Synthesis, Characterization and Biological Activity of Two New Copper (II) Complexes with N-sulfonamide Ligand

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Despite the fact that a large number of chemotherapeutic anticancer agents have been discovered, cancer still remains a great cause of deaths worldwide. The purpose of our researches is to discover a new antitumor drug. In this aim, two new Cu(II) complexes, \([\text{Cu(L)}_2(\text{py})_2(H_2O)_2] \) (C1) and \([\text{Cu(L)}_2(\text{phen})] \) (C2) with a new ligand, N-(5-trifluoromethyl-[1,3,4]-thiadiazole-2-yl)-benzenesulfonamide (HL) were synthesized. The complexes were characterized by elemental analysis, spectral and magnetic determinations. The nuclease activity studies of the complexes confirm their capacity to cleavage the DNA molecule. Both complexes have in vitro antioxidant activity (DPPH, FRAP methods), in vitro (using xanthine/xanthine oxidase system) and in vivo (using S.cerevisiae) SOD mimetic activity. The results of MTT assay on two carcinoma cell lines (HeLa and WM35) indicate that both complexes have antitumor activity, but (C1) has a superior activity compared with (C2) and with Cisplatin. On normal fibroblast (HDFa), (C1) showed toxicity comparable with Cisplatin, but (C2) showed a lower one. Bacterial assays were also performed (by the disk diffusion method) and both complexes have antibacterial activity against S. aureus, E. coli, P. aeruginosa and B. cereus. All the biological studies are in concordance and show that both complexes have biologic activity but (C2) is much more active.

Keywords: oxidative DNA cleavage, antioxidant capacity, SOD-mimetic activity, cytotoxicity, antibacterial properties

Metal ions are important for the course of the living organisms’ vital functions where they occur under the form of complex or chelated combinations, as well as for the drugs control and analyses [1-4]. Copper exhibits considerable biochemical action either as an essential trace metal or as a constituent of various exogenously administered compounds in humans [5,6].

Current interest in Cu complexes is stemming from their potential use as antimicrobial, antiviral, anti-inflammatory, antitumor agents, enzyme inhibitors, or chemical nuclease [7-11].

The study of N-substituted heterocyclic sulfonamides ligands showed their ability to coordinate biologically important metal ions. The sulfonamides structures are varied and complex, and offer multiple possibilities to coordinate metallic ions depending on the type of donor atoms in the molecule (O, S, N), the stereochemistry of the compounds, the nature of the metallic center that can form complexes, the nature of the anion of the salt that offers the central ion, the synthesis parameters [12].

Copper complexes as artificial nuclease have been shown to possess in vitro anticancer activity and a lower toxicity than platinum drugs. Many of them are active against platinum drug resistant tumor cell lines. In contrast to platinum compounds which covalently bind DNA nucleobases, copper derivatives usually form non-covalent interactions with DNA (intercalation, electrostatic forces of attraction or major/minor groove binding). For the majority of copper complexes reported as DNA cleavage agents, their mode of action is mediated via the formation of ROS generated either by oxidation of Cu(II) to Cu(III) or by reduction from Cu(II) to Cu(I) in the presence of oxidizing (dihydrogen peroxide or molecular oxygen) or reducing agents (ascorbic acid) [13-15].

The synthesis of Cu(II) complexes with N-substituted heterocyclic sulfonamides has greatly increased in the past twenty years, due to the diversity of biological activity of the resulting compounds. In this context, for the past several years our group worked on the synthesis of such compounds in order to obtain new antitumor agents and we described the DNA cleavage, SOD-like and antitumor activity of several copper sulphonamide complexes [16-21].

In this paper we report the synthesis, the physicochemical characterization of two new Cu(II) complexes with a N-substituted sulfonamide and we demonstrate their antioxidant, SOD-mimetic, antitumor and antibacterial activities.

Experimental part

Materials and physical measurements

2-amino-5-fluoromethyl-[1,3,4]-thiadiazole, sulfonyl chlorides, pyridine and phenantroline were provided by Aldrich and were used without further purification.

Elemental analyses (C, N, H, S) were performed with Perkin-Elmer elemental analyser. IR spectra were recorded using KBr disks on a Perkin-Elmer FT-IR 1730 instrument, in the 4000-400 cm⁻¹ range. Electronic paramagnetic spin

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resonance spectrum was performed at room temperature with a Bruker ER 200D instrument. Solid UV-Vis spectra were recorded on a Shimadzu UV-2101 PC spectrophotometer. Mass spectra for the ligand were recorded using the FAB technique, on a VG Autospectrometer. Magnetic susceptibility measurements were performed at room temperature according to the Faraday method, using Hg(CO)(SCN)$_2$ as standard.

**Synthesis**

Synthesis of the ligand N-(5-trifluoromethyl-[1,3,4]-thiadiazole-2-yl)-benzensulfonamide (HL) (C$_7$H$_{10}$N$_2$O$_2$F)

The ligand was prepared by reacting 2-amino-5-trifluoromethyl-[1,3,4]-thiadiazole (1 mmol) with benzene-sulfonil chloride (2 mmol) in a pyridine solution (6 mL). The mixture was refluxed at 60°C for 60 min; 10 mL of very cold water was then added in the mixture, continuously stirring. The white precipitate formed was filtered and further recrystallized using ethanol.

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The working FRAP solution was freshly prepared by mixing 50 mM acetate buffer; 270 mg FeCl$_3$·6 H$_2$O dissolved in 50 mL distilled water; 150 mg TPTZ and 300 µM, having a correlation coefficient $R^2 = 0.9956$ and the regression equation ($y = 0.0017x + 0.0848$), where $x$ represents absorbance detected at 595 nm. The results were expressed as µmol Trolox equivalents (TE)/100 mL extract [23,24].

**In vitro SOD mimetic activity**

The in vitro SOD mimetic activities of the complexes were assayed using the Oberley and Spitz method with some minor modifications [25]. Xanthine (1.5 x 10$^{-4}$ M) and xanthine oxidase in 50 mM potassium phosphate buffer 0.1 M, pH 6 (cyclic, cyclic) buffer, pH = 7.8 were used to generate a reproducible and constant flux of superoxide anions. These were detected by the reduction of nitro blue tetrazolium (NBT) (5.6 x 10$^{-5}$ in base pairs), and 6µL of activating agent solution (H$_2$O - ascorbic acid) in a threefold molar excess relative to the concentration of the complex. The resulting solutions were incubated for 1 h at 37°C, after which a quench buffer solution (3µL) consisting of bromphenol blue (0.25%), xylene cyanole (0.2%) and glycerol (30%) was added.

The solution was then subjected to electrophoresis on 0.8% agarose gel in 0.5 x TBE buffer (0.045 M Tris, 0.045 M boric acid, and 1 mM EDTA) containing 5μL/100 mL of a solution of ethidium bromide (10 mg/mL) at 100 V for 2 h. The bands were photographed on a capturing system (Gelprinter Plus TDI).

To test for the presence of reactive oxygen species (ROS) generated during strand scission and for possible complex-DNA interaction sites, various reactive oxygen intermediate scavengers and groove binders were added to the reaction mixtures. The scavengers used were 2,2,6,6-tetramethyl-4-piperidone (0.5 M), dimethylsulfoxide (DMSO) (14 M), tert-butyl alcohol (10.5 M), sodium azide (NaN$_3$) (400 mM), superoxide dismutase (SOD) (15 units).

In addition, a chelating agent of copper (I), neocuprine (36μM), along with the groove binder distamycin (80μM) were also assayed. Samples were treated as described above [16,19].

In vitro antioxidant activity

DPHH radical scavenging assay

The radical scavenging activity of the (C$_7$) and (C$_7$)$_2$ complexes towards the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured according to Kikuzaki et al. [22]. The sample solution (50 µL, 20 mg/mL in DMSO) was mixed thoroughly with a solution of DPPH in methanol (1 mL, 0.4 mM) and brought up with methanol to 5 mL. The mixture was shaken vigorously using a vortex and left to stand for 30 min at room temperature, in the dark. After that, the absorbance was measured at 517 nm using a Genesys-10 spectrophotometer. The DPPH solution without sample solution was used as control sample (A$_0$). The ability to scavenge the DPPH radical was calculated using the following formula:

$$\% \text{Inhibition} = \frac{[(A_0 - A_{\text{sample}}) / A_0]}{\times 100}$$

**FRAP method (ferric reducing antioxidant power)**

FRAP method is a simple spectrophotometric method that assesses the antioxidant power of the complexes, being based on the reduction of ferric tripyridyltriazine complex [Fe(III)-TPTZ] by a reductant, at an acid pH. The stock solutions included: 300 mM acetate buffer; 270 mg FeCl$_3$·6 H$_2$O dissolved in 50 mL distilled water; 150 mg TPTZ and 300 µM, having a correlation coefficient $R^2 = 0.9956$ and the regression equation ($y = 0.0017x + 0.0848$), where $x$ represents absorbance detected at 595 nm. The results are expressed as µmol Trolox equivalents (TE)/100 mL extract [23,24].

Biological assays

DNA cleavage

Reactions were performed by mixing 7 µL of cacodylate buffer 0.1 M, pH 6 (cyclic acid/sodium cacodylate), 6 µL of complex solution (final concentrations: 3, 6, 9, 12 and 15µM), 1µL of pUC18 DNA solution (0.25µg/µL, 750μM in base pairs), and 6μL of activating agent solution (H$_2$O - ascorbic acid) in a threefold molar excess relative to the concentration of the complex. The resulting solutions were incubated for 1 h at 37°C, after which a quench buffer solution (3µL) consisting of bromphenol blue (0.25%), xylene cyanole (0.2%) and glycerol (30%) was added.

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In addition, a chelating agent of copper (I), neocuprine (36µM), along with the groove binder distamycin (80µM) were also assayed. Samples were treated as described above [16,19].
which has the ability to delete/insert the SOD 1 gene.

In vivo SOD mimetic activity

The protective effect of the complexes (C1) and (C2) against free radicals produced by oxidative agents has been determined.

Three cell lines were used for determining the antibacterial activity of the complexes against two Gram-positive bacteria species: Staphylococcus aureus ATCC 6538P and Pseudomonas aeruginosa ATCC 14579 and against two Gram-negative bacteria species: Escherichia coli ATCC 10536 and P aeruginosa ATCC 27853 was done using the disk diffusion method [29–32]. As positive control Norfloxacin (Sigma-Aldrich) was used [33]. The reference microbial strains were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

The values of the minimum inhibitory concentration were determined using the serial micro-broth dilution method with Müller-Hinton broth, described by Carson et al. [34]. All tests were duplicated and the MIC values were expressed as mean ± standard deviation.

Statistics

All the experiments were conducted in triplicates and data are displayed as Mean ± SD, representing the average of independent experiments performed in triplicate. The IC50 values representing the complexes concentration required to inhibit 50% of cell proliferation were calculated from the dose-response curves using nonlinear regression [18,20,27,28].

In vivo SOD mimetic activity

The protective effect of the complexes (C1) and (C2) against free radicals produced by oxidative agents has been determined.

The SOD-like activities of the complexes where evaluated using a strain of Saccharomyces cerevisiae his3Δ1 leu2-3,112 sod1: URA3 (ATCC66867), which has the ability to delete/insert the SOD, gene encoding the synthesis of Cu2Zn2SOD. The characteristics of Saccharomyces cerevisiae his3Δ1 (ATCC66867) are: MAT aura3-52 trpl1-289 his3-1 leu2-121 sod1: URA3. The Cu2Zn2SOD is the main SOD in the cell and it is localized in the cytoplasm. Yeast cells were grown in YPD reach medium (1% yeast extract, 2% peptone and 2% glycerol). The used culture medium does not contain glucose, it contains glycerol instead, because in its presence the levures can grow. This is determinant, as free radicals are going to be generated during the breath processus taking place in the mithocondria. Solid media contained 1.5%agar. Cell density from cultures grown overnight was determined by cell counting in a Neubauer hematematik. 106 cells were suspened in 15 mL of melted solid YPD media kept at 45 °C. Solutions of the (C1) and (C2) complexes in a mixture of DMSO:EtOH (1:4) at increasing concentrations (30, 50, 70 µM) were added to the growth medium. Cell suspensions were poured into Petri dishes and allowed to solidify at room temperature. Paper disks measuring 6 mm in diameter (Antibiotica test Blättchen) containing 5 µL of a 5 mM menadione solution in ethanol or 5 µL of 17.5% H2O2 were added to the growth medium. The absorbance was read at 550 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA). The IC50 values representing the concentration of complex required to inhibit 50% of cell proliferation were calculated from the dose-response curves using nonlinear regression [18,20,27,28].

Antibacterial activity

Preliminary screening of (C1) and (C2) complexes against two Gram-positive bacteria species: S. aureus ATCC 6538P, B. cereus ATCC 14579 and against two Gram-negative bacteria species: E. coli ATCC 10536 and P. aeruginosa ATCC 27853 was done using the disk diffusion method [29–32]. As positive control Norfloxacin (Sigma-Aldrich) was used [33]. The reference microbial strains were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

The values of the minimum inhibitory concentration were determined using the serial micro-broth dilution method with Müller-Hinton broth, described by Carson et al. [34]. All tests were duplicated and the MIC values were expressed as mean ± standard deviation.

Statistics

All the experiments were conducted in triplicates and data are displayed as Mean ± SD, representing the average of independent experiments performed in triplicate. The IC50 values representing the complexes concentration required to inhibit 50% of cell proliferation were calculated from the dose response curve using non-linear regression. Statistical values were generated using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

Results and discussions

The chemical structures of the complexes (C1), (C2) and of the ligand (HL) are given in Figure 1. The complexes structures

In complex (C1), CuN4O2 chromophore type, the coordination geometry of the Cu(II) ion is slightly distorted octahedral. The two deprotonated ligands by Nh2 coordinates the metal ion through the sulfonamide moiety. Each ligand coordinates the metal ion through the one Nh2 group. Each ligand contains two nitrogen atoms Nh2 and one phenantrolite in a square-planar environment (C2). The sulfonamide ligand (HL) coordinates the Cu(II) ion upon the deprotonation of the NH2-SO2 moiety. Each ligand coordinates the metal ion through the one Nh2 atom. The phenantrolite’s molecules also participate at the Cu(II) coordination as bidentate ligand by two nitrogen atoms Nh2.
Spectroscopic properties

Comparing the IR spectra of the complexes and of the ligand, the most remarkable modifications appear in the vibrational bands of the thiadiazolic ring, \( \nu(S-O) \) and \( \nu(S-N) \) (in the sulfonamidic group). The band corresponding to the stretching vibrations of the thiadiazolic ring is shifted towards lower frequencies for both complexes: from 1550 cm\(^{-1} \) for \((HL)\) to 1493 cm\(^{-1} \) for \((C_1)\) and from 1490 cm\(^{-1} \) for \((C_2)\). The modifications prove the involvement of the thiadiazolic nitrogen in the coordination of the cooper ion; an electronic transfer between the thiadiazolic and sulfonamidic nitrogen atoms may occur upon deprotonation of the sulfonamidic nitrogen -N(-)-SO\(_2\). The bands attributed to \((S=O_{as})\) and \((S=O_{os})\) in the structure of both complexes, are shifted towards lower frequencies (15-20 cm\(^{-1} \)); these modifications can be related to the deprotonation of the sulfonamidic nitrogen and subsequent conjugation between the atoms of the group (N, S, O). The phenomenon is confirmed by the shifting of the S-N vibrational band toward higher frequencies for both complexes as compared to the ligand (932 cm\(^{-1} \) and 938 cm\(^{-1} \) for the complexes and 915 cm\(^{-1} \) for the ligand). The IR spectra, with all the modifications mentioned above, display a similar pattern to all Cu(II) complexes described in literature where N-substituted sulfonamides act as ligands [35]. The deprotonation of the sulfonamidic nitrogen, and the coordination of the cooper ion through the nitrogen atoms in the heterocyclic ring are characteristic for this type of complexes [35]. The characteristic bands for the pyridine molecule that coordinates the metal ion cannot be identified due to the superposition of these bands with those of the ligand. The spectrum of the \((C)\) complex presents bands at 1125, 1087 and 730 cm\(^{-1} \), which are attributed to the coordinated phenanthroline molecule [36]. In general, the IR spectra are similar to those for the other copper N-sulfonamide compounds [37]. The changes of the IR characteristic bands are mainly due to the sulfonamide deprotonation.

EPR and magnetic properties

The complexes display effective magnetic moments of 1.93 MB \((C_1)\) and 1.83 MB \((C_2)\). This behavior is characteristic for monomers, with no metal-metal interactions (1.72 - 2.20 MB) and indicates the presence of a single unpaired electron in a 3d orbital for the metal ion [39].

The polycrystalline X-band EPR spectrum of the complex \((C_1)\) is axial and for the complex \((C_2)\) is rombic. The parallel region is resolved with hyperfine splitting from cooper. The EPR parameters calculated by simulation are \(g|| = 2.26, g = 2.05\) and \(A|| = 154 \times 10^{-4} \) cm\(^{-1} \) for complex \((C_1)\) and \(g|| = 2.030, g = 2.070, g = 2.140\) and \(A|| = 190 \times 10^{-4} \) cm\(^{-1} \) for complex \((C_2)\) [40]. The calculated parameters suggest that the unpaired electron is in a d\(_{xy}\) or a d\(_{x^2-y^2}\) orbital [41]. According to the Bertini classification, the value of \(A||\) can be correlated with the geometry of the complex [42].

The structures of the complexes \((C_1)\) and \((C_2)\) resulting from the elemental analysis and the spectral data are shown in Figure 1.

DNA cleavage

The ability of the complexes to cleave DNA was studied using supercoiled pUC18 DNA in DMF: cacodylate buffer (0.1 M, pH 6.0) a molar proportion (1:39) in the presence of H\(_2\)O\(_2\)/ascorbic acid, 3.0-fold excess relative to the complex concentration. Control experiments with CuSO\(_4\) were also carried out under the same experimental conditions. The results are presented in Figure 2(a), 2(b).

Complex \((C_1)\) at 18 \(\mu\)M (Figure 2(a), lanes 10) produced a partial conversion of closed circular (NC) conformation DNA into its supercoiled form (SC). At 24 \(\mu\)M, degradation of the supercoiled form to open circular DNA (Figure 2(a), lanes 11) was observed. Under the same conditions, CuSO\(_4\) exhibited less nucleolytic activity than either of the complexes (lanes 4-7). As for the bis(o-phenanthroline)
copper(II) complex, which is a well-known and very efficient chemical nuclease, we found that at a dose of 24 µM, complex (C₁) was less active than [Cu(phen)]²⁺.

It appears that complex (C₂) can destroy DNA in stages, forming the circular form at a concentration of 18 µM and then the linear form at a concentration of 24 µM. At the last concentration, the circular and linear forms coexist (lane 11), while at 18 µM the circular and the helicoidal form coexist (lane 10). Initially, the cleavage is realized at a single point of the DNA chain, leading to the circular form. A second cleavage then occurs at another point of the chain, leading to the linear form.

For complex (C₁) (Figure 2b), the DNA cleavage starts at 6 µM, giving an appreciable amount of plasmid Form II (lane 10). Then, at 9 µM the nicked circular and linear forms appear (lane 11). At higher concentrations (lanes 12 and 13), smearing is observed. CuSO₄, at the highest concentration, 15 µM, does not induce any DNA cleavage. Overall, the activity of complex (C₁) is higher than that of the bis(o-phenanthroline) copper complex (compare line 11 with line 14). As a consequence, complex (C₁) can be defined as a strong nuclease agent in the presence of H₂O₂/ascorbic acid. When the concentrations of complexes (C₁) and (C₂) are increased whilst keeping those of reducing agents and DNA pUC18 plasmid constant, the amount of SC (Form I) decreases whereas that of NC (Form II) increases. If we compare complex (C₂), containing sulfonamide ligands, with complex (C₁), which also incorporates 1,10-phen ligand, we can say that the introduction of the phenantroline ligand increases the nuclease activity of the resulting complex. Phenantroline also enhances the activity of the complex for complete destruction of the DNA molecule (smearing). In the presence of an oxidizing agent like hydrogen peroxide, Cu(II) complexes with phenantroline ligand are known to initiate an oxidative attack on C-1' and C-4' of the 2-deoxyribose moiety, leading to the destruction of DNA [43]. The planar aromatic rings of the sulfonamide ligand may allow the complex molecules to be intercalated between the DNA base pairs. This would be followed by the destruction of the nucleic acid, caused by the production of ROS in its close vicinity [44,45].

The involvement of ROS in the nuclease mechanism was determined by monitoring the quenching of DNA cleavage in the presence of certain ROS scavengers: DMSO, t-butyl alcohol, sodium azide, 2,2,6,6-tetramethyl-4-piperidone, distamycin, superoxide dismutase and neocuproine. We chose a concentration of 24 µM for (C₁) and 12 µM for (C₂) (in view of the difference in nuclease activity for the two complexes). The assays were performed with H₂O₂/ascorbic acid (in 3.0-fold molar excess relative to the complex concentration) as an activating agent and a concentration 3 times higher for the redox reagents ascorbic acid/H₂O₂. Samples were incubated for one hour, at 37°C. The resulting electroferograms are presented in Figure 3.

In the presence of both DMSO and of t-butyl alcohol (lanes 4 and 5), we observed a decrease in degradation of the DNA. This can be explained by the presence of HO· radicals in the degradation of DNA, since these inhibitors act by quenching these free radicals.

The influence of inhibitors on the nuclease activity of the complexes was observed in the fact that sodium azide and 2,2,6,6-tetramethyl-4-piperidone (lanes 6 and 7) do not greatly modify the nuclease activity of the complexes. This indicates that singlet oxygen does not participate in the destruction of the DNA.

By adding distamycin (lane 8), the nuclease activity of the complex is slightly increased. This suggests that the activity is not similar to that of distamycin, which is a binder of the minor groove of DNA.

The SOD enzyme leads to a significant increase of the nuclease activity of the complexes (line 9), manifested by an increase of the amount of linear DNA in this experiment (lane 3). SOD catalyzes the dismutation of the superoxide anion radical O₂⁻, leading to H₂O₂ and O₂, which can further produce active species which participate in the destruction of DNA.

In the presence of neocuproine (lane 10), the capacity of the complexes to destroy DNA is much reduced (the helicoidal and circular form coexist). This can be explained by the reduction of the Cu(II) ion to Cu(I) as an intermediate step in the DNA degradation process. By adding neocuproine, a stable neocuproine complex of Cu(I) is formed, thus inhibiting the subsequent reactions required for DNA degradation.

We can conclude that, for these complexes, the destruction of DNA follows a Fenton or Haber-Weiss reaction mechanism [46,47]. A possible pathway for ROS generation would be:

\[
\text{Cu(II)L + e}^{-} \rightarrow \text{Cu(I)L} \\
\text{Cu(I)L} + \text{O}_2 \rightarrow \text{Cu(I)L} + \text{O}_2^{-} \\
2\text{O}_2^{-} + 2\text{H}^+ \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \\
\text{Cu(II)L} + \text{H}_2\text{O}_2 \rightarrow \text{Cu(II)L} + \text{OH}^- + \text{HO}_2^-. 
\]

**In vitro antioxidant activity**

**DPPH-radical scavenging assay**

DPPH is a well-known radical which demonstrates a strong absorption band centered at 517 nm, and it becomes colorless or pale yellow when it is neutralized. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH, and it is a well-known radical which demonstrates a strong absorption band centered at 517 nm, and it becomes colorless or pale yellow when it is neutralized. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH, and it is commonly used to evaluate the radical scavenging capacity of antioxidants [48,49]. The scavenging ability (%) of the complexes was found 69±0.23, for (C₁) complex and 80±0.34, for (C₂) complex. Comparing the scavenging ability, both complexes are less active than ascorbic acid (97.08±0.52) [50] and are more active than some Cu complexes with flavonoid ligands: [Cu₂(quercetin)(H₂O)₄]Cl₂ (61.83±0.14) and [Cu₃(rutin)₂(H₂O)₄]Cl₂ = 24.70±0.53, values taken from literature [51].

**FRAP method (ferric reducing antioxidant power)**

The measurement of reducing power defines an important aspect of the antioxidant activity of the complexes. FRAP (mgTE/mL) for (C₁) complex was found, 80±0.14 and for (C₂) complex, 112±0.14, results that Fig. 3.Electroferogram in agarose gel of the pUC18 plasmid treated with the (C₁) and (C₂) complexes and various inhibiting agents.
confirm the superior antioxidant capacity of \((C_1)\) complex, fact demonstrated also by DPPH radical scavenging assay.

**In vitro SOD mimetic activity**

The SOD-like activities of \((C_1)\) and \((C_2)\) complexes were tested by an indirect method using the xanthine/xanthine oxidase system as the source of superoxide radicals. Complex \((C_1)\) was more potent than \((C_2)\); the IC\(_{50}\) values were found to be 0.115 µM \((C_1)\) and 0.223 µM \((C_2)\). These values are higher than that for native Cu\(_2\)Zn\(_2\)SOD (0.006 µM) (that means that the complexes are less active than native SOD) but similar to those of complexes with related sulfonamide ligands [18,37,52].

**In vivo SOD mimetic activity**

The in vivo SOD mimetic activity of \((C_1)\) and \((C_2)\) complexes was quantified by a method based on the protection against free radicals provided by the extracts to the yeast S. cerevisiae [23,26,52]. The SOD-mimetic activity of the complexes on cell growth with a \(\Delta sod1\) mutant treated with menadione or H\(_2\)O\(_2\) had been evaluated. The oxidative stress was produced by two oxidative agents: menadione which toxicity is due to the superoxide radical production and H\(_2\)O\(_2\) which toxicity is registered due to HO\(^{–}\) radicals.

It was been considered that the complex has a SOD-mimetic activity if a decrease of the diameter of the inhibition zone is registered versus the control zone. The efficacy was evaluated by comparison of the diameter of the inhibition area for the complex and control (Figure 4).

![Complex C1 and Complex C2](image)

Fig. 4. Effect of \((C_1)\) and \((C_2)\) complexes on the growth of the \(\Delta sod1\) mutant against free radicals produces by H\(_2\)O\(_2\) (disk at the top of each Petri disk) and menadione (disk at the bottom of each Petri disk).

In the presence of \((C_1)\) and \((C_2)\) complexes at 30, 50 and 70µM a significant reduction of the inhibition area was observed when the oxidative stress is produced by both menadione and H\(_2\)O\(_2\). The diameter of the inhibition area for both complexes in different concentrations, using menadione and H\(_2\)O\(_2\), are given in Table 1.

The reduction of the inhibition area is between 25-30% for the complex \((C_1)\) and 41.25-45% for the complex \((C_2)\) against oxidative stress generated by menadione. The protective activities of the complexes do not seem to be dependent by increasing the complexes concentration.

| Complex (µM) | Diameter of the inhibition area (cm) | Complex (µM) | Diameter of the inhibition area (cm) |
|--------------|-------------------------------------|--------------|-------------------------------------|
| Control (Menadione 3 mM; H\(_2\)O\(_2\) 17.5%) | Menadione: 8 H\(_2\)O\(_2\): 7.5 | Menadione: 8 H\(_2\)O\(_2\): 7.5 |
| 30           | 6                                   | 6.5          | 4.7                                 |
| 50           | 5.6                                 | 6.3          | 4.5                                 |
| 70           | 5.6                                 | 6.1          | 4.4                                 |

The protection of the complexes against free radicals generated by H\(_2\)O\(_2\) is lower than in the case of free radicals generated by menadione. \((C_1)\) complex produce a reduction of the inhibition diameter about 18.75-23.75% while \((C_2)\) complex between 30-33.75%. Nor in this case the protective action produced by the complexes does not depend on complexes concentration.

Both complexes are able to protect efficiently against superoxide anions, but \((C_2)\) complex register a higher SOD-mimetic activity compared with \((C_1)\) complex. They could be considered as promising effective agents against toxicity of superoxide anion, improving significantly the growth of \(\Delta sod1\) strain. They supply the Cu-Zn-SOD deficiency of the mutant. These levels of protection are superior to those obtained for other complexes of Cu (II) with sulfonamide ligand cited in the literature [22,53,54]. For this reason both complexes are potential therapeutic agents in the prevention and treatment of diseases mediated by free radicals.

**Cytoxicities of the complexes**

\((C_1)\) and \((C_2)\) complexes were examined for their antiproliferative properties. The in vitro cytotoxicity of \((C_1)\) and \((C_2)\) copper complexes were tested on two human carcinoma cells, a cervical line (HeLa) and a melanoma cell line (WM35); as well as on human normal fibroblastic epithelial cell line (HDFa), using Cisplatin as positive control. The response was quantified using 3-(4,5-dimethyl-2-thiazoloyl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The cells were exposed to various concentrations of each complex (ranging between 0.2 and 50µM) at 24, 48 and 72 hours.

The results revealed a promising profile, the complexes expressing different levels of cytotoxicity. On both tumor cell lines: HeLa and WM35, complex \((C_1)\) was more potent as compared to \((C_2)\) and more potent than Cisplatin, the IC\(_{50}\) values being lower than those of Cisplatin. \((C_1)\) complex evoked also cytotoxicity, but a weaker one. No significant differences were found between IC\(_{50}\) values obtained for the two complexes at 24, 48 and 72 hours (Table 2).

The presence of phenanthroline in \((C_1)\) complex clearly increases the activity of the compound toward DNA cleavage. Due to the greater hydrophobic character of phenanthroline, the binding of \((C_1)\) complex to DNA is expected to be stabilized through greater hydrophobic interactions, which may contribute to its higher toxicity [18].

On human normal fibroblast (HDFa line), \((C_1)\) complex showed a toxicity comparable with Cisplatin, but \((C_2)\) complex showed a lower toxicity than Cisplatin (Table 2).

In this study we demonstrated that \((C_2)\) complex has a superior antitumor activity on both HeLa and WM35 human cell lines comparative with Cisplatin. In addition complex \((C_1)\) is less toxic on human HDFa cells compared to Cisplatin.

| Table 1 | THE DIAMETER OF INHIBITION AREA FOR \((C_1)\) and \((C_2)\) COMPLEXES, IN DIFFERENT CONCENTRATIONS, USING MENADIONE AND H\(_2\)O\(_2\) |
Antibacterial activity

Both (C₁) and (C₂) complexes were found active against the four microorganisms, the MIC values are given in Table 3.

The results reveal that both complexes have similar antibacterial activity against S. aureus, E. coli and P. aeruginosa, but less than Norfloxacin. Against B. cereus both complexes have shown a better antibacterial activity and superior to Norfloxacin activity.

The antibacterial activity may be due to the following mechanisms: inhibition of ribonucleoside diphosphate reductase enzyme which helps in DNA synthesis; by oxidative rupture, creation of lesions in DNA strand or by binding to the nitrogen base of DNA or RNA results in inhibit base replication [55].

Conclusions

Two new Copper (II) complexes with N-substituted sulfonamide were synthesized. The molecular structures for the complexes were attributed using the data obtained from elemental analysis, spectral (IR, UV-Vis, EPR) and magnetic determinations. The complexes have a superior nuclease activity as compared to the non-coordinated Cu(II) ion. The use of scavengers of ROS indicates that the hydroxyl and the superoxide anions are the main radicals that break the DNA strands. Both complexes have antioxidant, SOD mimetic and antibacterial activities. The hydroxyl and superoxide anions are the main radicals that break the DNA strands. Both complexes have shown a better antibacterial activity and superior to Norfloxacin activity.

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