Kragujevac J. Sci. 45 (2023) 79-91.
doi: 10.5937/KgJSci2345079A

UDC 54-386:546.57:57.085
Original scientific paper

DINUCLEAR SILVER(I) COMPLEXES WITH PHTHALAZINE: DNA/BSA BINDING AND *IN VIVO* TOXICITY STUDY

Darko P. Ašanin[1]*, Tina P. Andrejević[2], Dušan Milivojević[3], Nevena LJ. Stevanović[2], Biljana Đ. Glišić[2]

[1]*University of Kragujevac, Institute for Information Technologies Kragujevac, Department of Science, Jovana Cvijića bb, 34000 Kragujevac, Serbia*
[2]*University of Kragujevac, Faculty of Science, Department of Chemistry, Radoja Domanovića 12, 34000 Kragujevac, Serbia*
[3]*University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, 11042 Belgrade, Serbia*
*****Corresponding author; E-mail: darko.asanin@uni.kg.ac.rs

(Received March 03,2023; Accepted April 08, 2023)

ABSTRACT. The present study reports the synthesis and spectroscopic characterization of dinuclear silver(I) complexes, $\{[Ag(X\text{-}O)(phtz\text{-}N)]_2(\mu\text{-}phtz\text{-}N,N')_2\}$ (X = NO_3^- (**Ag1**) and $CF_3SO_3^-$ (**Ag2**), and phtz is phthalazine). The interaction of these two complexes with calf thymus DNA (ct-DNA) and bovine serum albumin (BSA) was investigated to assess their binding affinity to these biomolecules. The binding constants of complexes **Ag1** and **Ag2** to BSA are found to be higher than those for ct-DNA, being in accordance with their higher affinity toward the studied protein. The values of partition coefficient (log*P*) for the investigated complexes indicate higher cellular uptake efficiency of **Ag1** than **Ag2**. The *in vivo* toxicity of the complexes **Ag1** and **Ag2** against the model organism, *Caenorhabditis elegans*, was studied to check their therapeutic potential.

Keywords: silver(I) complexes, phthalazine, *Caenorhabditis elegans*, DNA interaction, protein interaction.

INTRODUCTION

Silver(I) compounds have a long tradition as effective antimicrobial agents in the treatment of human diseases due to their higher toxicity to microbes and lower toxicity to mammals (MELAIYE and YOUNGS, 2005). One of many examples includes the use of 1% solution of silver(I) nitrate in the form of eye drops to prevent gonorrheal infection in newborn infants and this was common practice until the last century (SILVER *et al*., 2006). In 1968, silver(I) sulfadiazine (Silvadene®) was obtained in the reaction of silver(I) nitrate and a sulfonamide and is still used as a cream for the treatment of burn wounds (SILVER *et al*., 2006). Nowadays, with the increasing resistance of bacteria to widely used antibiotics, special attention has been devoted to synthesis and biological evaluation of silver(I) complexes as potential agents for the treatment of microbial infections (MEDICI *et al*., 2016). In comparison to silver and silver(I) nitrate, silver(I) complexes represent the formulations with tunable antimicrobial properties. The crucial factors which determine the antimicrobial activity of silver(I) comp-

lexes are the nature of the ligand coordinated to Ag(I) ion and the ease of its substitution (MEDICI *et al*., 2016). In general, silver(I) complexes containing Ag−O and Ag−N bonds have shown a more effective and wider spectrum of antimicrobial activity than those containing Ag−P and Ag−S bonds (MEDICI *et al*., 2016; AHMAD *et al*., 2022). Their effectiveness can be attributed to the presence of Ag–O/N bonds, which are weak and can be easily cleaved in the reactions of these complexes with different biomolecules, including DNA and proteins contaninng cysteine residues (MEDICI *et al*., 2016; AHMAD *et al*., 2022).

The antimicrobial silver(I) complexes have a unique mechanism of action, which is different from that of antibiotics and other biologically active metal complexes (CLAUDEL *et al*., 2020). The exact pathways of silver(I) complexes have not been completely clarified; nevertheless, it is proposed that a slow dissociation of the complexes to free Ag(I) ions is a key step responsible for their activity (MEDICI *et al*., 2019). Ag(I) ions can induce the death of bacterial cells as a result of impairment of enzymes that coordinate these ions on the surface of cell wall or through impairment of permeability of the cell wall. In the cells, Ag(I) ions can react with the enzymes, contribute to proteins denaturation, inhibit the bacterial respiration and ATP oxidation, bind to cellular components, induce formation of reactive oxygen species (ROS), and inactivate DNA and RNA molecules (MEDICI *et al*., 2019).

Aromatic nitrogen-containing heterocycles are an attractive class of ligands for the synthesis of silver(I) complexes showing antimicrobial activity (MEDICI *et al*., 2016; CLAUDEL *et al*., 2020). Among them, phthalazine has gained attention in design of therapeutic agents due to its biological potential (SANGSHETTI *et al*., 2019). Phthalazine scaffold represents a pharmacophoric feature of different therapeutics showing antimicrobial, anti-inflammatory, analgesic, antihypertensive, antidepressant, antidiabetic, antithrombotic and anticancer activities (SANGSHETTI *et al*., 2019). Considering this, we have used phthalazine (phtz) as a ligand for the synthesis of three dinuclear silver(I) complexes of the formula {[Ag(X-*O*)(phtz-*N*)]$_2$(μ-phtz-*N,N'*)$_2$}, X = NO_3^-, $CF_3SO_3^-$ and ClO_4^-, and evaluated their antimicrobial activity against various microbial strains (GLIŠIĆ *et al*., 2016). All these complexes have shown significant activity against the tested bacterial strains, whereas the highest activity was observed against Gram-negative bacterium *Pseudomonas aeruginosa* (GLIŠIĆ *et al*., 2016). The complexes were also remarkably active against Gram-positive bacterium *Staphylococcus aureus*, while their antifungal activity against yeast *Candida albicans* was marginal. It is important to mention that phtz itself did not show any significant activity against the investigated microbial strains (GLIŠIĆ *et al*., 2016). Moreover, two silver(I) complexes, {[Ag(NO_3-*O*)(phtz-*N*)]$_2$(μ-phtz-*N,N'*)$_2$} and {[Ag(CF_3SO_3-*O*)(phtz-*N*)]$_2$(μ-phtz-*N,N'*)$_2$}, have shown an ability to disrupt biofilms of *P. aeruginosa* strains, which are resistant to the widely used antibiotics.

In a continuation of this study, herein the interactions of the silver(I)-phthalazine complexes which were most active across the range of *P. aeruginosa* strains with calf thymus DNA (ct-DNA) and bovine serum albumin (BSA) were investigated by fluorescence emission spectroscopy to gain an insight on their behaviour towards these biologically important molecules. Moreover, to investigate therapeutic potential of the silver(I) complexes as antibacterial agents, their *in vivo* toxicity on the *Caenorhabditis elegans* (Maupas, 1900), which shares a high homology with the mammalian system (HUNT, 2017), was studied.

MATERIALS AND METHODS

Materials and instrumentation

Silver(I) salts ($AgNO_3$ and $AgCF_3SO_3$), phthalazine, methanol, ethanol, acetonitrile, dimethyl sulfoxide (DMSO), deuterated acetonitrile, phosphate buffered saline (PBS), calf

thymus DNA (ct-DNA), ethidium bromide (EthBr), bovine serum albumin (BSA) and *n*-octanol were purchased from the Sigma-Aldrich (St. Louis, Missouri, USA). All chemicals were of reagent-grade quality or higher and used as supplied.

Elemental microanalysis for carbon, hydrogen, and nitrogen of the silver(I) complexes was done by the Microanalytical Laboratory at the University of Belgrade-Faculty of Chemistry. NMR spectroscopic measurements were performed at ambient temperature in acetonitrile-d_3 as a solvent on a Bruker Avance II 400 MHz spectrometer, in a 5 mm NMR tube. The sample contained 5.0 mg of the complex in 0.6 mL of acetonitrile-d_3. Chemical shifts (δ) are given in ppm. IR spectra were recorded by using KBr pellet technique on a Perkin Elmer Spectrum 2 spectrometer over 4000–450 cm^{-1} range. A Shimadzu double-beam spectrophotometer was used for the measurement of the UV-Vis spectra, which were recorded after dissolving the complexes in DMSO over 1100–200 nm range at ambient temperature. Moreover, the UV-Vis spectra of the complexes were recorded immediately after their dissolution in PBS and during 2 days at ambient temperature to follow their solution stability. The fluorescence emission spectra were measured on a Jasco FP-6600 spectrophotometer.

Synthesis of Ag1 and Ag2

Silver(I) complexes, {[Ag(X-*O*)(phtz-*N*)]$_2$(μ-phtz-*N,N'*)$_2$} (X = NO_3^- (**Ag1**) and $CF_3SO_3^-$ (**Ag2**), phtz is phthalazine) were obtained by the previously reported method (GLIŠIĆ *et al*., 2016). The purity of these complexes was confirmed by the results of elemental microanalysis and NMR spectroscopy. These data agreed with those previously published for the same silver(I) complexes (GLIŠIĆ *et al*., 2016).

The solution of 0.5 mmol of the silver(I) salt (84.9 mg of $AgNO_3$ for **Ag1** and 128.5 mg of $AgCF_3SO_3$ for **Ag2**) in 2.5 mL of ethanol (**Ag1**) or methanol (**Ag2**) was added to the solution of an equimolar amount of phtz (65.1 mg) in 10.0 mL of hot ethanol (**Ag1**) or methanol (**Ag2**). The obtained reaction mixture was stirred in dark at ambient temperature for a period of 3 h. Complex **Ag1** was obtained after recrystallization of the obtained solid product in 10.0 mL of acetonitrile, while complex **Ag2** crystallized from the mother methanol solution after its standing in refrigerator for 3 days. Yield: 61.3 mg (57%) for **Ag1** and 82.8 mg (64%) for **Ag2**.

Anal. Calcd. for **Ag1** = $C_{32}H_{24}Ag_2N_{10}O_6$ (MW = 860.36): C, 44.67; H, 2.81; N, 16.28. Found: C, 44.53; H, 2.89; N, 16.14%. ^{1}H NMR (400 MHz, CD_3CN): δ = 8.04 (*m*, H6 and H7), 8.12 (*m*, H5 and H8), 9.59 ppm (*s*, H1 and H4). ^{13}C NMR (101 MHz, CD_3CN): δ = 126.9 (C5 and C8), 127.0 (C4a and C8a), 133.6 (C6 and C7), 152.0 ppm (C1 and C4). IR (KBr, ν, cm^{-1}): 3035 ($\nu(C_{ar}–H)$), 1618, 1568, 1488, 1440 ($\nu(C_{ar}=C_{ar})$, $\nu(C_{ar}=N)$ and ν(N=N)), 1377 and 1362 ($\nu(NO_3)$), 650 ($\gamma(C_{ar}–H)$).

Anal. Calcd. for **Ag2** = $C_{34}H_{24}Ag_2F_6N_8O_6S_2$ (MW = 1034.47): C, 39.48; H, 2.34; N, 10.83. Found: C, 39.33; H, 2.45; N, 10.91%. ^{1}H NMR (400 MHz, CD_3CN): δ = 8.07 (*m*, H6 and H7), 8.14 (*m*, H5 and H8), 9.60 ppm (*s*, H1 and H4). ^{13}C NMR (101 MHz, CD_3CN): δ = 127.1 (C5 and C8), 127.1 (C4a and C8a), 133.9 (C6 and C7), 152.4 ppm (C1 and C4). IR (KBr, ν, cm^{-1}): 3041 ($\nu(C_{ar}–H)$), 1619, 1564, 1489, 1445 ($\nu(C_{ar}=C_{ar})$, $\nu(C_{ar}=N)$ and ν(N=N)), 1274 ($\nu_{as}(SO_3)$), 1256 ($\nu_s(CF_3)$), 1150 ($\nu_{as}(CF_3)$), 1031 ($\nu_s(SO_3)$), 636 ($\gamma(C_{ar}–H)$).

BSA binding experiment

The protein (BSA) binding study was carried out at pH = 7.4 in PBS by recording fluorescence spectra over the range of 280–500 nm with an excitation wavelength of 275 nm. The quenching of the BSA (16 μM) emission intensity at 366 nm was followed after addition

of an increasing concentration of the silver(I) complexes (0–160 μM). The following equation was used for calculation of the Stern–Volmer constant (K_{sv}):

$$F_0/F = 1 + K_q\tau_0[\text{complex}] = 1 + K_{sv}[\text{complex}]. \quad (1)$$

In this equation, F_0 and F represent the fluorescence intensities in the absence and presence of the complex, respectively, while K_q is a bimolecular quenching constant. τ_0, which amounts 10^{-8} s, represents the fluorophore lifetime, when the quencher is absent (SMOLEŃSKI *et al*., 2015). The binding constant of the complex to BSA (K_A) and the number of binding sites (n) were calculated by the following equation (WOLFE *et al*., 1987):

$$\log(F_0 - F)/F = \log K_A + n\log[\text{complex}]. \quad (2)$$

Lipophilicity assay

The flask-shaking method by PUCKETT and BARTON (2007) was used for determination of the lipophilicity of the complexes **Ag1** and **Ag2**. These complexes were dissolved in DMSO and added to water/*n*-octanol system. After that, the solutions were vortexed for 1 h at ambient temperature and left to stand for an additional 24 h allowing the separation of the two phases. The values of absorbance were determined from the previously determined calibration curves and used for calculation of the concentration of the complexes in two phases (c_o is the concentration of the complexes in *n*-octanol phase, while c_w represents their concentration in water phase). The following equation was applied for calculation of the log*P* values:

$$\log P = \log(c_o/c_w) \quad (3)$$

DNA binding experiment

PBS was used for the preparation of a stock solution of ct-DNA. Its concentration (1.19×10^{-2} M) was determined from the values of UV absorbance at 260 nm and molar extinction coefficient ($\varepsilon = 6.6 \times 10^3$ M^{-1} cm^{-1}; BERA *et al*., 2008). Stock solutions of ethidium bromide (EthBr; 1.10×10^{-2} M) and silver(I) complexes **Ag1** and **Ag2** (10 mM) were prepared in DMSO.

The binding studies were performed at pH = 7.4 in PBS, whereas the ratio [DNA] : [EthBr] was 10 : 1, and concentration of the complexes gradually increased. The spectra were measured in the range of 525 – 750 nm, while the excitation wavelength was 520 nm. The K_{sv} and K_A constants, and the n number were calculated as described for BSA binding study (WOLFE *et al*., 1987; SMOLEŃSKI *et al*., 2015).

In vivo toxicity assay

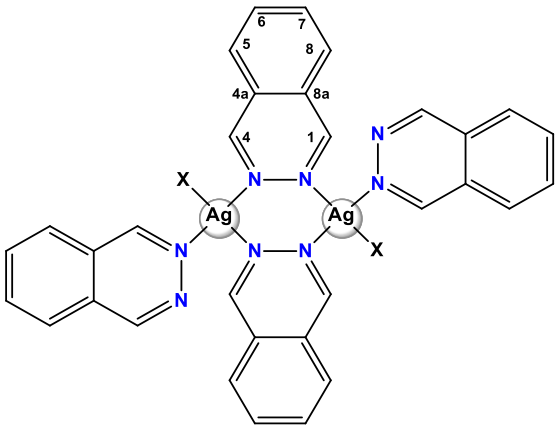
Toxicity was tested in *in vivo* model system *C. elegans* by measuring survival of nematodes after 48 h. *Caenorhabdites elegans* N2 (*glp-4*; *sek-1*) was propagated under standard conditions, synchronized by hypochlorite bleaching, and cultured on nematode growth medium using *Escherichia coli* OP50 as a food source, as reported by STIERNAGLE (1999). The *C. elegans* survival assay was performed as described previously with some modifications (BRACKMAN *et al*., 2011; SCOFFONE *et al*., 2016). An experiment was performed in 96-well flat-bottomed microtiter plates (Sarstedt, Germany) in 100.0 μL per well. 25.0 μL of the suspension of nematodes, which contained 25–35 nematodes, were transferred to the wells, in which 50.0 μL of the medium was previously added. After that, 25.0 μL of DMSO solvent (used as a control) or of a concentrated solution of the studied complexes was added to the wells. The concentrations of the complexes were 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.781 μg/mL and were made from their stock solutions. The plates were incubated at ambient tem-

perature for 48 h. After that time, the fraction of dead worms was calculated by dividing the number of dead worms and their total number in each well. These numbers were determined by a stereomicroscope (SMZ143-N2GG, Motic, Germany). The complexes were evaluated three times in each assay and each assay was repeated twice ($n \geq 6$). Nematodes exposed to the medium containing 1% (v/v) DMSO represented a negative control experiment. The calculated IC_{50} values represent the concentrations which are required for inhibition of the nematode growth for 50%.

RESULTS AND DISCUSSION

The results obtained from our previous study showed that silver(I)-phthalazine complexes manifest a good antibacterial activity as well as excellent ability to disrupt biofilms of *P. aeruginosa* strains, which are highly resistant to antibiotics (GLIŠIĆ *et al.*, 2016). In the present study, the synthesis of two silver(I) complexes with phthalazine (phtz) of the general formula {[Ag(X-*O*)(phtz-*N*)]$_2$(μ-phtz-*N,N'*)$_2$}, X = NO_3^- (**Ag1**) and $CF_3SO_3^-$ (**Ag2**) (Scheme 1), which showed the best antimicrobial potential, was repeated in order to investigate their binding affinity towards calf thymus DNA (ct-DNA) and bovine serum albumin (BSA). The *in vivo* toxicity of **Ag1** and **Ag2** on the *C. elegans* was investigated to determine their therapeutic potential as antibacterial agents.

Complexes **Ag1** and **Ag2** were obtained in the reactions of phthalazine with $AgNO_3$ or $AgCF_3SO_3$ in 1 : 1 molar ratio in ethanol or methanol, respectively, at ambient temperature. The purity of these complexes was checked by elemental microanalysis, while their structure was confirmed by comparing their NMR and IR data with those previously reported for these complexes of the known crystal structures (GLIŠIĆ *et al.*, 2016). From these data, it was found that structures of **Ag1** and **Ag2** complexes agree with those previously determined by X-ray crystallography (GLIŠIĆ *et al.*, 2016). As can be seen in Scheme 1, in these complexes, two phtz molecules are bridging ligands between Ag(I) ions, while the other two phtz act as monodentate ligands. In addition, the X anion (NO_3^- in **Ag1** and $CF_3SO_3^-$ in **Ag2**) is monodentately coordinated to the Ag(I) *via* one oxygen atom.



{[Ag(X-*O*)(phtz-*N*)]$_2$(μ-phtz-*N,N'*)$_2$}
X = NO_3^- (**Ag1**), $CF_3SO_3^-$ (**Ag2**)

Scheme 1. Structural representation of **Ag1** and **Ag2** complexes analyzed in this study. Carbon atoms are numbered in accordance with IUPAC recommendation.

The stability of the complexes **Ag1** and **Ag2** was confirmed by measurements of their UV-Vis spectra in PBS solution during 2 days at ambient temperature (Fig. 1). A very slight decrease in the absorption intensity of **Ag1** and **Ag2** was observed after 2 days (3 and 5%,

respectively). Nevertheless, the shape of the UV-Vis spectra and position of the absorption maxima for these complexes remained unchanged, being in accordance with their stability in solution.

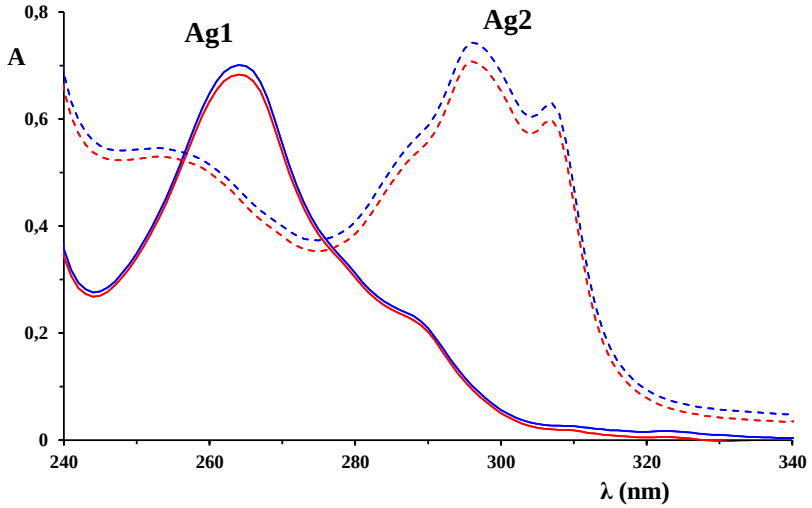


Figure 1. UV-Vis spectra of **Ag1** and **Ag2** complexes recorded right after their dissolution (blue/upper lines) and after 2 days (red/lower lines) in PBS at ambient temperature.

BSA binding experiment

Serum albumin (SA) has a significant role in distribution, transport, and deposition of different drugs and metabolites (LIU *et al.*, 2011; SATHYADEVI *et al.*, 2012). This protein is also important for the maintenance of the pH and osmotic pressure of plasma, and it brings different compounds to target cells (PETERS, 1995). Hence, investigation of interaction mechanisms between this plasma protein and biologically active compounds is very important for understanding their pharmacodynamics and pharmacokinetics. Bovine serum albumin (BSA) has high homology and similarity to human serum albumin (HSA) and is the most investigated model for protein interactions (GHOSH *et al.*, 2009). Fluorescence emission spectroscopy is an efficient method for study the BSA interaction because this protein has tryptophan, tyrosine, and phenylalanine as three fluorophores in its structure that are responsible for its intrinsic fluorescence (NAIK *et al.*, 2010).

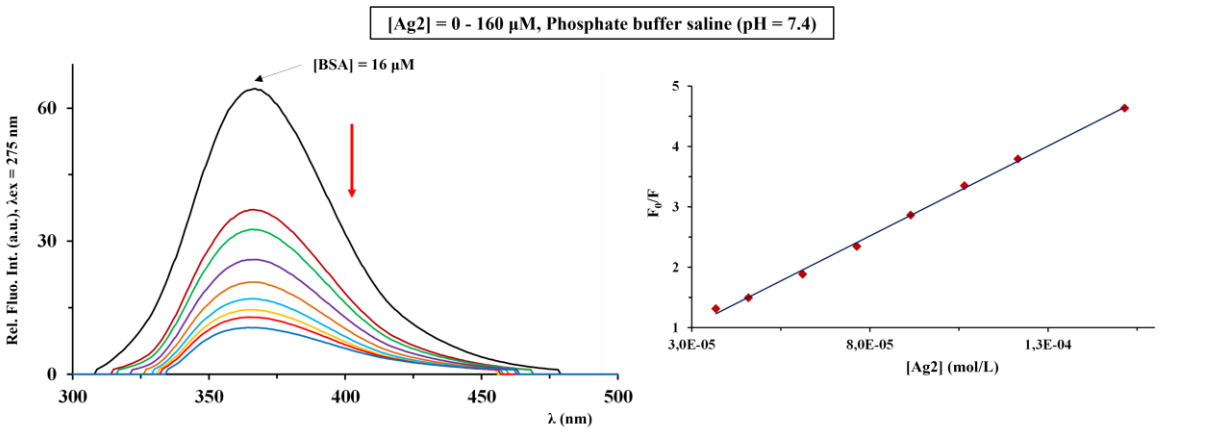


Figure 2. Fluorescence emission spectra of BSA in the presence of an increasing concentration of **Ag2** complex. The arrow shows the changes of fluorescence intensity after the addition of this complex. Inserted graph represents Stern-Volmer plot of F_0/F *vs.* [complex].

After adding **Ag1** and **Ag2** complexes to BSA solution (16 μM), a significant decrease of the BSA fluorescence intensity was observed (Fig. 2; 70 and 84% of its initial intensity, respectively), indicating that both complexes interact with this protein. In the case of interaction of BSA with **Ag1**, a red shift of 13 nm in the emission maximum was noticed as a result of its binding to the protein. This red shift is a consequence of the interaction of **Ag1** complex with hydrophilic site of the protein (MIN *et al*., 2004). On the other hand, a blue shift of 3 nm was observed after addition of **Ag2** complex and indicates its interaction with the BSA hydrophobic active site (YILMAZ *et al*., 2017).

The fluorescence quenching data for **Ag1** and **Ag2** complexes were studied using the Stern-Volmer and Scatchard equations. The calculated values of K_{sv}, K_q and K_A constants, and n number of binding sites per albumin are given in Tab. 1. The K_{sv} constants for **Ag1** and **Ag2** are comparable to those determined for silver(I) complexes containing 1,2-bis(4-pyridyl) ethene, thianthrene and 1,8-naphthyridine (ĐURIĆ *et al*., 2020; AŠANIN *et al*., 2021). The values of K_q constants for the investigated comlexes are higher than 2×10^{10} $M^{-1}s^{-1}$ (the maximum diffusion collision quenching rate constant), suggesting that the quenching mechanism is static (SHI *et al*., 2011). The K_A constants for both complexes are relatively high, and they show the affinity of the complexes to bind to BSA for their transport to the target cells. Nevertheless, the K_A constants are below 10^{15} M^{-1}, which represents the association constant of the interactions between avidin and ligands, as one of strongest non-covalent interactions (LAITINEN *et al*., 2006). This indicates that **Ag1** and **Ag2** can bind to BSA reversibly, and that these complexes are released from the protein upon arrival to the target sites. From the n values for the complexes, it can be concluded that there is one site accessible for their binding to the protein.

Table 1. BSA binding constants of the studied complexes **Ag1** and **Ag2**.

Complex	K_{sv} (M^{-1})	Hypochromism (%)	K_q ($M^{-1}s^{-1}$)	K_A (M^{-1})	n
Ag1	$(6.23\pm0.27) \times 10^4$	70.2	6.23×10^{12}	1.05×10^6	1.34
Ag2	$(2.21\pm0.11) \times 10^5$	83.7	2.21×10^{13}	1.37×10^6	1.41

Lipophilicity assay

Lipophilicity of a biologically active compound represents the most important factor that indicates its cellular uptake efficiency. A measure of lipophilicity is an *n*-octanol–water partition coefficient (log*P*), which can be determined by the flask-shaking method by PUCKETT and BARTON (2007). According to GHOSE *et al*., (1999), the log*P* values for a potentially new therapeutic agent are in the range from -0.4 to 5.6. The lipophilicity of complexes **Ag1** and **Ag2** was determined by measuring their concentration ratio in the two phases. The log*P* values for **Ag1** and **Ag2** are 0.0035 and -0.0063, respectively, suggesting that **Ag1** has a higher cellular uptake efficiency than **Ag2**.

In accordance with the Lipinski's rule of five, except log*P* value, molecular weight should be considered in design of new therapeutics (LIPINSKI *et al*., 2012). Thus, the molecular weight of oral drugs should not be higher than 500. The molecular weight of both **Ag1** and **Ag2** is higher than 500, nevertheless this does not represent a limitation of their potential application since it was found that many molecules having higher molecular weights can be transported into the cells (NEIDLE, 2012). Besides that, it was proposed that for metal-based drugs, molecular volume should be taken into consideration (ANTHONY *et al*., 2020).

DNA binding study

Previous studies have shown that one of the potential mechanisms of action of silver(I) complexes refers to their interaction with DNA (CLAUDEL *et al*., 2020). The DNA in-

teraction of silver(I) complexes is mainly dependent on their stability and the nature of the coordinated ligands (ZEGLIS *et al.*, 2007). For stable silver(I) complexes, non-covalent or electrostatic interactions between the complex and the negative phosphate groups of a DNA could be observed. On the other hand, if complexes contain labile ligands, they can be substituted by DNA donor atoms, leading to the formation of covalent bonds (STREKOWSKI *et al.*, 2007; KOMOR and BARTON, 2013).

DNA interactions of **Ag1** and **Ag2** complexes were investigated using fluorescence emission spectroscopy by measuring the intensity of ethidium bromide (EthBr) bound to ct-DNA after their addition (SMOLEŃSKI *et al.*, 2015). It is well known that EthBr intercalates between the adjacent ct-DNA base pairs, leading to the enhancement of its emission intensity, as a consequence of the formation of EthBr-ct-DNA system (SHI *et al.*, 2011; SMOLEŃSKI *et al.*, 2015). The emission spectra of the EthBr-ct-DNA ([ct-DNA] : [EthBr] = 10 : 1) were measured after the gradual addition of the studied complexes **Ag1** (Fig. 3) and **Ag2** (0 – 165 μM), which has caused a decrease of the emission intensities.

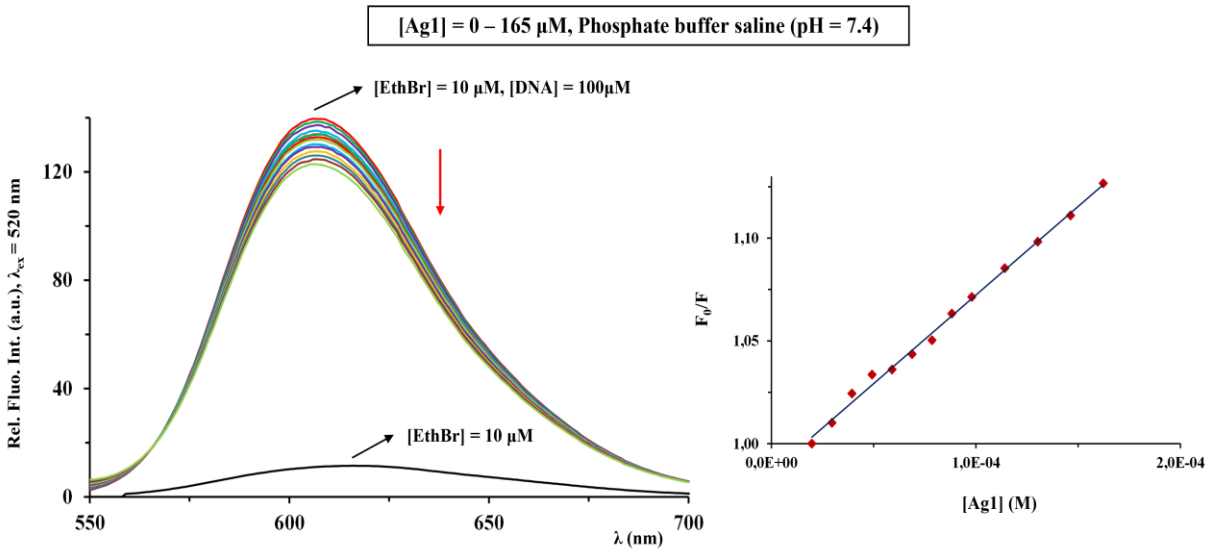


Figure 3. Fluorescence emission spectra of EthBr-ct-DNA system upon the gradual addition of **Ag1** complex. The arrow shows the change of fluorescence intensity after addition of the increasing concentration of the complex. Inserted graph represents Stern-Volmer plot of F_0/F *vs.* [complex].

The calculated binding constants (K_A, Tab. 2) are much lower than that of EthBr (K_A = 2×10^6 M^{-1}), suggesting that an explanation for the decrease in the fluorescence intensity of the EthBr-ct-DNA system after the addition of the silver(I) complexes is due to their interaction with this system, resulting in the formation of a new non-fluorescent product (SHI *et al.*, 2011). Moreover, the percentage of hypochromism is less than 15% (Tab. 2), while for the well-known DNA intercalator lucigenin is 50% (WU *et al.*, 2002), being in accordance with non-intercalative interactions of **Ag1** and **Ag2** complexes with ct-DNA. From the calculated K_{sv} values (Tab. 2), it could be concluded that **Ag2** show slightly higher affinity for ct-DNA than **Ag1**, whereas their affinity was found to be similar with that of the silver(I) complexes investigated previously by fluorescence spectroscopy (PETTINARI *et al.*, 2011; RENDOŠOVÁ *et al.*, 2017). The K_q constants for **Ag1** and **Ag2** are higher than 2×10^{10} $M^{-1}s^{-1}$ (Tab. 2), indicating that interaction between ct-DNA and these complexes is dominantly a static quenching process (SHI *et al.*, 2011).

Table 2. The ct-DNA binding constants of the studied complexes **Ag1** and **Ag2**.

Complex	K_{sv} (M^{-1})	Hypochromism (%)	K_q ($M^{-1}s^{-1}$)	K_A (M^{-1})	*n*
Ag1	$(8.73\pm0.01)\times10^2$	12.0	8.74×10^{10}	6.08×10^3	1.22
Ag2	$(1.67\pm0.01)\times10^3$	13.4	1.67×10^{11}	2.80×10^3	1.06

In vivo toxicity assay

The nematode *C. elegans* represents a model organism, having an extensive homology to mammals at the genetic level (HUNT, 2017). Accordingly, it can be used for toxicity evaluation and for the development of novel therapeutics (SEPÚLVEDA-CRESPO *et al*., 2020). As can be seen in Fig. 4, a dose dependent survival rate was observed for both investigated complexes **Ag1** and **Ag2**. At the highest tested concentration (50 μg/mL), around 70% of worms died within 48 h, while approximately only 5% of worms died at the lowest concentration (0.781 μg/mL). IC_{50} values against *C. elegans* for **Ag1** and **Ag2** complexes were found to be 12.5 and 6.25 μg/mL, respectively. Similar or lower IC_{50} values (12 – 55.5 μg/mL) were previously obtained for Ag(I) complexes with bidentate pyridine-4,5-dicarboxylate esters as ligands (ANDREJEVIĆ *et al*., 2020).

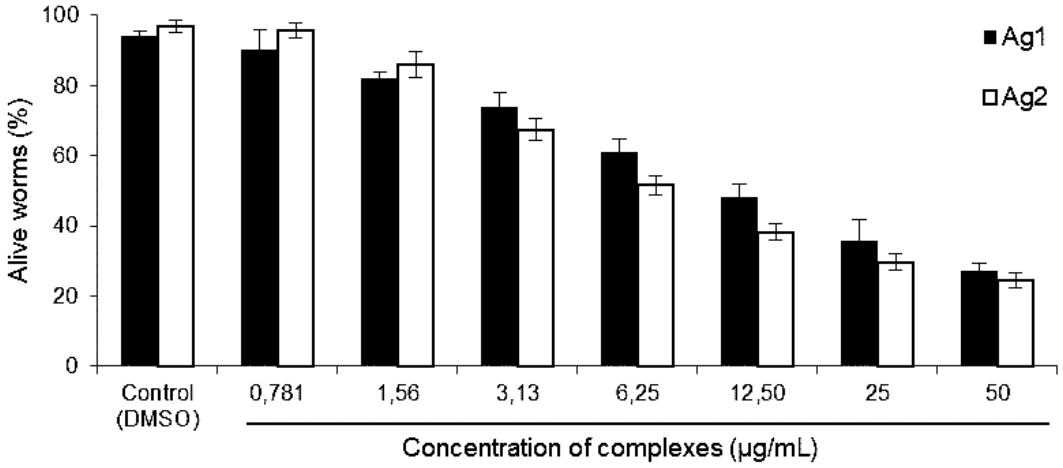


Figure 4. The influence of **Ag1** and **Ag2** complexes on the survival of *C. elegans* (%, average ± the SD) after 48 h treatment.

CONCLUSION

In the present study, silver(I)-phthalazine complexes, {[Ag(X-*O*)(phtz-*N*)]$_2$(*μ*-phtz-*N,N'*)$_2$}, X = NO_3^- (**Ag1**) and $CF_3SO_3^-$ (**Ag2**), were synthesized and their affinity toward ct-DNA and BSA was evaluated. These complexes can bind to BSA tightly and reversibly, while the interaction with DNA is rather non-covalent. The values of binding constants of silver(I)-phthalazine complexes to the studied protein are higher than those calculated for ct-DNA interaction, being in accordance with their greater binding affinity toward BSA. Given the antimicrobial resistance emergence, as well as the lack of novel antibacterial agents, the previously observed antibacterial activity of both complexes and their moderate cytotoxicity *in vivo* in *C. elegans* model system could be important for development of novel metal-based therapeutics.

Acknowledgments

This research has been financially supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Agreements No. 451-03-47/2023-01/200042, 451-03-47/2023-01/200122 and 451-03-47/2023-01/200378).

References:

[1] AHMAD, S., HANIF, M., MONIM-UL-MEHBOOB, M., ISAB, A.A., ALOTAIBI, M.A., AHMAD, T. (2022): Versatile coordination chemistry of mixed ligand silver(I) complexes of phosphanes and thioamides: Structural features and biological properties. *Polyhedron* **214**: 115643. doi: 10.1016/j.poly.2021.115643

[2] ANDREJEVIĆ, T.P., MILIVOJEVIC, D., GLIŠIĆ, B.Đ., KLJUN, J., STEVANOVIĆ, N.LJ., VOJNOVIC, S., MEDIC, S., NIKODINOVIC-RUNIC, J., TUREL, I., DJURAN, M.I. (2020): Silver(I) complexes with different pyridine-4,5-dicarboxylate ligands as efficient agents for the control of cow mastitis associated pathogens. *Dalton Transactions* **49** (18): 6084–6096. doi: 10.1039/D0DT00518E

[3] ANTHONY, E.J., BOLITHO, E.M., BRIDGEWATER, H.E., CARTER, O.W.L., DONNELLY, J.M., IMBERTI, C., LANT, E.C., LERMYTE, F., NEEDHAM, R.J., PALAU, M., SADLER, P.J., SHI, H., WANG, F.-X., ZHANG, W.-Y., ZHANG, Z. (2020): Metallodrugs are unique: opportunities and challenges of discovery and development. *Chemical Science* **11**: 12888–12917. doi: 10.1039/D0SC04082G

[4] AŠANIN, D.P., SKARO BOGOJEVIC, S., PERDIH, F., ANDREJEVIĆ, T.P., MILIVOJEVIC, D., ALEKSIC, I., NIKODINOVIC-RUNIC, J., GLIŠIĆ, B.Đ., TUREL, I., DJURAN, M.I. (2021): Structural characterization, antimicrobial activity and BSA/DNA binding affinity of new silver(I) complexes with thianthrene and 1,8-naphthyridine. *Molecules* **26** (7): 1871. doi: 10.3390/molecules26071871

[5] BERA, R., SAHOO, B.K., GHOSH K.S., DASGUPTA, S. (2008): Studies on the interaction of isoxazolcurcumin with calf thymus DNA. *International Journal of Biological Macromolecules* **42** (1): 14–21. doi: 10.1016/j.ijbiomac.2007.08.010

[6] BRACKMAN, G., COS, P., MAES, L., NELIS, H.J., COENYE, T. (2011): Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. *Antimicrobial Agents and Chemotherapy* **55** (6): 2655–2661. doi: 10.1128/AAC. 00045 -11

[7] CLAUDEL, M., SCHWARTE, J.V., FROMM, K.M. (2020): New antimicrobial strategies based on metal complexes. *Chemistry* **2** (4): 849–899. doi: 10.3390/chemistry2040056

[8] ĐURIĆ, S.Ž., VOJNOVIC, S., ANDREJEVIĆ, T.P., STEVANOVIĆ, N.LJ., SAVIĆ, N.D., NIKODINOVIC-RUNIC, J., GLIŠIĆ, B.Đ., DJURAN. M.I. (2020): Antimicrobial activity and DNA/BSA binding affinity of polynuclear silver(I) complexes with 1,2-bis(4-pyridyl) ethane/ethene as bridging ligands. *Bioinorganic Chemistry and Applications* **2020**: 3812050. doi: 10.1155/2020/3812050

[9] GHOSE, A.K., VISWANADHAN, V.N., WENDOLOSKI, J.J. (1999): A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. *Journal of Combinatorial Chemistry* **1** (1): 55–68. doi: 10.1021/cc9800071

[10] GHOSH, K.S., SEN, S., SAHOO, B.K., DASGUPTA, S. (2009): A spectroscopic investigation into the interactions of 3'-*O*-carboxy esters of thymidine with bovine serum albumin. *Biopolymers* **91** (9): 737–744. doi: 10.1002/bip.21220

[11] GLIŠIĆ, B.Đ., SENEROVIC, L., COMBA, P., WADEPOHL, H., VESELINOVIC, A., MILIVOJEVIC, D.R., DJURAN, M.I., NIKODINOVIC-RUNIC, J. (2016): Silver(I) complexes with phthalazine and quinazoline as effective agents against pathogenic *Pseudomonas aeruginosa* strains. *Journal of Inorganic Biochemistry* **155**: 115–128. doi: 10.1016/j.jinorgbio.2015.11.026

[12] HUNT, P.R. (2017): The *C. elegans* model in toxicity testing. *Journal of Applied Toxicology* **37** (1): 50−59. doi: 10.1002/jat.3357

[13] KOMOR, A.C., BARTON, J.K. (2013): The path for metal complexes to a DNA target. *Chemical Communications* **49** (35): 3617−3630. doi: 10.1039/C3CC00177F

[14] LAITINEN, O.H., HYTÖNEN, V.P., NORDLUND, H.R., KULOMAA, M.S. (2006): Genetically engineered avidins and streptavidins. *Cellular and Molecular Life Sciences CMLS* **63**: 2992–3017. doi: 10.1007/s00018-006-6288-z

[15] LIPINSKI, C.A., LOMBARDO, F., DOMINY, B.W., FEENEY, P.J. (2012): Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **64**: 4–17. doi: 10.1016/j.addr.2012.09.019

[16] LIU, H., SHI, X., XU, M., LI, Z., HUANG, L., BAI, D., ZENG, Z. (2011): Transition metal complexes of 2,6-di((phenazonyl-4-imino)methyl)-4-methylphenol: Structure and biological evaluation. *European Journal of Medicinal Chemistry* **46** (5): 1638–1647. doi: 10.1016/j.ejmech.2011.02.012

[17] MEDICI, S., PEANA, M., CRISPONI, G., NURCHI, V.M., LACHOWICZ, J.I., REMELLI, M., ZORODDU, M.A. (2016): Silver coordination compounds: A new horizon in medicine. *Coordination Chemistry Reviews* **327–328**: 349–359. doi: 10.1016/j.ccr.2016.05.015

[18] MEDICI, S., PEANA, M., NURCHI, V.M., ZORODDU, M.A. (2019): Medical uses of silver: History, myths, and scientific evidence. *Journal of Medicinal Chemistry* **62** (13): 5923–5943. doi: 10.1021/acs.jmedchem.8b01439

[19] MELAIYE, A., YOUNGS, W.J. (2005): Silver and its application as an antimicrobial agent. *Expert Opinion on Therapeutic Patents* **15** (2): 125–130. doi: 10.1517/13543776.15.2.125

[20] MIN, J., MENG-XIA, X., DONG, Z., YUAN, L., XIAO-YU, L., XING, C. (2004): Spectroscopic studies on the interaction of cinnamic acid and its hydroxyl derivatives with human serum albumin. *Journal of Molecular Structure* **692** (1–3): 71–80. doi: 10.1016/j.molstruc.2004.01.003

[21] NAIK, P.N., CHIMATADAR, S.A., NANDIBEWOOR, S.T. (2010): Interaction between a potent corticosteroid drug – Dexamethasone with bovine serum albumin and human serum albumin: a fluorescence quenching and fourier transformation infrared spectroscopy study. *Journal of Photochemistry and Photobiology B: Biology* **100** (3): 147–159. doi: 10.1016/j.jphotobiol.2010.05.014

[22] NEIDLE, S. (2012): *Therapeutic Applications of Quadruplex Nucleic Acids*. Elsevier Inc. Academic Press. doi: 10.1016/C2009-0-30510-3

[23] PETERS, T.J. (1995): *All about Albumin: Biochemistry, Genetics, and Medical Applications*. Academic Press, San Diego.

[24] PETTINARI, C., MARCHETTI, F., LUPIDI, G., QUASSINTI, L., BRAMUCCI, M., PETRELLI, D., VITALI, L.A., GUEDES DA SILVA, M.F.C., MARTINS, L.M.D.R.S., SMOLEŃSKI, P., POMBEIRO, A.J.L. (2011). Synthesis, antimicrobial and antiproliferative activity of novel silver(I) tris(pyrazolyl)methanesulfonate and 1,3,5-triaza-7-phosphadamantane complexes. *Inorganic Chemistry* **50** (21): 11173–11183. doi: 10.1021/ic201714c

[25] PUCKETT, C.A., BARTON, J.K. (2007). Methods to explore cellular uptake of ruthenium complexes. *Journal of the American Chemical Society* **129** (1): 46–47. doi: 10.1021/ja 0677564

[26] RENDOŠOVÁ, M., VARGOVÁ, Z., KUCHÁR, J., SABOLOVÁ, D., LEVOČA, Š., KUDLÁČOVÁ, J., PAULÍKOVÁ, H., HUDECOVÁ, D., HELEBRANDTOVÁ, V., ALMÁŠI, M., VILKOVÁ, M., DUŠEK, M., BOBÁLOVÁ, D. (2017). New silver complexes with bioactive glycine and nicotinamide molecules – characterization, DNA binding, antimicrobial and anticancer evaluation. *Journal of Inorganic Biochemistry* **168**: 1–12. doi: 10.1016/j.jinorgbio.2016. 12.003

[27] SANGSHETTI, J., PATHAN, S.K., PATIL, R., ANSARI, S.A., CHHAJED, S., AROTE, R., SHINDE, D.B. (2019): Synthesis and biological activity of structurally diverse phthalazine derivatives: A systematic review. *Bioorganic and Medicinal Chemistry* **27** (18): 3979 −3997. doi: 10.1016/j.bmc.2019.07.050

[28] SATHYADEVI, P., KRISHNAMOORTHY, P., JAYANTHI, E., BUTORAC, R.R., COWLEY, A.H., DHARMARAJ, N. (2012): Studies on the effect of metal ions of hydrazone complexes on interaction with nucleic acids, bovine serum albumin and antioxidant properties. *Inorganica Chimica Acta* **384**: 83–96. doi: 10.1016/j.ica.2011.11.033

[29] SCOFFONE, V.C., CHIARELLI, L.R., MAKAROV, V., BRACKMAN, G., ISRAYILOVA, A., AZZALIN, A., FORNERIS, F., RIABOVA, O., SAVINA, S., COENYE, T., RICCARDI, G., BURONI, S. (2016): Discovery of new diketopiperazines inhibiting *Burkholderia cenocepacia* quorum sensing *in vitro* and *in vivo*. *Scientific Reports* **6**: 32487. doi: 10.1038/srep32487

[30] SEPÚLVEDA-CRESPO, D., REGUERA, R.-M., ROJO-VÁZQUEZ, F., BALAÑA-FOUCE, R., MARTÍNEZ-VALLADARES, M. (2020): Drug discovery technologies: *Caenorhabditis elegans* as a model for anthelmintic therapeutics. *Medicinal Research Reviews* **40** (5): 1715−1753. doi: 10.1002/med.21668

[31] SHI, Y., GUO, C., SUN, Y., LIU, Z., XU, F., ZHANG, Y., WEN, Z., LI. Z. (2011): Interaction between DNA and microcystin-LR studied by spectra analysis and atomic force microscopy. *Biomacromolecules* **12** (3): 797−803. doi: 10.1021/bm101414w

[32] SILVER, S., PHUNG, L.T., SILVER, G. (2006): Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. *Journal of Industrial Microbiology and Biotechnology* **33** (7): 627−634. doi: 10.1007/s10295-006-0139-7

[33] SMOLEŃSKI, P., PETTINARI, C., MARCHETTI, F., GUEDES DA SILVA, M.F.C., LUPIDI, G., PATZMAY, G.V.B., PETRELLI, D., VITALI, L.A., POMBERIO, A.J.L. (2015): Syntheses, structures, and antimicrobial activity of new remarkably light-stable and water-soluble tris(pyrazolyl)methanesulfonate silver(I) derivatives of *N*-methyl-1,3,5-triaza-7-phosphaadamantane salt - [mpta]BF_4. *Inorganic Chemistry* **54** (2): 434–440. doi: 10.10 21/ic501855k

[34] STIERNAGLE, T. (1999): Maintenance of *C. elegans*. *In*: Hope. I. (ed.) *C. elegans: A Practical Approach*. Oxford University Press: 51–67.

[35] STREKOWSKI, L., WILSON, B. (2007): Noncovalent interactions with DNA: an overview. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **623** (1–2): 3–13. doi: 10.1016/j.mrfmmm.2007.03.008

[36] WOLFE, A., SHIMER JR., G.H., MEEHAN, T. (1987): Polycyclic aromatic hydrocarbons physically intercalate into duplex regions of denatured DNA. *Biochemistry* **26** (20): 6392–6396. doi: 10.1021/bi00394a013

[37] WU, H.-L., LI, W.-Y., HE, X.-W., MIAO, K., LIANG, H. (2002): Spectral studies of the binding of lucigenin, a bisacridinium derivative, with double-helix DNA. *Analytical and Bioanalytical Chemistry* **373**: 163–168. doi: 10.1007/s00216-002-1309-8

[38] YILMAZ, V.T., ICSEL, C., BATUR, J., AYDINLIK, S., CENGIZ, M., BUYUKGUNGOR, O. (2017): Synthesis, structures and biomolecular interactions of new silver(I) 5,5-diethylbarbiturate complexes of monophosphines targeting Gram-positive bacteria and breast cancer cells. *Dalton Transactions* **46**: 8110–8124. doi: 10.1039/c7dt01286a

[39] ZEGLIS, B.M., PIERRE, V.C., BARTON, J.K. (2007): Metallo-intercalators and metallo-insertors. *Chemical Communications* (44): 4565−4579. doi: 10.1039/B710949K