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Losartan and its interaction with copper(II): Biological effects

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Abstract—Losartan, the potassium salt of 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazol, is an efficient antihypertensive drug. The vibrational FTIR and Raman spectra of Losartan (its anionic and protonated forms) are discussed. In addition, the copper(II) complex of Losartan was obtained and characterized as a microcrystalline powder. The metal center is bound to the ligand through the nitrogen atoms of the tetrazolate moiety as determined by vibrational spectroscopy. The compound is a dimer with the metal centers in a tetragonal distorted environment but the presence of a monomeric impurity has been determined by EPR spectroscopy. The antioxidant properties of the complex (superoxide dismutase mimetic activity) and its effect on the proliferation and morphology of two osteoblast-like cells in culture are reported. The new compound exerted more toxic effects on tumoral cells than the copper(II) ion and Losartan.

1. Introduction

Losartan, the potassium salt of 2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1H-tetrazol-5-yl)biphenyl-4-yl)-methyl]imidazol (Fig. 1), is the prototype of a new generation of effective and orally active pharmaceutical products used for the treatment of arterial hypertension. It acts by selectively binding to and blocking the angiotensin II type 1 receptor, thus interfering with the rennin angiotensin system, an important regulator of normal blood pressure.[1–5] Structurally, Losartan is a biphenyltetrazol ring system attached to a substituted imidazol ring through a methylene spacer. Recently, single-crystal X-ray determinations of the structures of Losartan and its potassium salt were reported.[6,7] Copper is an essential transition element that plays a fundamental role in the biochemistry of all aerobic organisms. Proteins exploit the unique redox nature of this metal to undertake a series of facile electron transfer reactions using copper as a cofactor in a select number of critical enzymatic pathways. The function of these enzymes is essential for cellular respiration, iron homeostasis, pigment formation, neurotransmitter production, peptide biogenesis, connective tissue biosynthesis, and antioxidant defense.[8,9] The inherited alteration of copper homeostasis produces the pathologies of Wilson[10] (excess in copper storage) or Menkes[11–13] (defect in copper enzymes). The clinical features of Wilson disease generate liver damage because of copper cytotoxicity. In Menkes disease copper deficiency

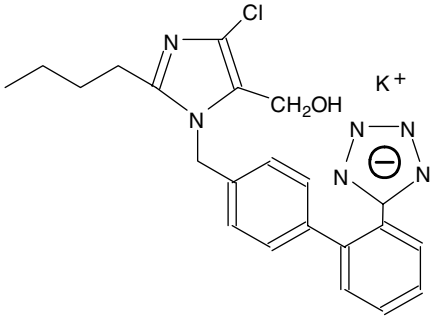

Figure 1. Losartan potassium salt.

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mainly affects enzymes involved in collagen metabolism.[14] Collagen is one of the most important components of connective tissue and its alteration can explain some of clinical features of this disease such as laxity of the skin and metaphyseal dysplasia.[15,16] On the other hand, Losartan contains functional groups capable to bind the copper biometal in stable complexes. From a pharmacological point of view part of the effect of medications could be connected with complexation of biometals and changes in homeostasis.[17]

Besides, the classical signs of copper deficiency, first carefully recorded in a series of malnourished infants in Peru, include hypocupremia, decreased serum ceruloplasmin concentration, neutropenia, anemia, metaphyseal dysplasia, osteoporosis, and fracture of the long bones.[18] It is well known that once absorbed many metal compounds are distributed among tissues in the organisms and stored mainly in bone.[19,20] For this reason it is interesting to investigate the effects of the biometals, the organic active drugs, and their metal complexes on bone related cells.

The aim of this study is the synthesis and characterization of a new copper complex of Losartan and its potential biological activities. In order to determine the antioxidant effects the superoxidedismutase (SOD) like activity of the complex has been investigated. Moreover, the biological effects of the new complex have been tested on two osteoblastic cell lines in culture, one normal and the other tumoral. We have analyzed the effect of the copper Losartan complex and its components on cell proliferation and cellular morphology.

2. Results and discussion

2.1. Vibrational spectroscopy

In Table 1 the main vibrational Raman frequencies of Losartan (LosH), Losartan Potassium salt (LosK), and Copper Losartan (LosCu) complex are shown. It can be observed that the very strong band at ca. 1615 cm^{-1} corresponding to the stretching C=C of aromatic groups disappeared by complexation with copper(II). Protonation of the tetrazol moiety produced the localization of the electronic density generating a double bond between nitrogen atoms causing a strong absorption at 1489 cm^{-1}(ν(N=N)[21]). Besides, the spectrum of Losartan presents a strong band at 808 cm^{-1} that is absent in the spectra of LosK and LosCu and it corresponds to a ring breathing vibrational mode.[22]

Table 1. Assignment of the main bands of the Raman spectra of protonated Losartan (LosH), Losartan potassium salt (LosK), and the copper complex (LosCu) (band positions in cm^{-1})

LosH	LosK	LosCu	Assignment
1615 vs	1612 vs	—	$\nu(C{=}C)_{aromat}$
1489 s	—	—	$\nu(N{=}N)$[21]
808 s	—	—	Ring breathing[22]

vs, very strong; s, strong.

Table 2. Assignment of the main bands of the infrared spectra of protonated Losartan (LosH), Losartan potassium salt (LosK), and the copper complex (LosCu) (band positions in cm^{-1})

LosH	LosK	LosCu	Assignment
3374 vs			ν(N–H)
1434 s, 1412 s	1423 s	1423 s	ν_{ring}, δ_{ip} (N–H)[23]
1117 m	1108 m	1100 w	$\nu(C_{tz}–N_1)$, $\delta(C_{tz}–H)$[27,28]
1087 s	1072 m	1070 m	ν_{ring}, δ_{ip}(C–H), δ_{ip}(N–H)[23]

vs, very strong; s, strong; m, medium; w, weak.

Table 2 displays the FTIR spectra of the ligand and the complex. The protonation of the ligand (LosH) can be realized by the presence of strong stretching and bending N–H bands located at 3374 cm^{-1}, 1434–1412 cm^{-1} (split of 1423 cm^{-1}), and 1087 cm^{-1}, respectively.[23] On the contrary, the band corresponding to the strong N–H stretching mode appears as a very weak band in the Raman spectrum as it has been previously reported in the literature.[24] Besides, the band related to the N–H bending mode decreases its intensity from strong (FTIR) to medium (Raman) as it is expected.[25]

The position of the bands associated to the ring vibration modes of the imidazol[26] and tetrazol moieties is difficult to assign unambiguously because they are superimposed. Nevertheless, as it is known, the band at 1105 cm^{-1} in the free ligand corresponds only to the tetrazol group.[27,28] The decrease of the intensity of this band in the FTIR spectrum may be due to the coordination of this fragment of the Losartan molecule to the metal center. A similar effect is also observed upon protonation of the same fragment in the LosH spectrum.

Furthermore, some shifts and changes in the intensities of several bands (vibrations or deformations modes) corresponding to the aromatic rings in the range between 1000 and 750 cm^{-1} are observed.[23–28]

2.2. Electron paramagnetic spectroscopy

With the aim to obtain a deeper insight into the environment around copper ions in the complex, spectroscopic studies by EPR were undertaken. Electron Spin Resonance is often employed to characterize binuclear systems. Particularly, in the case of copper(II)–copper(II) complexes EPR allows the recognition of a magnetic coupling between the two ions with $S = 1$ state and a characteristic half-field signal $\Delta M_s = \pm 2$ feature. At room temperature (Fig. 2, inset), the spectra show a dimeric type of signal. An absorption at 1650 G corresponding to forbidden half-field $\Delta M_s = \pm 2$ transition between $I - 1>$ and $I + 1>$ levels of a triplet state is observed.[29]

The nature of exchange coupling within Cu–Cu pairs is evident from the thermal dependence of EPR intensity. At 4.2 K (Fig. 2) the triplet state becomes depopulated as the temperature is lowered and so the signal intensity drops. The observed signal of the EPR spectrum shows that small amounts of isolated copper ions exist in the compound. As it is expected, beyond the transitions of the copper(II) pairs, also a feature to simple $S = 1/2$

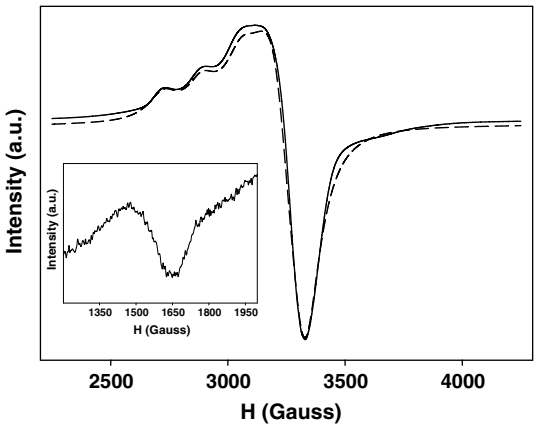

Figure 2. Band X-EPR spectra of LosCu (——) experimental and (- - - -) simulated at 4.2 K. (Inset) $\Delta M_s = \pm 2$ signal, 290 K.

species is observed because of the presence of a 'mononuclear' impurity (probably due to the rupture of the dinuclear complex).[29] This observation correlates with a monomeric species having axial symmetry in a square bipyramidal copper(II) geometry being consistent with $d_{x^2-y^2}$ ground state.[30] Spin Hamiltonian parameters obtained by carefully simulating experimental spectra are: $g_{\|} = 2.27$, $g_{\perp} = 2.08$; $A_{\mathrm{II}} = 175 \times 10^{-4}\ \mathrm{cm}^{-1}$ and $A_{\perp} = 20 \times 10^{-4}\ \mathrm{cm}^{-1}$. The low $g_{\|}$-value obtained is in concordance with a strongly elongated octahedral environment and the value of the parallel component of the hyperfine coupling constant is similar to that found in copper complexes with chromophores containing N and O donor atoms.[31]

Altogether, taking into account the experimental data the structure of the complex can be proposed. The complex has a dimeric structure with a distorted tetragonal configuration around each metal center. Two copper(II) atoms are linked by two bridges of the tetrazol moieties of each Losartan ligand, and the coordination sphere is completed with one N atom of the tetrazol moiety of another Losartan molecule and three oxygen atoms of three water molecules (Fig. 3).

2.3. Superoxide dismutase activity

Measurements of SOD-like activities are a good marker of the antioxidant properties of copper compounds. It has been reported that the presence of coordination sites belonging to nitrogen heteroaromatic rings such as imidazol, pyridine, and pyrazole is important for high SOD activity.[32] Therefore, we have selected losartan to generate an active site similar to that of copper(II) in the native enzyme. The determinations were carried out using the xanthine/xanthine oxidase/NBT assay system. NBT acts as superoxide detecting agent through its reduction to methylformazan (MF^+). In the presence of the copper complex the mechanism of the reduction of NBT to MF^+ comprises several reactions. The system xanthine/xanthine oxidase produces superoxide that reacts with NBT to produce MF^+. At the same time the copper complex interacts with $O_2^{\cdot -}$ catalyzing its dismutation to molecular oxygen and hydrogen peroxide; this reaction reduces the superoxide concentration in solution and, thus, the MF^+ production rates (i.e., slowing down absorbance variation). The experimental determination of SOD activity was performed at pH 10.2 and the complex produced a 50% inhibition (IC_{50}) at $72.1 \pm 5\ \mu M$. Under the present experimental conditions the IC_{50} value of SOD enzyme (from bovine erythrocytes) was 4 nM, similar to previously reported values.[33]

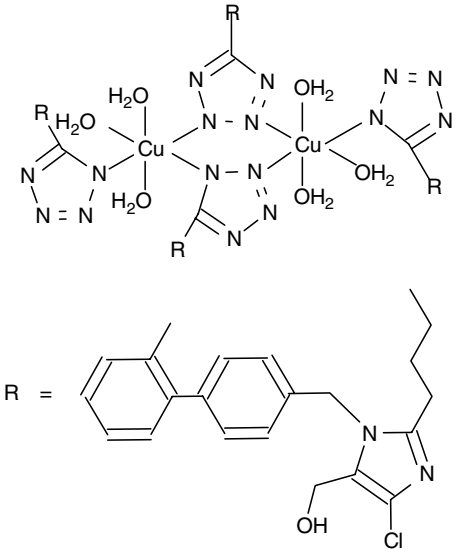

Figure 3. Schematic drawing of the complex.

If compounds have an IC_{50} value below 20 μM they show SOD-like activity but they are inactive at higher concentrations. The experimental IC_{50} values obtained for complex were found to be in the lower limit of the active compounds[34] (sometimes called moderate behavior toward the dismutation of superoxide).

2.4. Cellular proliferation

Cell proliferation was estimated through the crystal violet assay on two osteoblast-like cells in culture: UMR106, derived from a rat osteosarcoma, and MC3T3E1, derived from mouse calvaria. The effects of the Losartan, copper(II), and the complex LosCu were investigated (Figs. 4 and 5). The results showed that the ligand was slightly inhibitory causing a decrease of ca. 10% at 500 μM in both cell lines. The free cation Cu(II) behaved in a similar way in the tumoral cells but it was more deleterious for the normal osteoblasts inhibiting cell proliferation ca. 25% at 100 μM and ca. 50% at 500 μM. On the other hand, for the complex a similar injurious action on both cell lines can be observed in a dose response manner with values of ca. 60% at 500 μM. Altogether these results point to the fact that the complex formation modulates the action of the free copper. The greater antiproliferative effect of the complex in both cell lines in comparison with the free ligand and copper ion might be due to the higher lipophilicity of the neutral complex in relation to the ionic species formed by losartan potassium salt and copper(II) chloride. Nevertheless, cytotoxicity is a complex phenomenon only partially influenced by the

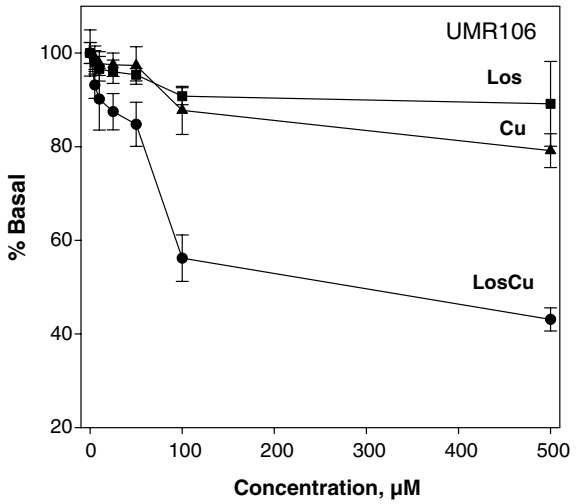

Figure 4. Effect of Losartan, copper(II), and Losartan/copper compounds on UMR106 cell proliferation. Cells were incubated in serum-free DMEM alone (basal) or with different concentrations of the compounds at 37 °C for 24 h. Basal values are 5.2×10^4 cells/well. Results are expressed as % basal and represent means ± SEM, $n = 9$.

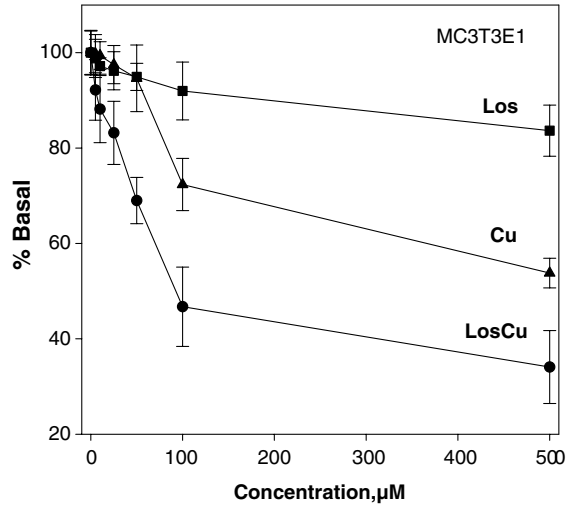

Figure 5. Effect of Losartan, copper(II), and Losartan/copper compounds on MC3T3E1 cell proliferation. The experimental conditions were similar to those of Figure 3. Basal values are 4.0×10^4 cells/well. Results are expressed as % basal and represent the means ± SEM, $n = 9$.

lipophilicity of the compounds as it has previously been reported.[35]

2.5. Cell morphology

Figure 6 shows the results of the effect on cell morphology for the treatment of the osteoblast-like cells with copper and LosCu in comparison with basal or control conditions (without any compound added). In Figure 6a MC3T3E1 osteoblast-like cells are shown while Figure 6b displays the effects on the UMR106 osteosarcoma cells.

Control dish shows polyhedral MC3T3E1 cells with slender lamellar expansions that join each cell with its neighboring cells. The nuclei show moderately thick chromatin granules. The perinuclear cytoplasm has numerous organelles and vacuoles, while the exoplasm is diffuse and homogeneous. Preparations with 500 μM $CuCl_2$ show a gradual loss of connections and an increase in cytoplasm condensation. Cells become spindle-like and condensed. Besides, a sticking decrease in the cell number/field can be observed since cells died and detached from the monolayers. A similar effect more marked at 500 μM can be observed for LosCu. On the other hand, the tumoral cells (UMR106) under basal conditions (medium alone) display a polygonal morphology with well-stained nuclei and cytoplasms with processes connecting neighboring cells. After the treatment with Cu^{2+} ions a slight decrease in the number of cells can be observed. In the case of the copper complex (LosCu), a significant decrease in the number of surviving cells has been induced. The cytoplasm shows a strong condensation with loss of connections between cells. Losartan, potassium salt did not cause any morphological change in both cell lines at the maximum tested concentration value (500 μM).

3. Conclusion

A new complex $[Cu(Los)_2(H_2O)_3]_2$ derived from the antihypertensive agent Losartan and copper(II) cation has been synthesized and characterized. The metallic ion is coordinated to the ligand through the nitrogen atoms of the tetrazol moiety as it was demonstrated by FTIR and Raman spectroscopies. The solid phase of the new compound contains a dinuclear complex as the main species and a monomeric impurity according to the room temperature EPR measurements. At 4 K an antiferromagnetic coupling between the metallic centers takes place and the EPR signal is only due to the monomeric impurity. According to the determined parameters the copper ions are located in a tetragonal distorted octahedral environment surrounded by nitrogen and oxygen atoms, the latter provided by water molecules.

Even though Losartan does not show antioxidant activity, its copper complex has displayed a moderately superoxidedismutase activity. Besides, the complex behaves as a deleterious agent when tested on the tumoral osteoblasts (UMR106). This effect has not been produced by the copper(II) ion or the Losartan ligand in a broad range of concentrations. On the other hand, both the metal and the complex behave as inhibitory agents on the non-transformed osteoblasts since both of them produce a decrease in cellular proliferation being the metal complex the more toxic compound. The results also show that the toxic action of the complex has a similar magnitude on both cell lines. Morphological alterations such as cytoplasm condensation, loss of the connections between neighboring cells, and a cellular spindle like shape are produced in both cell lines only by the complex. Altogether these results indicate that the complexation of the antihypertensive drug with the biometal copper modulates the biological

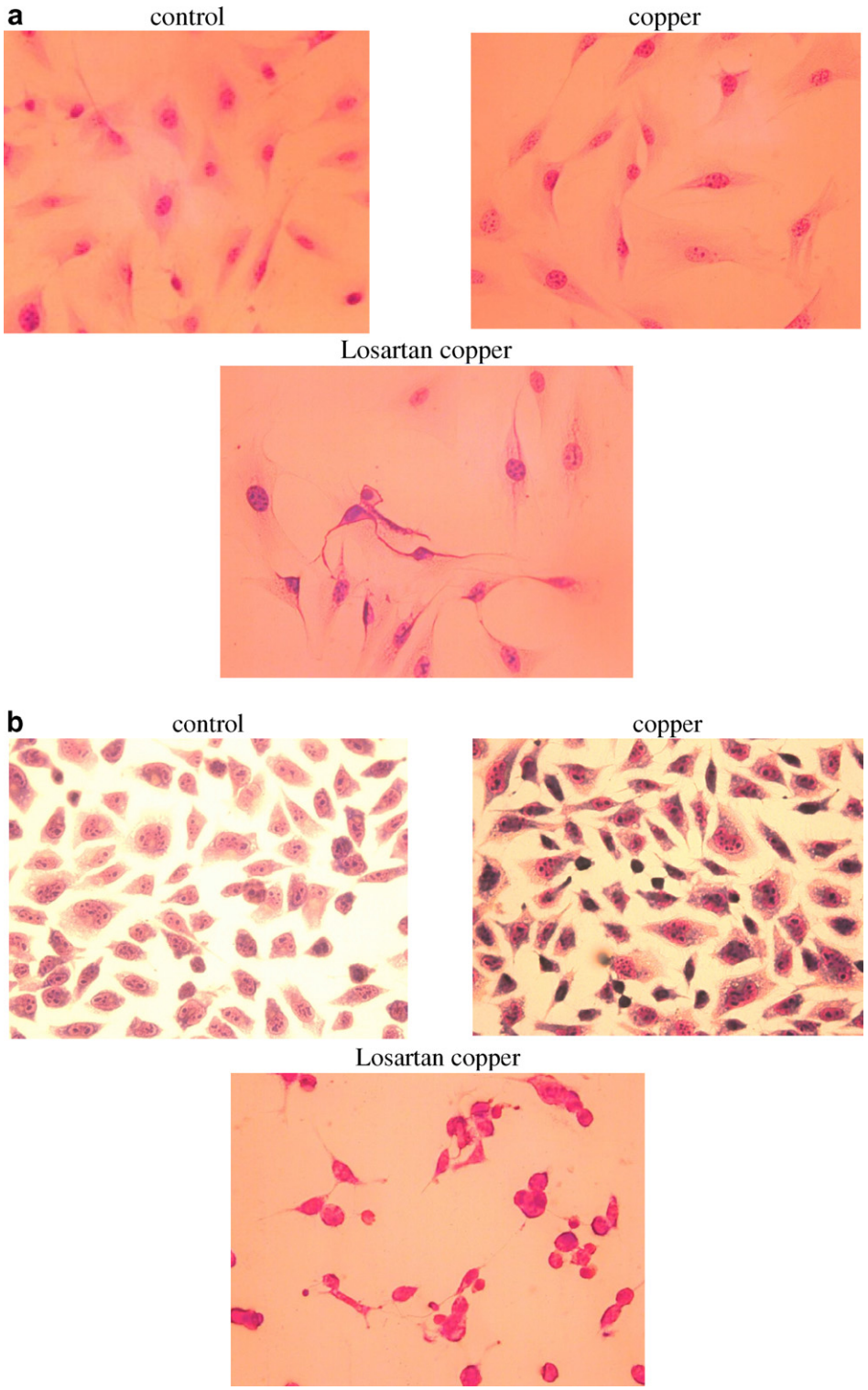

Figure 6. Effect on cell morphology of the treatment of the osteoblast-like cells with copper (500 μM) and Losartan/copper (500 μM) in comparison with basal or control conditions (without any compound added). (a) MC3T3E1 osteoblast-like cells; (b) UMR106 osteosarcoma cells.

effects of both moieties. Above all, it has been demonstrated the harmless effect of the antihypertensive drug. Losartan did not cause any morphological transformation in both cell lines and its action on cell proliferation was very weak since it only produced inhibition of ca. 10% at high doses.

4. Materials and methods

Losartan, potassium salt (Parafarm), and Copper(II) acetate (Merck) were used as supplied. Losartan was obtained as monocrystals using the potassium salt by lowering the pH of the solution to 2.0 with 1 M HCl. The

structure of the sample is the same to that previously reported.[6] Corning or Falcon provided tissue culture materials. Dulbecco's modified Eagle's medium (DMEM) and trypsin–EDTA were purchased from Gibco (Gaithersburg, MD, USA) and fetal bovine serum (FBS) from GibcoBRL (Life Technologies, Germany). All other chemicals used were of analytical grade. UV–vis spectra for SOD determinations were recorded on a Hewlett-Packard 8453 diode-array spectrophotometer. Diffuse reflectance spectrum was recorded on a Shimadzu UV-300 spectrophotometer. Infrared spectra were recorded on a Bruker IFS 66 FTIR-spectrophotometer from 4000 to 400 cm^{-1} using the KBr pellet technique. Raman spectra were obtained with a fast Fourier transformed FRA106 Bruker spectrometer, using the 1064 nm line of Nd–YAG ion laser for excitation. A Bruker ESP300 spectrometer operating at X-band and equipped with standard Oxford low temperature devices (ESR900/ITC4) was used to record the EPR spectra of the compounds at different temperatures. The magnetic field was measured with a Bruker BNM 200 gaussmeter, and the frequency inside the cavity (rectangular ER4102ST) was determined by using a Hewlett-Packard 5352B microwave frequency counter. A computer simulation of the EPR spectra was performed using the program SimFonia (WINEPR SimFonia v1.25, Bruker Analytische Messtecnik GmbH, 1996). Elemental analysis for carbon, hydrogen, and nitrogen was performed using a Carlo Erba EA 1108 analyzer.

4.1. Synthesis of $[Cu(Los)_2(H_2O)_3]_2$

A slight blue losartan complex was prepared upon slowly mixing aqueous solutions of copper(II) acetate (0.5 mmol) and losartan potassium salt (1 mmol) with constant stirring. The complex was filtered, washed with water and dried in an oven at 60 °C.

Anal. Calcd for $C_{44}H_{50}N_{12}O_5Cl_2Cu$: C, 55.0; H, 5.2; N, 17.5. Exp.: C, 55.0; H, 5.1; N, 17.4%. Diffuse reflectance spectrum: 370 nm, 730 nm (br).

4.2. SOD assays

The superoxide dismutase activity was examined indirectly using the nitroblue tetrazolium (NBT) assay. The indirect determination of the activity of SOD and the copper complexes was assayed by their ability to inhibit the reduction of NBT by the superoxide anion generated by the system xanthine/xanthine oxidase, at pH 10.2 (carbonate buffer), reported previously.[36,37] As the reaction proceeds, the formazan color was developed and a change was observed from yellow to blue which was associated with an increase in the absorption spectrum at 560 nm. The reaction system contained different concentrations of the native SOD from bovine erythrocytes or the copper complex. The reaction was started by the xanthine–xanthine oxidase system in a concentration needed to yield the absorbance change between 0.2 and 0.4. Copper(II) chloride aqueous solution, 0.2 mM, was added in order to stop the NBT reduction. Free copper(II) ion is able to interact with the superoxide anion producing its dismutation. Ethylenediaminetetraacetic acid (EDTA), 0.1 mM, was included due to the formation of a copper chelate (CuEDTA) that has no SOD activity. Each experiment was performed in triplicate and at least three independent experiments were performed in each case. All the reagents (Sigma) were used as purchased. The amount of complex (or SOD) that gives a 50% inhibition (IC_{50}) was obtained by plotting the percentage of inhibition versus the log of the concentration of the tested solution.

4.3. Cell culture

Rat osteosarcoma UMR106 and osteoblastic non-transformed mouse calvaria derived MC3T3E1 cells were grown in DMEM supplemented with 10% (v/v) FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) in a humidified atmosphere of 95% air/5% CO_2. Cells were grown at near-confluence (70–80%) and were subcultured using 0.1% trypsin–1 mM EDTA in Ca^{2+}–Mg^{2+}-free phosphate-buffered saline (PBS). For experiments, about 5.5×10^4 cells/well (UMR 106) and 3.3×10^4 cells/well (MC3T3E1) were plated into 24-well plates. After the culture reached 70% confluence, the cells were washed twice with DMEM. Cells were incubated overnight with the compounds at different doses in serum-free DMEM.

4.4. Cell proliferation assay

A mitogenic bioassay was carried out as described by Okajima et al.[38] with some modifications. Briefly, cells in 24-well plate were washed with PBS and fixed with 5% glutaraldehyde/PBS at room temperature for 10 min. Cells were then stained with 0.5% crystal violet/25% methanol for 10 min. After that, the dye solution was discarded and the plate was washed with water and dried. The dye in the cells was extracted using 0.5 mL/well 0.1 M glycine/HCl buffer, pH 3.0/30% methanol and transferred to test tubes. Absorbance was read at 540 nm after a convenient sample dilution. The correlation between cell number/well and the absorbance at 540 nm of diluted extraction sample after crystal violet staining has been previously established.[39] Data are expressed as means ± SEM. Statistical differences were analyzed using Student's t test. t Tests were done to compare treated cultures with the untreated cultures. Recently prepared solutions of Losartan, $CuCl_2$, and LosCu (DMSO 0.5%-serum-free DMEM 99.5%) were added to the culture dishes at the following concentrations: 0, 5, 10, 25, 50, 100, and 500 μM.

4.5. Cell morphology

MC3T3E1 osteoblast-like cells and UMR106 osteosarcoma cells were grown in six-well plates, at an initial density of 2×10^4 and 1×10^4 cells/well for MC3T3E1 and UMR106 osteoblast-like cells, respectively. The cells were incubated overnight with fresh serum-free DMEM plus 0 (basal), 500, and 1000 μM of copper complex and Cu^{2+} ($CuCl_2$). The monolayers were subsequently washed twice with PBS, fixed with methanol, and stained with 1:10 dilution of Giemsa for 10 min. After that, the monolayers were washed with water

and the morphological changes were examined by light microscopy.

Acknowledgments

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