Synthesis, characterization, antimicrobial and cytotoxic activity and DNA-binding properties of d-metal complexes with hydrazones of Girard’s T and P reagents

Nevena Stevanović1 · Paolo Pio Mazzeo2,3 · Alessia Bacchi2,3 · Ivana Z. Matić4 · Marija Đorđić Crnogorac4 · Tatjana Stanojković4 · Miroslava Vujčić5 · Irena Novaković5 · Dušanka Radanović5 · Maja Šumar-Ristović1 · Dušan Sladić1 · Bofžidar Čobeljić1 · Katarina Andelković1

Received: 17 March 2021 / Accepted: 16 August 2021 / Published online: 6 September 2021
© Society for Biological Inorganic Chemistry (SBIC) 2021

Abstract
In this work synthesis, characterization and crystal structures of 1, Zn(II) complex ([ZnL1(NCS)2]), with (E)-1-(2-oxo-2-(2-(quinolin-2-ylmethylene)hydrazinyl)ethyl)pyridin-1-ium chloride (HL1Cl) and 2, Bi(III) complex ([BiHL2Cl4] × 1/2CH3OH), with (E)-N,N,N-trimethyl-2-oxo-2-(2-(1-(thiazol-2-yl)ethylidene)hydrazinyl)ethan-1-aminium chloride (HL2Cl), have been reported. Zn(II) complex possesses a distorted trigonal bipyramidal geometry while surroundings around Bi(III) ion are extended pentagonal bipyramidal. Antimicrobial activity, brine shrimp assay and DPPH radical scavenging activity of both complexes, including previously synthesized complexes with HL1Cl ligand (Zn(II) and Ni(II)) and complexes with (E)-N,N,N-trimethyl-2-oxo-2-(2-(1-(pyridin-2-yl)ethylidene)hydrazinyl)ethan-1-aminium chloride (HL3Cl) (Zn(II), Cu(II), Cd(II), Co(II), Fe(III), Ni(II)), were evaluated. For the most active complexes, cytotoxic activity against five malignant cancer cell lines (HeLa, A375, MCF7, PC-3 and A549) and normal cell line HaCaT, as well as generation of reactive oxygen species (ROS), was tested.

Graphic abstract

Biological study:
- antimicrobial activity
- brine shrimp assay and DPPH radical scavenging activity
- cytotoxic activity
- determination of reactive oxygen species (ROS) levels
- DNA binding activity

Extended author information available on the last page of the article
Keywords Hydrazone complexes · X-ray · Antimicrobial activity · Cytotoxic activity · ROS · DNA-binding experiments

Introduction

Hydrazones are a class of organic compounds that have general formula R₁R₂C=NRR₃R₄. The general method for the synthesis of a hydrazone is the condensation reaction of hydrazine/hydrazide with aldehyde/ketone in solvents like ethanol, methanol, butanol, etc. Hydrazones can form stable chelates with transition metals in the cells, thus some vital enzymatic reactions cannot take place in their presence [1, 2]. They are known to exhibit diverse interesting biological activities, such as antioxidant, [3] anticonvulsant, [4] analgesic, [5] anti-inflammatory, [5] anti-parasitic, [6] antimicrobial [7] and antitumor activities [8–10].

The hydrazones that have hydrazides as precursor molecules, are known to have an additional ligand donor site, that is C=O, which make them more versatile and more interesting as possible ligands in coordination chemistry, especially is C=O, which make them more versatile and more interesting molecules, are known to have an additional ligand donor site, that is C=O, which make them more versatile and more interesting as possible ligands in coordination chemistry, especially knowing that their complexes can show biological activity [11, 12]. In many cases, activity of hydrazones increases upon coordination to metal centers [13, 14]. Coordination reduces the hydrophilicity of the metal ion because of the partial sharing of its positive charge with the donor atom within the chelate ring system. The increased lipophilicity of the central metal atom favors its more efficient permeation through the lipid membranes of microorganisms or tumor cells, thus destroying them more aggressively [2, 14].

Girard’s reagents are quaternary ammonium acetohydrazides which form water-soluble hydrazones with carbonyl compounds. Girard’s reagent-based hydrazones can have three or more potential ligator atoms [15, 16], and can exhibit keto-enol tautomerism and coordinate metal ions in non-deprotonated positively charged form or deprotonated formally neutral zwitter-ionic form. The presence of positively charged group in the metal complexes of Girard’s reagents hydrazones increases their water solubility, thus affecting their biological activity [17]. Some of these compounds and their metal complexes showed biological activity, indicating their pharmacologically interesting properties [18].

Bismuth compounds are considered safe and relatively non-toxic. Bismuth compounds have been used for various microbial infections, such as syphilis, colitis, wound infection, diarrhea and peptic ulcers [19]. These compounds are used worldwide to treat various gastrointestinal diseases which are related to the infection caused by Helicobacter pylori bacteria [20]. Several bismuth(III) complexes show antitumor activities both in vitro and in vivo [21–24].

Zinc is one of the most important bioelements, taking a significant part in metabolic pathways, gene expression, and signal transduction. It is the second-most abundant d-metal in the human body, an essential trace element and is extensively present in proteins (ca. 10% proteins in the human body) [25, 26]. In cell biology, it is regarded as an active signaling ion. Many enzymes have Zn(II) ion in the active sites, due to the fact that it has very high Lewis acidity, non-redox properties, ease of ligand exchange and flexibility of coordination environment [27]. A small concentration of Zn(II) ion is important for normal metabolism of bacteria, while at high concentration Zn(II) inhibits bacterial growth [28]. Zinc ion has three main mechanisms for antimicrobial activity (binding to microbial proteins, interacting with the vital microbial membranes or with nucleic acids) [13]. Zn(II) complexes can interact with DNA molecules, either by intercalation or cleavage, thus showing antitumor properties [29, 30].

In continuation of our previous investigations on synthesis and characterization of complexes with Girard’s reagent T-based hydrazones, which showed moderate biological activities, generally higher than ligands themselves [31–34] in this paper, a novel Bi(III) complex, from this series, and Zn(II) complex as first of the series with Girard’s reagent P-based hydrazones, are described. Also, comprehensive analysis of biological activity of previously synthesized and characterized complexes with Girard’s T reagent-based hydrazones ([ZnL₂(NCS)₂]×0.5MeOH (5) [35, 36], [ZnL₂(NCS)₂]×2Η₂Ο (3) [36], [CdHL₁(NCS)₂] (7) [35], [FeL₁(NCS)₂] (12) [37], [CoL₆]([Co(NCS)₄]BF₄ (10) [38], [CuL₁(NCS)₂] (9) [39] [CuL₁Cl](ClO₄) (8), [Cu₂L₂(μ₁-1,N₃)₂](ClO₄)₂ (6) [40], [Ni₂L₃(μ₁-1,N₃)₂(N₃)₂]×6Η₂Ο (11) [41] and [Ni₂L₃(μ₁-1,N₃)₂(N₃)₂]×4Η₂Ο (4) [42]) including the novel Zn(II) complex, was performed.

Results and discussion

Synthesis of HL₁Cl and HL₂Cl ligands, and the corresponding complexes 1 and 2

The HL₁Cl ligand, (E)-1-(2-oxo-2-(2-(quinolin-2-ylmethyl)hydrazinyl)ethyl)pyridin-1-ium chloride, was obtained from the condensation reaction of 2-quinolinecarboxaldehyde and Girard’s P reagent in ethanol (Scheme S1). Reaction of the ligand HL₁Cl with Zn(BF₄)₂×6Η₂Ο and NH₄SCN in molar ratio 1: 1: 2 in methanol/acetonitrile/water mixture results in formation of mononuclear isothiocyanoato Zn(II) complex (1) with composition [ZnL₁(NCS)₂] (Scheme S1).

The HL₂Cl ligand, (E)-N,N,N,N-trimethyl-2-oxo-2-(2-(1-thiazol-2-yl)ethylidene)hydrazinyl)ethan-1-aminium chloride, was obtained from the condensation reaction of
2-acetylthiazole and Girard’s T reagent in water (Scheme S2) in a mixture with its Z-isomer (1:1). Reaction of this ligands mixture with BiCl₃ in molar ratio 1:1 in methanol/acetonitrile mixture results in formation of mononuclear Bi(III) complex (2) with composition [BiHL₂Cl₄] × 1/2CH₃OH (Scheme S2). In the complex, the ligand has (E)-conformation.

Detailed spectroscopic characterization (IR and NMR (¹H and ¹³C spectra) of complexes 1 and 2 including the stability of complexes in solution used for the biological study (1–12) investigated by UV–Vis spectroscopy and molar conductivity measurements can be found in ESI.

**Single crystal X-ray analysis of complexes 1 and 2**

Complex 1 crystallizes with two independent molecules in the asymmetric unit of the triclinic P-1 space group. The two molecules, displayed in Fig. 1, differ for the ligand conformation, but present the same coordination geometry (Table S2). In both molecules the zinc coordination is five-fold and can be described as a distorted trigonal bipyramid, with two NCS⁻ and one N of the ONN chelating system in the equatorial plane, and the trans N and O atom at the apical positions. In both cases, the coordinated zwiterionic L¹ ligand forms two five-membered chelation rings which result practically co-planar with the quinoline moiety, apart for a slight deviation of the oxygen atom out of the plane (deviating 0.15 and 0.27 Å for O1 and O2, respectively). In fact, this chelation system is quite common in the CSD, with 176 occurrences (version August 2019), of which 63 present five coordination and 3 involve two NCS⁻ counter anions, all displaying the same distorted trigonal bipyramidal arrangement (CSD refcodes: MJINJEB [35], OMOCOK [43], HOPHEC [44]). The terminal pyridinium of L¹ is in both cases almost perpendicular to the rest of the ligand skeleton, with remarkably different orientations for the two molecules (+86° and –89°, respectively).

The most striking aspect of the crystal packing of is the presence of a S···N chalcogen bond [45] connecting one terminal thiocyanate of one independent molecule with the iminic nitrogen of the second independent molecule (S1···N9 = 3.182(5)Å). Otherwise, stacking assembly especially between quinoline rings is generally preponderant (Fig. S1).

Complex 2 crystallizes in the monoclinic C2/c space group; the asymmetric unit contains one whole complex and one half of a methanol molecule, disordered on a symmetry plane. The complex, displayed in Fig. 2, presents a loose NNO chelation of the cationic ligand to a bismuth(III) cation, which completes an extended seven-fold pentagonal bipyramidal coordination and attains neutrality by bonding to four chloride ions. The NNO chelation is highly distorted, as the Bi1–O1 (2.7638(1) Å) and Bi1–N2 (2.8121(2) Å) distances are significantly longer than the average Bi–O (2.5(2) Å) and Bi–N (2.54(6)Å) found in the Cambridge Structural Database (CSD) [46] for BiOCl₄ and BiNCl₄ fragments, respectively, and can be ascribed to secondary bonding effect [47]. This secondary coordination builds two chelation rings, both with a slight envelope distortion with the

![Fig. 1](image1.png) Molecular structures of the two independent molecules observed in the crystal structure of 1, with atom labeling and thermal displacement ellipsoids displayed at the 50% probability level.

![Fig. 2](image2.png) Molecular structure of 2 as determined by X-ray diffraction. Atom labeling is reported, and thermal displacement ellipsoids are at the 50% probability level. The solvation methanol molecule is omitted. Elongated bonds are dashed.
metal at the flap. A search in the CSD revealed only one similar complex displaying an ONO chelation combined with four chloride ions in a pentagonal bipyramidal coordination (CSD refcode: VIGCET [48]), where the chelation involves two elongated Bi–N and Bi–O bonds. These long bonds results in weak chelation and can explain hydrolysis of the Bi(III) complex in solution.

Interestingly, the crystal packing is based on a combination of hydrogen bonds between the NH of the HL2 protonated ligand and the chloride of an adjacent molecule (N–H···Cl(1/2 + x, 1/2 + y, z) = 3.408(2)Å, 148(2)°), together with a chalcogen bond [45] assembling the apical chloride of a complex with the sulfur atom of a vicinal molecule (Cl4···S1(3/2 − y, 1/2 − y, 1 − z) = 3.331(1)Å); these two supramolecular motifs generate one-dimensional molecular ribbons (Fig. S2), decorated with the solvation methanol which is hydrogen bonded to the second apical chloride of each complex.

### Biological activity

The antibacterial activity of the complexes 1–12 and their precursors towards a panel of five Gram-negative and five Gram-positive bacteria is given in Table 1. The most active complex 1, obtained from HL3Cl, NH4SCN and Zn(BF4)2 × 6H2O, shows antibacterial activity against all tested bacterial strains. It is particularly interesting that the precursor compounds for this complex, either do not have or show low antibacterial activity. This means that either the complex itself is an active species, or it serves as a vehicle for transmembrane transport of the cationic ligand which could otherwise not cross the bacterial cell membrane. The activity of this complex towards P. aeruginosa and C. sporogenes was better than control antibiotic chloramphenicol.

The complex 7, obtained from inactive precursors, ligand HL3Cl and NH4SCN and from active salt Cd(NO3)2 × 4H2O, shows very good activity towards E. coli, M. luteus (ATCC 4698) and S. enterica and good activity towards the other tested bacteria. The lower activity of the complex, in comparison with the salt, indicates that the antibacterial activity is manifested by the Cd ion itself, which is trapped in the inner sphere of the complex. Other tested complexes showed weak antibacterial activity. It should be pointed out that neither ligand HL3Cl nor its complexes showed any significant antibacterial activity indicating the importance of pyridine moiety.

The results of the antifungal activity are given in Table 2. In contrast to antibacterial activity, all ligands showed antifungal activity, the activities of ligands HL2Cl and HL3Cl being comparable to the control compound amphotericin B. From the obtained results, it can be noticed that most of the precursors and complexes showed very good antifungal activity as well as some selectivity towards A. brasiliensis.

The complex with the greatest activity against this strain was 11, while the complexes 4, 6 and 10 show remarkable activity. Other complexes also show significant activity. Against strain C. albicans, the complex 10 showed the best activity, while against S. cerevisiae the complex 4 manifested the greatest activity. In contrast to antibacterial activity, both HL2Cl and its complexes showed a remarkable antifungal activity. Even Bi(III) complex, for which it was shown by NMR spectroscopy that undergoes hydrolysis in aqueous solution, exhibited significant activity against all three strains, probably acting as a vehicle for the active ligand.

The results of toxicity of complexes and their precursors against nauplii of the Artemia salina as well as radical scavenging activity are given in ESI.

### Cytotoxic activity

Based on the results of the brine shrimp test, five complexes were selected for evaluation of cytotoxic activity. The intensities of cytotoxic activity of the tested complexes and their precursor compounds against malignant cancer cell lines and normal human keratinocytes are presented in Table 3. The examined complexes exerted selective concentration-dependent cytotoxic effects against malignant cells.

The highest intensity of cytotoxicity on melanoma A375 and cervical adenocarcinoma HeLa cell lines was found for complex 12 with IC50 values of 22.98 µM and 37.63 µM, respectively. The complex exhibited lower cytotoxicity on lung carcinoma A549 cells (IC50 value of 83.66 µM). The lowest cytotoxicity of this complex was observed on breast adenocarcinoma MCF7 and prostate adenocarcinoma PC-3 cells (IC50 values 135.06 µM and 125.92 µM, respectively). The cytotoxic activity of the complex was higher against melanoma A375 cells when compared with this activity against normal keratinocytes HaCaT (IC50 value of 40.17 µM) with selectivity index of 1.75.

Complex 6 showed the strongest cytotoxic effects against HeLa and A375 malignant cells with similar IC50 values of 45.87 µM and 44.99 µM. The complex showed moderate cytotoxic activity against MCF7, PC-3 and A549 malignant cell lines (obtained IC50 values of 62.48 µM, 66.65 µM, and 72.16 µM, respectively). Moderate cytotoxicity of this complex was observed against normal keratinocytes HaCaT cell line with IC50 value of 59.16 µM.

Complex 7 exhibited the strongest cytotoxic activity on prostate adenocarcinoma PC-3 cells with IC50 value of 45.55 µM. This complex showed moderate cytotoxicity against A375, A549 and HeLa cells (IC50 values: 51.04 µM, 59.62 µM, and 61.72 µM). Similar intensity of cytotoxicity was observed against HeLa and normal keratinocyte cell line HaCaT. The complex showed the lowest cytotoxic effect on MCF7 breast cancer cells (IC50 value of 70.17 µM).
### Table 1 Antibacterial activity of tested compounds

| MIC (mM) | E. coli | P. aeruginosa | P. hauseri | K. pneumoniae | S. enterica | S. aureus | M. luteus ATCC 10,240 | M. luteus ATCC 4698 | B. subtilis | C. sporogenes |
|----------|---------|---------------|------------|----------------|-------------|-----------|----------------------|-------------------|-----------|-------------|
| **HL₁Cl** | 7.65   | 3.83          | 7.65       | 7.65           | 7.65        | 7.65      | 3.83                 | 7.65              | 7.65      | 7.65        |
| 1        | 0.33   | 0.66          | 0.66       | 1.32           | 0.66        | 0.66      | 0.33                 | 0.33              | 0.33      | 0.66        |
| **HL₂Cl** | –      | –             | –          | –              | –           | –         | –                    | –                 | –         | –           |
| 2        | –      | –             | –          | –              | –           | –         | –                    | –                 | –         | –           |
| 3        | 2.74   | 2.74          | 5.48       | 2.74           | 5.48        | 5.48      | 2.74                 | 5.48              | 5.48      | 5.48        |
| 4        | 1.56   | 3.12          | 3.12       | 3.12           | 1.56        | 3.12      | 1.56                 | 3.12              | 3.12      | 3.12        |
| **HL₃Cl** | –      | –             | –          | –              | –           | –         | –                    | –                 | –         | –           |
| 5        | 2.89   | 5.78          | 2.89       | 2.89           | 5.78        | 5.78      | 2.89                 | 5.78              | 5.78      | 5.78        |
| 6        | 1.42   | 1.42          | 1.42       | 1.42           | 0.71        | 1.42      | 1.42                 | 1.42              | 1.42      | 1.42        |
| 7        | 0.30   | 2.40          | 1.20       | 1.20           | 0.60        | 1.20      | 1.20                 | 0.15              | 1.20      | 2.40        |
| 8        | 2.89   | 5.78          | 5.78       | 5.78           | 2.89        | 5.78      | 5.78                 | 5.78              | 5.78      | 5.78        |
| 9        | 3.16   | 6.32          | 6.32       | 6.32           | 3.16        | 6.32      | 6.32                 | 6.32              | 6.32      | 6.32        |
| 10       | 0.69   | 1.38          | 1.38       | 1.38           | 1.38        | 1.38      | 0.69                 | 1.38              | 1.38      | 1.38        |
| 11       | 1.55   | 1.55          | 3.10       | 3.10           | 1.55        | 3.10      | 1.55                 | 3.10              | 3.10      | 3.10        |
| 12       | 5.38   | 5.38          | 5.38       | 5.38           | 2.69        | 5.38      | 5.38                 | 5.38              | 5.38      | 5.38        |
| NH₄SCN   | –      | –             | –          | –              | –           | –         | –                    | –                 | –         | –           |
| NaN₃     | 4.81   | 9.61          | –          | –              | 9.61        | 19.23     | 19.23                | 38.46             | 38.46     | –           |
| Zn(BF₄)₂×6H₂O | 0.45 | 7.19          | 7.19       | 7.19           | 3.60        | 3.60      | 7.19                 | 1.80              | 3.60      | 3.60        |
| Zn(OAc)₂×2H₂O  | 3.41 | 6.82          | 6.82       | 6.82           | 6.82        | 6.82      | 6.82                 | 6.82              | 6.82      | 6.82        |
| Ni(BF₄)₂×6H₂O  | 3.67 | 3.67          | 3.67       | 3.67           | 3.67        | 3.67      | 6.32                 | 6.32              | 6.32      | 6.32        |
| Cd(NO₃)₂×4H₂O | 0.13 | 0.51          | 0.26       | 0.51           | 0.51        | 0.51      | 0.13                 | 0.51              | 0.51      | 0.51        |
| Fe(NO₃)₃×9H₂O | –   | –             | –          | –              | –           | –         | –                    | –                 | –         | –           |
| Co(BF₄)₂×6H₂O | 0.92 | 1.84          | 0.46       | 3.68           | 1.84        | 0.46      | 0.46                 | 0.46              | 0.46      | 0.46        |
| Cu(ClO₄)₂×6H₂O | 6.75 | 6.75          | 6.75       | 6.75           | 6.75        | 6.75      | 6.75                 | 6.75              | 6.75      | 6.75        |
| Cu(NO₃)₂×3H₂O  | 5.17 | 10.34         | 10.34      | 5.17           | 10.34       | 10.34     | 5.17                 | 10.34             | 10.34     | 10.34       |
| BiCl₃   | ND     | ND            | ND         | ND             | ND          | ND        | ND                   | ND                | ND        | ND          |
| Chloramphenicol | 0.19 | 0.77          | 0.39       | 0.19           | 0.10        | 0.05      | 0.10                 | 0.05              | 0.05      | 0.77        |

ND not determined since BiCl₃ forms insoluble hydroxylated species in aqueous media
Complex 10 had the highest cytotoxic activity against melanoma A375 cells with IC\textsubscript{50} value of 45.48 µM. The lower intensity of cytotoxicity was observed against normal human keratinocytes HaCaT when compared with cytotoxicity against melanoma cells (IC\textsubscript{50} value of 70.52 µM). The complex demonstrated similar intensity of cytotoxic activity on HeLa and PC-3 cells (IC\textsubscript{50} values of 72.38 µM and 69.63 µM). The lowest cytotoxic effects were observed against MCF7 and A549 cell lines (IC\textsubscript{50} values of 88.21 µM and 84.45 µM).

All four complexes exerted remarkably higher cytotoxic effects against malignant cell lines in comparison with the effects of their ligand HL\textsubscript{3}Cl. The measured IC\textsubscript{50} values for HL\textsubscript{3}Cl were in the range of 161.08–199.23 µM, and even higher than 200 µM for A549 cells.

The novel complex 1 showed moderate cytotoxic activity against HeLa, A375 and A549 malignant cells (IC\textsubscript{50} values of 59.13 µM, 57.35 µM and 54.79 µM). This complex showed lower cytotoxicity against normal keratinocytes HaCaT when comparing its activity against these three malignant cell lines (IC\textsubscript{50} value of 69.29 µM). The complex exerted lower cytotoxic effects on PC-3 cells with IC\textsubscript{50} value of 87.23 µM, while the lowest cytotoxicity was observed against MCF7 cells (IC\textsubscript{50} value of 106.17 µM). The complex exhibited higher cytotoxic effects on examined cell lines in comparison with its ligand HL\textsubscript{1}Cl, with the exception of the effect on MCF7 cells. The obtained IC\textsubscript{50} values for HL\textsubscript{1}Cl were in the range of 74.05–183.95 µM.

Cervical adenocarcinoma HeLa cells were the most sensitive to the cytotoxic effects of the complexes 6 and 12. Melanoma A375 cells showed the highest sensitivity to the activity of complex 12. Metastatic breast adenocarcinoma MCF7 cells were the most sensitive to the cytotoxicity of

### Table 2: Antifungal activity of tested compounds

|            | A. brasiensis | C. albicans | S. cerevisiae |
|------------|---------------|-------------|---------------|
| HL\textsuperscript{1}Cl | 3.83          | 7.65        | 7.65          |
| 1          | 1.32          | 1.32        | 1.32          |
| HL\textsuperscript{2}Cl | 0.07          | 0.14        | 4.48          |
| 2          | 0.26          | 1.04        | 0.26          |
| 3          | 0.34          | 1.37        | 0.17          |
| 4          | 0.20          | 3.12        | 0.10          |
| HL\textsuperscript{3}Cl | 0.04          | 0.16        | 0.36          |
| 5          | 0.36          | 2.89        | 0.36          |
| 6          | 0.18          | 1.42        | 0.18          |
| 7          | 0.30          | 1.20        | 0.15          |
| 8          | 0.36          | 5.78        | 0.72          |
| 9          | 0.40          | 6.32        | 0.80          |
| 10         | 0.17          | 0.69        | 0.35          |
| 11         | 0.10          | 1.55        | 0.19          |
| 12         | 0.67          | 1.35        | 0.34          |
| NH\textsubscript{4}SCN | 0.26          | 0.52        | 16.42         |
| Na\textsubscript{3} | 0.21          | 0.42        | 1.68          |
| Zn(BF\textsubscript{4})\textsubscript{2}× 6H\textsubscript{2}O | 3.60          | 3.60        | 3.60          |
| Zn(OAc)\textsubscript{2}× 2H\textsubscript{2}O | 3.41          | 6.82        | 6.82          |
| Ni(BF\textsubscript{4})\textsubscript{2}× 6H\textsubscript{2}O | 3.67          | 1.84        | 1.84          |
| Cd(NO\textsubscript{3})\textsubscript{2}× 4H\textsubscript{2}O | 0.13          | 0.07        | 0.13          |
| Fe(NO\textsubscript{3})\textsubscript{3}× 9H\textsubscript{2}O | 12.38         | 12.38       | 12.38         |
| Co(BF\textsubscript{4})\textsubscript{2}× 6H\textsubscript{2}O | 0.46          | 0.46        | 0.46          |
| Cu(ClO\textsubscript{4})\textsubscript{2}× 6H\textsubscript{2}O | 6.75          | 6.75        | 3.38          |
| Cu(NO\textsubscript{3})\textsubscript{2}× 3H\textsubscript{2}O | 5.17          | 10.24       | 2.59          |
| BiCl\textsubscript{3} | ND            | ND          | ND            |
| Amphotericin B | 0.04          | 0.02        | 0.01          |

*ND* not determined since BiCl\textsubscript{3} forms insoluble hydroxylated species in aqueous media

### Table 3: Cytotoxic activity of the complexes and their precursor compounds

|            | HeLa | A375 | MCF7 | PC-3 | A549 | HaCaT |
|------------|------|------|------|------|------|-------|
| IC\textsubscript{50} [µM] average ± SD |
| HL\textsuperscript{1}Cl | 78.17 ± 6.35 | 86.01 ± 3.43 | 74.05 ± 5.21 | 183.95 ± 2.56 | 156.93 ± 1.98 | 83.82 ± 7.65 |
| 1 | 59.13 ± 4.31 | 57.35 ± 1.45 | 106.17 ± 8.84 | 87.23 ± 5.83 | 54.79 ± 0.65 | 69.29 ± 5.35 |
| HL\textsuperscript{2}Cl | 199.23 ± 1.34 | 161.08 ± 8.64 | 197.55 ± 3.46 | 196.34 ± 5.17 | > 200 | 193.47 ± 9.24 |
| 6 | 45.87 ± 1.74 | 44.99 ± 2.79 | 62.48 ± 2.40 | 66.65 ± 4.41 | 72.16 ± 5.08 | 59.16 ± 5.89 |
| 7 | 61.72 ± 5.12 | 51.04 ± 4.81 | 70.17 ± 3.04 | 45.55 ± 3.17 | 59.62 ± 4.34 | 61.52 ± 4.43 |
| 10 | 72.38 ± 4.36 | 45.48 ± 1.37 | 88.21 ± 4.16 | 69.63 ± 0.72 | 84.45 ± 5.94 | 70.52 ± 7.66 |
| 12 | 37.63 ± 4.25 | 22.98 ± 2.32 | 135.06 ± 5.10 | 125.92 ± 15.37 | 83.66 ± 1.17 | 40.17 ± 0.44 |
| NH\textsubscript{4}SCN | > 200 | > 200 | > 200 | > 200 | > 200 | > 200 |
| Cd(NO\textsubscript{3})\textsubscript{2}× 4H\textsubscript{2}O | 70.59 ± 3.96 | 51.00 ± 4.41 | 70.52 ± 7.39 | 66.88 ± 1.53 | 82.50 ± 1.71 | 75.72 ± 8.09 |
| Fe(NO\textsubscript{3})\textsubscript{3}× 9H\textsubscript{2}O | > 200 | > 200 | > 200 | > 200 | > 200 | 177.36 ± 1.28 |
| Co(BF\textsubscript{4})\textsubscript{2}× 6H\textsubscript{2}O | 152.94 ± 2.79 | 67.16 ± 2.08 | 152.84 ± 3.06 | 113.21 ± 6.69 | 172.36 ± 9.57 | 90.78 ± 5.73 |
| Na\textsubscript{3} | > 200 | > 200 | > 200 | > 200 | > 200 | > 200 |
| Cu(ClO\textsubscript{4})\textsubscript{2}× 6H\textsubscript{2}O | 124.08 ± 10.06 | 130.79 ± 8.00 | 149.23 ± 6.47 | 119.07 ± 10.99 | ≈200 | 145.84 ± 0.57 |
| Zn(BF\textsubscript{4})\textsubscript{2}× 6H\textsubscript{2}O | 171.06 ± 2.85 | 131.85 ± 11.88 | 198.78 ± 1.72 | 192.77 ± 2.63 | 199.22 ± 1.10 | 114.15 ± 8.75 |
complex 6. Among examined complexes, prostate adenocarcinoma PC-3 cells exerted the highest sensitivity to the activity of 7. Considering the effects of the complexes on lung carcinoma A549 cells, 1 and 7 were the most active against this cell line. All complexes were more cytotoxic to all cell lines then their respective salts, except for Cd complex. It should be pointed out that all complexes were more active to melanoma cell line that to normal HaCaT cells. The same applies for Zn(II) and Cu(II) complexes against HeLa cells, Cd(II) complex against PC-3 cells and Zn(II) complex against A549 cells.

Effects of complexes on cell cycle phase distribution

To explore mechanisms of cytotoxic activity of the selected complexes 1 and 12, their effects on cell cycle phase distribution of cervical adenocarcinoma HeLa cells treated with IC_{50} and 2IC_{50} concentrations of the complexes for 24 h were examined (Fig. 3). Complex 12 applied at IC_{50} and 2IC_{50} concentrations induced prominent increase in the percentages of HeLa cells in the subG1 and G2/M cell cycle phases when compared with those percentages in control cell sample. These changes were accompanied with decrease in the percentage of HeLa cells in G1 phase.

Complex 1 applied at IC_{50} concentration caused small increase in the percentage of HeLa cells in subG1 cell cycle phase. However, treatment of HeLa cells with 2IC_{50} concentration of this complex led to increase in the percentage of cells in G2/M phase when compared with control cells. The percentages of HeLa cells within G1 phase were decreased after 24 h incubation with both concentrations of this complex.

Cell cycle analysis showed the ability of complex 12 to trigger cell death in HeLa cells, demonstrated by high number of cells within subG1 phase of the cell cycle. Both of the selected complexes induced G2/M cell cycle phase arrest in treated HeLa cells. The accumulation of HeLa cells within G2/M cell cycle phase caused by tested complexes might be explained by preventing cells to enter mitosis through G2/M checkpoint or by M-phase arrest, finally leading to apoptosis of arrested cells.

Effects of complexes on intracellular ROS levels

To further examine whether cytotoxic effects of the complexes are connected with ROS generation, the levels of intracellular ROS in HeLa cells treated with IC_{50} concentrations of the complexes 1 and 12 for 24 h were determined by flow cytometry. As it could be seen in Fig. 4, both complexes lowered the intracellular ROS levels in HeLa cells in comparison with this level in control untreated cells, pointing to possible radical scavenging activity of these complexes in cell culture. This is in accordance with the results of DPPH test. For that reason, the possible cytoprotective effects of two complexes against generation of oxidative stress triggered by hydrogen peroxide were explored in normal keratinocyte HaCaT cell line and these results are shown in Fig. 5. Pretreatment of HaCaT cells with subtoxic IC_{20} concentrations of complexes 1 and 12 for 24 h led to decrease in ROS production induced by H_{2}O_{2} when compared with HaCaT cells grown in complete nutrient medium without the complexes for 24 h before being exposed to H_{2}O_{2}. Complex 1 was more effective in reducing ROS generation in HaCaT
cells in comparison with 12. The ROS scavenging activity in normal cells is an important cancer-suppressive property shown by these complexes, since high levels of intracellular ROS may damage DNA, proteins and lipids, and may promote malignant transformation, cancer cell growth and invasiveness [49].

**Effects of complexes on angiogenesis in vitro**

To examine possible in vitro anti-angiogenic effects of two selected complexes, endothelial cell tube formation assay was performed. The representative photomicrographs of control EA.hy926 cells and EA.hy926 cells incubated for 24 h with sub-toxic IC_{20} concentrations of the complexes are presented in Fig. 6. Tested complexes 1 and 12 exerted strong anti-angiogenic activity in vitro. Both complexes effectively inhibited elongation, sprouting and formation of capillary-like tube structures of EAhy926 cells grown on the surface of matrigel basement membrane matrix in comparison to control untreated EA.hy926 cells.

**Effects of complexes on gene expression levels**

To further investigate mechanisms of anticancer activity of complexes 1 and 12, their possible anti-invasive and anti-angiogenic effects were explored by measuring their effects in HeLa cells on expression levels of genes involved in angiogenesis and malignant cells metastasis: matrix metalloproteinase-2 (MMP2), matrix metalloproteinase-9 (MMP9) and vascular endothelial growth factor A (VEGFA). Both complexes applied at sub-toxic IC_{20} concentrations for 24 h reduced MMP9 gene expression levels in HeLa cells when compared to this level in control HeLa cells (Fig. 7). This may suggest their ability to exert suppressive effects on growth and metastasis of cervical cancer cells. However, in contrast to MMP9, the complexes increased expression levels of MMP2 in treated HeLa cells.

The complexes were especially effective in decreasing VEGFA gene expression levels in HeLa cells. This ability of both complexes to decrease expression levels of crucial proangiogenic factor VEGFA in malignant cells in addition to their ability to strongly inhibit in vitro angiogenesis of EA.hy926 cells, suggest prominent anti-angiogenic properties of 1 and 12. Targeting angiogenesis represents an important strategy in therapy of cancer since anti-angiogenic drugs may inhibit further progression of malignant tumors [49].

**DNA-binding activity**

Electronic absorption spectra of the compounds HL^3Cl, 12, HL^1Cl and 1 recorded at different concentrations without or with fixed concentration of CT-DNA are shown in Fig. S5. It was found that the maximum absorption of the ligand HL^3Cl was centered at 283 nm. Upon interaction with CT-DNA the peaks at 283 nm shifted with an increase of concentration of
HL₃Cl, while DNA absorption maximum was shifted from 258 to 281 nm, indicating the formation of a CT-DNA–compound HL₃Cl complex. Absorption changes induced by binding compound HL₃Cl were further calculated (inset in Fig. S5a). The absorption value of the sum of absorbances at 258 nm of a free compound HL₃Cl and free CT-DNA was not very different from the absorbance of a CT-DNA–compound HL₃Cl complex. Only at the highest concentration of HL₃Cl, hypochromism of about 6% was observed. Absorption spectra of 12 (complex of Fe(III) formed with ligand HL₃Cl) are shown in Fig. S5b. In UV region, the complex 12 presented a broad peak with maximum absorbance at 264 nm assigned to metal-coordinated pyridyl group, and a shoulder at about 280–320 nm (assigned to the intra-ligand π→π* transition). After the interaction with CT-DNA, the shoulder disappeared, while the observed maximum at 260 nm could be associated with overlapping of the absorption of nucleic base pairs and pyridyl group from complex 12, suggesting an interaction of the complex with CT-DNA. Analysis of the absorption changes induced upon binding of 12 to CT-DNA is shown in the inset in Fig. S5b. By comparison of the sum of absorbances at 258 nm of free complex HL₃Cl and free CT-DNA with the observed absorbance of the HL₃Cl–CT-DNA, the absorbance of the complex was increased up to 23% with the highest concentration of the compound. It can be attributed either to non-covalent binding in minor groove or to electrostatic interaction between CT-DNA and HL₃Cl.

The observed reduction of hyperchromism and insignificant blue shift in electronic absorption spectra, as well as the presence of pyridinium moiety in the structure of HL₃Cl suggest electrostatic mode of interaction.

The interactions of ligand HL₃Cl and complex 1 with CT–DNA were monitored by UV–Vis spectroscopy under the same experimental conditions, Fig. S5c and Fig. S5d, respectively. Ligand HL₃Cl displayed two absorption bands at 266 nm and at 316 nm with the shoulder at 327–352 nm. After the interaction with CT-DNA, changes in HL₃Cl–CT-DNA spectra were detected: decrease in absorption band at 266 nm with increase in ligand HL₃Cl concentration and decrease in absorption band at 316 nm in concentration-dependent way. Comparing the sum of absorbances at 258 nm of free complex HL₃Cl and free CT-DNA with the observed absorbance of the HL₃Cl–CT-DNA (Fig. S5c, inset), the result showed hypochromisms of 7.5%, 10.9% and 19.4% for 25 µM, 50 µM and 100 µM ligand 3, respectively. A small blue shift of 3 nm was observed for the band at 316 nm. Hyperchromism of about –42% observed with lower concentration of HL₃Cl was increased up to –23% with the highest concentration of the compound. It can be attributed either to non-covalent binding in minor groove or to electrostatic interaction between CT-DNA and HL₃Cl.

The observed reduction of hyperchromism and insignificant blue shift in electronic absorption spectra, as well as the presence of pyridinium moiety in the structure of HL₃Cl suggest electrostatic mode of interaction.

In the case of complex of ligand HL₃Cl (compound 1), UV–Vis spectrum displayed three absorbance peaks: at 263 nm, 293 nm and 363 nm. After interaction, the bands which correspond to CT-DNA showed small changes: hypochromisms of 2.9% and 5.1% for 25 µM and 50 µM, respectively, and hyperchromism of –5.6% for 100 µM of 1 (Fig. S5d, inset). Significant changes in spectra of 1–CT-DNA were observed for bands at 293 nm as evidenced by hypochromisms of 24.0%, 30.6% and 19.4% (for 25 µM, 50 µM and 100 µM of the complex, respectively) and for bands at 364 nm with hypochromisms of 32.0%, 48.5% and 39.4% (for 25 µM, 50 µM and 100 µM of the complex, respectively). In this case, hypochromism probably originates from the stabilization of the DNA duplex by the electrostatic effect of small molecules. [50, 51]

To obtain information on stability of these compound–CT-DNA complexes, spectroscopic titrations of solutions of constant concentration of the compounds (50 µM) with increasing concentration of CT-DNA were performed. Results are shown in Fig. S5. A quantitative determination of the binding strength of the compound to DNA is made by calculation of binding constants Kbinding of the compound from Eq. (1) [52]. The intrinsic binding constant

![Fig. 7](https://via.placeholder.com/150)

Changes in gene expression levels of MMP2 (a), MMP9 (b), and VEGFA (c), in HeLa cells treated with sub-toxic IC₅₀ concentrations of complexes 1 ([ZnL₁(NCS)₂]) and 12 ([FeL₃(NCS)₃]) for 24 h.
The fluorescence intensity of the band at 441 nm, which depended on the increase in concentration of the compounds. The obtained fluorescence quenching data were analyzed according to the Stern–Volmer Eq. (2) [54]. The fluorescence intensity of the band at 441 nm of the H−CT-DNA system decreased only by 12.5% with maximal used concentration of ligand HL$^3$Cl (Fig. S7a) with the quenching constant calculated by linear regression of a plot $I_0/I$ against $[Q]$ (Inset in Fig. S7a) as $K_{sv} = 1.59 \times 10^{-3}$ M$^{-1}$. In contrast, the enhancement of fluorescence of the H−CT-DNA system upon binding complex 12 to CT-DNA in a concentration-dependent manner was observed (Fig. S7b). The fluorescence intensity of the band at 441 nm of the H−CT-DNA system increased by 64.4% with maximal applied concentration of 12 and the corresponding constant $K_{sv} = -2.43 \times 10^{-3}$ M$^{-1}$ was calculated from the ratio of the slope to the intercept from the plot of $I_0/I$ against $[Q]$ (Inset in Fig. S7b). The reason for increase in fluorescence might be a consequence of fluorescence of 12 (Fig. S8).

As shown in Fig. S7c and Fig. S7d, the competition of HL$^3$Cl and 1 with H 33,258 in binding to DNA was more efficient than of the previous two compounds. An addition of HL$^3$Cl to H−CT-DNA system caused appreciable reduction in the fluorescence intensity of the band at 441 nm (about 66% with maximal performed concentration of HL$^3$Cl) accompanied by blue shift of emission maximum from 441 to 435 nm. The quenching constant calculated by linear regression of a plot $I_0/I$ against [HL$^3$Cl]/[CT-DNA] (Inset in Fig. S7c) was $K_{sv} = 1.20 \times 10^3$ M$^{-1}$. The plot displayed a good linear relationship for the investigated concentration ranges of HL$^3$Cl indicating the displacement of H33258 from H−CT-DNA. In view of fluorescence and UV/Vis absorption spectral results, interactions of the compound with DNA occurred possibly due to minor groove binding and electrostatic interactions between a positively charged pyridinium ion and negatively charged phosphate groups of DNA backbone. In case of interaction of complex 1 (prepared by coordination Zn(II) with ligand HL$^3$Cl and two thiocyanato groups) with DNA, fluorescence spectra measurements revealed that reduction in the fluorescence intensity of the emission maximum to 443 nm was 63% and was followed by formation of a clearly defined isosbestic point at 498 nm. The value of quenching constant $K_{sv} = 1.04 \times 10^4$ M$^{-1}$ was calculated from the ratio of the slope to the intercept from the plot of $I_0/I$ versus [1]/[CT-DNA] as demonstrated in inset in Fig. S7d. However, the observed broadening of the bands at 441 nm which depended on the increase in concentration of 1, and the occurrence of may be attributed to the fluorescence of the complex 1 (Fig. S8).

To clarify mutual interactions among the ligands, complexes, Hoechst and CT-DNA, the additional experiments were performed. As shown in Fig. S8, compound 12 (curve 6), HL$^3$Cl (curve 3) and 1 (curve 10) exhibited a fluorescence. The enhancement of fluorescence intensity of absorption bands suggested the interaction of compound with H (curves 7, 5, 11, respectively). The whole set of results indicates no displacement of H from its complex with CT−DNA by neither HL$^3$Cl nor its iron complex. On the other hand, the results indicate minor groove binding by HL$^3$Cl and its Zn(II) complex.

### Plasmid DNA interaction study

The interactions of plasmid pUC18 and compounds HL$^3$Cl, 12, HL$^3$Cl and 1 were analyzed by agarose gel electrophoresis (Fig. 8a, b). Result of agarose electrophoresis could be a retardation of the migration of plasmid DNA through gel due to covalent binding or change of different forms of DNA (supercoiled, nicked circular and linear) as the consequence of nuclease activity of an investigated compound. As shown in Fig. 8, no strand scission was observed upon addition of the compound to the plasmid at the concentration used in comparison to the control plasmid (lanes 1 in Fig. 8) showing that DNA cleavage does not present a mode of action of the studied ligands and complexes. Only complex 1 produced slight quenching of fluorescence of electrophoretic bands (lanes 5, 6 and 7) but independent of the concentration.
The results of interaction with BSA of ligands HL\textsubscript{1}Cl and HL\textsubscript{3}Cl, their corresponding complexes 1 and 12 are given in ESI.

**Conclusion**

The complexes 1, and 2 have been synthesized and characterized by X-ray crystallographic analysis, elemental analysis and IR spectroscopy. NMR spectroscopy results for Zn(II) complex showed its stability in solution, while in the case of Bi(III) complex, NMR results indicated that hydrolysis occurred. The distorted trigonal–bipyramidal geometry of the Zn(II) complex consists of the deprotonated form of the hydrazone ligand coordinated through the quinoline nitrogen, azomethine nitrogen and carbonyl oxygen atoms with two isothiocyanato ligands in the remaining coordination places. The geometry around Bi(III) ions is pentagonal–bipyramidal, with highly distorted NNO chelation from HL\textsubscript{2}Cl ligand and four chloride ligands.

The novel complex 1 showed a significant antibacterial activity especially towards Gram-negative bacteria, with intensity similar to chloramphenicol. As for the antifungal activity, the best results were shown for ligands HL\textsubscript{2}Cl and HL\textsubscript{3}Cl, although most complexes exerted a remarkable activity.

Among tested complexes, the strongest cytotoxic effects were exerted by complex 12 against melanoma A375 and cervical adenocarcinoma HeLa cells. The novel complex 1 showed moderate cytotoxic activity against HeLa, A375 and A549 malignant cell lines. Two selected complexes induced increase of the percentage of treated HeLa cells within G2/M cell cycle phase, while 12 induced remarkable increase in percentage of HeLa cells within subG1 phase of the cell cycle. Tested complexes showed cytoprotective effects against generation of ROS activated by hydrogen peroxide in normal keratinocytes. Both complexes exerted in vitro anti-angiogenic effects. Taken together, results of our study demonstrated anticancer properties of tested complexes.

The interactions of ligands HL\textsubscript{1}Cl and HL\textsubscript{3}Cl, and their corresponding complexes 1 and 12 with CT–DNA were monitored by UV–Vis spectroscopy. Results showed that in the case of HL\textsubscript{1}Cl and 1 electrostatic interaction was the binding mode. Similar results were observed with HL\textsubscript{3}Cl and 12, but in this case, electrostatic interactions were weak. Additional confirmation of these findings was evaluated by displacement study with Hoechst 33,3258 and calculation of corresponding quenching constants.

**Experimental**

**Materials and methods**

2-Acetylthiazole (99%) was obtained from Acros, 2-quinolinecarboxaldehyde (97%) from Flurochem and Girard’s T reagent (99%) from Aldrich. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer using the ATR technique in the region 4000-400 cm\textsuperscript{-1} (vs-very strong, s-strong, m-medium, w-weak). \textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on Varian 400/54 PS spectrometer (\textsuperscript{1}H at 400 MHz; \textsuperscript{13}C at 125 MHz) at room temperature using TMS as internal standard in DMSO-\textsubscript{d}\textsubscript{6}. Chemical shifts are expressed in ppm (\(\delta\)) values and coupling constants (J) in Hz. Elemental analyses (C, H, and N) were performed by standard micro-methods using the ELEMENTAR Vario ELIII C.H.N.S.O analyzer. Molar conductivities of DMSO solutions of the complexes (C = 10\textsuperscript{-3} mol/dm\textsuperscript{3}) were measured at room temperature (26°C) on a digital conductivity-meter JENWAY-4009.

**Synthesis**

**Synthesis of (E)-1-(2-oxo-2-(2-quinolin-2-ylmethylene) hydrazinyl)ethyl)pyridin-1-ium chloride (HL\textsubscript{1}Cl)**

The ligand HL\textsubscript{1}Cl was synthesized by the reaction of 2-quinolinecarboxaldehyde (0.629 g, 4 mmol) dissolved in ethanol (40 mL) and solid Girard’s P reagent (0.750 g, 4 mmol). The mixture was refluxed for 3 h. After cooling to the room temperature, light yellow precipitate was filtered and washed with ethanol. Yield: 1.201 g (92%). Elemental analysis calc. for C\textsubscript{17}H\textsubscript{15}N\textsubscript{4}OCl: C 62.48%, H 4.63%, N 17.15%; found: C 62.50%, H 4.65%, N 17.11%. IR (cm\textsuperscript{-1}): 3418.5 (s), 3132.9 (s), 3065.2 (s), 2957.7 (s), 2865.9 (m), 1695.5 (vs), 1634.6 (m), 1597.2 (m), 1487.5 (s), 1428.2 (w), 1389.4 (s), 1273.8 (s), 1216.8 (w), 1125.3 (m), 838.9 (w), 773.0 (m), 750.4 (w), 688.8 (w). \textsuperscript{1}H NMR (400 MHz, DMSO-\textsubscript{d}\textsubscript{6}), \(\delta\) (ppm):
Synthesis of complex 1 ([ZnL1(NCS)2]) The Zn(II) complex was synthesized by the reaction of HL1 (98 mg, 0.30 mmol) and Zn(BF4)2·6H2O (104 mg, 0.30 mmol) in solvent mixture of MeOH/CH3CN (25/5 mL). After complete dissolution of Zn(BF4)2·6H2O in reaction mixture, NH4SCN (46 mg, 0.60 mmol) dissolved in water (10 mL) was added. The mixture was refluxed for 2 h. After slow evaporation of solvent at room temperature for 1 day, yellow crystals were obtained. Crystals suitable for X-ray analysis were obtained after recrystallization in methanol. Yield: 28 mg (20%). Elemental analysis calcd. for C19H15N6OS2Zn: C 48.26%, H 3.20%, N 17.75%, S 13.49%. IR (cm⁻¹): 3129.2 (w), 3091.8 (m), 3017.9 (m), 2955.5 (s), 1783.6 (w), 1656.3 (vs), 1620.3 (s), 1529.4 (vs), 1475.7 (s), 1443.3 (s), 1389.1 (s), 1260.0 (s), 1193.5 (m), 1143.4 (s), 1066.3 (s), 1021.1 (s), 971.9 (m), 927.5 (w), 753.2 (s), 717.2 (w), 694.3 (s), 666.3 (s), 611.7 (w), 599.5 (s), 589.2 (s), 578.1 (s), 551.7 (w). (HL2Cl–E). 1H NMR (500 MHz, DMSO-d6), δ (ppm): 2.41 (s, 3H, C5-H), 3.30 (s, 9H, C8-H), 4.82 (s, 2H, C7-H), 7.85 (d, 1H, J2, NH=-C3-H = 5.0 Hz, C2-H), 7.93 (d, 1H, J2, NH=-C3-H = 5.0 Hz, C3-H), 11.61 (s, 1H, N-H). 13C NMR (125 MHz, DMSO-d6), δ (ppm): 13.90 (C5), 53.65 (C8), 61.92 (C11), 117.57 (C3), 128.04 (C6), 128.01 (C7), 128.40 (C6), 129.74 (C4a), 132.52 (C7), 134.73 (C6), 146.05 (C9), 146.79 (C14), 147.77 (C8a), 153.33 (C2), 167.43 (C6).

Synthesis of complex 2 ([BiHL2Cl4]·1/2CH3OH) The Bi(III) complex was synthesized by the reaction of HL2Cl (69 mg, 0.25 mmol) and BiCl3 (79 mg, 0.25 mmol) in solvent mixture of MeOH/CH3CN (12/12 mL). The mixture was stirred for 1 h at 60 °C. After slow evaporation of solvent at room temperature for 1 day, white crystals were obtained. Yield: 89 mg (57%). Elemental analysis calcd. for C10H17N4OSCl4Bi1/2CH3OH: C 20.72%, H 3.12%; found: C 20.61%, H 3.16%. IR (cm⁻¹): 3498.5 (m), 3210.3 (s), 3111.2 (m), 2955.5 (s), 1666.9 (m), 1656.3 (vs), 1620.3 (s), 1529.4 (vs), 1475.7 (s), 1443.3 (s), 1389.1 (s), 1260.0 (s), 1193.5 (m), 1143.4 (s), 1066.3 (s), 1021.1 (s), 971.9 (m), 927.5 (w), 753.2 (s), 717.2 (w), 694.3 (s), 666.3 (s), 611.7 (w), 599.5 (s), 589.2 (s), 578.1 (s), 551.7 (w). (HL2Cl–E). 1H NMR (500 MHz, DMSO-d6), δ (ppm): 2.53 (s, 3H, C5-H), 3.34 (s, 9H, C8-H), 4.82 (s, 2H, C7-H), 7.85 (d, 1H, J2, NH=-C3-H = 5.0 Hz, C2-H), 7.93 (d, 1H, J2, NH=-C3-H = 5.0 Hz, C3-H), 11.86 (s, 1H, N-H). 13C NMR (125 MHz, DMSO-d6), δ (ppm): 13.90 (C5), 53.65 (C8), 63.36 (C7), 123.33 (C2), 143.97 (C3), 150.80 (C4), 166.78 (C1), 167.34 (C6).

Synthesis of complex 3 [BiHL2Cl4](OH)2 The Bi(III) complex was synthesized by the reaction of HL2Cl (69 mg, 0.25 mmol) and BiCl3 (79 mg, 0.25 mmol) in solvent mixture of MeOH/CH3CN (12/12 mL). The mixture was stirred for 1 h at 60 °C. After slow evaporation of solvent at room temperature for 1 day, white crystals were obtained. Yield: 89 mg (57%). Elemental analysis calcd. for C10H17N4OSCl4Bi1/2CH3OH: C 20.72%, H 3.12%; found: C 20.61%, H 3.16%. IR (cm⁻¹): 3498.5 (m), 3210.3 (s), 3111.2 (m), 2955.5 (s), 1666.9 (m), 1656.3 (vs), 1620.3 (s), 1529.4 (vs), 1475.7 (s), 1443.3 (s), 1389.1 (s), 1260.0 (s), 1193.5 (m), 1143.4 (s), 1066.3 (s), 1021.1 (s), 971.9 (m), 927.5 (w), 753.2 (s), 717.2 (w), 694.3 (s), 666.3 (s), 611.7 (w), 599.5 (s), 589.2 (s), 578.1 (s), 551.7 (w). (HL2Cl–E). 1H NMR (500 MHz, DMSO-d6), δ (ppm): 2.53 (s, 3H, C5-H), 3.34 (s, 9H, C8-H), 4.82 (s, 2H, C7-H), 7.85 (d, 1H, J2, NH=-C3-H = 5.0 Hz, C2-H), 7.93 (d, 1H, J2, NH=-C3-H = 5.0 Hz, C3-H), 11.86 (s, 1H, N-H). 13C NMR (125 MHz, DMSO-d6), δ (ppm): 13.90 (C5), 53.65 (C8), 63.36 (C7), 123.33 (C2), 143.97 (C3), 150.80 (C4), 166.78 (C1), 167.34 (C6).

Single crystal X-ray

SCXRD analysis was performed on single crystal samples on a Bruker D8 Venture diffractometer equipped with a kappa goniometer and an Oxford cryostream. Microfocused MoKα radiation (λ = 0.71073 Å) was used for data collection. Data were reprocessed using Bruker APEX v3 software. Lorentz polarization and absorption corrections were applied. Structures were solved by direct methods using SHELXT [55] and refined by full-matrix least-squares on all F² using SHELXL [56] implemented in Olex2.21 [57]. For complexes 1 and 2, anisotropic displacement parameters were refined except for hydrogen atoms. Table S1 reports crystal data collection and refinement results. ORTEP diagrams are reported.

Synthesis of ligand HL2Cl and complexes 3-12 including analytical data confirming their purities are presented in ESI.
in Figs. 1 and 3. Crystallographic data for complexes 1 and 2 have been deposited with the Cambridge Crystallographic Data Centre as supplementary material CCDC 2,064,600–2,064,601. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Antimicrobial activity

Antimicrobial activity was tested against a panel of microorganisms including: Gram-negative bacteria *Escherichia coli* (ATCC 25,922), *Pseudomonas aeruginosa* (ATCC 9027), *Proteus hauseri* (ATCC 13,315), *Klebsiella pneumoniae* (ATCC 10,031), *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13,076), Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Clostridium sporogenes* (ATCC 19,404), *Micrococcus luteus* (ATCC 4698), *Micrococcus luteus* (ATCC 10,240), yeasts *Candida albicans* (ATCC 10,231), *Saccharomyces cerevisiae* (ATCC 9763) and fungal strain *Aspergillus brasiliensis* (ATCC 16,404).

Antimicrobial activity was evaluated using broth microdilution method according to NCCLS. [National Committee for Clinical Laboratory Standards, Approval Standard Document M7-A5, Villanova, Pa, USA, 2000] The 96-well plates were prepared by dispensing 100 µl of Mueller–Hinton broth for bacteria and Sabouraud dextrose broth for yeasts and fungi, into each well. A 100 µL aliquot from the stock solution of the tested compounds (concentration 10 mg/mL in DMSO) was added into the first row of the plate and double diluted using multi-channel pipette. The direct colony method was used in preparation of suspension of bacteria and yeasts in sterile 0.9% saline, while the process of preparing the suspension of fungal spores included gentle stripping of spore from agar slants with growing aspergilli into sterile 0.9% saline. Suspension turbidity evaluation was conducted by comparison with 0.5 McFarland’s standard. A 10 µL of diluted bacterial, yeast or spores suspension was added to each well to give a final concentration of 5 × 10^3 CFU/mL for bacteria and 5 × 10^3 CFU/mL for fungi and yeast. Chloramphenicol served as positive control for bacteria, while amphotericin B served as positive control for yeasts and fungi.

The inoculated plates were incubated at 37 °C for 24 h for bacteria and at 28 °C for 48 h for the yeasts and fungi. The bacterial growth was visualized by adding 20 µL of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) aqueous solution [58]. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compounds that inhibited bacterial growth (red-colored pellet at the bottom of the wells after the addition of TTC).

Determination of cytotoxic activity

The cytotoxic effects of the complexes and their precursor compounds were examined against five human malignant cell lines: cervical adenocarcinoma HeLa, melanoma A375, breast adenocarcinoma MCF7, prostate adenocarcinoma PC-3, and lung carcinoma A549, as well as against normal human keratinocyte cell line HaCaT. Stock solutions of the compounds were made in DMSO at concentration of 10 mM. The cell lines were maintained in complete nutrient medium RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin–streptomycin solution (100 U/mL of penicillin and 0.1 mg/mL of streptomycin). HeLa (3000 cells per well), A375 (3000 cells per well), MCF7 (7000 cells per well), PC-3 (5000 cells per well), A549 (5000 cells per well), and HaCaT cells (7000 cells per well) were seeded into 96-well microtiter plates and after 20 h the cells were treated with complexes and their precursor compounds (five increasing concentrations tested, ranging from 12.5 to 200 µM). Complete nutrient medium was added to control cell samples. Cell cultures were incubated with compounds for 72 h. Cell survival was measured using MTT test according to the method of Mosmann [59], which was modified by Ohno and Abe [60] and described in our previous studies [61]. Each of the three independent experiments was performed in triplicate.

Cell cycle analysis

The effects of the complexes 1 and 12 on the cell cycle of HeLa cells were examined by flow cytometry. HeLa cells were incubated with IC_{50} and 2IC_{50} concentrations of two selected complexes for 24 h. After 24 h treatment, HeLa cells were collected by trypsinization, washed with phosphate buffered saline (PBS) and fixed in 70% ethanol on ice, according to standard procedure [62]. Cell samples were stored at –20 °C for at least 1 week before analysis. Before staining, the cells were washed, re-suspended in PBS containing RNase A and incubated for 30 min at 37 °C. Subsequently, propidium iodide solution was added to the cell samples. Percentages of HeLa cells within cell cycle phases were determined using a BD FACSCalibur flow cytometer. The analyses of acquired data (10,000 events collected for each gated cell sample) were performed by BD CellQuest software. Cell cycle data are presented as mean ± SD of three independent experiments.

Determination of intracellular reactive oxygen species (ROS) levels in HeLa and HaCaT cell lines

HeLa cells were treated with IC_{50} concentrations of the selected complexes 1 and 12 for 24 h. Afterwards, the HeLa cells were collected by trypsinization, washed with PBS...
and incubated in a solution of 30 µM 2′,7′-dichlorodihydrofluorescein diacetate (Sigma Aldrich) in PBS for 45 min at 37 °C, according to previously described procedure [63]. The cells were then washed with PBS and analyzed.

HaCaT cells were incubated with sub-toxic IC₂₀ concentrations of the selected complexes 1 and 12 for 24 h (applied concentration was 50 µM for both complexes, as determined by MTT test for 24 h treatment). Afterwards, the HaCaT cells were collected, washed and incubated in a solution of 30 µM 2′,7′-dichlorodihydrofluorescein diacetate (Sigma Aldrich) in PBS for 45 min at 37 °C. After incubation and washing, the cell samples were exposed to 4 mM hydrogen peroxide solution (H₂O₂) to induce generation of ROS for 30 min at 37 °C. After 30 min, these cells were washed with PBS and analyzed.

The intensity of green fluorescence emitted by the dichlorofluorescein in HeLa and HaCaT cells was determined by flow cytometry. Data are presented as mean ± SD of two independent experiments.

Endothelial cell tube formation assay

The possible anti-angiogenic effects of the selected complexes were examined on human umbilical vein EA.hy926 cells using endothelial cell tube formation assay [64, 65]. The EA.hy926 cells were seeded on the surface of Corning® Matrigel® basement membrane matrix and incubated with sub-toxic IC₂₀ concentrations of the complexes 1 and 12 for 24 h (applied concentrations were 50 µM and 70 µM, respectively, as determined by MTT test for 24 h treatment). After 20 h incubation with the complexes the photographs of EA.hy926 cells were captured under the inverted phase-contrast microscope.

Gene expression analyses

HeLa cells were seeded into 25 cm² cell culture flasks (1.5 × 10⁶ cells/flask). After 24 h, the cells were treated with sub-toxic IC₂₀ concentrations of the complexes 1 and 12 (applied concentrations were 25 µM and 50 µM, respectively, as determined by MTT test for 24 h treatment). Control cells were incubated in nutrient medium only. After 24 h incubation with selected complexes, HeLa cells were collected by trypsinization, washed with PBS, and stored at −80 °C. Total RNA was isolated from HeLa cells using TRI Reagent® (Sigma) according to the manufacturer’s protocol. Concentration and quality of isolated RNA for each sample were determined spectrophotometrically (BioSpec-nano, Shimadzu). High-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) was used for reverse transcription of 1 µg total RNA into single-stranded cDNA. Measurement of mRNA expression levels of selected genes was done by real-time quantitative PCR (RT-qPCR) and TaqMan® gene expression assays (MMP2—Hs01548727_m1, MMP9—Hs00957562_m1, and VEGFA—Hs00900055_m1) using LightCycler® 480 II system (Roche Diagnostics GmbH, Mannheim, Germany). Gene expression values were normalized to GAPDH (Hs02758991_g1) and were obtained by comparative ΔΔCt method, analyzed with LightCycler® 480 Software.

UV−visible measurements experiments

For DNA-binding experiments, calf thymus DNA (CT-DNA, lyophilized, highly polymerized, obtained from Serva, Heidelberg) was dissolved in Tris buffer (10 mM Tris–HCl pH 7.9) overnight at 4 °C. This stock solution was stored at 4 °C and was stable for several days. A solution of CT-DNA in water gave a ratio of UV absorbance at 260 and 280 nm, A₂₆₀/A₂₈₀ of 1.89–2.01, indicating that DNA was sufficiently free of protein. The concentration of DNA (3.15 mg/mL) was determined from the UV absorbance at 260 nm using the extinction coefficient ε₂₆₀ = 6600 M⁻¹ cm⁻¹ [66]. All compounds (HL₁Cl, HL₁Cl and I) was dissolved in dimethyl sulfoxide at concentrations of 10 mM and these solutions were used as stock solutions.

Reaction mixtures (1 mL in 40 mM bicarbonate buffer, pH 8.4) consisting of different concentrations of compounds (25 µM, 50 µM, and 100 µM) and 97 µM of CT-DNA (calculated per phosphate) were incubated at 37 °C for 60 min with occasional vortexing. The absorbance titrations were performed at a fixed concentration of the compound (50 µM) and gradually increasing the concentration of double-stranded CT-DNA (4.85, 5.82, 6.79, 7.76, 8.73, 9.73, 10.67, 11.64, 12.61, 13.58, 14.55, 15.32, 16.49 and 17.46 × 10⁻⁵ M). The absorbance at 282, 273, 266 and 260 nm for HL₁Cl, HL₂Cl and I, respectively, was monitored for each concentration of DNA. The binding constant Kₘ was determined using the Eq. (1) [52]:

\[
[DNA] \times (ε_a - ε_i)^{-1} = [DNA] \times (ε_b - ε_i)^{-1} + K_m^{-1} \times (ε_b - ε_i)^{-1},
\]

where ε_a, ε_i, ε_b are absorbance/[compound], extinction coefficient of the free compound and extinction coefficient of the bound compound, respectively.

Fluorescence measurements

The competitive interactions of the compound and the fluorescent stain Hoechst 33,258 (H) with CT-DNA have been studied by measuring the change of fluorescence intensity of the probe–DNA solution after addition of the compound. Reaction mixtures containing 97 µM of CT-DNA (calculated per phosphate) in 1 mL of 40 mM bicarbonate solution (pH 8.4) were pretreated with 1 µL of the probe solution (28 µM of Hoechst stain, final concentration) for 20 min and the
mixture was analyzed by fluorescence measurement. Then, the gradually increasing concentrations of the complexes (2, 4, 6, 8, 10, 12, 16, and 20 μM, final concentrations) were successively added and the change in the fluorescence intensity was measured using a Thermo Scientific Lumina Fluorescence spectrometer (Finland) equipped with a 150 W Xenon lamp. The slits on the excitation and emission beams were fixed at 10 nm. All measurements were performed by excitation at 350 nm in the range 390–650 nm. The control was the H−CT-DNA solution. The obtained fluorescence quenching data were analyzed according to the Stern–Volmer Eq. (2)

\[ I_0/I = 1 + K_{sv}[Q], \]

where \( I_0 \) and \( I \) represent the fluorescence intensities of H−CT-DNA in the absence and the presence of the compound, respectively, \( K_{sv} \) is quenching constant and \([Q]\) is the concentration ratio of the compound to DNA ([compound]/[CT-DNA]). The \( K_{sv} \) value was calculated from the ratio of the slope to the intercept from the plot of \( I_0/I \) versus \([Q]\).

Primary spectra for all spectrometric measurements were imported into OriginPro 8.0.

**DNA cleavage experiments**

For DNA cleavage experiments the plasmid pUC18 (pUC18, 2686 bp, purchased from Sigma-Aldrich, USA) was prepared by its transformation in chemically competent cells *Escherichia coli* strain XL1 blue. Amplification of the clone was done according to the protocol for growing *E. coli* culture overnight in LB medium at 37 °C [67] and purification was performed using Qiagen Plasmid plus Maxi kit. Finally, DNA was eluted in 10 mM Tris–HCl buffer and stored at −20 °C. The concentration of plasmid DNA (131 ng/µL) was determined by measuring the absorbance of the DNA-containing solution at 260 nm. One optical unit corresponds to 50 µg/mL of double-stranded DNA.

The cleavage reaction of supercoiled pUC18 DNA by the compounds (0.5, 1 and 2 mM) was investigated by incubation with 460 ng of pUC18 in a 20 µL reaction mixture in 40 mM bicarbonate buffer pH 8.4, at 37 °C, for 90 min. The reaction mixtures were vortexed from time to time. The reaction was terminated by short centrifugation at 10,000 rpm and addition of 5 µL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in TAE buffer, pH 8.24 (40 mM Tris–acetate, 1 mM EDTA)). The samples were subjected to electrophoresis on 1% agarose gel (Amersham Pharmacia-Biotech, Inc) prepared in TAE buffer pH 8.24. The electrophoresis was performed at a constant voltage (80 V) until bromophenol blue had passed through 75% of the gel. A Submarine Mini-gel Electrophoresis Unit (Hoeffer HE 33) with an EPS 300 power supply was used.

After electrophoresis, the gel was stained for 30 min by soaking it in an aqueous ethidium bromide solution (0.5 µg/mL). The stained gel was illuminated under a UV transilluminator Vilber-Lourmat (France) at 312 nm and photographed with a Panasonic DMC-LZ5 Lumix Digital Camera through filter DEEP YELLOW 15 (Tiffen, USA).

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00775-021-01893-5.

**Acknowledgements** The authors are grateful to the Ministry of Education, Science and Technological Development of the Republic of Serbia for the financial support (Grant numbers: 451-03-9/2021-14/20004, 451-03-68/2021-14/200026 and 451-03-9/2021-14/200168). The Laboratorio di Strutturistica “M. Nardelli” of the University of Parma and Chiesi Farmaceutici SpA are thanked for the X-ray diffraction data collection. This work has benefited from the equipment and framework of the COMP-HUB Initiative, funded by the “Departments of Excellence” program of the Italian Ministry for Education, University and Research (MIUR, 2018-2022).

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**References**

1. Qin W, Long S, Panunzio M, Biondi S (2013) Schiff bases: a short survey on an evergreen chemistry tool. Molecules 18:12264–12289. https://doi.org/10.3390/molecules18102264
2. Shakodfa MME, Shtaiwi MH, Morsy N, Abdel-rassel TMA (2014) Metal complexes of hydrazones and their biological, analytical and catalytic applications: a review. Main Group Chem 13:187–218. https://doi.org/10.3233/MGC-140133
3. Belkheiri N, Bouguerne B, Bedos-Belval F et al (2010) Synthesis and antioxidant activity evaluation of a syringic hydrazones family. Eur J Med Chem 45:3019–3026. https://doi.org/10.1016/j.ejmech.2010.03.031
4. Kaushik D, Khan SA, Chawla G, Kumar S (2010) N’-[(5-chloro-3-methyl-1-phenyl-1H-pyrazol-4-yl)methylene] 2/4-substituted hydrazides: synthesis and anticonvulsant activity. Eur J Med Chem 45:3943–3949. https://doi.org/10.1016/j.ejmech.2010.05.049
5. Júnior WB, Alexandre-Moreira MS, Alves MA et al (2011) Analgesic and anti-inflammatory activities of salicylaceladehyde 2-chlorobenzoyl hydrazone (H2LASSBio-466), salicylaldehyde 4-chlorobenzooyl hydrazone (H2LASSBio-1064) and their Zinc(II) complexes. Molecules 16:6902–6915. https://doi.org/10.3390/molecules16086902
6. Catto M, Aliano R, Carotti A et al (2010) Design, synthesis and biological evaluation of indane-2-aryldiazimethylene-1,3-di-ones and indol-2-aryldiazenymethylene-3-ones as β-amyloid aggregation inhibitors. Eur J Med Chem 45:1359–1366. https://doi.org/10.1016/j.ejmech.2009.12.029
7. Altintop MD, Sever B, Ekilioglu OA et al (2020) A series of furan-based hydrazones: design, synthesis, and evaluation of antimicrobial activity, cytotoxicity and genotoxicity. Lett Drug Des Discov 17:312–322. https://doi.org/10.2174/1570180816666190325163948
8. Kocyigit-Kaymakcioglu B, Yazici SS, Tok F et al (2019) Synthesis and anticancer activity of new hydrazide-hydrazone and their
Authors and Affiliations

Nevena Stevanović1 · Paolo Pio Mazzeo2,3 · Alessia Bacchi2,3 · Ivana Z. Matić4 · Marija Đorđić Crnogorac4 · Tatjana Stanojković4 · Miroslava Vujčić5 · Irena Novaković5 · Dušanka Radanović5 · Maja Šumar-Ristović1 · Dušan Sladić1 · Bofžidar Čobeljić1 · Katarina Andelković1

1 University of Belgrade-Faculty of Chemistry, Studentski trg 12–16, 11000 Belgrade, Serbia
2 Dipartimento di Scienze Chimiche, della Vita e Della Sostenibilità Ambientale, Università Degli Studi di Parma, Viale delle Scienze, 17A, 43124 Parma, Italy
3 Biopharmanet-TEC, Università Degli Studi di Parma, via Parco Area delle Scienze 27/A, 43124 Parma, Italy
4 Institute of Oncology and Radiology of Serbia, 11000 Belgrade, Serbia
5 University of Belgrade-Institute of Chemistry, Technology and Metallurgy, Department of Chemistry, Njegoševa 12, 11000 Belgrade, Serbia