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Scientific paper

Interaction of Copper(II) Complexes of Bidentate Benzaldehyde Nicotinic Acid Hydrazones with BSA: Spectrofluorimetric and Molecular Docking Approach

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Abstract

Two copper(II) complexes of 4-chloro- and 4-dimethylaminobenzaldehyde nicotinic acid hydrazones were prepared and characterized by elemental analysis, mass spectrometry, infrared and electron spectroscopy and conductometry. These rare examples of bis(hydrazonato)copper(II) complexes are neutral complex species with copper(II) center coordinated with two monoanionic bidentate O,N-donor hydrazone ligands coordinated in enol-imine form. The interaction of hydrazone ligands and corresponding copper(II) complexes with CT DNA and BSA was investigated. Copper(II) complexes are slightly effective in binding the DNA than pristine hydrazones. The results indicate groove binding or moderate intercalation which are not significantly affected by the nature of substituent at hydrazone ligands. On contrary, affinities of two copper(II) complexes toward BSA significantly differs and depends on the nature of the substituent, however in absence of thermodynamic data difference in nature of binding forces cannot be excluded. The complex bearing electron-withdrawing 4-chloro substituent has larger affinity toward BSA compared to 4-dimethyamino analogue. These findings were theoretically supported by molecular docking study.

Keywords: Copper, nicotinic acid, hydrazone, bidentate, CT DNA, BSA

1. Introduction

Since the discovery of the cytostatic properties of cisplatin more than half a century ago, research into metal-based chemotherapeutics has increased exponentially. Essentially, today there is no metal whose complexes had not been tested as potential antitumor drugs. Although these researches were not always postulated on clear scientific motivations, these colossal expanses of progress in medicinal inorganic chemistry provided a couple of promising candidates with antitumor properties.[1] In addition to compounds of the platinum group metals, complex compounds of copper attracted a lot of attention. Copper, as a vital trace element, is cofactor of many metalloenzymes and has important functional and metabolic roles in growth, function and development of living organisms.[2,3] Compared to platinum group metals, it has a very well-studied physiological role and there are well defined pathways of its intake, transportation and elimination.[4,5] Moreover, copper compounds possess promising anticancer properties while the low cost and ease of their synthesis make it of huge interest.[6,7] Furthermore, hydrophobic nature of neutral copper(II) complexes with aromatic ligands improves its peculiar chemotherapeutic role in depositing in tumor tissues due to a selective cell membrane permeability.[8]

Hydrazones are type of Schiff base ligands which are discerned from oximes and imines by having two adjacent nitrogen atoms. Their biological activity is well documented and includes potential antibacterial,[9] antiviral,[10] antioxidant,[11] antifungal[12] and anticancer activity.[13,14] From the aspect of the coordination chemistry, aroylhydrazones are good candidates for chelation of many metal ions since their versatile coordinating ability allows coordination as bidentate or tridentate neutral, monoanionic or dianionic ligands thus providing complexes with attractive structural, magnetic, electrochemical, catalytic or biological properties.[15] Additionally, coordination of organic ligands to metal ions regularly results with improved biological properties of corresponding complexes making hydrazones as tempting choice for metal ligation. Coordination of hydrazones to copper(II) results in complexes of different nuclearities ranging from mononuclear, binuclear, tetranuclear , cuban-like or polymeric, depending on the number and sterical availability of functional groups on hydrazone core.[16–19] Hydrazones having phenolic group readily form binuclear complexes with phenolic oxygen acting as bridging ligand. Complexes of higher nuclearities most often have lower solubility compared to their mononuclear analogues and thus weaker potential for application in biological purposes. As a contribution to copper(II) hydrazone complexes chemistry we prepared two novel bis(hydrazonato)copper(II) complexes of 4-substituted benzaldehyde nicotinic acid hydrazones. The hydrazones featuring heterocyclic moieties are known for their strong biological activity,[20] while substitution on a hydrazone moiety can affect biological activity of compounds.[21,22] From biological point of view benzaldehyde derivatives including imines, hydrazones, (thio)semicarbazones and chalcones, along with their metal complexes, are well documented in literature as promising synthetic agents having wide range of biological activities including anticancer, antimicrobial, antifungal etc.[23–26] From synthetic point of view, benzaldehyde is convenient synthetic precursor for substitution to obtain substituted derivatives which can serve as models to elucidate structure-activity relationship in terms of substituent directed biological activity. These results would provide information how substitution patterns and substituents could be potentially used for manipulation and improvement of the biological activity of more structurally complex ligands.

We aimed to test how electron-accepting or electron-donating properties of substituent on benzaldehyde component of hydrazone ligand can affect the binding of ligands and corresponding copper(II) complexes to nucleic acid or albumins. Clear understanding of factors that govern the reactivity of copper(II) complexes toward biomolecules can lead to rational design of novel compounds with desirable properties, high selectivity and efficiency in disease treatments.

2. Experimental

2. 1. Chemicals

All chemicals used for syntheses, spectroscopic characterization and interaction with CT DNA and BSA were of analytical grade unless otherwise stated. Tetra-μ_2-acetatodiaquadicopper(II) (>98%) was obtained from Fluka. Nicotinic hydrazide (97%) and 4-substituted benzaldehyde (>98%) were purchased from Sigma. Highly polymerized deoxyribonucleic acid sodium salt from calf thymus (CT DNA), Type I, fibers (A_{260}/A_{280}>1.8) and lyophilized powder of Bovine serum albumin (BSA, ≥98%) were acquired from Sigma. Organic solvents were used as received from commercial sources, while dimethylsulfoxide was dried over molecular sieves (3 Å).

2. 2. Physical Measurements

Fourier transformed infrared spectra were collected in transmission mode using KBr pellet technique on a Perkin Elmer BX FTIR. Electronic spectra of complexes and ligands were recorded in DMSO (5×10^{-5} M) using Perkin Elemer BioLambda 35 in 260–1100 nm range. Fluorimetric measurements were done on PerkinElmer LS55 Luminescence. Elemental analyses of C, H and N content were performed on a Perkin Elmer 2400 Series II CHNS analyzer. Copper content in complexes was determined by spectrophotometry using neocuproin method.[27] Mass spectra were collected from DMSO solution of complexes using Shimadzu LCMS-2020. Conductometry measurements were done in DMSO solution using Phywe conductometer.

2. 3. Syntheses of Hydrazone Ligands

Hydrazone ligands were prepared following previously reported procedure.[28,29] Nicotinic hydrazide (10 mmol, 1.37 g) dissolved in absolute ethanol (15 mL) was added to a stirring solution of equimolar amount of appropriate 4-substituted benzaldehyde (10 mmol, 1.41 g for 4-chlorobenzaldehyde nicotinic acid hydrazone, hereinafter HL^1, 1.49 g for 4-(dimethylamino)benzaldehyde nicotinic acid hydrazone, hereinafter HL^2) in ethanol (10 mL). One drop of concentrated sulfuric acid was added and the reaction mixture was kept under reflux for 3 hours. Upon cooling to room temperature corresponding hydrazone ligands separated out and were filtered off, washed with ice-cold ethanol and dried in vacuum. Yields: 1.87 g (72%) for HL^1 and 2.02 g (75%) for HL^2.

2. 4. Syntheses of Copper(II) Complexes

To a vigorously stirred methanol solution (20 mL) of tetra-μ_2-acetatodiaquadicopper(II) ($[Cu_2(OAc)_4(OH_2)_2]$, 100 mg, 0.25 mmol) a methanol solution of appropriate hydrazone ligand was added (1 mmol, 260 mg for $[Cu(L^1)_2]$, hereinafter (**1**) and 268 mg for $[Cu(L^2)_2]$, here-

inafter (**2**)). The reaction mixture was refluxed for 2 hours and left standing overnight. Green-brown powders of copper(II) complexes were collected by suction filtration, washed thoroughly with cold methanol and dried in vacuum overnight.

bis(*N*'-4-chlorobenzylidenenicotinohydrazonato-κ^2*N*',*O*) copper(II) dihydrate (**1**). Green powder. Yield: 222 mg (72%). *Anal. Calc.* (%) for $C_{26}H_{22}Cl_2CuN_6O_4$ (M_r = 616.94): C, 50.62; H, 3.59; N, 13.62; Cu, 10.30; *Found* (%): C, 50.70; H, 3.44; N, 13.64; Cu, 10.11. MS for $[C_{26}H_{19}Cl_2CuN_6O_2]^+$ *Found* (*Calc.*): 582.05 (582.03). IR (KBr) ν(cm^{-1}): 3437 ν(O–H), 1608 ν(C=N'N), 1526 ν(C=NN'), 1340 ν(C–O_{enol}), 1158 ν(N–N). UV/Vis (DMSO) λ_{max} / nm (log ε): 316 (4.58).

bis[*N*'-4-(dimetylamino)benzylidenenicotinohydrazonato-κ^2*N*',*O*]copper(II) dihydrate (**2**). Green-brown powder. Yield: 203 mg (64%). *Anal. Calc.* (%) for $C_{30}H_{34}CuN_8O_4$ (M_r = 634.19): C, 56.82; H, 5.40; N, 17.62; Cu, 10.02; *Found* (%): C, 57.40; H, 4.90; N, 17.40; Cu, 9.94. MS for $[C_{30}H_{31}CuN_8O_2]^+$ *Found* (*Calc.*): 598.20 (598.20). IR (KBr) ν (cm^{-1}), intensity: 3435 ν(O–H), 1598 ν(C=N'N), 1509 ν(C=NN'), 1317 ν(C–O_{enol}), 1163 ν(N–N). UV/Vis (DMSO) λ_{max} / nm (log ε): 403 (4.67).

2. 5. Interaction with CT DNA

Interaction of hydrazone ligands and corresponding copper(II) complexes was carried out in 10 mM Tris-HCl buffer pH 7.42 using method of spectroscopic titration. Stock solution of CT DNA was prepared in 10 mM Tris-HCl buffer pH 7.42 by continuous stirring of CT DNA during 24 hours to assure proper hydratation and to obtain millimolar concentration of stock solution. The concentration and purity of CT DNA was determined based on extinction coefficient of 6,600 M^{-1} cm^{-1} at 260 nm (7.20 mM, A_{260}/A_{280} = 1.83, A_{230}/A_{260} = 0.39). Stock solutions of complexes and ligands (1 mM) were prepared in dry DMSO. Working solutions of compounds (5×10^{-5} M) were always freshly prepared by diluting aliquot (100 μL) of DMSO stock solution of compounds by Tris-HCl buffer up to a volume of 2000 μL. Thus, obtained solutions were used for spectroscopic titration of compound with DNA by successive addition of 10-microliter amounts of CT DNA stock solution and recording the electronic spectra in 250–500 nm range. The equilibration time after each addition of DNA was 2 minutes.

2. 6. Interaction with BSA

The interaction of compounds with BSA was investigated in 10 mM Tris-HCl buffer solution by spectrofluorimetric titration of BSA with ligands and complexes. The working solution of BSA was prepared by dissolving BSA in 10 mM Tris-HCl buffer until 1 μM solution was obtained. The concentration of BSA was determined by electron spectroscopy based on extinction coefficient of 43,824 M^{-1} cm^{-1} at 280 nm. Stock solution of ligands and complexes were prepared in methanol (1×10^{-4} M). Spectrofluorimetric titration was carried out by titrating solution of BSA (2000 μL, 1 μM) with 10-μL amounts of ligand or complex and recording emission spectra in 290–420 nm range with 278 nm as excitation wavelength. Synchronous emission spectra were collected similarly as mentioned above only in 250–310 nm range with Δλ= 15 nm and Δλ= 60 nm.

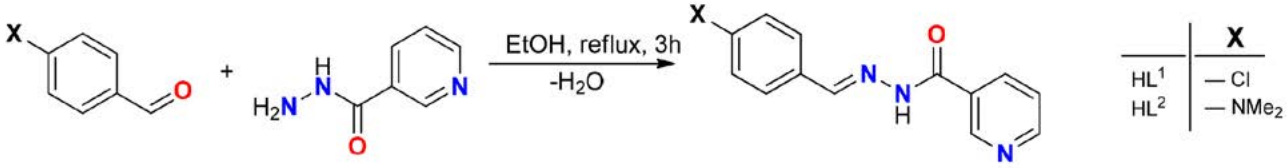
2. 7. Molecular Docking Study

The docking study was set up in YASARA Structure 19.12.14[30,31] and performed using AutoDock 4.2.[32] The crystal structure of BSA (PDB ID: 3V03)[33] from Protein Data Bank was used as the target molecule. The protein structure was prepared by removing water molecules, adding polar hydrogen atoms and optimizing in the AMBER03 force field.[34] The 3D structures of the copper(II) complexes and ligand molecules were prepared and geometries optimized by the density functional theory (DFT) (B3LYP/LanL2DZ and B3LYP/6-31G* basis sets) using Gaussian 09.[35] A blind docking approach was used, searching the whole protein for potential binding sites, with a grid point spacing of 0.375 Å for all compounds. During the docking process, copper(II) complex molecules were kept rigid, to maintain their square planar molecular geometries. The Lamarckian genetic algorithm was employed with the following parameters: 150 docking runs with a maximum of 17,500,000 energy evaluations and 27,000 generations for each run, providing this way the lowest energy docked structures.

3. Results and Discussion

3. 1. Syntheses

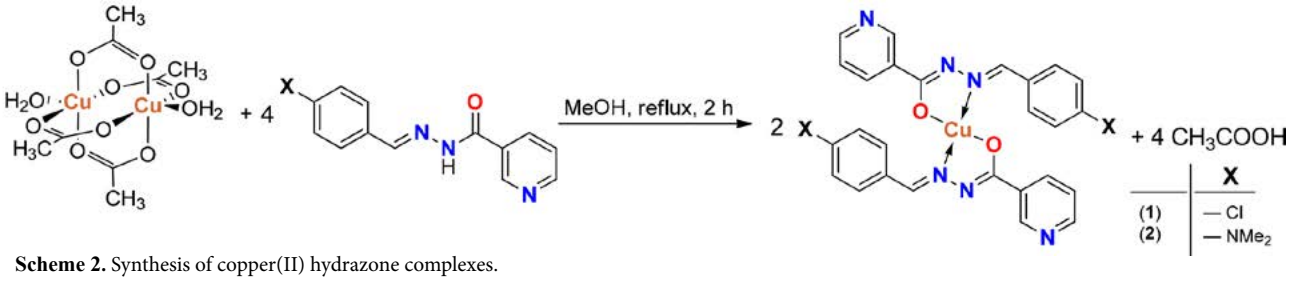
Preparation of hydrazone ligands HL^1 and HL^2 was carried out using standard condensation procedure of appropriate 4-substitued benzaldehyde with nicotinic acid



Scheme 1. Synthesis of hydrazone ligands.

hydrazide in ethanol (**Scheme 1**). The products were identified by infrared and electronic spectroscopy.[28,29]

Copper(II) hydrazone complexes were prepared by refluxing tetra-μ_2-acetatodiaquadicopper(II) and appropriate hydrazone ligand in methanol in molar ratio 1:4 (Scheme 2). Besides the fact that acetic anion is a good leaving group, in preparative chemistry of copper complexes it also behaves as a base deprotonating hydrazone ligand. The copper compounds isolated are neutral complex species having copper(II) coordinated by two monoanionic bidentate O,N-donor hydrazone ligands in enol-imine form based on elemental and spectroscopic analysis. Complexes are moderately soluble in DMSO and DMF, weakly soluble in lower alcohols, acetonitrile, dichloromethane and insoluble in water and non-polar organic solvents. All of our attempts to prepare single crystals of suitable quality for diffraction were unfortunately unsuccessful, except in case for complex **1** from pyridine. Recrystallization of **1** from pyridine afforded green prismatic single crystals of suitable size for SCXRD. However, our attempts to resolve the structure were unsuccessful due to a dynamic process in a crystal lattice during the measurements even at temperatures of liquid nitrogen.



Scheme 2. Synthesis of copper(II) hydrazone complexes.

3. 2. Characterization

Characterization of bis(hydrazonato)copper(II) complexes **1** and **2** was carried out by elemental analysis, infrared and electronic spectroscopy, mass spectrometry and conductometry. The results of elemental analysis of carbon, hydrogen, nitrogen and copper content in copper(II) complexes **1** and **2** confirm formulation, composition and purity of the prepared complexes. Theoretical and experimental mass spectra of copper(II) complexes are shown in ESI (Figures S2–S5). Isotopic distribution pattern arising from two stable copper isotopes (^{63}Cu and ^{65}Cu) is clearly seen for $[M+H]^+$ ion of both bis(hydrazonato)copper(II) complexes thus confirming the $[CuL_2]$ composition.

Infrared spectra of hydrazone ligands and copper(II) complexes **1** and **2** are shown in Figure 1. Hydrazones generally show tautomerism and can be present in keto-amine or enol-imine form. Infrared spectra of hydrazone ligands HL^1 and HL^2 in solid state showed absence of band corresponding to stretching vibrations of O–H bond and showed vibrations assigned to N–H and C=O vibrations indicating that both ligands are present in keto-amine form (Table 1). Upon coordination to copper(II) hydrazone ligands are deprotonated and bands corresponding to stretching of N-H (~3190 cm^{-1}) and deformation of N-H (1298–1305 cm^{-1}) disappear in spectra of copper(II) complexes. Moreover, the band located in spectra of hydrazone in 1663–1685 cm^{-1} range, predominantly attributed to C=O stretching, is absent in spectra of copper(II) complexes, while new band in 1317–1340 cm^{-1} range, assigned to $C–O_{enol}$ stretching, arises. If we add the fact that a new band appears in the spectra of the complex in 1598–1608 cm^{-1} region, which is attributed to $C=N_{hyd}N$ bond stretching, then the coordination of the hydrazone through the deprotonated enol oxygen is beyond a doubt. The band in 1524–1544 cm^{-1} range in spectra of hydrazone ligands arises from $C=NN_{hyd}$ stretching and upon coordination to Cu(II) it is shifted to lower wavenumbers for 15–18 cm^{-1} appearing in spectra of complexes in 1509–1526 cm^{-1} region, thus indicating coordination of hydrazone through imine nitrogen. Infrared spectra clearly demonstrate deprotonated enol-imine coordination of Cu(II) with HL^1 and HL^2 hydrazone ligand. Beside enol oxygen and imine nitrogen both ligands, HL^1 and HL^2, possess additional coordination sites – pyridine nitrogen and in case of HL^2 dimethylamino nitrogen. Considering the fact that copper(II) is coordinated by two bidentate ligands occupying four equatorial positions, two axial positions remain available for cross coordination of copper(II) centers *via* pyridine and dimethylamino nitrogen atoms thus creating polymeric structures, which are immanent to copper(II) complexes of ligands with additional side chain donors.[36–38] However, solid state infrared spectra of copper(II) complexes **1** and **2** do not indicate this is likely to occur here, since band shifting corresponding to bonds which would be affected by bridging is not observed in spectra of complexes compared to free ligands. The C–N bonds of $Me_2N–Ar$ and ArN–Me are intact upon coordination indicating no interaction of Cu(II) with nitrogen atom of $–NMe_2$ (Table 1). Vibrations of pyridine ring, as the most sensitive for coordination to metal center, are not affected by ligating Cu(II) with hydrazone ligands HL^1 and HL^2 demonstrating no coordination of Cu(II) through pyridine nitrogen of hydrazone ligand occurred (Table 1). Although there is no infrared evidence of polymeric na-

ture of copper(II) complexes **1** and **2** in solid state this reasonable possibility cannot be excluded *a priori* in the absence of crystal structure.

Trans configuration of donor atoms in copper coordination sphere of **1** and **2** is indicated by the appearance of only one new band corresponding to Cu–N vibration in low energy region of infrared spectra of complexes. *Cis* isomer, *cis*-[CuN_2O_2] with C_{2v} symmetry, is expected to exhibit two bands for each bond (Cu–N and Cu–O), while *trans* isomer, *trans*-[CuN_2O_2] with C_{2h} symmetry, has only one infrared active vibration for each bond.[39] In our case, new bands in infrared spectrum of complexes **1** and **2**, with purely Gaussian shape and without any obvious splitting, were found at 462 and 485 cm^{-1}, respectively indicating *trans* configuration of [CuN_2O_2] entity in **1** and **2**.

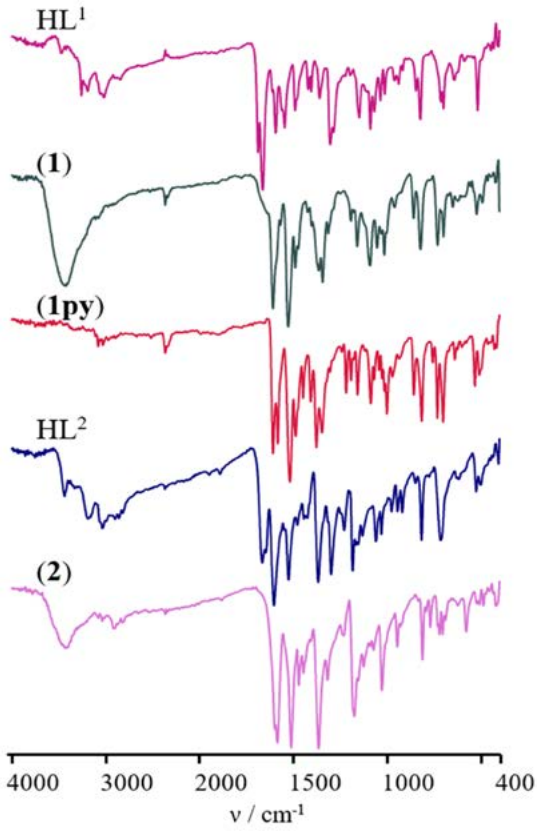


Figure 1. Infrared spectra of hydrazone ligands and corresponding copper(II) complexes.

Electronic spectra of ligands and bis(hydrazonato) copper(II) complexes were recorded in DMSO (Figure S6). As expected for heavily electron populated d^9 system, the electronic spectrum of copper(II) complexes is dominated by ligand-centered transition. The band assigned to n→π* transition, initially positioned in spectra of ligands in 309–359 nm range is red shifted in spectra of complexes thus confirming hydrazone deprotonation and coordination to copper(II) center. Low intensity band arising from Laporte forbidden *d-d* transition, related to d^9 copper(II) center in complexes, is seen in low energy red part of the electronic spectrum and is found in 680–750 nm range. The molar conductivity of **1** and **2** in DMSO were measured as 9 and 11 S cm^2 mol^{-1}, respectively and are in accordance with non-electrolyte nature of bis(hydrazonato) copper(II) complexes. Complexes **1** and **2** are stable toward hydrolysis in aqueous solution and thus all their biological reactivity can be attributed to authentically formulated species.

3. 3. Interaction with CT DNA

Evaluation of the biological activity of new complexes that have potential anticancer activity almost always relies on their interaction with biomolecules that are potential targets *in vivo*. Following the concept of anticancer activity of platinum-based chemotherapeutics, such as cisplatin, which exhibits cytotoxicity by preventing DNA replication by forming covalent bonds with neighboring intrastrand guanines, DNA molecule remains target of focus in gaining the insights of complexes biological activi-

Table 1. Assignment of the most important vibrational bands in FTIR spectra of hydrazone ligands and corresponding copper(II) complexes.

Compound	ν(O–H)	ν(N–H)	ν(C–H)$_{sat.}$	ν(C=O)	ν(CO)+ ν(CN)+δ(NH)	ν(C=N$_{hyd}$N)	ν(C=NN$_{hyd}$)	δ(py) ring deformation	ν(Ar–NMe$_2$)	ν(C–O$_{enol}$)	δ(N–H)	ν(N–N)	ν(ArN–Me)	ν(C–Cl)	δ(C–H)py out of plane	ν(Cu–N)
								cm^{-1}								
HL^1	–	3189	*n.a*	1685	1661	–	1544	1421	*n.a*	–	1305	1146	*n.a*	846	821	*n.a*
1	3437	–	*n.a*	–		1608	1526	1421	*n.a*	1340	–	1158	*n.a*	857	821	462
HL^2	–	3190	2898	1663	1645	–	1524	1422	1366	–	1298	1163	1029	*n.a*	815	*n.a*
2	3435	–	2914	–		1598	1509	1425	1366	1317	–	1172	1027	*n.a*	811	485

n.a – not applicable

ties. The condition for the formation of covalent interactions with DNA is the existence of a coordination position on the metal that can be activated in physiological conditions to ensure the replacement of the easily removable ligand with the donor atoms of the ligating group of biomolecules. Moreover, unoccupied coordination position on metal center is also opportune. In this context, non-sterically closed copper complexes of square-planar or square-pyramidal geometries are of interest. However, covalent binding of DNA remains the most prominent feature of platinum-based complexes while many other metal complexes bind to DNA by non-covalent interactions such as groove binding, intercalation and hydrophobic or electrostatic interactions. Planar aromatic systems that can achieve a π-interaction by inserting between pairs of adjacent bases are said to intercalate DNA. This type of interaction is typical for planar organic molecules and complex compounds that have condensed aromatic systems such as bipyridine, phenanthroline, terpyridine, etc. as ligands, but it is not limited to them, since the coordination of the ligand to the metal limits the conformational freedom of the ligand, which can result in aromatic systems that are bridged with a non-planar bridge to become planar after coordination, which is often the case with the coordination of bidentate ligands to copper(II) in a square-planar geometry. Furthermore, if hydrophobic interactions of ligands with DNA is possible, groove binding cannot be excluded. Bis(hydrazonato)copper(II) complexes **1** and **2** meet all the above mentioned conditions to bind the DNA. The interaction of pristine hydrazone ligands and the corresponding copper(II) complexes with DNA was investigated by the method of spectroscopic titration with DNA. Typical spectroscopic titration is shown in **Figure 2**. Additional data on titration experiments can be found in ESI (Tables S3–S6 and Figures S7–S9). The binding constants were determined graphically using equation (1):

$$\frac{[\mathrm{DNA}]}{(\varepsilon_a - \varepsilon_f)} = \frac{[\mathrm{DNA}]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_b - \varepsilon_f)} \quad (1)$$

by plotting [DNA] *vs* [DNA]/(ε_f – ε_a), where [DNA] is concentration of DNA, and ε_f and ε_a are extinction coefficients of complex in free and bound form at apparent concentration of DNA, respectively. The binding constant (K_b) is obtained as a slope to intercept ratio. The obtained values of binding constants are summarized in Table 2.

The spectroscopic titration showed moderate hypochromism without the obvious shifting of the peak maximum. The obtained values of the binding constants are of 10^3 M^{-1} magnitude for pristine ligands and 10^4 M^{-1} for corresponding bis(hydrazonato)copper(II) complexes. The values of K_b for complexes are approximately twice as those for uncoordinated hydrazone ligands indicating that complexes show grater affinity toward DNA (Table 2). The values of binding constants of 10^4 M^{-1} and the fact that redshift was not observed suggests that the groove binding could be a mode of interaction of these complexes with

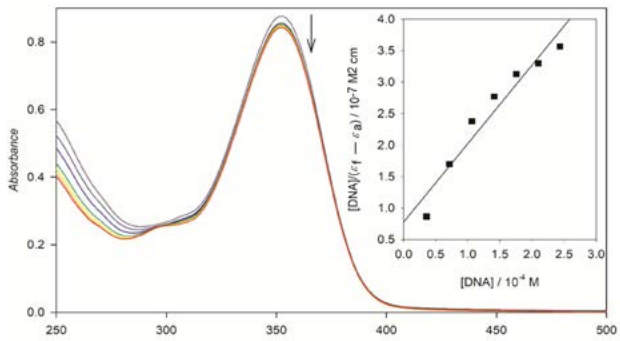


Figure 2. Spectroscopic titration of 1 (5.00×10^{-5} M) with CT DNA in 10 mM Tris-HCl buffer. *Inset*: Graphical determination of binding constant.

DNA, however due the presence of hydrazone ligands capable of π-binding, intercalation cannot be *a priori* excluded. Literature reveals that many copper(II) hydrazone complexes moderately intercalate or bind DNA through grooves with binding constants of 10^4 M^{-1}. Indeed, copper(II) complexes of bidentate hydrazones are not numerous as those of tridentate hydrazones, and even for those reported the interaction with DNA was not investigated,[22,40,41] so the rational comparison is hard to make. The rare examples of copper(II) complexes with bidentate hydrazones, whose interaction with DNA was investigated, are those of dimedone-derived hydrazones[42] with K_b = $(3.67–6.84) \times 10^5$ M^{-1}, which are considered as DNA intercalators and mononuclear cooper(II) complexes of 4-aminobenzoylhydrazones bearing quaternary alky ammonium salts[43] with $K_b = (3–4) \times 10^4$ M^{-1}, which are considered as moderate intercalators and groove binders. On the other hand, interaction of copper(II) complexes of tridentate hydrazones with DNA is extensively documented and data reveal that bis(hydrazonato)copper(II) complexes **1** and **2** have binding constant comparable to those of square-planar copper(II) complexes with hydrazones derived from salicylaldehyde and ibuprofen- and naproxen-hydrazides ($K_b = (1.02–3.50) \times 10^4$ M^{-1})[44] and 1,1,1-trifluoropentanedione and 4-chlorobenzhydrazide (K_b = 4.02×10^4 M^{-1}).[45] Also, complexes **1** and **2** have K_b comparable to mixed square-planar square-pyramidal and octahedral copper(II) complexes of tridentate hydrazone of 2-acetylpyridine and benzhydrazide (K_b = $(1.88–4.66) \times 10^4$ M^{-1}).[20] The cited paper also reports that uncoordinated hydrazone ligand shows binding constant ($K_b = 9.50 \times 10^3$ M^{-1}), which is comparable to HL^1 and HL^2, and that corresponding copper(II) complexes have 2–5 fold higher binding constants, which agrees well with our findings. The similar 2-fold increase in binding constant of the complex compared to native hydrazone ligand was reported for copper(II) complexes of 2-acetylpyridine thiophene-2-carboxylic acid hydrazone and 2-acetylpyridine benzoyl hydrazone.[46] All the above mentioned complexes are considered as moderate DNA intercalators and/or

groove binders. Copper(II) complexes that exclusively intercalate DNA have higher K_b values such acetylhydrazone copper(II) complexes of 2-acetylpyridine and 2-benzoylpyridine ($K_b = (1.42–8.08) \times 10^5$ M^{-1}).[47]

Table 2. Binding constant values of copper(II) complexes and hydrazone ligands with CT DNA.

Compound	K_b / M^{-1}
1	1.19×10^4
2	1.61×10^4
HL^1	6.36×10^3
HL^2	9.14×10^3

3. 4. Interaction with BSA

Albumins, as the most important proteins in the blood, liable for normal osmotic pressure, transportation of fatty acids and hormones and neutralization of free radicals, are the first biological target of many drugs. Considering that proteins have nucleophile rich side-chains of amino acids, such as imidazole of histidine, thiol of cysteine, sulfide of methionine, carboxylate of aspartic and glutamic acid and amide group of asparagine and glutamine, it is not surprising that metal complexes readily bind to proteins. Indeed, some of the drugs become deactivated after binding to proteins, while for others binding to proteins is an essential aspect of their biological activity and target specific delivery. For laboratory studies bovine serum albumin (BSA) is most convenient as it has high structural and functional similarity to human serum albumin (HSA). When excited at 278 nm, BSA shows strong emission near 340 nm, owing to fluorescence of endogenous aromatic amino acids tryptophan, tyrosine and phenylalanine. The interaction of copper(II) complexes and hydrazone ligands with BSA was investigated by spectrofluorimetric titration. The linear decrease of fluorescence with increasing concentration of complex 1 and ligands HL^1 and HL^2 obeys to Stern-Volmer relation (Eq. 2) and K_{SV} values were graphically obtained as a slope of the linear regression of an emission intensities ratio in the absence (I_0) and presence (I) of the compound versus concentration of the compound (Table 3).

$$\frac{I_0}{I} = 1 + K_{SV}[compound] \quad (2)$$

Typical decrease of fluorescence intensity is shown in **Figure 3**a while additional data can be found in ESI (Tables S7–S9, Figures S10–S12). For bis(hydrazonato)copper(II) complex **2**, bearing dimethylamino substituent, continual decrease of fluorescence was not observed. Initial decrease of BSA fluorescence is followed with an increase of fluorescence as the concentration of the complex increases (Figure S10). The increase in fluorescence can occur when complex has its own intrinsic fluorescence, which is not case for **2**, or when the tryptophan intrinsic fluorescence enhances due to conformational change that leads to better stacking in hydrophobic pockets of BSA as observed for nickel(II) hydrazone complex.[48] For complex **1** and the corresponding hydrazone ligand decrease of the fluorescence results with significant blueshifting of the peak maximum for 13 and 5 nm, respectively indicating

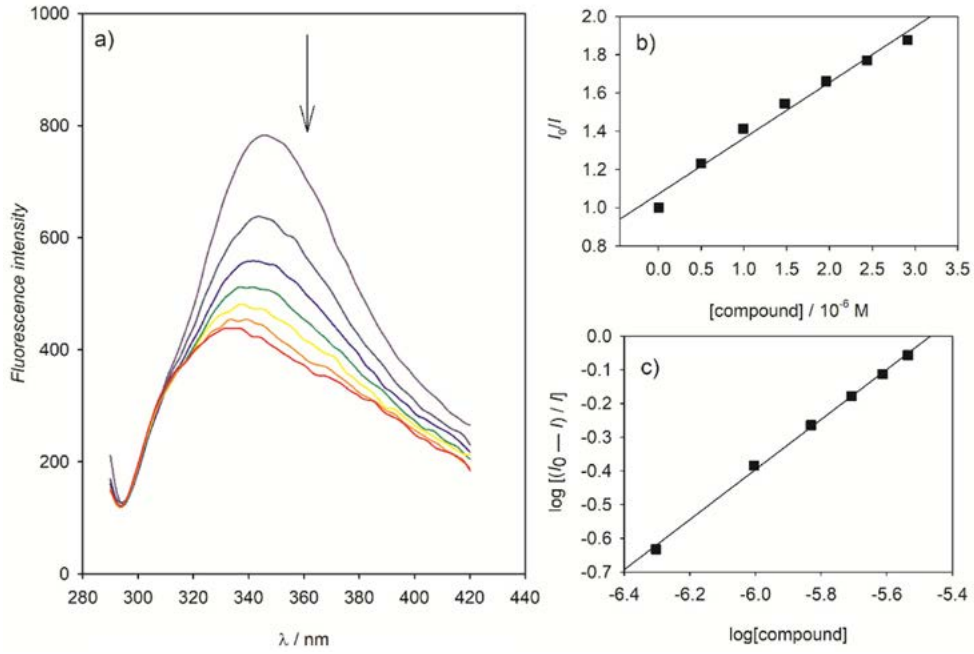


Figure 3. Interaction of 1 with BSA (1 μM) in 10 mM Tris-HCl buffer: a) Decrease of BSA fluorescence with increasing concentration of 1, b) graphical determination of Stern-Volmer constant, c) graphical determination of binding constant and number of binding sites.

significant change in hydrophobicity of tryptophan residues.[49] For ligand HL^2 the minor blueshifting ($\Delta\lambda$ = 2 nm) of the peak was observed suggesting no significant change in the local dielectric environment of BSA occurs.[49]

Further quantification of the interaction was carried out by determining the values of the binding constant (K_b) and the number of the binding sites (n) by plotting $\log[(I_0 - I)/I]$ *vs* [compound] (Eq. 3).

$$log\frac{I_0 - I}{I} = logK_b + n\log[compound] \quad (3)$$

Hydrazone ligands HL^1 and HL^2 showed low affinity to BSA ($K_b \leq 10^3$ M^{-1}) with binding constants that differ significantly from each other. The ligand having dimethylamino substituent (HL^2) showed ~75-fold lower binding constant to BSA compared to the one having chloro substituent (HL^1). This indicates that the nature of the substituent on hydrazone ligand has reasonable impact on the BSA binding properties of the compounds and can be exploited in designing the compounds of desirable affinity to BSA. Electron-accepting nature of chlorine substituent, compared to electron-donating nature of dimethylamino substituent, decreases π-electron density of hydrazone ligands making it more susceptible for interaction with negatively charged BSA. The coordination of HL^1 to Cu(II) center results with complex **1** having 2-fold larger binding constant ($K_b = 1.15 \times 10^4$) compared to corresponding ligand. The value of the binding constant suggests moderate affinity to BSA which is very favorable in designing new bioactive compounds, since the interaction is strong enough that the compound can be transported by albumin, but weak enough that release of the compound at the target site can occur.[50] Bis(hydrazonato)copper(II) complex **1** has larger binding constant compared to copper(II) 2-benzoylpyridine benzhydrazone complex ($K_b = 6.68 \times 10^3$ M^{-1})[48] but is comparable to mononuclear [Cu(hydrazone)Cl_2] complexes of nitrogen-rich hydrazone ligands derived from pyrazine-2-carbaldehyde and isonicotino-based hydrazides ($K_b \sim 5 \times 10^4$ M^{-1}).[51] It is also comparable to copper(II) complexes of dehydroacetic acid benzoyl hydrazone featuring DMSO as coligand ($K_b = 7.59 \times 10^4$ M^{-1}), while it has significantly lower K_b compared to analogous complexes having water or imidazole as coligands ($K_b = 2.68 \times 10^6$ M^{-1} and 1.50×10^7 M^{-1}, respectively).[52] Complex **1** has 10-fold lower binding constant compared to monomeric square-pyramidal copper(II) complexes of tridentate NNO neutral acetylhydrazones of 2-acetylpyridine and 2-benzoylpyridine.[47]

The values of quenching rate constant (k_q), obtained as K_{SV}/τ_0 ($\tau_0 = 10^{-8}$ s), are larger than 2×10^{12} M^{-1} s^{-1}, thus indicating that hydrazone ligands and copper(II) complex quench BSA fluorescence through static mechanism.[53]

More detailed insight into the changes of the microenvironment of tryptophan and tyrosine residues of BSA can be gathered using synchronous spectrofluorimetry. The difference between excitation and emission wavelengths ($\Delta\lambda = \lambda_{ex} - \lambda_{em}$) results in spectra of different fluorophores. If the difference is large ($\Delta\lambda$ = 60 nm) tryptophan synchronous spectra are collected, but if the difference is small ($\Delta\lambda$ = 15 nm) emission arises from tyrosine residues. The synchronous spectra are shown in **Figure 4** and Figs. S13–S15. The typical features of the spectra are decrease of tryptophan fluorescence, significantly lower decrease of tyrosine florescence and blueshifting of both peak maximums. Only for

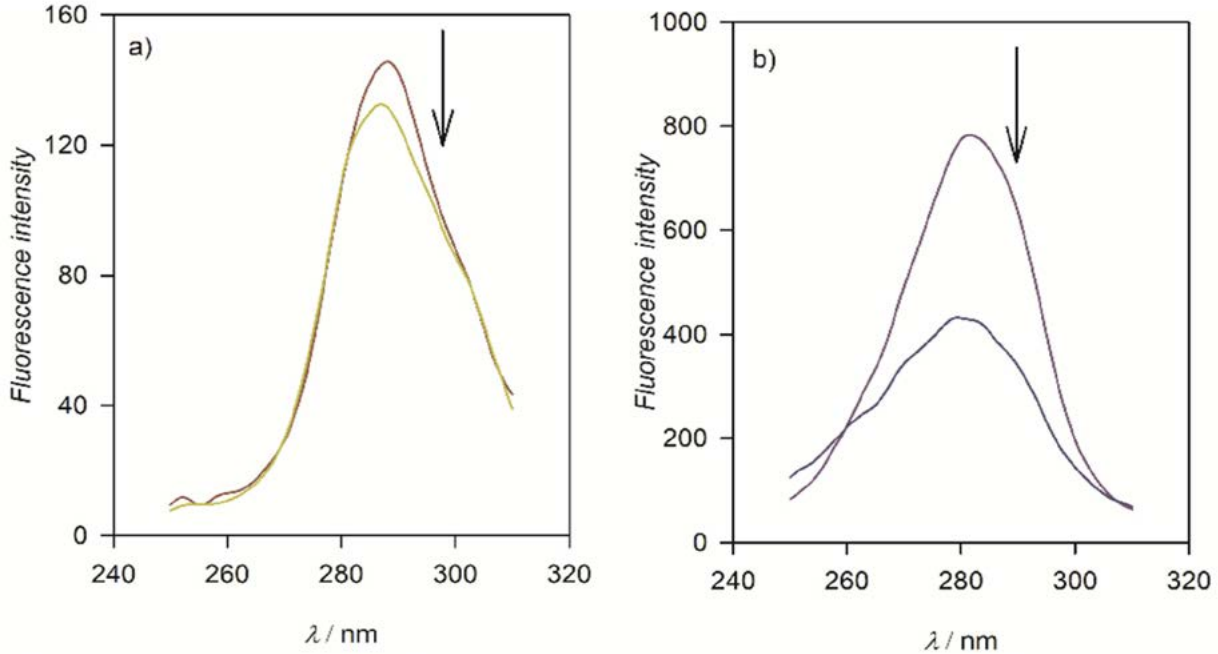


Figure 4. Changes in synchronous emission spectra of BSA (1 μM) in 10 mM Tris-HCl buffer pH 7.42 in absence and presence of 1 at [complex]/[BSA] = 3: a) $\Delta\lambda$ = 15 nm; b) $\Delta\lambda$ = 60 nm.

complex **2** synchronous spectra showed increase of tryptophan fluorescence and no obvious change in fluorescence of tyrosine followed with blue shift. This is consistent with previously commented data (*vide supra*).

Molecular docking was further used to analyze the interaction and binding modes between bis(hydrazonato) copper(II) complexes and BSA, as well as between pristine hydrazone ligands and BSA. The docking results are shown in **Table 4** and Figure 5Figure 6. Calculations indicate that both copper(II) complexes occupied the same domain of BSA (Pro-110, His-145, Ala-193, Glu-424, Arg-458, etc.). This was also observed previously for the copper(II) complexes of similar structures.[54] However, the binding energies and dissociation constants of two copper(II) complexes were quite different in comparison to each other (**Table 4**). Complex **1** showed much lower/better binding energy and dissociation constant of 3.42 μM indicating better binding affinity toward BSA, while **2** with a higher binding energy value showed a 100-fold higher dissociation constant. No hydrogen bonds were observed in the interactions.

Molecular docking parameters obtained for the ligands alone were in relatively good accordance with the docking parameters of the complexes. Ligand HL^1 showed quite similar values to those of **1** including the same BSA domain, with observed hydrogen bonds between the ligand and amino acid residues. Ligand HL^2 showed higher binding energy and dissociation constant, same as **2**, although the dissociation constant was about 7.5 times higher compared to ligand HL^1. The biggest difference was actually in the occupied domain of BSA, where ligand HL^2 alone occupied the outer domain of BSA, and had no overlapping contacting amino acids in comparison to interactions of other compounds (**Table 4**), and showed hydrogen bonding to Phe508 (**Figure 5**). This finding indicates the influence of the ligand's nature on the lower overall binding affinity of the **2** to BSA. Docking parameters also indicate the influence and importance of electron-withdrawing chlorine substituent in **1** for binding to BSA, while electron-donating dimethyl amino substituent greatly decreased binding affinity in both, ligand HL^2 and complex

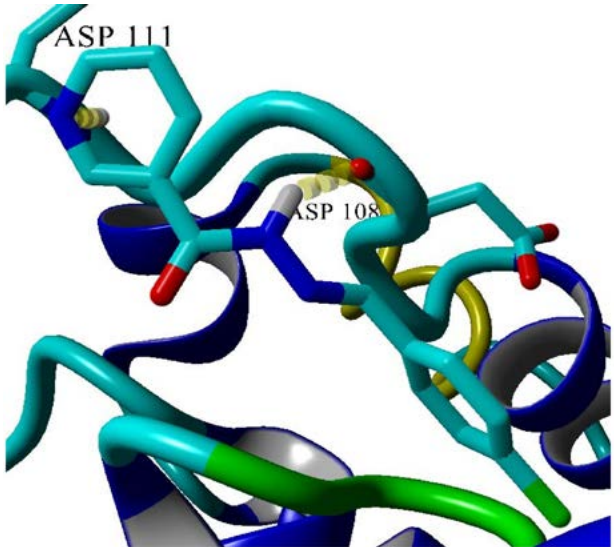


Figure 5. Binding pose of ligand HL^1 to BSA.

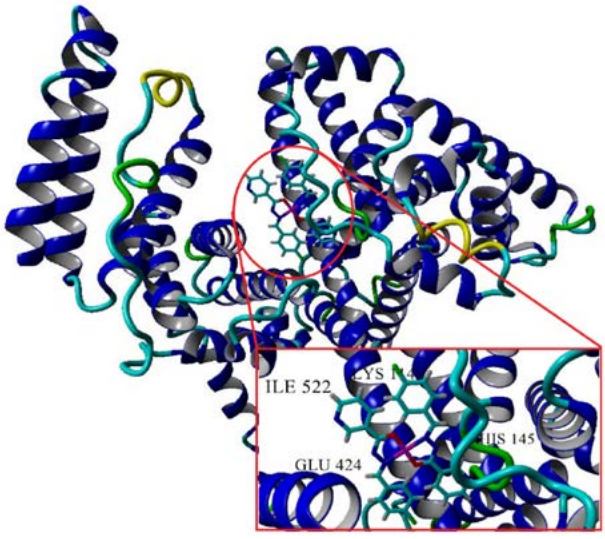


Figure 6. Binding of 1 to BSA. *Inset*: Close-up projection of binding pose.

Table 4. Molecular docking parameters of analyzed interaction between copper(II) complexes and BSA.

Compound (amino acids)	Binding energy (kcal mol^{-1})	Contacting receptor residues
1	−7.46	Asp 108, Pro 110, Leu 112, Pro 113, Lys 114, Arg 144, His 145, Pro 146, Tyr 147, **Arg 185**, **Leu 189**, Ser 192, Ala 193, Arg 196, Leu 197, Glu 424, Ser 428, Leu 454, Ile 455, Asn 457, Arg 458, Ile 522
2	−4.75	Asp 108, Pro 110, Leu 112, Pro 113, Lys 114, Arg 144, His 145, Pro 146, Tyr 147, Ser 192, Ala 193, **Arg 194**, Arg 196, Leu 197, Glu 424, **Arg 427**, Ser 428, Leu 454, Ile 455, Asn 457, Arg 458, **Thr 518**, **Glu 519**, Ile 522
HL^1	−7.77	**Asp 107**, Asp 108*, **Ser 109**, Pro 110, **Asp 111***, Leu 112, Arg 144, His 145, Pro 146, Tyr 147, **Phe 148**, Ser 192, Ala 193, **Gln 195,** Arg 196, Glu 424, Arg 458
HL^2	−6.56	**Phe 506, Thr 507, Phe 508*, His 509, Ala 510, Phe 550, Phe 553, Glu 564, Phe 567, Ala 568, Gly 571, Pro 572, Leu 574, Val 575**

Bolded amino acids are involved in only one compound-BSA interaction. Asterisk indicates a hydrogen bond to specified amino acid.

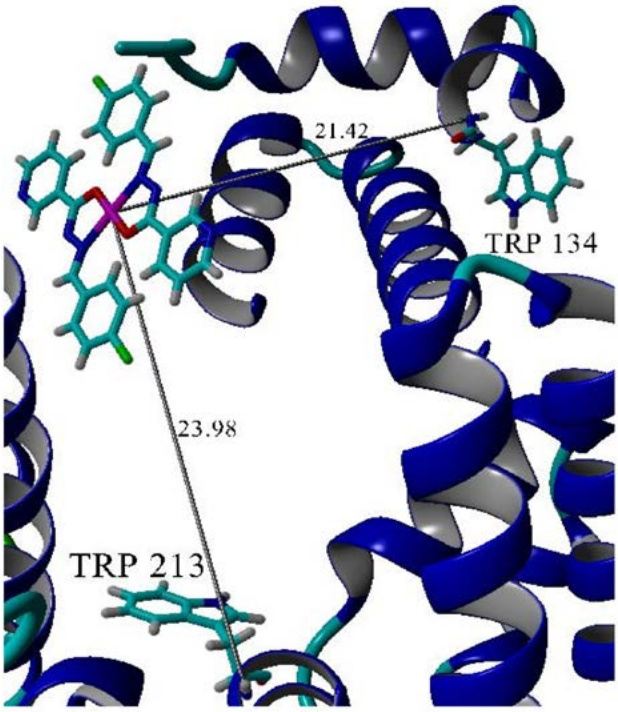


Figure 7. Distance from copper center in complex 1 from two tryptophan residues in complex-BSA adduct.

2. However, the observed change in the binding constant cannot be exclusively addressed to electron-withdrawing or electron-accepting properties of substituents based only on these two examples, especially not in the absence of thermodynamic data that indicate nature of the binding forces responsible for the interaction. A decrease in the binding affinity could also be considered as the effect of the steric hindrance and polar properties of the dimethyl amino group. The molecular docking results suggest that binding of complexes to BSA is weaker compared to pristine hydrazone ligands, however this is not experimentally confirmed. Moreover, coordination of small organic ligands to metal center generally results in larger binding constants to BSA as found in our case.[8,46,53,55–58]

The distances between copper(II) complexes with tryptophan residues in BSA were also determined from the docking analysis as shown for complex **1** in **Figure 7**. Obtained results showed that distances between two tryptophan residues and complexes were as follows: 23.98 Å (Trp-213) and 21.42 Å (Trp-134) with an average of 22.70 Å for **1**, and 25.18 Å (Trp-213) and 22.48 Å (Trp-134) with an average of 23.83 Å for **2**.

4 Conclusion

Solution synthesis afforded two novel copper(II) hydrazone complexes of general composition $[CuL_2]$. Hydrazone ligands act as bidentate monoanionic chelators coordinating Cu(II) through oxygen and nitrogen of deprotonated enol-imine form of hydrazones. The interaction with CT DNA revealed that complexes have nearly 2-fold higher affinity toward DNA. The binding constant of copper(II) complexes to DNA is not significantly affected by the nature of the substituent at hydrazone ligand and groove binding or moderate intercalation are indicative as interaction modes which is in agreement with previously reported data for hydrazone complexes of copper(II) binding to DNA. The interaction of complexes and ligands with BSA revealed that substituent effect on hydrazone ligand has much more impact to binding with BSA compared to DNA. The complex bearing hydrazone ligand with electron-withdrawing chlorine substituent shows stronger quenching of tryptophan fluorescence and causes significant perturbation in microenvironment of tryptophan and increases the hydrophobicity. In all cases static mechanism of fluorescence quenching was observed suggesting that tested compounds interact with BSA in ground state. The substituent effect of hydrazone ligand on BSA binding affinity was confirmed for pristine ligands, experimentally and theoretically through molecular docking study. We found that benzaldehyde nicotinic acid hydrazone ligand having a chloro substituent has ~75-fold larger binding constant to BSA compared to its 4-dimethyamino analogue, which is supported by theoretical calculations that dissociation constant for these two ligands significantly differs with chloro analogue being more tightly bound to BSA. Results of molecular docking suggest that ligands interact with BSA mostly through hydrogen bonding while interaction of copper(II) complexes is more hydrophobic in nature. These results indicate that the nature of the substituent on benzaldehyde component of hydrazone ligand has reasonable impact on the BSA binding properties of the compounds and can be exploited in designing the compounds of desirable affinity toward BSA. However, in the absence of thermodynamic data on the nature of the binding forces between complex and BSA, electron-donating and electron-accepting properties of the substituent cannot be claimed as governing factor in these interactions.

Declaration of Competing Interest

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.

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Supplementary materials

Supplementary material associated with this article can be found in the online version.

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Povzetek

Pripravili smo dva bakrova(II) kompleksa s 4-kloro- in 4-dimetilaminobenzaldehidnim hidrazonom nikotinske kisline in ju okarakterizirali z elementno analizo, masno spektrometrijo, infrardečo in elektronsko spektroskopijo ter konduktometrijo. Ta dva redka primera bis(hidrazonato)bakrovih(II) kompleksov sta nevtralni kompleksni vrsti z bakrovim(II) centrom, koordiniranim z dvema monoanionskima bidentatnima O,N-donorskima hidrazonskima ligandoma, koordiniranima v enol-iminski obliki. Proučevali smo interakcijo hidrazonskih ligandov in njunih bakrovih(II) kompleksov s CT DNA in BSA. Bakrova(II) kompleksa sta nekoliko učinkovitejša pri vezavi na DNK kot prosta hidrazona. Rezultati kažejo na vezavo ali interkalacijo, na katero narava substituenta na hidrazonskih ligandih ne vpliva bistveno. Nasprotno pa se afiniteti dveh bakrovih(II) kompleksov do BSA bistveno razlikujeta in sta odvisni od narave substituenta, vendar zaradi pomanjkanja termodinamskih podatkov ni mogoče izključiti razlike v naravi veznih sil. Kompleks z elektronakceptorskim 4-kloro substituentom ima večjo afiniteto do BSA v primerjavi s 4-dimetiaminskim analogom. Te ugotovitve so bile teoretično podprte s študijo molekularnega dockinga.