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ARTICLE

Silver(I) complexes with quinazoline and phthalazine: synthesis, structural characterization and evaluation of biological activities†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Nada D. Savić,[a] Biljana Đ. Glišić,[*a] Hubert Wadepohl,[b] Aleksandar Pavic,[c] Lidija Senerovic,[c] Jasmina Nikodinovic-Runic[c] and Miloš I. Djuran[a]

New silver(I) complexes with quinazoline (qz) and phthalazine (phtz), $[Ag(NO_3)(qz)]_n$ (**1**) and $\{[Ag(CH_3CN)]_2(\mu\text{-phtz})_2\}[BF_4]_2$ **(2)**, have been synthesized and structurally characterized by using different spectroscopic and single crystal X-ray diffraction techniques. The obtained results revealed that the reaction of $AgNO_3$ with qz at room temperature in a 2 : 1 molar ratio led to the formation of the polynuclear complex **1**. However, the reaction of $AgBF_4$ with phtz under the same experimental conditions resulted in the formation of the dinuclear complex **2**. The solution behaviour and air/light stability of these silver(I) complexes have been investigated. The complexes **1** and **2**, alongside with the silver(I) salts used for their synthesis, were evaluated by *in vitro* antimicrobial studies against a panel of microbial strains that lead to many skin and soft tissue, respiratory, wound, and nosocomial infections. The obtained results indicate that all tested silver(I) compounds have good antibacterial activity with MIC values in the range from 1.5 to 15.6 μg mL^{-1} against the investigated strains. On the other hand, their antifungal activity against *Candida albicans* was moderate. In order to determine the therapeutic potential of **1** and **2**, their antiproliferative effect on the normal human lung fibroblast cell line MRC5, the hemolytic effect on red blood cells and embryotoxicity on zebrafish (*Danio rerio*) have also been evaluated.

Introduction

The bioactivities of silver, especially its antimicrobial properties, are widely exploited in numerous products and medical devices. For this purpose colloidal and nanocrystalline silver and different forms of silver(I) compounds (oxide, salts and complexes) have been used.[1,2] The therapeutic potential and toxicity of silver highly depend on the chemical species. Up to now, numerous studies have been conducted in attempts to elucidate the mechanism of activity of the abovementioned silver preparations and although a general consensus has not been reached, the highest activity seems to be due to ionic Ag^+.[3-5] Several different mechanisms are thought to be responsible for the antimicrobial activity of this ion: (*i*) Ag^+ interacts with thiol groups of L-cysteine residues of proteins, inactivating their functions; (*ii*) Ag^+ binds to nucleic acids; (*iii*) Ag^+ causes potassium release and (*iv*) Ag^+ generates superoxide intracellularly.[6] Therefore, free Ag^+ has plenty of possibilities to disturb biochemical processes on multiple levels. For example, the interaction of Ag^+ with bacterial proteins and nucleic acids causes structural changes in membranes by blocking respiration or nucleic acids transcription.[6]

Among other biologically active silver formulations, in the last decades, silver(I) complexes came into focus as an alternative formulation of silver with desirable and tunable antimicrobial properties.[7-17] Numerous silver(I) complexes with nitrogen-, oxygen-, phosphorus- and sulphur-donor ligands, as well as with *N*-heterocyclic carbenes (NHC) have been synthesized and evaluated as potential antibacterial and antifungal agents against different Gram-positive and Gram-negative bacteria and various yeasts and molds. The results show that one of the key factors determining the antimicrobial potential of silver(I) complexes is the nature of the donor coordinated to Ag^+, rather than the complexes' solubility, charge, chirality or degree of polymerization.[12] Thus, silver(I) complexes with nitrogen and oxygen donors exhibit an effective and wide spectrum of antimicrobial activities.[12,16] Their effectiveness was ascribed to the presence of weak Ag(I)–O/N bonds, which are cleaved in their reactions with different biomolecules, especially with those containing thiol groups.[6] However, silver(I) complexes with a sulphur atom in the coordination sphere have demonstrated a narrower spectrum of antimicrobial activity than those with nitrogen- and oxygen-donor ligands. Moreover, silver(I) complexes with NHC ligands, especially those containing electron withdrawing

[a] Department of Chemistry, Faculty of Science, University of Kragujevac, R. Domanovića 12, PO Box 60, 34000 Kragujevac, Serbia; *E-mail: bglisic@kg.ac.rs
[b] Anorganisch-Chemisches Institut, University of Heidelberg, Im Neuenheimer Feld 270, 69120 Heidelberg, Germany
[c] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11000 Belgrade, Serbia

†Electronic Supplementary Information (ESI) available: [Fig. S1 and Table S1-S3]. See DOI: 10.1039/x0xx00000x

CCDC 1426003 and 1426004 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

groups attached to the imidazole ring (such as (1,3-dimethyl-4,5-dichloroimidazole-2-ylidene)silver(I) acetate), have manifested activity against bacterial strains associated with cystic fibrosis and chronic lung infections.[13,17] On the other hand, most of the investigated silver(I) complexes having coordinated phosphorus donor atom have shown no antimicrobial activity.[12]

An attractive class of ligands for the synthesis of antimicrobial silver(I) complexes comprises aromatic nitrogen-containing heterocyclic ligands (*N*-heterocycles).[7,9,14-16] These compounds are one of the most important classes of ligands in coordination and bioinorganic chemistry, and have also found use in the rapidly evolving field of metallosupramolecular chemistry.[18] Although some promising activities were described against biofilms of *Pseudomonas aeruginosa* and, more importantly, silver(I) complexes of 1,10-phenanthroline (phen) and coumarin-4-carboxylate ligands have been shown to be active against clinically relevant methicillin-resistant *Staphylococcus aureus* (MRSA) strains,[15] more work is needed to determine the therapeutic potential of silver(I) complexes. Furthermore, the cytotoxic properties of silver(I) complexes have been rarely addressed,[16] and their embryotoxic and teratogenic effects have not been examined. Therefore, in a continuation of our studies on the synthesis, characterization and evaluation of silver(I) complexes with aromatic *N*-heterocycles,[16] we have prepared and characterized new silver(I) complexes with quinazoline (qz) and phthalazine (phtz) and comprehensively examined their antimicrobial activity, the effect on the viability of human cell line, as well as the effect on the development of zebrafish embryos, a model of the vertebrate development that is increasingly important for the evaluation of compounds and pharmaceuticals that may pose a risk to human health or to the environment. It is worth noting that both investigated *N*-heterocycles and their derivatives embedded with a variety of functional groups are important structural moieties of many natural products and biologically active compounds, and show diverse pharmacological properties, such as antimicrobial, antitumor, anticonvulsant and anti-inflammatory.[19]

Results and discussion

Synthesis and structural characterization of 1 and 2

The silver(I) complexes with aromatic nitrogen-containing heterocycles, $[Ag(NO_3)(qz)]_n$ (**1**) and $\{[Ag(CH_3CN)]_2(\mu\text{-phtz})_2\}[BF_4]_2$ (**2**) (qz is quinazoline and phtz is phthalazine), have been synthesized. Schematic presentation of the reactions for the synthesis of **1** and **2** is shown in Scheme 1. These complexes were prepared by reacting the *N*-heterocyclic ligand and the corresponding AgX salt (X = NO_3^- for **1** and BF_4^- for **2**) in a 1 : 2 molar ratio in methanol/acetone (1 : 1, v/v) at room temperature. The crystals of **1** and **2** were obtained after the white precipitates from these reactions were recrystallized in acetonitrile. The ^{1}H NMR spectra of the precipitates formed in the investigated reactions show the same resonances for qz and phtz as those for these ligands in **1** and **2**, respectively. However, in complex **2** with BF_4^- counter-anion, acetonitrile is coordinated to Ag(I) ion, while in nitrate-containing complex **1**, no coordination of this solvent was observed. This is in accordance with the previous finding that the ability of acetonitrile to coordinate Ag(I) ion is inversely proportional to the order of the coordination ability of these polyatomic anions, *i.e.* NO_3^- >> BF_4^-.[20] Thus, the coordinating nature of NO_3^- represents an obstacle to the acetonitrile coordination, while weakly coordinating BF_4^- anion afford acetonitrile-abundant product **2**. The stoichiometries of the complexes **1** and **2** were confirmed by elemental microanalysis, and the structures were elucidated by spectroscopic (IR, ^{1}H NMR and UV-vis) and crystallographic methods.

Description of crystal structures. The asymmetric unit of coordination polymer **1** comprises two crystallographically independent Ag(I) centers, Ag1 and Ag2, as well as two quinazoline heterocycles and two NO_3^- anions (Fig. 1; an extended view of **1** is shown in Fig. S1). As expected for pyrimidine-type heterocycles,[21,22] in complex **1**, quinazoline acts as a bridging ligand bonded to two Ag(I) ions through its two nitrogen atoms (Fig. 1). The Ag—N(qz) bond lengths fall in the range of 2.255(3) – 2.322(4) Å (Table 1) and are in accordance with those previously reported for other polymeric silver(I) complexes with aromatic *N*-heterocycles.[16] The Ag—O bond distances in **1** are in range of 2.377(3) – 2.451(3) Å (Table 1), being slightly longer than usual silver(I)-oxygen bond of about 2.3 Å.[23] However, Ag—O bonds in this complex are shorter than those in the previously reported nitrate-containing dinuclear $\{[Ag(NO_3)(phtz)]_2(\mu\text{-phtz})_2\}$ complex (2.576(4) Å).[16] It is worth to mention that Ag···Ag interactions are not observed in **1**, *i.e.* the distances between two Ag(I) ions are much larger than the sum of the van der Waals radii (3.44 Å).[24]

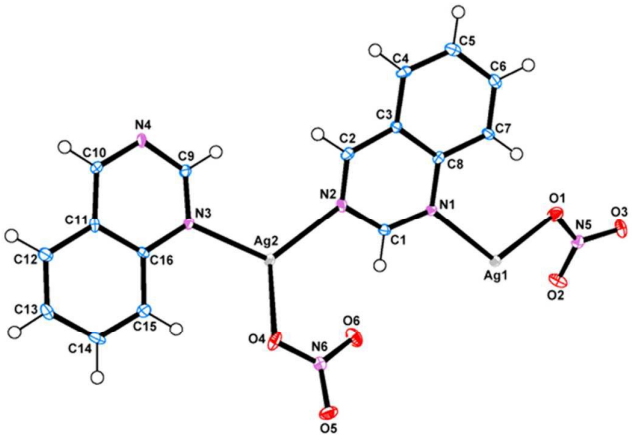


Fig. 1 The asymmetric unit of polymeric complex **1**. Displacement ellipsoids are drawn at 50% probability level and H atoms are represented by spheres of arbitrary size.

Table 1 Selected bond distances (Å) and valence angles (°) of the silver(I) complexes **1** and **2**

1		2	
Ag1—O1	2.377(3)	Ag—N1	2.3173(13)
Ag1—N1	2.266(3)	Ag—N2^{iii}	2.2267(15)
Ag1—N4^{i}	2.255(4)	Ag—N3	2.2447(15)
Ag2—O4	2.451(3)	N2—Ag^{iii}	2.2267(14)
Ag2—N2	2.322(4)		
Ag2—N3	2.255(3)		
N1—Ag1—O1	113.19(11)	N2^{iii}—Ag—N1	127.44(4)
N4^{i}—Ag1—O1	121.85(11)	N2^{iii}—Ag—N3	135.49(5)
N4^{i}—Ag1—N1	121.21(13)	N3—Ag—N1	96.99(5)
C1—N1—Ag1	119.4(3)	N2—N1—Ag	117.88(8)
C1—N1—C8	117.5(3)	C8—N1—Ag	122.87(9)
C8—N1—Ag1	123.1(2)	C8—N1—N2	119.26(12)
O3^{ii}—Ag2—O4	100.56(8)	N1—N2—Ag^{iii}	114.12(9)
N2—Ag2—O4	123.42(10)	C1—N2—Ag^{iii}	125.67(9)
N3—Ag2—O4	103.56(10)	C1—N2—N1	119.65(12)
N3—Ag2—N2	119.69(13)	C9—N3—Ag	174.77(13)
C1—N2—Ag2	114.7(3)		
C2—N2—Ag2	127.8(3)		
C2—N2—C1	116.8(4)		
C9—N3—Ag2	117.7(3)		
C9—N3—C16	117.3(3)		
C16—N3—Ag2	124.7(3)		

Symmetry code(s): (i) *x*, *y*+1, *z*; (ii) -*x*+1, -*y*+2, *z*+1/2; (iii) -*x*+2, -*y*+2, -*z*+2

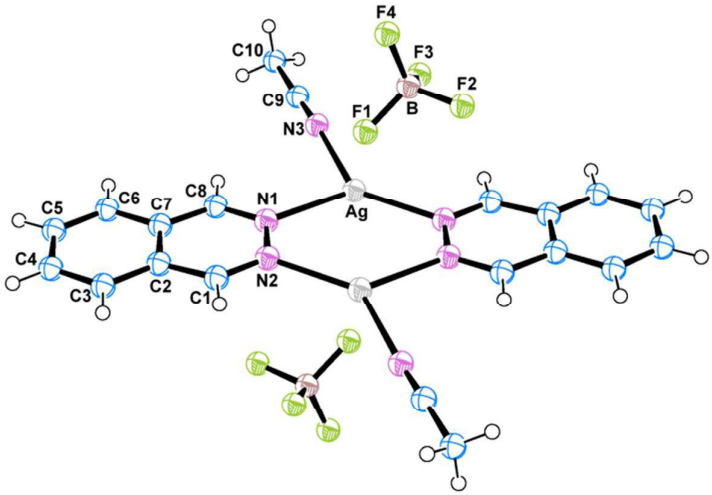
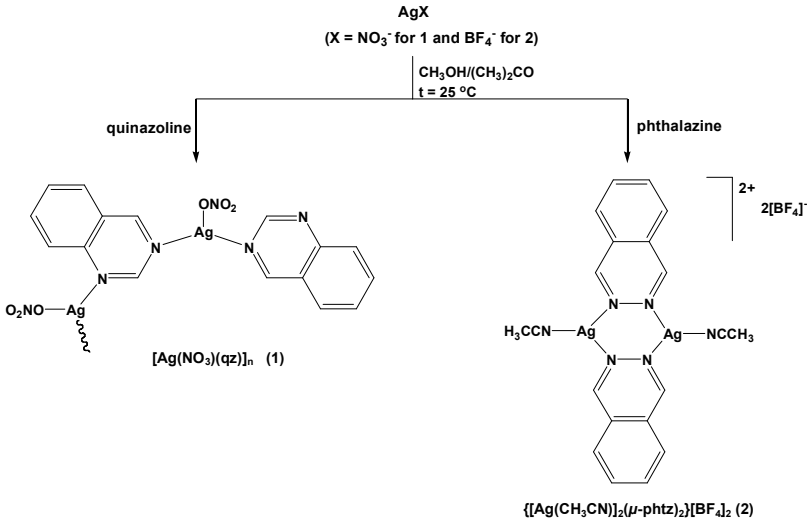


Fig. 2 Molecular structure of dinuclear complex **2**. Displacement ellipsoids are drawn at 50% probability level and H atoms are represented by spheres of arbitrary size.



Scheme 1 Schematic presentation of the reactions for the synthesis of silver(I) complexes **1** and **2**. The complexes crystallized from acetonitrile.

Similar to the previously characterized {[Ag(X)(phtz)]$_2$(μ-phtz)$_2$} complexes (X = NO_3^-, $CF_3SO_3^-$ and ClO_4^-),[16] the molecular structure of the dinuclear complex **2** is composed of a six-membered dimetallic ring, which is formed by two Ag(I) ions and four nitrogen atoms of two phthalazines (Fig. 2). These two phtalazines act as bridging ligands between two Ag(I) ions, while the third coordination site of these ions is occupied by acetonitrile. In complex **2**, the coordination of the BF_4^- anion to Ag(I) is not observed, and this is in contrast to {[Ag(X)(phtz)]$_2$(μ-phtz)$_2$} complexes with a weakly coordinated *O*-bound anion X.[16] The Ag–N1/N2(phtz) bond distances in **2** (Table 1) adopt values of 2.317(1) and 2.227(2) Å, respectively, and are comparable with other *N*-heterocycle-silver(I) complexes.[16,22,25-27] The Ag–N3(acetonitrile) bond distance of 2.245(2) Å falls in the range of 2.18 – 2.33 Å, reported previously.[28] The Ag(I) ion in **2** adopts a distorted trigonal planar geometry, with N2—Ag—N1, N2—Ag—N3 and N3—Ag—N1 angles of 127.44(4), 135.49(5) and 96.99(5)°, respectively (Table 1), *i.e.* both angles involving the nitrogen atom of acetonitrile (N3) significantly deviate from 120°. Contrary to complex **1**, a week Ag···Ag intramolecular interaction is observed in **2**, with an Ag···Ag distance of 3.386(1) Å. This is longer than the commonly reported Ag···Ag interaction range of 2.85 – 3.29 Å,[29] but slightly shorter than the sum of van der Waals radii (3.44 Å).[24]

Contrary to the dinuclear phtz-silver(I) complex **2**, the previously characterized {[Ag(μ-CH_3COO)(μ-phtz)(H_2O)$_2$]$_2$}$_n$ complex has polymeric structure, which comprises stacks of

phtz-bridged dimeric $Ag_2(phtz)_2$ subunits; these subunits are further connected by the acetates which bridge these stacks to form a polymer.[30] On the other hand, reaction of silver(I) triflimide ($AgNTf_2$) with phtz led to the formation of tetranuclear $[Ag_4(\mu\text{-phtz})_7](NTf_2)_4$ complex, while in the presence of 2,2':6',2''-terpyridine (terpy), dinuclear $[Ag_2(\mu\text{-phtz})(terpy)_2](NTf_2)_2 \cdot 2CH_3CN$ complex was obtained as the final product.[31]

IR spectroscopic characterization. Except for the peaks ascribed to the coordinated phthalazine and quinazoline (see Experimental section), the IR spectra of **1** and **2** also show the typical peaks of NO_3^- and BF_4^-, respectively. Thus, in the spectrum of **1**, the strong bands at 1377 and 1352 cm^{-1} and two very week transitions in the overtone region (1750 and 1738 cm^{-1}) can be ascribed to the asymmetric and symmetric stretching modes and a combination of symmetric stretching and in-plane bending, respectively, of the coordinated nitrate group.[32,33] On the other hand, the presence of the strong band at 1058 cm^{-1} and a medium one at 767 cm^{-1} in the IR spectrum of **2** indicates no coordination of BF_4^- to the Ag(I) center.[16,34]

Stability of 1 and 2

Solution stability. In order to investigate the solution behaviour of complexes **1** and **2**, they were dissolved in DMSO, and their UV-Vis spectra were recorded immediately after dissolution, as well as after 24 and 96 h of incubation at 37 °C. As it was found previously for the similar silver(I) complexes,[16,35] the absorbance peaks at 306.0 and 298.0 nm in **1** and **2**, respectively, correspond to $\pi \rightarrow \pi^*$ transitions in the aromatic *N*-heterocycles. These peaks show red shifts in comparison to those in the spectra of the free *N*-heterocycles recorded in the same solvent (λ_{max}= 304.0 nm for quinazoline and λ_{max} = 288.0 nm for phthalazine). A slight decrease in intensity of the absorption maxima of **1** and **2** was noticed after 96 h at 37 °C (11 and 7%, respectively, in comparison with the maxima determined immediately after dissolution of these complexes). However, the shape of spectra of **1** and **2** remained unmodified during 96 h, implying their substantial stability in solution. Moreover, on the basis of proton NMR spectroscopy, no coordination of DMSO to Ag(I) ion was observed during this time.

Air/light stability. In order to investigate the air/light stability of the silver(I) complexes **1** and **2** which might be of importance for their use as antimicrobial agents in the form of ointments and gels,[7] sterile cellulose discs impregnated with these complexes and the corresponding silver(I) salts (200 μg per disc, using 50 mg mL^{-1} DMSO stock solution) were exposed to air and light for 48 h. The obtained results revealed that complexes **1** and **2** are more stable to air and light than $AgNO_3$ and $AgBF_4$ salts. The investigated silver(I) compounds started to be a little beige after 12 h, and after 48 h all were slightly darker, indicating that slow light decomposition processes occurred during this time. Among the investigated Ag(I) compounds, complex **2** qualitatively showed slightly higher air/light stability.

Evaluation of biological activities of 1 and 2

Antimicrobial potential. The newly synthesized silver(I) complexes **1** and **2** were evaluated against a panel of six microorganisms that included three Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* PAO1 and *Salmonella typhimurium*), two Gram-positive bacteria (*Staphylococcus aureus* and *Lysteria monocytogenes*) and the fungus *Candida albicans*. All these microorganisms are human pathogens, which can cause a variety of diseases such as skin, wound and burn infections, diarrhea, pneumonia, typhoid fever, and infections of the central nervous system and urinary tract.[36,37] The antimicrobial activity of **1** and **2** and the respective silver(I) salts, $AgNO_3$ and $AgBF_4$, against the abovementioned strains is expressed as minimal inhibitory concentration (MIC, μg mL^{-1}) and compared to the effect on the viability on the human fibroblast cell line (MRC5), to properly evaluate the therapeutic index (Table 2).

The obtained results indicate that all tested silver(I) compounds have good antibacterial activity with MIC values in the range of 1.5 to 15.6 μg mL^{-1} against the investigated strains. The activity of **1** and **2** is comparable and generally lower than that of the $AgNO_3$ and $AgBF_4$ salts. Among all investigated silver(I) compounds, $AgBF_4$ appears to be the most active against bacterial strains with MIC values 10- and 5- fold lower than those of **2** for *P. aeruginosa* and *S. aureus*, respectively (Table 2). However, it is important to note that the molar ratio of Ag(I) ions in salts ($n(Ag^+)/M_r$) is considerably higher than in complexes; with $AgNO_3$ it is 1.8-fold higher than in complex **1**, while with $AgBF_4$ it is 2.0-fold higher than in **2**.

From the obtained results, it could not be concluded whether the investigated silver(I) compounds were more active against Gram-positive or Gram-negative species. However, all compounds exhibited lower antifungal activity, in comparison to antibacterial, with Ag(I) salts being more active than the corresponding complexes (2.5-fold difference in MIC values; Table 2). This was also the case with the previously synthesized silver(I) complexes with phthalazine, $\{[Ag(X)(phtz)]_2(\mu\text{-phtz})_2\}$ (X = NO_3^-, $CF_3SO_3^-$ and ClO_4^-), and quinazoline, $\{[Ag(CF_3SO_3)(qz)]_2\}_n$ and $\{[Ag(qz)][BF_4]\}_n$; nevertheless these complexes were more active against *C. albicans* in comparison to complexes **1** and **2**.[16] Similarly, silver(I) complexes obtained in the reactions of $AgNO_3$ and $AgBF_4$ with metronidazole (mtz), $[Ag(mtz)_2(NO_3)]$ and $[Ag_2(mtz)_4][BF_4]_2$, showed better antibacterial activity, especially towards Gram-negative bacteria *E. coli* and *P. aeruginosa* (MIC = 8.7 – 60.1 μg mL^{-1}; 17 – 98 μM), than antifungal towards *C. albicans* (MIC ≥ 220 μg mL^{-1}; 215 μM).[7] Contrary to this, three silver(I) complexes with coordinated 4-(hydroxymethyl)pyridine, 2,6-di(hydroxymethyl)pyridine and 2-(hydroxymethyl)benzimidazole exhibited better activity towards *C. albicans* (MIC = 10 – 20 μg mL^{-1}; 21 – 52 μM) than Gram-positive bacteria *S. aureus* and *S. epidermidis* (MIC = 40 – 90 μg mL^{-1}; 103 – 201 μM).[9]

Although good antimicrobial activities were detected for **1** and **2**, these complexes together with salts exhibited stronger negative effects on the viability of the normal human lung fibroblast cell line MRC5, which is not a desirable property for

application of these complexes as antibiotics (Table 2). All investigated compounds showed stronger toxicity on the human cell line, with salts being 3-fold more toxic in comparison to the complexes, judged from the IC_{50} values. A therapeutic index lower than 1 is seriously limiting application potential of compounds as antibacterial agents and opening the possibility to consider them as agents for external applications (treatment of medicinal and other devices, etc.). On the other side, certain medical conditions, such as lung and gastric cancer, are often associated with serious bacterial infections.[38,39] Infections are also the major cause of morbidity and mortality in children with cancer.[40] Therefore, dual activity of compounds, antiproliferative and antibacterial, such as observed with **1** and **2** may be further exploited.

According to the literature data, many silver(I) complexes with *N*-heterocyclic compounds have a strong antibacterial activity, however, their cytotoxicity has rarely been examined. The cytotoxic activity of $\{[Ag(X)(phtz)]_2(\mu\text{-phtz})_2\}$ (X = NO_3^-, $CF_3SO_3^-$ and ClO_4^-), $\{[Ag(CF_3SO_3)(qz)]_2\}_n$ and $\{[Ag(qz)][BF_4]\}_n$ against the human normal cell line MRC5 was moderate; therefore, these complexes can be further evaluated as possible antimicrobial agents.[16] On the other hand, di- and polynuclear silver(I) saccharinate complexes with tertiary monophosphines showed significant activity towards different bacterial strains (MIC = 14.5 – 114.6 μM), however they were also highly cytotoxic toward the human fibroblast cell line WI-38 (IC_{50} = 0.74 – 1.41 μM).[41]

Table 2 Minimal inhibitory concentrations (MIC, μg mL^{-1}) against a panel of microorganisms and antiproliferative activity (IC_{50}, μg mL^{-1}) of **1** and **2** in comparison to $AgNO_3$ and $AgBF_4$, respectively

Silver(I) compound / Organism	1	$AgNO_3$	2	$AgBF_4$
Escherichia coli	7.8	7.8	15.6	5
Pseudomonas aeruginosa PAO1	7.8	3.1	15.6	1.5
Salmonella typhimurium	7.8	7.8	7.8	5
Staphylococcus aureus	15.6	3.1	15.6	3.1
Lysteria monocytogenes	15.6	5	15.6	7.8
Candida albicans	125	50	125	50
MRC5 (human lung fibroblasts)[a]	4	1.5	3	1

[a] IC_{50} is defined as the concentration inhibiting 50% of cell growth after the treatment with the tested compounds. Results are from three independent experiments, each performed in triplicate. Standard deviations were within 1-3%.

We have also tested whether these silver(I) compounds had the ability to cause hemolysis using sheep red blood cells in the concentration range from 1-50 μg mL^{-1}, covering concentrations that showed activity against bacteria (Fig. 3). When applied in 50 μg mL^{-1}, all silver(I) compounds caused lysis of red blood cells between 50-58%, within 1 h after the treatment, indicating that at this concentration, disruption of the membrane may be the mode of action of these compounds. At lower concentrations (≤ 25 μg mL^{-1}) complex **2** exhibited a lower hemolytic effect in comparison to all other compounds. Further study into determining the exact mode of activity of silver(I) complexes is needed; however it emerges that, with certainty, silver(I) complexes have multiple cellular targets.

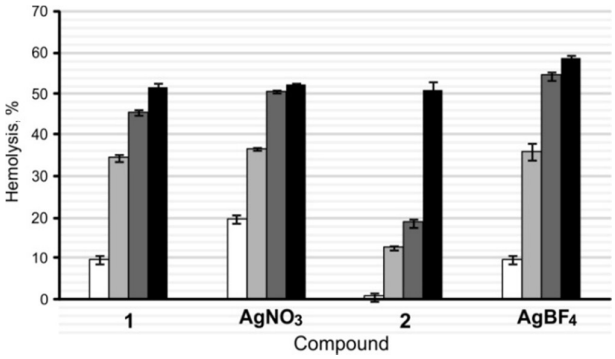


Fig. 3 Hemolytic effect of silver(I) compounds at 1 (□), 5 (▨), 25 (■) and 50 μg mL^{-1} (■).

***In vivo* toxicity on zebrafish**. A higher cytotoxicity in comparison to the antimicrobial activity of all investigated silver(I) compounds led us to further examine their toxicity and safety profiles using the zebrafish model. The zebrafish model is a widely accepted model for mechanism-based toxicological studies, as the embryonic zebrafish offers a rapid, high-throughput platform to assess chemical and biological system interactions.[42]

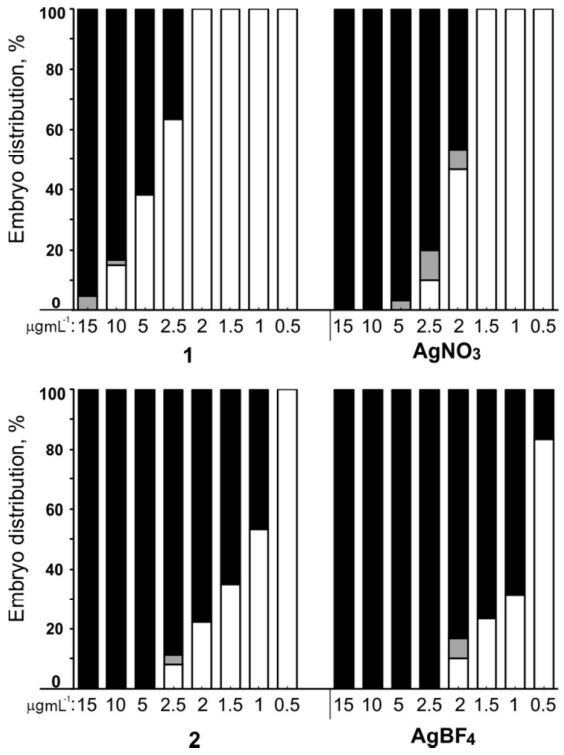


Fig. 4 Comparison of toxicity of complexes **1** and **2**, $AgNO_3$ and $AgBF_4$ on development of zebrafish embryos after 96 h exposure to different concentrations. White bars – normal embryos, black bars – lethal embryos, grey bars – teratogenic embryos.

Acute toxicity of the silver(I) complexes **1** and **2** to zebrafish (*Danio rerio*) was investigated in a 96 h exposure study and

compared with the toxicity of the silver(I) salts, $AgNO_3$ and $AgBF_4$. We have exposed 4 h old embryos to various concentrations of silver(I) compounds, and the results of the toxicity assay showed that all tested compounds were toxic, causing more than 80% lethality of zebrafish embryos, when applied in concentrations ≥10 μg mL^{-1} (Table S1, Fig. 4). However, both silver complexes showed lower overall toxicity in comparison to the salts, with **1** being less toxic than **2** (Table S1, Fig. 4); the IC_{50} values were 4.35 and 2.09 μg mL^{-1} for **1** and $AgNO_3$, 1.2 and 0.85 μg mL^{-1} for **2** and $AgBF_4$, respectively. Thus, the difference in the toxicity profiles were more pronounced between **1** and $AgNO_3$, then between **2** and $AgBF_4$. At concentrations of **1** ≥ 10 μg mL^{-1}, majority of embryos were killed, while embryos survived at 5 μg mL^{-1} of **1** (c.a. 40%) showed no signs of teratogenicity. In turn, the most of zebrafish embryos exposed to ≥ 5 μg mL^{-1} of $AgNO_3$ were dead, while alive embryos at 5 μg mL^{-1} (c.a. 3%) had serious skeletal abnormalities (lordosys and small head), weak pericardial edema and weakly resorbed yolk (Fig. 5), that may be caused by impaired yolk sack circulation. On the other hand, complex **2** and $AgBF_4$ were toxic at ≥ 2.5 μg mL^{-1}, whereas embryos surviving 2 μg mL^{-1} of **2** had no developmental defects, and those at 2 μg mL^{-1} of $AgBF_4$ suffered of serious pericardial edema and lordosys, much reduced head, as well as mostly unresorbed yolk (Fig. 6). None of the tested compounds had effect on heart beating, body circulation rate (data not shown) or hatching.

Results obtained in this study in the toxicity assay on zebrafish revealed that the new silver(I) complexes, especially **1**, are less toxic then the corresponding silver(I) salts. The number of dead embryos upon treatment with **1** was significantly lower than the number of embryos following $AgNO_3$ treatment. In addition, signs of teratogenicity were rarely observable in alive embryos treated with **1**, while alive $AgNO_3$–treated embryos developed serious cardiovascular and skeletal abnormalities, similarly to those reported by other researchers.[43,44]

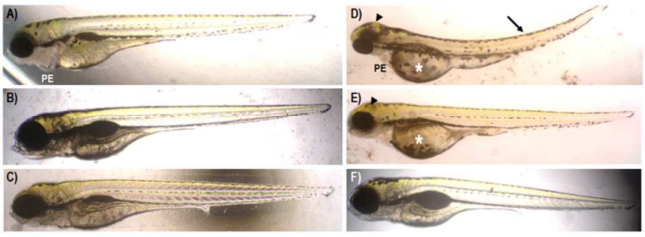


Fig. 5 Images of zebrafish embryos after 96 h exposure to **1** (A - C) and $AgNO_3$ (D – F). At concentration of 15 μg mL^{-1} of **1** (A) only small pericardial edema (PE) was observable, while at 10 μg mL^{-1} (B) and 5 μg mL^{-1} (C), embryos were not affected. On the other hand at 5 μg mL^{-1}, $AgNO_3$ induced serious malformations of head (arrowhead), lordosys (arrow), small pericardial edema (PE) and reduced yolk absorption (asterisk) (D), while at 2.5 μg mL^{-1} (E), embryos were affected at lesser degree. (F) Untreated embryos.

These effects may be due to the stability of complex **1** and therefore reduced releasing of Ag^+ ions by this complex in solution, and this has already been the proposed toxicity mechanisms of diverse Ag-nanoparticles.[45] On the other hand, complex **2** was significantly more toxic than **1**, exerting a 3.5 times smaller IC_{50} value, what may be attributed to its chemical structure, involving Ag^+ and BF_4^- ions. Similarly to free silver(I) ions, fluoride and fluoroborate anions were shown to be toxic on zebrafish embryos, causing developmental deformities.[46]

Our results also confirmed the toxicity of $AgNO_3$. Ag-nanoparticles and Ag^+ ions were shown to induce toxicity in zebrafish embryos as well as oxidative stress, with Ag^+ ions being more toxic, but both leading to death and delayed hatching in surviving embryos in concentrations from 0.03 to 1.55 μg mL^{-1} of total Ag^+.[47]

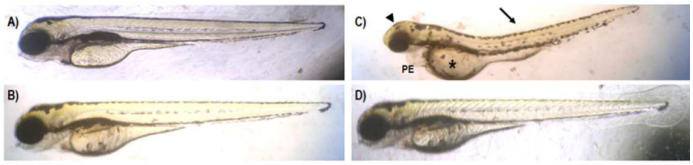


Fig. 6 Images of zebrafish embryos after 96 h exposure to **2** (A, B) and $AgBF_4$ (C, D). Living embryos at 2.5 μg mL^{-1} (A) and 2 μg mL^{-1} (B) of **2** had no observable developmental defects. Embryos exposed to 2 μg mL^{-1} of $AgBF_4$ (C) had serious pericardial edema (PE), small head (arrowhead), lordosys (arrow) and unresorbed yolk (asterisk), while at 1.5 μg mL^{-1} (D) embryos were not affected.

Experimental

Materials

Distilled water was demineralized and purified to a resistance of greater than 10 $M\Omega \cdot cm^{-1}$. Silver(I) salts, $AgNO_3$ and $AgBF_4$, methanol, acetone, acetonitrile and dimethyl sulfoxide were purchased from Sigma-Aldrich. Phthalazine (phtz) and quinazoline (qz) were obtained from ABCR. All reactants were of analytical reagent grade and used without further purification.

Synthesis of silver(I) complexes 1 and 2

Silver(I) complexes $[Ag(NO_3)(qz)]_n$ (**1**) and $\{[Ag(CH_3CN)]_2(\mu\text{-phtz})_2\}[BF_4]_2$ (**2**) were synthesized according to a modified procedure for the preparation of silver(I) complexes with methylcamphorquinoxaline.[48] The solution of 1.0 mmol of the corresponding silver(I) salt (169.9 mg of $AgNO_3$ for **1** and 194.7 mg of $AgBF_4$ for **2**) in 10 mL of warm methanol was added slowly under stirring to the solution containing 0.5 mmol (65.1 mg) of quinazoline (for **1**) or phthalazine (for **2**), dissolved in 10 mL of warm acetone. A white precipitate was formed immediately after addition of the *N*-heterocyclic ligand. The reaction mixture was stirred in the dark at room temperature for 3 h, and then the precipitate was filtered off and dissolved in 20 mL of acetonitrile at room temperature. The obtained solution was left in a refrigerator at +4 °C and after a few days colorless crystals of **1** and **2**, suitable for single crystal X-ray crystallography were formed. Yield: 117.0 mg (78%) for **1** and 133.5 mg (73%) for **2**.

Anal. Calcd. for **1** = $C_8H_6AgN_3O_3$ (M_r = 300.03): C, 32.03; H, 2.02; N, 14.01. Found: C, 31.72; H, 2.12; N, 14.08%. ^{1}H NMR (200 MHz, DMSO): δ = 7.82 (*ddd*, *J* = 8.1, 5.2, 2.9 Hz, 1H), 8.05-8.11 (*m*, 2H), 8.22 (*dt*, *J* = 8.2, 1.2 Hz, 1H), 9.32 (*s*, 1H, qz), 9.67 ppm (*s*, 1H). IR (KBr, ν, cm^{-1}): 3441(br), 3029(w), 2923(w), 1750(w), 1738(w), 1619(m), 1581(m), 1568(m), 1491(m), 1437(s), 1377(vs), 1352(vs), 1312(m), 1278(s), 1212(m), 1036(w), 932(w), 871(w), 832(w), 815(w), 785(m), 758(m), 647(w), 633(w), 510(w), 482(w), 454(w). UV-Vis (DMSO, λ_{max}, nm): 306.0 (ε = 1.3·10^3 M^{-1}cm^{-1}).

Anal. Calcd. for **2** = $C_{20}H_{18}Ag_2B_2F_8N_6$ (M_r = 731.76): C, 32.83; H, 2.48; N, 11.48. Found: C, 32.98; H, 2.63; N, 11.75%. ^{1}H NMR (200 MHz, DMSO): δ = 2.08 (*s*, 6H), 8.01-8.13 (*m*, 4H), 8.14-8.27 (*m*, 4H), 9.72 ppm (*s*, 4H). IR (KBr, ν, cm^{-1}): 3450(br), 3038(w), 2984(w), 2922(w), 2174(br), 1618(w), 1489(m), 1438(m), 1377(m), 1307(w), 1278(m), 1247(w), 1217(w), 1159(m), 1058(s), 1036(s), 916(m), 767(m), 751(m), 649(m), 512(w), 474(m). UV-Vis (DMSO, λ_{max}, nm): 298.0 (ε = 2.1·10^3 M^{-1}cm^{-1}).

Measurements

Elemental analyses for carbon, hydrogen and nitrogen were performed on a Vario EL instrument by the Microanalytical Laboratory, Department of Organic Chemistry, University of Heidelberg. ^{1}H NMR spectra were recorded at 25 °C in DMSO-d_6 on a Varian Gemini 2000 spectrometer at 200 MHz. Chemical shifts are reported in ppm (δ) and scalar couplings are reported in Hertz. 10 mg of each complex was dissolved in 0.7 mL of DMSO-d_6, and the solution transferred into a 5 mm NMR tube. Infrared spectra were recorded as KBr pellets on a Perkin Elmer Spectrum One spectrometer over the range of 450 – 4000 cm^{-1}. The UV-Vis spectra were recorded on a Cary 100 spectrophotometer (Varian, USA), after dissolving of the corresponding silver(I) complex in DMSO as well as after 24 and 96 h incubation in the dark at 37 °C, over the wavelength range of 200 – 600 nm. The concentration of the silver(I) complexes was 0.50 mg mL^{-1}.

Air/light stability of silver(I) complexes and salts

Air/light stability of complexes **1** and **2**, and the starting $AgNO_3$ and $AgBF_4$ salts was studied by indirect light in air atmosphere at room temperature. Sterile cellulose discs were impregnated with the silver(I) complexes and salts (200 µg per disc, using 50 mg mL^{-1} DMSO stock solution) and exposed to air and light. The stability was monitored visually within 48 h.

X-ray crystal structure determinations

Crystal data and details of the structure determinations are compiled in Table S2. Full shells of intensity data were collected at low temperature with a Bruker AXS Smart 1000 CCD diffractometer (Mo-K_α radiation, sealed X-ray tube, graphite monochromator). Data were corrected for air and detector absorption, Lorentz and polarization effects;[49] absorption by the crystal was treated with a semiempirical multiscan method.[50,51] The structures were solved by intrinsic phasing[52] (complex **1**) and by the charge flip procedure[53] (complex **2**) and refined by full-matrix least squares methods based on F^2 against all unique reflections.[54] All non-hydrogen atoms were given anisotropic displacement parameters. Hydrogen atoms were generally input at calculated positions and refined with a riding model. When justified by the quality of the data the positions of some hydrogen atoms were taken from difference Fourier syntheses and refined. MERCURY and ORTEP computer graphics programs[55] were used to prepare drawings.

Antimicrobial studies

MIC concentrations of **1** and **2**, and the starting silver(I) salts were determined according to standard broth micro dilution assays, recommended by the National Committee for Clinical Laboratory Standards (M07-A8) for bacteria and Standards of European Committee on Antimicrobial Susceptibility Testing (EDef7.1.).[56] The tested compounds were dissolved in DMSO. The highest concentration used was 500 µg mL^{-1}. The inoculums were 10^5 colony forming units, cfu mL^{-1}, for bacteria and 10^4 cfu mL^{-1} for *Candida albicans*. The MIC value corresponds to the lowest concentration that inhibited the growth after 24 h at 37 °C. The $AgNO_3$ salt was considered as positive control, as it has been used clinically.[57]

Cytotoxicity and hemolysis assays

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[58] The assay was carried out using human lung fibroblasts (MRC5) after 48 h of cell incubation in the media, containing compounds at concentrations ranging from 0.1 - 100 µg mL^{-1}. The MRC5 cell line was maintained in the RPMI-1640 medium, supplemented with 100 µg mL^{-1} streptomycin, 100 U mL^{-1} penicillin and 10% (v/v) fetal bovine serum (FBS) (all from Sigma, Munich, Germany) as a monolayer (1 x 10^4 cells per well) and grown in humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

The extent of MTT reduction was measured spectrophotometrically at 540 nm using a Tekan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland), and the cell survival was expressed as percentage of the control (untreated cells). The percentage viability values were plotted against the log of concentration and a sigmoidal dose response curve was calculated by non-linear regression analysis, using the Graphpad Prism software, version 5.0 for Windows (Graphpad Software, CA, USA). Cytotoxicity is expressed as the concentration of the compound inhibiting growth by 50% (IC_{50}).

***In vivo* zebrafish toxicity assay**

The assessment of toxicity (lethality and teratogenicity) of silver(I) complexes and salts on zebrafish embryos have been performed following general rules of the OECD Guidelines for the Testing of Chemicals[59] (Fish Embryo Acute Toxicity (FET)

Test. OECD: Paris, France, 2013; Test No. 236). Adult zebrafish (*Danio rerio*, wild type) were maintained in the fish medium (2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM $NaHCO_3$, 0.07 mM KCl) at 27 ± 1 °C and a 12 h light/12 h dark cycle, and regularly fed twice daily with commercial dry flake food supplemented with *Artemia nauplii* (TetraMin™ flakes; Tetra Melle, Germany). Eggs at 4 h post fertilization (hpf) were treated with eight different concentrations (0.5, 1, 1.5, 2, 2.5, 5, 10 and 15 μg mL^{-1}) and 0.25% DMSO as negative control. Embryos were then individually transferred into 24-well plates containing 1000 μL test solution, 10 embryos per well, and incubated at 28 °C. These experiments were repeated twice, using 30 embryos per concentration.

Apical endpoints (Table S3) for toxicity evaluation were recorded at 24, 48, 72, and 96 hpf using an inverted microscope (CKX41; Olympus, Tokyo, Japan). At 96 hpf, the embryos were anesthetized by addition of 0.1% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO), photographed and killed by freezing at −20 °C for ≥ 24 h. The experiment was valid if the percentage of normal embryos or larvae in the control group (0.25% DMSO) was at least 90%.

All experiments involving zebrafish were performed in compliance with the European directive 86/609/EEC and the ethical guidelines of Guide for Care and Use of Laboratory Animals of the Institute for Molecular Genetics and Genetic Engineering, University of Belgrade.

Conclusions

In this study, new silver(I) complexes with quinazoline and phthalazine, $[Ag(NO_3)(qz)]_n$ (**1**) and $\{[Ag(CH_3CN)]_2(\mu\text{-phtz})_2\}[BF_4]_2$ (**2**), have been synthesized and characterized by using spectroscopic and single crystal X-ray diffraction techniques. The obtained results together with those previously reported for silver(I) complexes with these ligands[16] showed that the reaction of AgX salts (X = NO_3^-, $CF_3SO_3^-$, ClO_4^- and BF_4^-) with quinazoline resulted in the formation of polynuclear complexes, while with phthalazine, dinuclear silver(I) complexes were formed. The obtained results of biological investigations showed that silver(I) complexes **1** and **2** have manifested good activity against different bacterial strains, while the activity against the fungus *C. albicans* was moderate. However, these complexes exhibited a negative effect on *in vitro* proliferation of the normal human lung fibroblast cell line and also toxicity on zebrafish embryos, although significantly lower than the $AgNO_3$ and $AgBF_4$ salts. The latter finding offers the possibility to consider silver(I) complexes **1** and **2** as antimicrobial agents for external applications or their dual activity, both antiproliferative and antibacterial, may be further exploited for the treatment of some cancers, which are often associated with serious bacterial infections.

Acknowledgements

This work has been financially supported by the Ministry of Education and Science, Republic of Serbia, under Grant No. 172036 and 173048. B.Đ.G. gratefully acknowledges financial support from the German Academic Exchange Service (DAAD) during postdoctoral stay at the University of Heidelberg, Germany, and to Prof. Dr. Peter Comba and his group for the hospitality and support.

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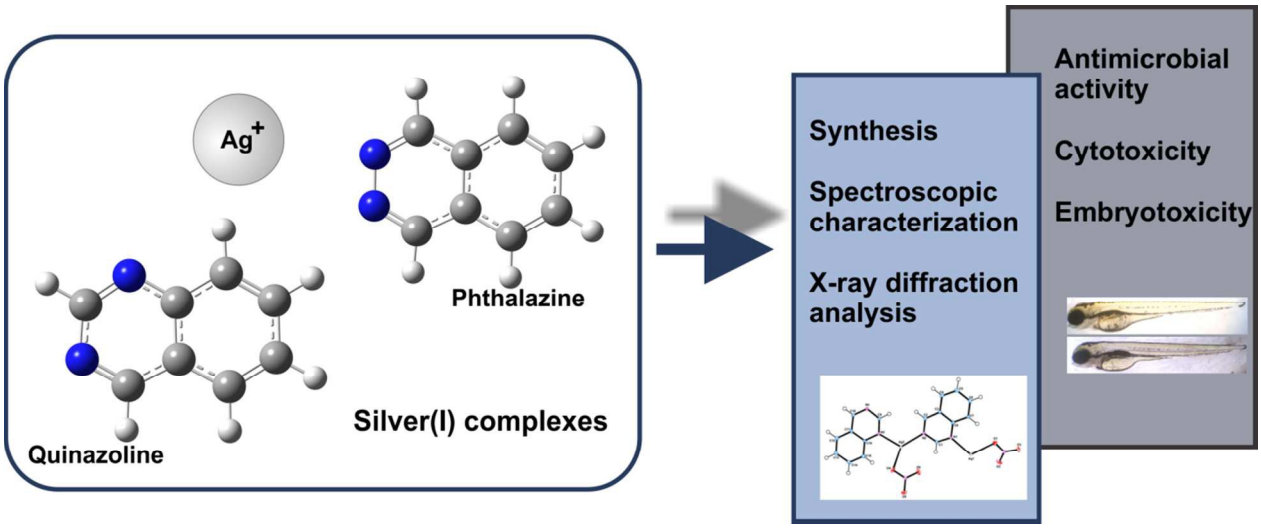
Graphical abstract

Silver(I) complexes with quinazoline and phthalazine: synthesis, structural characterization and evaluation of biological activities

Nada D. Savić, Biljana Đ. Glišić, Hubert Wadepohl, Aleksandar Pavic, Lidija Senerovic, Jasmina Nikodinovic-Runic and Miloš I. Djuran



New silver(I) complexes with aromatic *N*-heterocycles phthalazine and quinazoline have been synthesized, characterized and comprehensively evaluated for their antimicrobial activity, the effect on the viability of human cell line, as well as the effect on development of zebrafish embryos.