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Macrocyclic Copper (II) Complexes: Superoxide Scavenging Activity, Structural Studies and Cytotoxicity Evaluation

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Key words
Superoxide scavenging activity; Macrocycles; Copper (II) complexes; Superoxide dismutase; Antioxidant; Cytotoxicity evaluation

Abstract
Synthetic superoxide dismutase mimetics have emerged as a potential noval class of drug for the treatment of oxidative stress related to diseases. Among these agents, metal complexes with macrocyclic ligands constitute an important group. In this work we synthesized six macrocyclic copper (II) complexes and evaluated their ability to scavenge the super oxide anions generated by the xanthine-xanthine oxidase system. Two different endpoints were used, the nitro blue tetrazolium (NBT) reduction assay (colorimetric method) and the dihydroethidium (DHE) oxidation assay (fluorimetric method). IC_{50} values in the low micromolor range were found in 5 out of six macrocyclic complexes studied, demonstrating their effective ability to scavenge the superoxide anion. The IC_{50} values obtained with the NBT assay for the macrocyclic copper (II) complexes, were consistently higher, approximately three fold, than those obtained with the DHE assay. Spectroscopic and electrochemical studies were performed in order to correlate the structure features of the complexes with their superoxide scavenger activity. Cytotoxicity assays were also performed using the MTT method in MCF-7 breast cell lines and we found that the complexes, in the range of concentrations tested in the superoxide scavenging assays were not considerably toxic. In summary, some of the present macrocyclic copper (II) complexes, especially those with a high stability constant and low IC_{50} appear to be promising superoxide scavenger agents, and should be considered for further biological assays.

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INTRODUCTION

Reactive oxygen species (ROS) are implicated in several human pathological processes including tissue injury, inflammation, ageing, cancer, cardiovascular, pulmonary and neurodegenerative diseases.[1] Superoxide anion ($O_2^{\cdot-}$) may cause several harmful effects, leading to tissue injury and inflammation.[2,3] By catalyzing the conversion of $O_2^{\cdot-}$ to H_2O_2 and O_2, superoxide dismutases (SOD) represent the first line of defence against $O_2^{\cdot-}$ Preclinical studies have revealed that SOD enzymes play a protective effect in animal models of several diseases.[3] MnSOD has shown to suppress cancer phenotypes in a large number of cancer models.[4] However, the therapeutic use of the native SOD has several limitations related with low cell permeability and short half-life. In addition, bovine CuZn- SOD was tested in clinical trials but immunological problems lead to its withdrawal from the market.[5] To overcome this problem, synthetic SOD mimetic compounds have emerged as a potential novel class of drugs. Transition metal complexes [e.g. complexes of Mn(II), Mn(III), Cu(II) and Fe(III)] have notably shown important antioxidant properties, namely SOD mimetic activity.[6,7] The metal containing SOD mimetic agents more extensively studied are manganese (III) metalloporphyrins, manganese (III) salen complexes and Manganese(II) macrocyclic complexes.[1] Copper is an essential element involved in several biological functions, acting as a catalytic component of many metalloenzymes, including SOD. Therefore, copper(II) complexes can enclose a SOD mimetic activity hindering increased levels of reactive oxygen species. Biological effects of the SOD mimics are related to their structures. High stability constants are required to avoid the dissociation of the complex *in vivo*. This could be achieved by using macrocyclic ligands.[6] The macrocyclic nature of the ligand seems important for the SOD mimetic activity of the corresponding Complexes as well as for their stability in the presence of proteins, even if the metal ion does not lie inside the cavity.[8] Several macrocyclic copper complexes have been reported to scavenge the superoxide anion.[7-11] Previous studies have demonstrated that chemical modifications in the ring size, donor atoms and substituents on the macrocycles, may have profound effects both on the stability and the SOD-like activities of the respective complexes.[10,11] A copper(II) complex which possesses SOD mimetic activity should have a flexible arrangement of the ligands around the copper(II) ion in order to allow an easy reduction to copper(I). In addition, a copper(II) SOD mimetic complex should enclose a certain stability, avoiding thus dissociation in the acid region and should possess an accessible site in order to easily bind the $O_2^{\cdot-}$ radical and, hence to give a quick reduction to copper(I). Finally, an equatorial field of medium strength is required because strong ones do not favour the attack of $O_2^{\cdot-}$ to the accessible apical sites.[12]

In the present work, we describe six low molecular weight copper (II) macrocyclic complexes, focusing on their possible application as superoxide scavenging agents. The ability of those copper (II) complexes to scavenge $O_2^{\cdot-}$ was evaluated by two different methods: the nitro blue tetrazolium (NBT) and the dihydroethidium (DHE). Spectroscopic studies were performed in order to correlate the structural features of the complexes with their scavenging activity. In addition, the cytotoxicity of these complexes was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-2H-tetrazolium bromide (MTT) assay in MCF - 7 cell line, a widely used and non-tumoral cell line.

EXPERIMENTAL

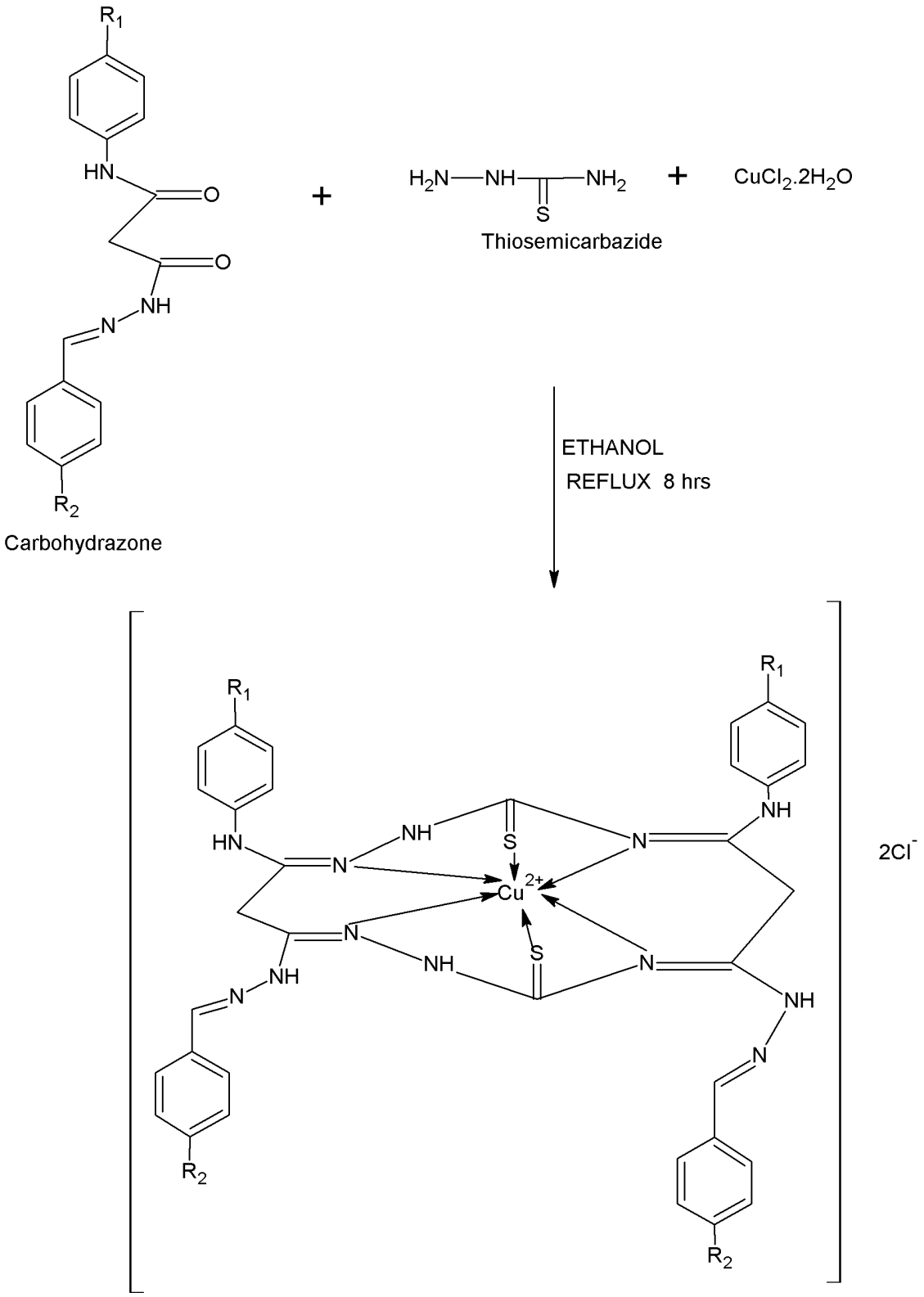
Materials

All the chemicals used in the study are of analytical grade unless reported. The organic chemicals viz. p-methoxy benzaldehyde, p-chloro benzaldehyde, p-nitro benzaldehyde etc. were purchased from merck pvt. Ltd. Thiosemicarbazide was obtained from SD Fine chemicals, Mumbai (India); copper (II) chloride and other organic solvents were purchased from the commercial sources and are used without further purification.

Synthetic Procedure

A series of three macrocyclic Cu (II) complexes were synthesized by following procedure previously described. To a mixture of the appropriate hydrated copper chloride (1 mmol) in absolute ethanol (10 mL) and Carbohydrazone (2 mmol) in absolute ethanol (20 mL) and thiosemicarbazide (2 mmol) in absolute ethanol (15 mL) was added slowly with stirring. After the addition of thiosemicarbazide, the reaction was carried out for 8 h under reflux. The solvent was evaporated under reduced pressure and the

residue obtained was quenched with ethanol. Precipitate was filtered off, washed with ether and dried *in vaccuo* (Scheme 1).



Where R_1 = alkyl group and R_2 = - Cl, -NO_3 and -OCH_3 groups

Scheme 1. Synthesis of macrocyclic copper (II) complexes

Superoxide Scavenging Activity

Superoxide scavenging activity of the complexes was studied by using their ability to scavenge $O_2^{\cdot-}$ generated by the xanthine–xanthine oxidase (X–XO) system, through two different endpoints: the reduction of NBT and the oxidation of DHE. Copper(II) and CuZnSOD from human erythrocytes were used as controls. The evaluation of the inhibition of XO by the complexes was performed following the production of uric acid.

NBT Assay

In this assay, while $O_2^{\cdot-}$ is generated, NBT is reduced, developing a blue formazan colour which is associated with an increase in the absorbance at 560 nm . When a scavenger compound is added, it competes with the NBT for the oxidation of the generated superoxide anions. Therefore, there is a decrease in the rate of the NBT reduction, which leads to lower absorbance increases. The more effective the compound, the lower the concentration which inhibits the NBT reduction in 50% (IC_{50}).[8]

DHE Assay

In the DHE assay, dihydroethidium is oxidized by $O_2^{\cdot-}$ giving a fluorescent compound. The fluorescence emission is related to the amount of superoxide anion present in the system. This assay was performed in 96-well microplates. Each well (200 ll) contained 0.2 mM of xanthine and phosphate buffer 0.1 M, pH 7.8. The tested compounds, diluted in phosphate buffer pH 7.8, were added to the reaction mixture (10 ll). Different concentrations up to 80 lM were tested for each compound (CuL1–CuL4). In what concerns CuL5, since it revealed no activity using the NBT assay, we only tested the highest concentration (80 lM).

Xanthine Oxidase Inhibition Assay

We also evaluated if the generating system X–XO could be inhibited by the copper (II) complexes in study. This was performed by following at 293 nm, during 5 min, the uric acid produced after xanthine was oxidized by XO in aerobic conditions concomitantly to the production of $O_2^{\cdot-}$. The assay was performed for each complex (80 lM) at the same experimental conditions described above, with the exception of the NBT solution, which was replaced for equal volume of phosphate buffer. Caffeic acid (50 lM) was used as positive control. Each experiment was performed in duplicate.

Structural Studies

Thin layer Chromatography was performed using silica gel 60 F_{254} plates with detecting agents iodine vapors spraying with 5% Sulphuric acid in ethanol followed by heating at 100°C, Tetra methyl silane (0.0 ppm) was used as an internal standard in ^{1}HNMR and $CDCl_3$ (77.0 ppm) was used in ^{13}CNMR. The abbreviations used to indicate the peak multiplicity were; S, singlet; bs, broad singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; Hz, Hertz. FAB MS was recorded on Jeol (Japan) / SX-102. Infrared spectrum was taken with KBr on Perkin – Elmer RX-1. Melting points were determined on a Buchi 535 digital melting point apparatus and were uncorrected. Elemental analysis was performed on a Perkin –Elmer 2400 C, H, N analyzer and values were within ± 0.5% of the calculated values. Anhydrous sodium sulphate (Na_2SO_4) was used as drying agent for the organic phases containing the compounds. Unless otherwise stated. All materials were obtained from commercial suppliers, sigma Aldrich Company, Lancaster, SRL and were used without further purification.

Cell Survival Evaluation (MTT Assay)

Cell viability was assessed by the MTT staining method. *In vitro* cytotoxicity assays on cultured human tumor cell line still represent the standard method for the initial screening of antitumor agents. Thus as a first step to assess their pharmacological properties, the synthesized copper (II) complexes were assayed against the human breast tumor MCF-7 cell line. The cells were routinely maintained at 37°C in a humidified 5% CO_2 atmosphere with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After reaching confluence, the cells were detached by tripsinization and counted. For the cytotoxicity assay, 4×10^4 cells well^{-1} were seeded in 100 µL of complete medium in 96-well plates (corning Costar). The plates were incubated at 37°C in 5% CO_2 for 24 h to allow cell adhesion prior to drug testing. The complex was dissolved in sterile dimethylsulfoxide (DMSO, stock solution with maximum concentration of 20 mmol L^{-1}) and dilute to 0.5 µM, 1 µM, 2 µM, 5 µM, 10 µM and 15 µM.

Two microliters of each complex sample were added to 100 μL medium (without FBS). In the control experiments, cells were grown in the same media without the compounds. Relative cell viability was evaluated by measuring the optical density at 570 nm on microplate reader (Quant Bio-tek Instruments, Inc.). At 20 μM concentration of the synthesized compounds, three different experiments were carried in triplicates and reported as the cell cytotoxicity for each compound.

RESULTS AND DISCUSSION

Macrocycles and their metal complexes have been suggested as promising agents for the diagnosis and treatment of different diseases. In addition, some macrocyclic complexes have been suggested as a potential class of SOD mimics, mainly because of their high thermodynamic stability.[6,8] Most of the catalytic antioxidants have a redox-active metal centre.[1,6], but only a few metal ions have the ability to catalyze the dismutation of O_2^{-} to hydrogen peroxide and oxygen. It is well known that copper(II) aqueous ion is a very potent superoxide scavenger.[6] The low IC_{50} values found using $Cu(NO_3)_2$ in this work are consistent with the values found for other Cu(II) salts[8] and show the efficacy of Cu(II) in the disproportionation of O_2^{-} In view of this, copper(II) must be enclosed in a stable ligand, which protects it from being chelated by serum and cellular components. Additionally, this ligand must allow copper(II) to switch its redox state and dismutate the O_2^{-}. It has been described that if the ligand is a macrocycle, the metal complex may have higher biological stability.[6,8] In this study, we present four macrocyclic copper(II) complexes possessing superoxide scavenging activity with IC50 in the low lM range. The superoxide scavenging effect of the macrocyclic copper(II) complexes determined by both the NBT and DHE methods. A very good correlation between the NBT and DHE assays was found (r = 0.979). The IC50 values obtained for the macrocyclic copper(II) complexes using the NBT assay, were consistently higher, approximately threefold, than those obtained with the DHE assay. The aforementioned differences in the sensitiveness and specificity between both methods may somehow explain these results. However, other authors have pointed out that DHE could enhance the rate of superoxide dismutation.

All macrocyclic copper (II) complexes were evaluated for their effectiveness against the breast tumor cell line MCF-7, for comparison purpose, the cytotoxicity of cisplatin was evaluated under the same experimental conditions. The values of cell viability were calculated after the tested compounds were incubated for 48 h. The IC_{50} values, calculated from the close survival curves from MTT assay (Fig 1). Comparing only the values of IC_{50} of all macrocyclic copper (II) complexes, the order of cytotoxic activity is increased by the presence of bulky groups bonded to $N_{(4)}$ of the thiosemicarbazone macrocyclic ligand. The good values of activity found for these complexes, around 5.0 μmol^{-1}, show that the complexation of macrocyclic thiosemicarbazone to Cu(II) may be a good strategy to obtain antitumor agents . The similarity of the values of IC_{50} found for the Cu(II) complexes is an evidence in favour of the same biochemical action mechanism but the different from those of the cisplatin inactive in this case. In fact, the literature reports that Cu (II) complexes of macrocyclic thiosemicarbazone derivatives are able to bind to DNA in-vitro and present enhanced capacity to form interstrand crosslinks when compared to cis-platin . All copper (II) complexes could present antitumor effect by inhibiting DNA synthesis through the blockage of the enzyme ribonucleoside diphospho reductase (RDR), which catalyses the conversion of ribonucleotides into deoxyribonucleotides, as proposed for other α (N) – heterocyclic thiosemicarbazone.

CONCLUSION

Different radiolabelled nano- and microcarriers have been elaborated for passive or active targeting of tumors with promising results in spite of the fact that not all these targeting mechanisms are actually clearly understood. Many of these carriers have been successfully applied in both preclinical and clinical studies showing a great conceivability to improve the quality of tumor detection and to enhance the therapy outcome in terms of tumor-selective radiation delivery. However, different major points will have to be addressed before moving forward to a full exploitation of their potential in clinical use. These points include, among others, the radiochemical stability and radioisotopes leakage, especially in liposomes and micelles, which induce severe toxicities and imply the necessity to find better chelating agents with higher affinity. Another concern is the practically limited control over isotope release profiles, synthesis methods, carrier size and size distribution and both the number and localization of functionalized branches in water soluble polymers. The carrier design and targeting strategies would vary according to

the type, location of tumor and the administration methods (local or systemic). Novel nano- and microcarriers design with high specificity towards defined tumors, improved pharmacokinetic data and simpler highly effective radiolabelling methods will constitute decisive steps for potential clinical applications in the battle against cancer. Furthermore, the combination of the more recent imaging multimodalities and the use of these specific tumor-targeting carriers can significantly contribute to the improvement of both cancer imaging and radiotherapy.

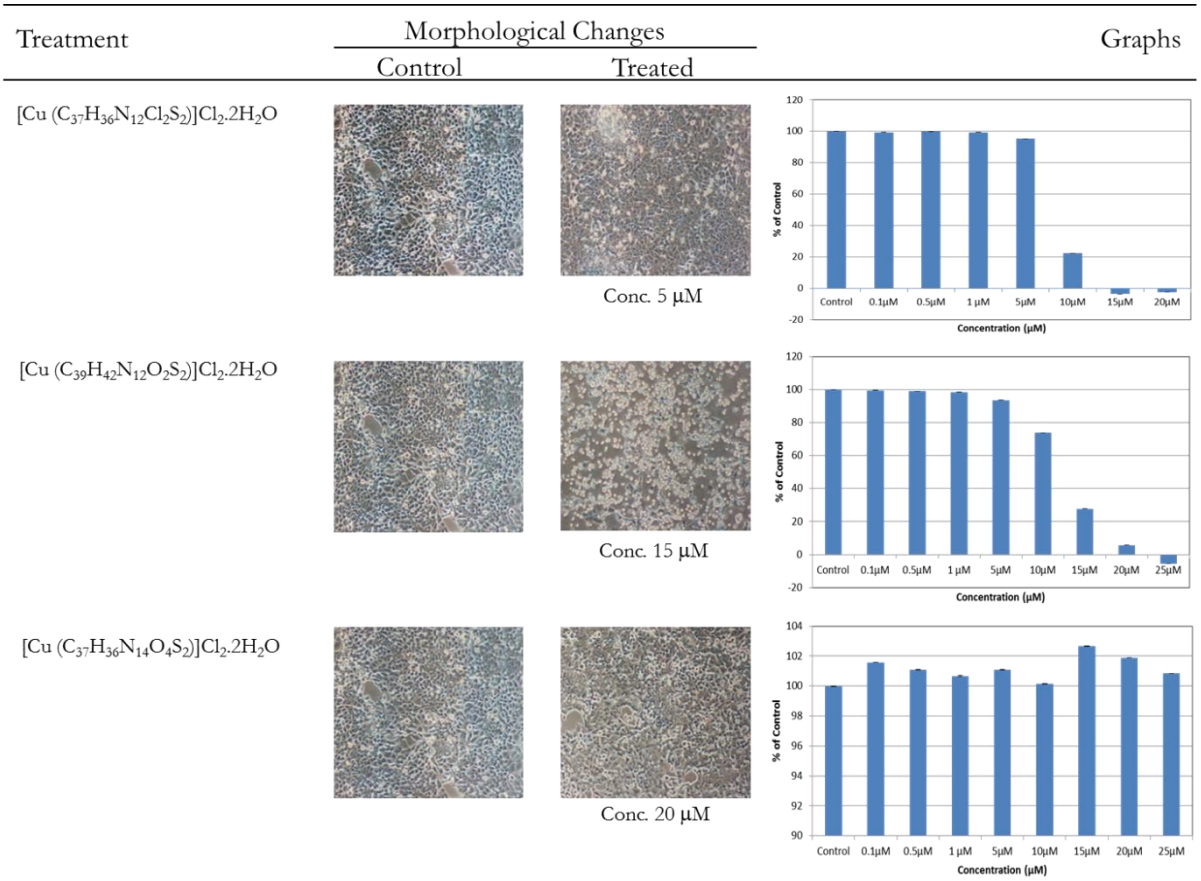


Fig 1. Cells treated with different concentrations of 1-3 complexes for 24 hrs (morphological changes indicate that complexes induced cell death is mostly via apoptosis)

DECLARATION OF INTEREST

It is hereby declared that this paper does not have any conflict of interest.

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