_b, were calculated using Eq. (S-1). Fig. 2 shows the changes in the UV–Vis spectra of complex **1** in the presence of the increasing ct-DNA concentrations. The observed results for complexes **2** and **3** are shown in Fig. S-6 of the Supplementary material.

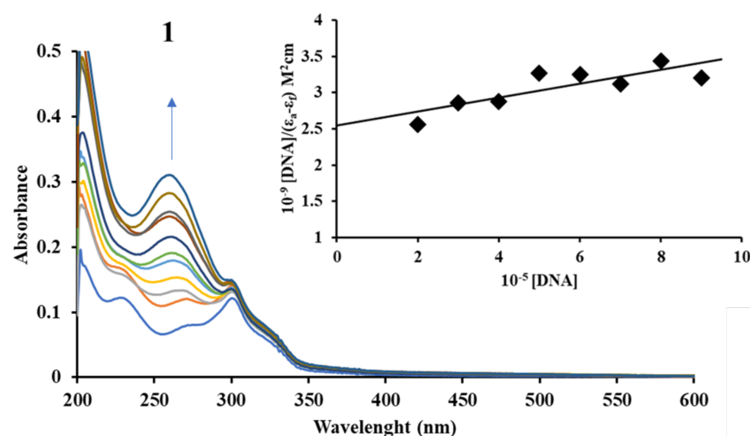


Fig. 2. UV–Vis titration spectra of 8 μM solution of complex 1 in 0.01 M PBS with the addition of the increasing ct-DNA concentration (0–40 μM). Arrow shows hyperchromism in the spectral band. Inset: plots of [DNA]/($\varepsilon_a - \varepsilon_f$) *vs*. [DNA] for the titration of the complex **1** with ct-DNA; with ■ are shown the experimental data points and the full line represents the exponential fitting of the data.

Addition of the ct-DNA solution to the solution of the studied complexes resulted in an intensity increase of the spectra shown in Figs. 2 and S-6 with a slight shift of the maximum peak (2-3 nm red shift). These observations indicate a moderate ability of the studied complexes to interact with ct-DNA, probably by external contact.[29] The intrinsic binding constants for DNA interactions of the complexes were calculated using Eq. (S1) and listed in Table I. The order of the reactivity for the complexes is **1** > **3** > **2**. Complex **1** has the highest value of K_b constant in the order of 10^4, while the other two complexes have similar values in the order of 10^3, indicating a moderate possibility for DNA interactions.

TABLE I. The obtained K_b and K_{sv} constant values for DNA and BSA interactions of complexes **1**–**3**

Complex	ct-DNA		BSA
	K_b / 10^3 M^{-1}	K_{sv} / 10^3 M^{-1}	K_{sv} / 10^4 M^{-1}
1	32±1	2.1±0.1	3.6±0.1
2	2.7±0.1	2.9±0.1	1.8±0.1
3	4.4±0.1	2.7±0.1	1.2±0.1

The fluorescence spectroscopic method is the most accurate and reliable method for studying the relative binding of small molecules to DNA. It is usually used to accurately determine if intercalation is the binding mode of complexes **1**–**3** toward ct-DNA. EtBr is a classical intercalator with significant fluorescence emission intensity at 612 nm[30] when bound to DNA. The changes observed in

the spectra of EtBr–ct-DNA are often used to study the DNA binding affinity of transition metal ion complexes. Namely, the quenching of the EtBr–ct-DNA fluorescence emission occurs upon addition of a compound capable to intercalate between the DNA strands displacing the EtBr from EtBr–ct-DNA. The fluorescence quenching curves of EtBr–ct-DNA in the absence and presence of the complex **2** is shown in Fig. 3, and for complexes **1** and **3** in Fig. S-7 of the Supplementary material. Addition of the increasing amounts (up to r = 1.0) of complexes (0–50 μM) resulted in an intensity decrease of the emission band at 612 nm, indicating competition of the complexes with EtBr in binding toward DNA. The obtained results indicate that complexes **1**–**3** displace EtBr from the aduct DNA–EtBr, exhibiting the possibility for intercalation.[31,32]

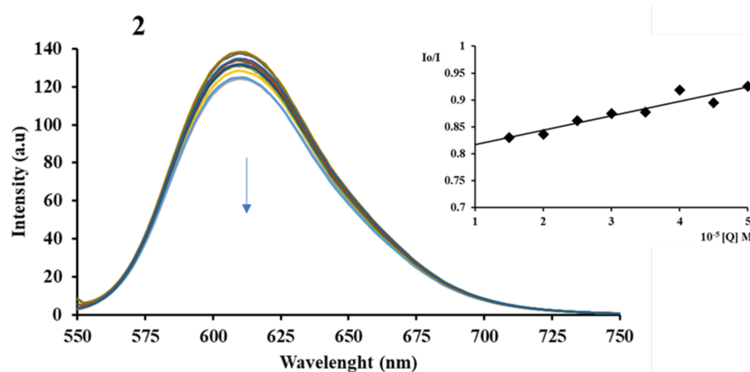


Fig. 3. Fluorescence titration spectra of EtBr–DNA (25 μM) in the presence of varying amounts of complex **2** (phosphate buffer solution = 0,01 M, pH 7.4). The arrow shows changes in fluorescence intensity upon increasing the concentration of complex (0–50 μM). Inset: plots of I_o/I *vs.* [Q]; with ■ are shown the experimental data points and the full line represents the exponential fitting of the data.

The dynamic quenching constant, K_{SV}, was determined from the slope of the linear plots by Eq. (S-2). The calculated K_{SV} values are listed in Table I. Complexes exhibited similar values of the quenching constant, indicating their moderate efficiency in the replacement of the EtBr from EtBr–DNA adduct.

BSA-binding studies

As it was mentioned before, serum albumin plays an important role in the transport and delivery of many pharmaceutical drugs to tissues and cells. Since albumin is the most abundant protein in the bloodstream, the studies of the binding of different biologically active compounds to albumin provide very useful information about their potential activity.[33,34] The interactions of metal drugs with proteins, related to the binding of complexes to albumin, are crucial for their biological distribution, toxicity and even mechanism of action. Moreover, the

binding of drugs to proteins can affect (either enhance or reduce) their biological properties. BSA is intensively studied serum albumin due to its structural homology with human serum albumin (HSA). A BSA solution shows intense fluorescence emission at 352 nm when excited at 295 nm.[35] Addition of complexes **1**–**3** to a BSA solution affects the BSA fluorescence spectra, Figs. 4 and S-8.

The observed quenching can be attributed to the changes in the tertiary structure of the protein after binding of complex. The values of the dynamic quenching constant, K_{sv}, and quenching constant, kq (M^{-1} s^{-1}) for the interaction with BSA were derived according to the Stern–Volmer quenching equation (Eq. (S2)) and listed in Table I.

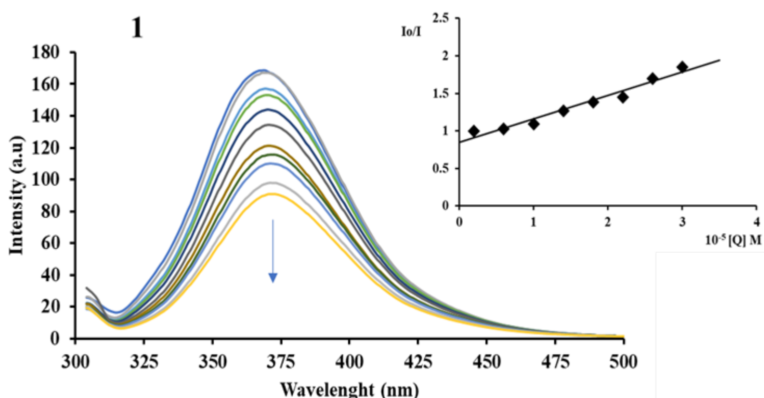


Fig. 4. Fluorescence titration spectra of BSA (2 μM) with different concentrations of complex 1 (phosphate buffer solution = 0,01 M, pH 7.4). Arrow shows the changes in the fluorescence intensity upon increasing the concentration of complex (0–30 μM). Inset: Stern–Volmer plots of the interaction of complex **1** with BSA.

The values presented in Table I are relatively high, suggesting that complexes **1**–**3** interact better with BSA than with DNA. The highest constant was determined for complex **1**, while complexes **2** and **3** showed similar values. The higher affinity of complexes **1**–**3** for the BSA molecule can also be seen from Fig. 5, which compares the K_{sv} values for the interactions with ct-DNA and BSA.

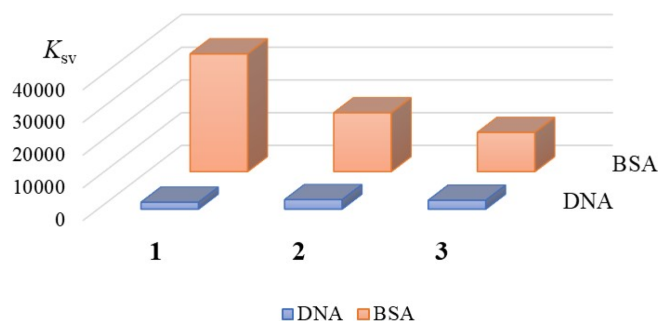


Fig. 5. Comparison of the obtained K_{sv} values for the interactions of complexes **1**–**3** with BSA and with ct-DNA.

The highest constant was determined for complex **1**, while the lowest was for complex **3**. Considering this, the greatest reactivity is shown by a complex that has two methyl groups in its structure while the lowest was shown by complex that has two CF_3 groups in the structures. Based on the obtained results it can be concluded that the binding of complexes strongly depends on steric effects of voluminous phenyl group as well as on electronic effects of CF_3 groups. Previously published results with similar Cu(II) complexes that differ in structure only by the ethylenediamine-bridge part, showed slightly higher reactivity to HSA, than the examined complexes to BSA with a similar order of reactivity.[36]

*Cytotoxicity of the complexes **1**–**3***

The observed results of the interactions of complexes **1**–**3** with DNA suggest that cells surviving treatment with these chemicals may be affected. Considering this, cancer cells and healthy cells were treated, and the results of cell viability are shown in Figs. S-9–S-14 and in Table II.

TABLE II. Cytotoxicity results (IC_{50} / μM) of the investigated Cu(II) complexes on HCT-116 and MRC-5 cells

Complex	HCT-116		MRC-5	
	Time, h		Time, h	
	24	72	24	72
1	>500	>500	>500	187.88
2	346.41	168.25	90.94	78.01
3	>500	>500	>500	>500

The complexes decreased cell viability in a concentration- and time-dependent manner. Complex **2** exerted the strongest effect on HCT-116 cells with an IC_{50} value of 168.25 μM at 72 h. Complexes **1** and **3** exerted no cytotoxic effects. On the other hand, in MRC-5 cells, we observed that complexes **2** red-

uced cell viability the most, complex 1 induced moderate effect, while complexes 3 had no effect.

CONCLUSION

Based on the results obtained by examining the interactions of complexes **1**–**3** with DNA and BSA molecules, it was found that they interact with ct-DNA molecule in a moderate manner. Moreover, the studied complexes showed good quenching of BSA fluorescence and good binding ability to this protein with relatively high binding constants. Among the tested complexes, the highest affinity for DNA and BSA molecules was shown by complex **1**, which has two methyl groups in the structure of the ligand. Cytotoxicity tests showed that complex **2** exerted the strongest effect on HCT-116 cells. These results could contribute to a better understanding of the interactions of novel transition metal ion complexes with biologically important molecules, such as DNA and proteins. In addition, this study could provide useful information as a basis for the design of new molecules with desired biological activities.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12435, or from the corresponding author on request.

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ИЗВОД

ДНК/BSA ИНТЕРАКЦИЈЕ И ЦИТОТОКСИЧНОСТ КОМПЛЕКСА БАКРА(II) СА ШИФОВИМ БАЗАМА КАО ЛИГАНДИМА

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Три комплекса Cu(II) јона са Шифовим базама као лигандима, (*N*,*N'*-*bis*-(ацетилацетон)пропилендиимин)бакар(II) комплекс, [Cu(acac$_2$pn)] (**1**), (*N*,*N'*-*bis*-(бензоилилацетон)пропилендиимин)бакар(II) комплекс, [Cu(phacac$_2$pn)] (**2**), и (*N*,*N'*-*bis*-(бензоилилацетон)пропилендиимин)бакар(II) комплекс, [Cu(tfacac$_2$pn)] (**3**) коришћени су за испитивање интеракција са ДНК (ct-ДНА) и говеђим серум албумином (BSA), користећи апсорпциону спектрофотометрију и флуоросценцију. Испитивања помоћу UV–Vis спектрофотометрије су показала да проучавани комплекси показују умерени афинитет за интеракцију са молекулом ДНК. Резултати испитивања флуоресценције комплекса **1**–**3** су показали да комплекси имају могућност интеркалације са молекулом ДНК, као и

релативно високу способност везивања за BSA. Од свих проучаваних комплекса, највећи афинитет за интеракцију са ДНК и BSA показао је комплекс **1**. Цитотоксичност комплекса испитивана је на ћелијским линијама људског колоректалног карцинома HCT-116 и на здравим фибробластима плућа MRC-5. Резултати су показали да комплекс **2** показује активност на обе ћелијске линије, док су комплекси **1** и **3** неактивни.

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