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THE SUPEROXIDE DISMUTASE-LIKE ACTIVITY OF SOME COPPER (II) COMPLEXES DERIVED FROM TRIDENTATE SCHIFF BASES

ĽUDOVÍT BERGENDI[‡], JURAJ KRÄTSMÁR-ŠMOGROVIČ[§], ZDENA ĎURAČKOVÁ[‡], and INGRID ŽITŇANOVA[†]

[‡] *Department of Chemistry and Biochemistry Faculty of Medicine*
[§] *Department of Inorganic and Organic Chemistry, Pharmaceutical Faculty, Comenius University, Bratislava, Czechoslovakia*

Oxygen free radicals are the final or intermediate products of many metabolic reactions. Of greatest significance to the organism are superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$) etc. A proper ratio between both production and breakdown of oxy-radicals is essential for the maintenance of a dynamic equilibrium of vital processes. The superoxide dismutases protect cells against toxic influence of the superoxide. In addition, some square-pyramidally pentacoordinated copper(II) complexes, derived from tridentate Schiff bases of the N-salicylideneaminoalcanoate type, show remarkable SOD-like activity. A selected set of complexes of this type have been tested: potassium [aqua-(N-salicylideneglutamato) cuprate] (L- and D,L-form), potassium [(isothiocyanato)-(N-salicylideneglycinato) cuprate], potassium [(isothiocyanato)-(N-salicylidene-D,L-alaninato) cuprate], potassium [(isothiocyanato)-(N-salicylidene-β-alaninato) cuprate] and potassium [(isocyanato)-(N-salicylideneglycinato) cuprate]. Our results suggest that the copper complexes are not only antioxidants, but may also possess anti-inflammatory, cytostatic and radioprotective properties.

KEY WORDS: Leukocyte, superoxide dismutase-like activity [(N-salicylideneaminoalcanoato) cuprates], xanthine oxidase, superoxide anion radical.

INTRODUCTION

Superoxide anion radical ($O_2^{\cdot-}$) is generated by aerobic cells during several enzymatic and nonenzymatic reactions.[1] The biological fate of $O_2^{\cdot-}$ and other free radicals[2] and their potentially deleterious effects on cell homeostasis are mediated by transition metals like copper, iron, manganese, etc. The reactions of these metals with $O_2^{\cdot-}$ are very complex and may result in two distinct, opposite processes.

The first process is brought about by Cu^{2+}, Mn^{3+}, or Fe^{3+} at the active site of various superoxide dismutases (SODs) which catalyze the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2.[3] As a common feature, the catalytic cycle of SOD consists of the reduction–oxidation of the metal cation with $O_2^{\cdot-}$.[4]

$$Me^{n+1} + O_2^{\cdot-} \rightarrow Me^{n} + O_2 \quad (1)$$

$$Me^{n} + O_2^{\cdot-} + 2H^+ \rightarrow Me^{n+1} + H_2O_2 \quad (2)$$

Other enzymes, mainly GSH peroxidase and catalase, reduce H_2O_2 to water or water

Correspondence: Professor Dipl. Ing. Ľudovit Bergendi, Department of Chemistry and Biochemistry, Faculty of Medicine, Comenius University, Sasinkova 2, 811 08 Bratislava, Czechoslovakia.

and molecular oxygen respectively and thus complete the detoxification chain initiated by SOD.[5]

The second process – enhancement of O_2^- toxicity – is thought to occur via the so-called iron-catalyzed Haber-Weiss[6] reaction. According to the proposed scheme, redox active metal in its high-valence state is first reduced by O_2^- and then reoxidized by H_2O_2.[1]

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad (3)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (5)$$

The reductive cleavage of H_2O_2 by Fe^{2+}, also referred to as Fenton's reaction[7] generates the $\cdot OH$ radical, which is more reactive than O_2^- and may attack a multitude of biomolecules, including proteins, nucleic acids, unsaturated lipids and carbohydrates.[8]

The excessive generation of O_2^- has been implicated in the pathogenesis of metabolic degenerative and inflammatory diseases. Thus, it is not surprising that the pharmacological administration of SOD has been proposed as a novel therapy for these diseases.[9]

The finding that the oxygen-tolerant, SOD-deficient Lactobacillus plantarum accumulates Mn^{2+} is extremely important.[10] The physiological significance of this phenomenon is to provide the cell with a pool of Mn^{2+} complexes that scavenge O_2^- and allows the microorganism to survive in oxygen-saturated environments even in the absence of "SOD". The characterization of these metallocomplexes would provide us with a physiological model for successful pharmacological approach to oxygen-mediated diseases. In the present work we have investigated the ability of some water soluble [(N-salicylideneaminoalcanoato) cuprates] of the composition: $K[Cu^{II}(TSBG)(H_2O)_2]$ or $K[Cu^{II}(TSBA)X]$, where TSBG = trianion of N-salicylideneglutamic acid (L-, or D,L-forms), TSBA = dianion of N-salicylideneaminoalcanoic acid, derived from glycine, D,L-α-alanine, or β-alanine and $X = NCS^-$ or NCO^- ligands.

The investigated Cu^{II} complexes exhibit a square-pyramidal structure. The base of their coordination polyhedra is formed by donor atoms of the tridentate Schiff base anion ligands and by an additional monodentate ligand (H_2O, or X). The apex of the pyramid is occupied by a donor atom from the neighbouring molecule in the crystal structure or by H_2O molecule.[11,12]

We have found that the *in vitro* tested Cu(II) complexes catalyze the dismutation of O_2^- at a different rate that corresponds on a molar basis to 1% of the activity of natural enzyme Cu/Zn-SOD. This activity determined in test systems by physiological conditions (pH 7–7.8) is higher than such activity of most simple Cu(II) complexes of different types.[13–16]

Using leukocytes[17] and an *in vitro* xanthine-xanthine oxidase model system we have also found that the apparently weak SOD-like activity of copper(II) complexes could play an important role in protecting a biological target from O_2^--dependent toxic reaction.

MATERIALS AND METHODS

Chemicals

Horse heart type VI cytochrome *c* were purchased from Sigma Chemical Co (St. Louis, USA); Xanthine-Koch Light Lab. Ltd. England; Dextran obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); Zymosan-LIKO (Trenčín, Czechoslovakia); The other chemicals used were obtained from LACHEMA (Brno, Czechoslovakia).

The tested Cu(II) complexes
K[Cu(sal-L-glu)(H_2O)], signed (*C1*); K[Cu(sal-D,L-glu)(H_2O)], (*C2*); [where sal-L-, or sal-D,L-glu = L, or D,L-form of the (N-salicylideneglutamato)$^{3-}$ anion ligand]; K[Cu(salgly)(NCS)]. 2 H_2O, (*C3*); K[Cu(sal-D,L-ala)(NCS)], (*C4*); K[Cu(sal-β-ala)(NCS)], (*C5*) and K[Cu(salgly)(NCO)], (*C6*); [Where salgly = (N-salicylideneglycinato)]$^{2-}$, sal-D,L-ala = (N-salicylidene-D,L-α-alaninato)$^{2-}$ and sal-β-ala = (N-salicylidene-β-alaninato)$^{2-}$ anion ligands, were prepared according to refs.[18,19] All the complexes prepared are green or blue-green in colour and well soluble in water.

Enzymes

Xanthine oxidase (EC 1.1.3.22) (BOEHRING, Mannheim, FRG); Copper- and Zinc-containing SOD (EC 1.15.1.1) were purchased from Sigma Chemical Co (St. Louis, USA).

Isolation of Leukocytes

PMN leukocytes were isolated from human blood. Leukocytes were obtained by the modified method according to reference.[20]

Opsonization of Zymosan

A human serum was used, and zymosan particles were opsonized (7.5 mg/2 ml and 1 mg contained 8.10^7 particles at zymosan), diluted with PBS in a ratio of 3:7. The mixture was shaken 30 min in a water bath at 37°C. The opsonized zymosan was centrifuged.

The pellet was washed three times with PBS and resuspended in the known volume (2 ml) of HGPBS.[21]

Assay for Leukocyte Dependent Superoxide Formation

Leukocytes-induced phagocytosis of zymosan-dependent, O_2^- mediated reduction of cytochrome *c* was measured spectrophotometrically following the increase in absorbance at 550 nm.[22] This spectrophotometric assay, like the others presented in this paper, was carried out in an OPTON spectro-photometer PM-2 DL (GFR) using 1 cm pathlength cuvettes. Reaction mixtures (1 ml) contained 25 μM cytochrome *c*, 150 U SOD or without SOD; or 0.05 mg copper(II) complexes (C1–C6), or without Cu(II) complexes, 3.10^6 leukocytes. The reactions were started by the addition of zymosan and the cuvette chamber was maintained at 37°C. After the incubation period (15 min) the reaction mixture was placed into ice-cold water (4°C). Both parallel test tubes were centrifuged at 4°C for 5 min. The amount of O_2^- is expressed

as the difference in absorbances as found in parallel experiments with SOD or Cu(II) complexes and without SOD or Cu(II) complexes.

Assay for Superoxide Dismutase Activity

Xanthine–Xanthine oxidase-dependent, O_2^--mediated reduction of cytochrome *c* was measured as the increase in absorbance at 550 nm.[3] Reaction mixtures (1 ml) contained 5.10^{-5} M xanthine, 10^{-5} M cytochrome *c*, 0.015 U xanthine oxidase and 1 U SOD, or different weights of copper(II) complexes (*C1–C6*). The reaction was started by the addition of xanthine oxidase and the cuvette chamber was maintained at 22°C during 3 minutes. The SOD unit or copper(II) cmplexes was determined as 50% inhibition of O_2^--mediated reduction of cytochrome *c*.

RESULTS

The effect of SOD and tested Cu(II) complexes on the PMN leukocytes by zymosan stimulated formation of O_2^- were different. In phagocytizing PMN leukocytes the increase of O_2^- formation reflects the activation of the oxidative system which catalyses the reduction of molecular oxygen in the presence of NAD(P)H. There is considerable evidence that this system is located in both plasma and granule membranes of the cells.[23] Small molecules such as the Cu(II) complexes may successfully reach the active site of the membrane-bound system. However, the time required for the complexes to arrive at the active site of the system must be carefully taken into consideration in the experiments. Accordingly, preliminary investigations indicate that the preincubation time of PMN leukocytes in a medium containing Cu(II)

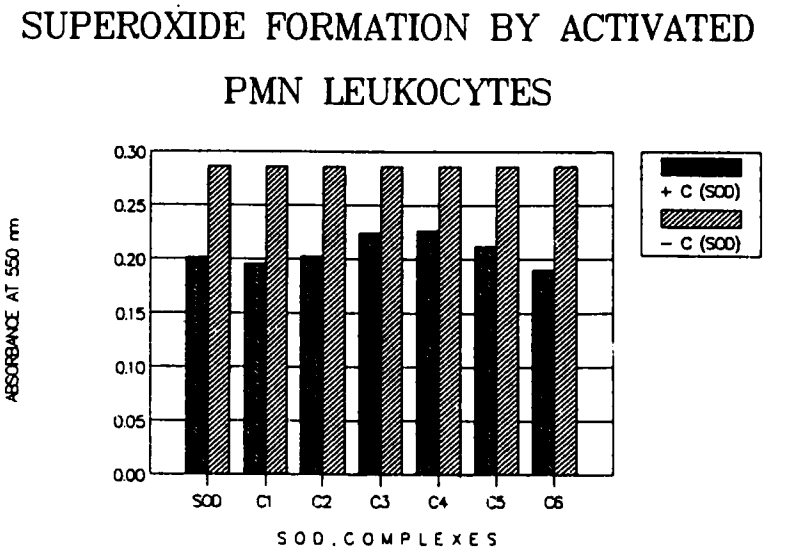

FIGURE 1 The effect os SOD and Cu(II) complexes *1–6* on the PMN leukocytes zymosan stimulated superoxide formation. Reaction mixture (1 ml) contained 25 μM cytochrome *c*, 150 U SOD (or without SOD) or 0.05 mg Cu(II) complexes (*C1–C6*) (or without Cu(II) complexes), 3.10^6 PMNL, pH 7.4, 37°C, 15 minutes. Reaction was initiated by addition of zymosan (20 zymosan particles per 1 PMNL).

complexes is determinant in the development of the complex-induced effect on the PMN leukocytes metabolic response associated with phagocytosis and O_2^- formation. Results reported in Figure 1 indicate that the addition of SOD and the tested Cu(II) complexes (*C1–C6*) just before the addition of zymosan has a relatively strong effect on the dismutation of O_2^-. However, the dismutation effects of SOD and the tested Cu(II) complexes are different. The highest dismutation effect was shown by SOD, the effects of complexes *C1*, *C2*, *C6* were comparable, but the effects of complexes *C3*, *C4*, *C5* were lower. Similar effects were observed by reduction of INT (unpublished results).

The assignment of a square-pyramidal pentacoordination Cu(II) in the studied *C1–C6* complexes, suggestive of a coordination polyhedron[30] in the active centre of the bovine Cu/Zn-SOD, follows from structural analysis by X-ray diffraction method.[11,12] Certain differences are, however, evident in sets of donor atoms. In the base of the square pyramid Cu/Zn-SOD[24] contains the chromophore [CuN_4] (strong Cu–N

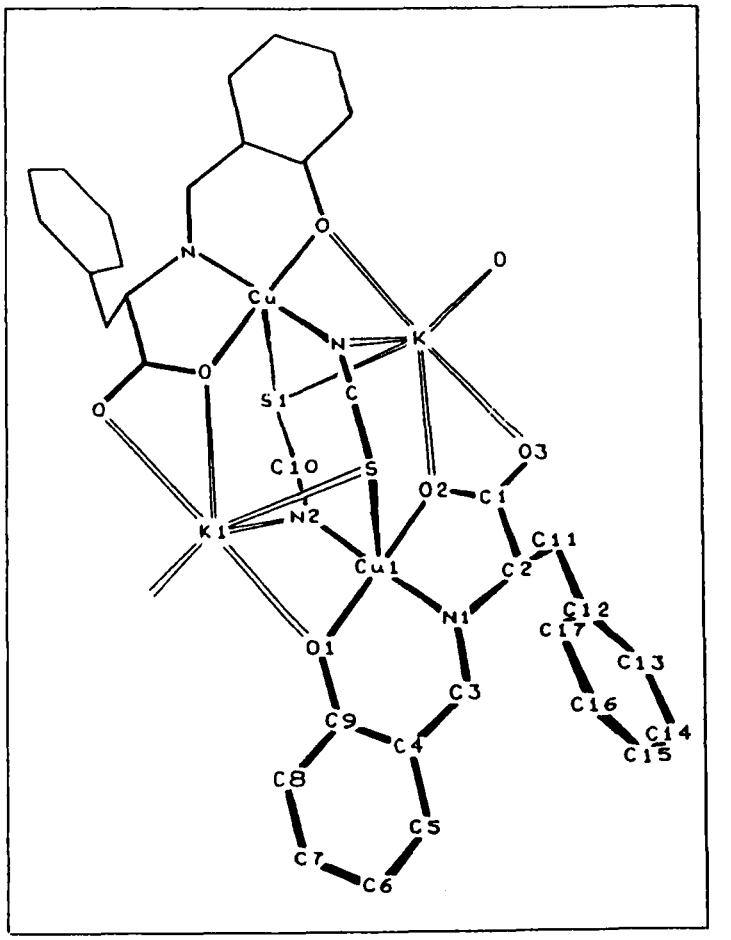

FIGURE 2 The perspective view on the part of the crystal structure of $K_2[Cu(sal\text{-}D,L\text{-}phe)_2(NCS)_2]$ which illustrates generally the structure of $[Cu_2(TSBA)_2X_2]^{2-}$ complex anions.

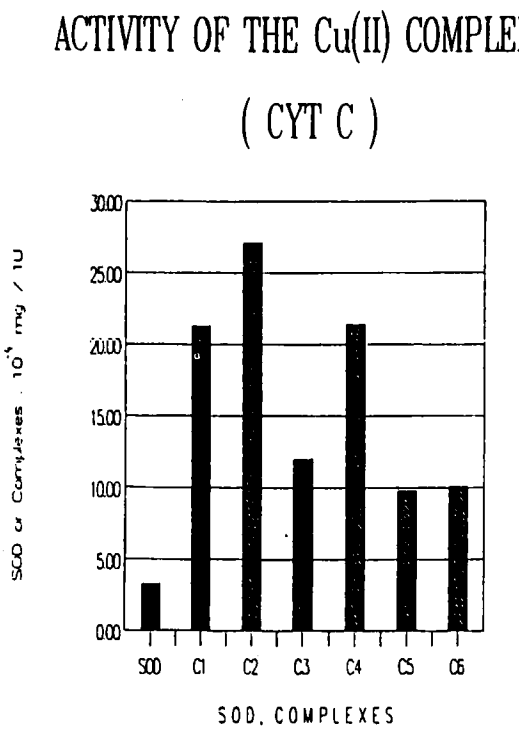

FIGURE 3 The effect of SOD and Cu(II) complexes *1–6* on xanthine oxidase-dependent cytochrome *c* reduction. Reaction mixture (1 ml) contained 5.10^{-5} M xanthine, 10^{-5} M cytochrome *c*, 1 U SOD or different weights of Cu(II) complexes (*C1–C6*) (pH 7.8, 22°C, 3 minutes). Reaction was initiated by addition of xanthine oxidase (0.015 U).

bonds $\simeq$ 0.21 nm). $[Cu(sal\text{-}L\text{-}glu)(H_2O)_2]\cdot H_2O$, used as parent compound in the preparation of $K[Cu(sal\text{-}L\text{-}glu)(H_2O)]$ has a chromophore $[CuO_3N]$ in this part of the coordination polyhedron.[11] The tested *C3–C6* complexes of the [Cu(TSBA)*X*] type contain the transplanary chromophore $[CuO_2N_2]$ in the pyramid base, as illustrated by the sketch of a fragment in the crystal structure of $K_2[Cu_2(sal\text{-}D,L\text{-}phe)_2(NCS)_2]$[12] (Figure 2). In the Cu/Zn—SOD structure, the chromophore $[CuN_4]$ is apically supplemented by a weakly bound water molecule (Cu–O $\simeq$ 0.3 nm), and it is this position of the coordination polyhedron which is considered as the place of introduction of O_2^- into the internal sphere of Cu(II).

Complexes (*C1–C6*) in solid state do not coordinate a water molecule apically but a donor atom from another complex unit, e.g., an S atom from NCS^- bridges while forming dimeric anions $[Cu_2(TSBA)_2(NCS)_2]$ (Figure 2). However, by dissolving these complexes in water, the weak apical bond (Cu–S $\simeq$ 0.3 nm) is evidently broken, and replaced by a water molecule, thus forming monomeric $[Cu(TSBA)(NCS)(H_2O)]^-$ units which then become active particles of the systems.

The courses of xanthine oxidase dependent, O_2^- mediated reduction of cytochrome *c* and its inhibition by SOD and the tested complexes are shown in Figure 3. Results reported in Figure 3 indicates that the addition of SOD and the complexes rapidly increase the dismution of O_2^-. The reoxidation phase of cytochrome *c* by H_2O_2 was eliminated by (a short?) incubation time (3 min).[25] The dismutation effects of SOD and

TABLE I
The effect of SOD and Cu(II) complexes on xanthine oxidase-dependent cytochrome *c* reduction[a]

Scavengers	SOD	*C1*	*C2*	*C3*	*C4*	*C5*	*C6*
% of activity (mg/l U)	100	16.17	12.18	27.39	15.56	33.35	32.49
% of activity (mol/ l U)	100	0.365	0.274	0.629	0.367	0.719	0.641
SOD, complexes 10^{-4} mg/l U	3.279	21.3	27.1	12.0	21.4	9.8	10.1

[a]Reaction mixture (1 ml) contained 10^{-5} M cytochrome *c*, 5.10^{-5} M xanthine, 1 U SOD of different weight of Cu(II) complexes (*C1–C6*), (pH 7.8, 22°C, 3 minutes). Reaction ws initiated by addition of xanthine oxidase (0.015 U).

of the complexes are different. The highest dismutation effect was shown by SOD, less by *C3*, *C5*, *C6* and the lowest by *C1*, *C2* and *C4*. In Table 1 we can see the inhibition effects of different weights of SOD and the complexes upon the reduction of cytochrome *c*. A titration – based determination of the inhibitory effects of SOD and complexes showed that under our experimental conditions, 50% of maximum inhibition (1 U) was achieved by either $3.28 .10^{-4}$ mg/l U of SOD; or $21.3 .10^{-4}$ mg/l U of *C1*, $27.1 .10^{-4}$ mg/l U of *C2*, $12.0 .10^{-4}$ mg/l U of *C3*, $21.4 .10^{-4}$ mg/l U of *C4*, $9.8 .10^{-4}$ mg/l U of *C5* and $10.1 .10^{-4}$ mg/l U of *C6*.

Table 1 also shows that addition of SOD and of the complexes caused a maximum inhibition effect as compared to SOD with *C5* (13.6%) and minimum inhibition effect compared to SOD with *C4* (6.4%) expressed in mg/l U.

From the viewpoint of the molar concentration of the complexes compared with 50% inhibition of SOD (1 U) the highest percentage of inhibition was 0.719 by *C5*.

DISCUSSION

Polymorphonuclear leukocytes are the predominant cells in the release of free oxygen metabolites which is one of the oxy-radicals postulated causes of tissue damage.[26] There is evidence of release of oxygen free radicals from PMN leukocytes during phagocytosis.[27] In this case the generation of oxygen free radicals is the result of increased oxidative metabolism of the cells. The extent of this metabolic activation may be accurately quantified by measuring various parameters such as O_2^- formation cytochrome *c* or INT reduction. These tests are considered as a good measure of the cytotoxic potential of these cells. Experimental data reported[17] show that PMN leukocytes treated with salicylato-copper(II) complexes sharply decrease the O_2^- formation and that this phenomenon is responsible for the anti-inflammatory activity of these compounds. If a relationship exists between the ability of anti-inflammatory drugs to inhibit the metabolism of PMN leukocytes and their pharmacological properties, results of our work suggest that the tested complexes are in fact an active form of salts. The mechanism by which the complexes affect the oxidative metabolism of the PMN leukocytes is not clear. However, it must be pointed out that the tested complexes exhibited a scavenging effect on O_2^- by an SOD-like activity. This means that the complexes may react with O_2^- by one of two possible mechanisms as an internal sphere process.[28]

SOD itself has been found to be anti-inflammatory. This property has been related

to the scavenging effect on $O_2^{\cdot-}$ and a protective effect against the autooxidation of stimulated PMN leukocytes. Our findings, regarding the effect of SOD on the functional activity of PMN leukocytes shows that PMN leukocytes behave quite differently in the presence of SOD as compared to PMN leukocyutes treated with the tested complexes. In summary, phagocytosis-induced formation of $O_2^{\cdot-}$ has been found strongly inhibited not only by SOD but also by the complexes *C1–C6*.

In this study we have shown that the investigated complexes may be considered as postential catalyst of $O_2^{\cdot-}$ dismutation. The ability of metal complexes to bring about the dismutation of $O_2^{\cdot-}$ may be tested in any one of the several assays originally developed for the measurement of the catalytic activity of the various SOD. Our results demonstrate that Cu(II) complexes display SOD-like activity. Indeed, Cu(II) complexes inhibited not only the PMN leukocytes zymosan stimulated $O_2^{\cdot-}$ formation, but also the xanthine oxidase-dependent $O_2^{\cdot-}$-mediated reduction of cytochrome *c*.

The assumption that Cu(II) complexes are a mimic of the active site of SOD implies that $O_2^{\cdot-}$ maintains these complexes within the continuous $Cu^{2+} \rightarrow Cu^{1+} \rightarrow Cu^{2+}$ conversion, but an alternate mechanism is also possible.[28] Using xanthine–xanthine oxidase as a simultaneous source of $O_2^{\cdot-}$ would be consistent with the view that Cu(II) complexes once reduced by $O_2^{\cdot-}$ are rapidly reoxidized by another molecule of $O_2^{\cdot-}$.[25]

Complexes of copper with histidine, thyrosine and lysine exhibit relatively high SOD-like activity, usually ranging from 4.9 to 7.5% of that of Cu/ZnSOD,[29] but the SOD-like activity of citrate-Fe^{3+} is only 0.03%.[25]

However it is important to point out that the utility of the copper complexes as *in vivo* biomimics of SOD is strictly limited by the presence (inside the cell) of many substances which may strongly bind Cu^{2+}.[30,31] Once displaced from its original carrier, Cu^{2+} may undergo dramatic changes in its redox potential and may unexpectedly start to catalyse the Haber-Weiss reaction rather than the dismutation of $O_2^{\cdot-}$.[32] We would, therefore, suggest that the SOD-like activity of the tested complexes although giving a first impression of weakness can be better appreciated in the context of these diverse multi-stepped, pathological processes.

Mammalian cells contain quite high concentrations of both Cu- Zn- SOD and MnSOD, the activity of which under physiological conditions, probably overshadows, that of trace amounts of Cu(II) complexes. However, there are certain disease states in which the integrity of the cell is threatened by an increase of the $O_2^{\cdot-}$ generation or by a decrease in SOD content. It has been suggested that in these particular situations, the toxicity may be stopped by water soluble Cu(II) complexes. Our results, which highlight the effects of Cu(II) complexes on the phagocytosis induced $O_2^{\cdot-}$-formation and also on xanthine oxidase $O_2^{\cdot-}$-dependent formation are in agreement with the hypothesis suggesting that the investigated complexes could be an active form of drug.

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