ORIGINAL ARTICLE

DNA CLEAVAGE STUDY AND SOD-MIMETIC ACTIVITY OF A NEW Cu(II) COMPLEX

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Abstract

The DNA cleavage capacity of a new Cu(II) complex with substituted N-sulphonamide ligand, $[Cu(L)_2(phen)(H_2O)]$ (HL = N-(5-ethyl-[1,3,4] thiadiazole-2-yl)-benzenesulphonamide) was studied by analysing the process of damaging the DNA molecule (pUC18 plasmid) in the presence of a reducing agent. Also, we tried to elucidate the reaction mechanism through which the complex damages the DNA molecule by studying the free radicals and ions involved in the degradation of the nucleic acid structure. The use of ROS (reactive oxygen species) scavengers indicates that the hydroxyl radicals and the superoxide radical anions are the main species capable of breaking the DNA strands by an oxidative mechanism. The SOD-like activity of the complex was tested by an indirect method using the xanthine/xanthine oxidase system as source for superoxide radicals. By this way, we demonstrated the SOD mimetic activity of the complex.

Rezumat

A fost studiată capacitatea de clivare a ADN pentru un complex al Cu(II) cu un ligand de tip N-sulfonamidic, $[Cu(L)_2(fen)(H_2O)]$ (HL = N-(5-etil-[1,3,4] tiadiazol-2-il)-benzenesulfonamidă) prin prisma capacității sale de a denatura molecula de ADN (pUC18 plasmid), în prezența unui agent reducător. S-a încercat, de asemenea, elucidarea mecanismului prin care complexul denaturează molecula de ADN, studiind care sunt radicalii liberi și ionii implicați în degradarea acidului nucleic. Utilizarea unor captatori ai ROS (speciilor de oxigen reactive) indică faptul că radicalii hidroxil și anionul radical superoxid sunt principalele specii implicate în ruperea ADN-ului printr-un mecanism oxidativ. A fost testată și activitatea SOD (superoxid dismutază) - mimetică a complexului, utilizând sistemul xantină/xantinoxidază ca sursă de radicali liberi. Pe această cale a fost demonstrată activitatea SOD - mimetică a complexului.

Keywords: Cu(II) complex, DNA cleavage, SOD-mimetic activity

Introduction

Metal ions are important for the course of the living organisms' vital functions where they occur under the form of complex or chelate combinations, as well as for the drugs control and analyses [1, 13, 16, 19, 22, 25].

Copper ions, as part of the active site of various metalloproteins, play an essential role in biological processes like electron transfer, oxidation and dioxygen transport. Among these metalloproteins, superoxide-dismutase (SOD) has the metal ion bound to three imidazole moieties from histidine structures and one imidazolato ion that acts as a bridging ligand between copper and zinc ions. Generally, the SOD-like activity depends on the structural and redox proprieties of the compounds coordination model [3].

The synthesis of Cu(II) complexes with N-sulphonamides ligands greatly increased in the past years and they have been used due to the diversity of the biological activity of the resulting compounds: DNA cleavage and anti-inflammatory, antimicrobial, SOD mimetic, antioxidant, antitumour activity [5, 7, 14].

In this context, we have described the DNA cleavage and antitumor activity of several copper-sulphonamide complexes [10-12]. In the present paper, we report the DNA cleavage capacity and SOD-mimetic activity for a new Cu(II) complex $[Cu(L)_2(phen)(H_2O)]$ (HL = N-(5-ethyl-[1,3,4] thiadiazole-2-yl)-benzenesulphonamide). The synthesis, the physico-chemical characterization and X-ray structure of the complexes were previously performed [13, 14].

Materials and Methods

Reagents and solvents

Complex synthesis: $CuSO_4 \cdot 5H_2O$; phenantroline; methanol

DNA cleavage capacity: TE buffer pH = 8.0 (Tris 10 mM, EDTA 1 mM); TBE buffer 0.5X (0.045 M Tris, 0.045 M boric acid, 1 mM EDTA); sample revealing buffer for (0.25% bromophenol, 0.25% xylenecyanol, 30% glycerol); caodylate buffer 0.1 M (pH = 6.0); agarose; ethidium bromide (10 mg/mL); commercial pUC18 0.25 μg/mL in TE buffer (pH = 8.0); activating agent solution (H_2O_2/ascorbic acid); dimethylsulfoxide 14 M; t-butyl alcohol 10.5 M; sodium azide 400 mM in caodylate buffer; 2,2,6,6-tetramethyl-4-piperidone 0.5 M in caodylate buffer; superoxide dismutase (15 units); distamicin 80 μM in caodylate buffer; neocuproine 36 μM in caodylate buffer.

All reagents and solvents were commercially available (Fluka and Merck) and were used without further purification. pUC18 was purchased from Roche Diagnostics.

The determinations were performed with a SPECORD 200 PLUS Spectrophotometer.

SOD mimetic activity: Tris-HCl buffer 50 mM pH = 7.8; potassium phosphate buffer 50 mM pH = 7.8; xanthine 0.15 mM and xanthine oxidase in potassium phosphate buffer 50 mM pH = 7.8; NBT (nitro blue tetrazolium) 0.056 mM in potassium phosphate buffer 50 mM pH = 7.8.

DNA cleavage capacity

Reactions were performed by mixing 7 μL of cacodylate buffer 0.1 M, pH = 6 (cacodylic acid/sodium cacodylate), 6 μL of complex solution in cacodylate buffer 0.1 M (final concentrations: 6, 9, 12, 15 and 18 μM), 1 μL of pUC18 DNA solution in TE buffer (0.25 μg/μL, 750 μM in base pairs), and 6 μL of activating agent solution (H_2O_2/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 (3 μL) consisting of bromophenol blue (0.25%), xylene cyanol (0.25%) and glycerol (30%) was added. The solution was then subjected to electrophoresis on 0.8% agarose gel in 0.5×TBE buffer (0.045 M Tris, 0.045 M boric acid, and 1 mM EDTA) containing 5 μL/100 mL solution of ethidium bromide (10 mg/mL) at 100 V for 2 h. The bands were photographed on a capturing system (Gelprinter Plus TDI).

The efficiency of the complex was compared with that of both copper sulphate and bis-(o-phenanthroline) Cu(II) complex under the same reactions conditions. The copper sulphate and bis-(o-phenanthroline) Cu(II) complex where considered control substances.

After electrophoresis, the proportion of both the cleaved and un-cleaved DNA in each fraction was quantitatively estimated on the basis of the band intensities using the AlphaEaseFC StandAlone software. The intensities of supercoiled DNA were corrected by a factor of 1.47 as a result of its lower staining capacity by ethidium bromide. The cleavage efficiency was measured by determining the ability of the complexes to convert the supercoiled DNA (SC) to the nicked circular (NC) [2].

In order to test 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, *t*-butyl alcohol (10.5 M), sodium azide (NaN_3) (400 mM), superoxide dismutase (SOD) (15 units). In addition, a chelating agent of copper(I), neocuproine (36 μM), along with the groove binder distamycin (80 μM) were also tested [9, 23].

SOD mimetic activity

The *in vitro* SOD mimetic activity of the complex was performed using the Oberley and Spitz method with some minor modifications [4, 20]. Xanthine 0.15 mM and xanthine oxidase in potassium phosphate buffer 50 mM pH = 7.8 were used to generate a reproducible and constant flux of superoxide anions. Superoxide radical anions generated by this system were detected by the reduction of nitro blue tetrazolium (NBT) 0.056 mM in potassium phosphate buffer 50 mM pH = 7.8, to formazan blue which was scanned spectrophotometrically at 560 nm. Complex solutions of different concentrations (50, 25, 12.5, 2 and 0.2 μM) were prepared in 50 mM Tris-HCl buffer pH = 7.8. For an experiment, 0.1 mL complex solution along with 0.1 mL xanthine oxidase were added to 0.8 mL solution containing 0.69 mL potassium phosphate buffer (pH = 7.8), 0.025 mL NBT and 0.085 mL xanthine. To determine the SOD activity of the complex, the percentage inhibition of NBT reduction was used. The formation of uric acid from xanthine was followed at 310 nm, in order to evaluate if the complex affects the generation of superoxide anions by direct interactions with the enzymatic system. The inhibition percentage of the enzyme activity was subtracted from that of NBT. IC_{50} (the complex concentration required to yield 50% inhibition of NBT reduction) was determined from a plot of percentage inhibition *versus* complex concentration. Statistic value was generated using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

Results and Discussion

The synthesis and physico-chemical characterization of the complex $[Cu(L)_2(phen)(H_2O)]$ have been performed at the Department of Inorganic Chemistry, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. In the complex, the copper(II) is five-coordinated, forming a CuN_4O chromophore. The coordination polyhedron of the copper (II) is square pyramidal. The coordination of the Cu(II) ion also involves a molecule of phenantroline as a bidentate ligand through its two nitrogen atoms, two deprotonated sulphonamide molecules as monodentate ligand, through a nitrogen atom from the thiadiazole heterocycle, and a water molecule through the oxygen atom. The crystal structure was confirmed by the spectroscopic data (IR, UV-Vis, EPR and magnetic determinations). In Figure 1 is represented the molecular structure of the complex (data not published).

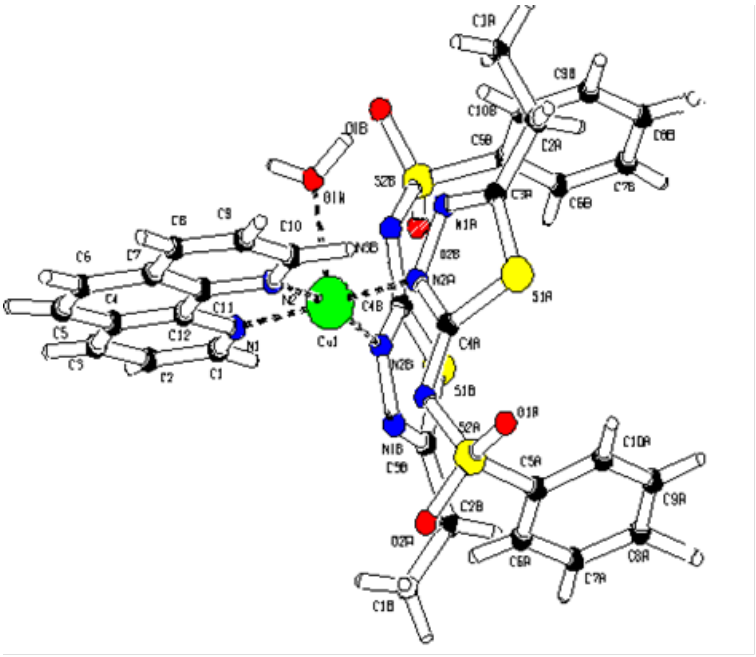

Figure 1.
Molecular structure of the $[Cu(L)_2(phen)(H_2O)]$

Study of the DNA cleavage capacity

The results presented in Figure 2, show that the complex exhibited high DNA cleavage capacity and was more effective at higher concentrations.

The Cu(II) complex manages to denaturate the nucleic acid from its helicoidally form to its circular form even when is used in small concentrations (6 and 9 µM, lanes 8 and 9). At these concentrations, the two forms of DNA coexist. This was proved by the presence of a higher amount of circular DNA and by the presence of the linear form (in a smaller amount at 15 µM) (lanes 10 and 11). At a complex concentration of 18 µM, the electroferogram shows, besides the linear form, a smearing phenomenon, determined by the fact that DNA was completely degraded to numerous fragments with small molecular weight (lane 12). The DNA maintained its helicoidally form when copper salt and reducing agents are added (lanes 3-7).

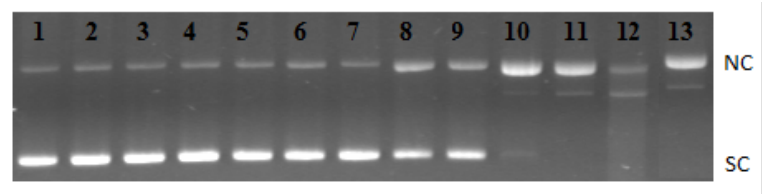

Figure 2.
Electropherogram in agarose gel of the pUC18 plasmid treated with the newly synthesised Cu(II) complex
1. control; 2. control with reducing agents; 3. $CuSO_4 \cdot 5H_2O$, 6 µM; 4. $CuSO_4 \cdot 5H_2O$, 9 µM; 5. $CuSO_4 \cdot 5H_2O$, 12 µM; 6. $CuSO_4 \cdot 5H_2O$, 15 µM; 7. $CuSO_4 \cdot 5H_2O$, 18 µM; 8. complex 6 µM; 9. complex 9 µM; 10. complex 12 µM; 11. complex 15 µM; 12. complex 18 µM; 13. $[Cu(phen)_2]^{2+}$ 6 µM + reducing agents

The last lane represents the DNA cleavage capacity of the $[Cu(phen)_2]^{2+}$ complex at a concentration of 6 μM, analysed by electrophoresis, in order to compare the DNA degradation capacity of the Cu(II) complex and $[Cu(phen)_2]^{2+}$. At 6 μM, we can see that the $[Cu(phen)_2]^{2+}$complex, known and consecrated as the first "chemical nuclease", has a greater DNA cleavage capacity than the tested complex.

The DNA cleavage capacity increases with the concentration of the complex. In Table I is shown the concentration-dependent cleavage data for SC pUC18 DNA by the complex in the presence of redox reagents (H_2O_2/ascorbic acid) in cacodylate buffer 0.1 M, pH = 6 at 37°C.

Table I

Concentration-dependent cleavage data for SC pUC18 DNA by the complex in the presence of redox reagents

Lane	Reaction condition	Form %	
		SC	NC
1	control	98.7	1.3
2	control + redox reagents	97.2	2.8
8	control + redox reagents + complex(6 μM)	55.3	44.7
9	control + redox reagents + complex(9 μM)	50,8	49.2
10	control + redox reagents + complex(12 μM)	7.5	92.5
11	control + redox reagents + complex(15 μM)	0.7	99.3
12	control + redox reagents + complex(18 μM)	Smearing	Smearing

SC = supercoiled forms NC = circular form

When the concentration of the complex is increased, while keeping those of reducing agents and DNA pUC18 plasmid constant, the amount of SC (supercoiled form) decreases, whereas that of NC (circular form) increases.

The presence of the phenantroline molecule in the structure of the Cu(II) complex increases the DNA cleavage capacity, and also contributes to the decrease of the complex concentrations at which it shows its DNA cleavage capacity to the complete degradation of the DNA molecule (smearing) [21].

The planar aromatic rings of the sulphonamide 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 [6, 18].

In the presence of an oxidizing agent like hydrogen peroxide, Cu(II) complexes with phenanthroline ligands are known to initiate an oxidative attack on C-1' and C-4' of the 2- deoxyribose moiety, leading to the destruction of DNA [10].

The influence of Potential Inhibiting Agents

The DNA cleavage capacity of the Cu(II) complex was studied in the presence of certain inhibiting agents (DMSO, t-butyl alcohol, sodium azide, 2,2,6,6-tetramethyl-4-piperidone, distamycin, SOD, neocuproine) trying to determine the reactive oxygen species involved in the degradation process of the DNA molecule. Thus, we studied the DNA cleavage capacity of the complex in the presence of inhibiting agents that can capture ROS (ex. $HO\cdot$, $O_2^{\cdot -}$, $^1O_2$, etc.) from their medium. For this purpose, we used the same protocol as the one presented above, the only difference being that it was considered a single complex concentration (the one with a significant cleavage capacity). In each sample with this concentration we added a different inhibiting agent of the DNA cleavage capacity of the copper complex. The resulting electropherogram is presented in Figure 3.

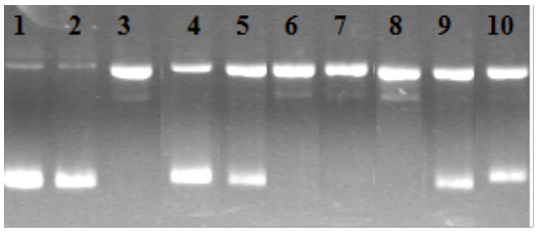

Figure 3.
Electropherogram in agarose gel of the pUC18 plasmid treated with the Cu(II) complex and various inhibiting agents

1. control; 2. control with reducing agents; 3. complex 15 μM without inhibitors; 4. complex 15 μM + DMSO; 5. complex 15 μM + t-butyl alcohol; 6. complex 15 μM + NaN_3; 7. complex 15 μM + piperidone; 8. complex 15 μM + distamycin; 9. complex 15 μM + SOD; 10. complex 15 μM + neocuproine

It was chosen a concentration of 15 μM for the complex and a concentration 3 times higher for the reducing agents ascorbic acid/H_2O_2. At this value, the DNA structure is broken to its circular form, obtaining also a small amount of the linear form (lane 3). DMSO and t-butyl alcohol (lanes 4 and 5) determined a decrease of the DNA cleavage capacity of the complex (helicoidal DNA coexists with circular DNA). This proves the contribution of the $HO\cdot$ radicals to the degradation of DNA [8]. Sodium azide and 2,2,6,6-tetramethyl-4-piperidone does not greatly influence the capability of the studied complex to denaturate the DNA molecule (lanes 6 and 7). It seems that the singlet oxygen $^1O_2$ does not participate as reactive species in the destruction of nucleic acid [17]. The capacity of

distamycin (as a binder of minor grove of DNA) to compete with the complex molecule is quite small, as the DNA cleavage capacity of the complex is not diminished (lane 8 as compared to lane 3). It seems that the interaction between the Cu(II) complex and the nucleic acid molecule is different than in the case of distamicin [10]. The presence of SOD determines a decrease of the DNA cleavage capacity of the complex (lane 9). This proves that the superoxide radical ions $O_2^{\cdot -}$ participate as reactive oxygen species to the destruction of the nucleic acid molecule [24]. The presence of neocuproine determines a decrease of the DNA cleavage capacity of the complex (lane 10), as it forms a more stable combination with the Cu(I) ion produced in a reaction step of the mechanism. As the Cu(I) ion is blocked, it cannot participate to future reactions generating active radicals which can denaturate DNA [15].

The reaction mechanism we propose for the Cu(II) complex comprises of the following steps: reduction of the Cu(II) ion to Cu(I) within the complex molecule, interaction of the ligands (phenantroline and deprotonated sulphonamide L) with the nitrogenous bases of the DNA molecule through π-stacking bonds and/or through hydrogen bonds and Fenton or Haber-Weiss reactions, producing active radicals (OH· and $O_2^{\cdot -}$), which will break the helicoidal chains of DNA in a single point, leading to the circular form, or in two points of the same chain, leading to the linear form. A possible pathway for ROS generation would be [9, 23]:

$$Cu(II)L + e^- \rightarrow Cu(I)L$$

$$Cu(I)L + O_2 \rightarrow Cu(II)L + O_2^{\cdot -}$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

$$Cu(I)L + H_2O_2 \rightarrow Cu(II)L + OH^- + HO\cdot$$

In vitro evaluation of SOD mimetic activity

The SOD-like activity of the complex was tested by an indirect method using the xanthine/xanthine oxidase system as the source for superoxide radicals. The complex possesses a SOD mimetic activity and the results of IC_{50} value is presented in Figure 4 Table II. This value is higher than the one found for native Cu_2Zn_2SOD (see Table II) but similar to those of complexes with related sulphonamide ligands [3, 4].

Table II

The IC_{50} values of the complex and of the native SOD

Complex	IC_{50} (μM)
C = $[Cu(L)_2(phen)(H_2O)]$	0.120
Cu_2Zn_2SOD	0.006

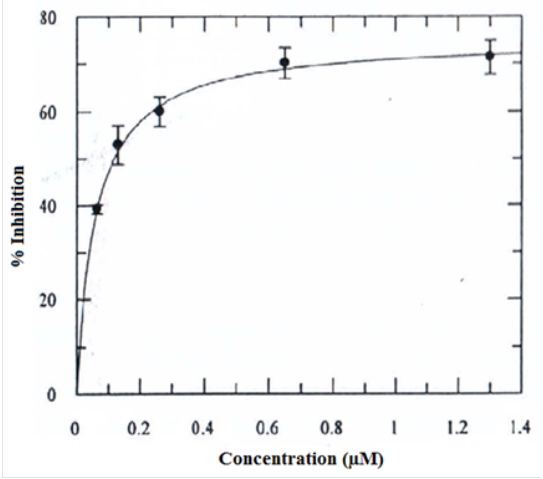

Figure 4.
SOD mimetic activity of the complex at different concentrations

Conclusions

The current study clearly suggests that the Cu(II) complex has a superior DNA cleavage capacity as compared to the non-coordinated Cu(II) ion. The use of ROS scavengers indicates that the hydroxyl radicals and the superoxide radical anions are the main species that break the DNA strands. The complex is able to protect efficiently against superoxide anions and it could be considered a therapeutic agent in the prevention and treatment of diseases mediated by free radicals. Our future projects concern the antitumour activity using different cell lines and also *in vivo* studies.

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