

Antioxidant Capacity of Binuclear Cu(II)-Cyclophanes, Insights from Two Synthetic Bioactive Molecules

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Received 13 October 2009; revised 19 February 2010; accepted 28 February 2010

ABSTRACT: The compounds 2,9,25,32-tetraoxo-4,7,27,30-tetrakis(carboxymethyl)-1,4,7,10,24,27,30,33-octaaza-17,40-dioxa[10.1.10.1]paracyclophane and 2,9,25,32-tetraoxo-4,7,27,30-tetrakis(carboxymethyl)-1,4,7,10,24,27,30,33-octaaza[10.1.10.1]paracyclophane binuclear copper complexes (Cu₂PO and Cu₂PC, respectively) were studied by determining their antioxidant capacity using the TROLOX equivalent antioxidant capacity (TEAC) assay, and their cytotoxicity on cultured cells, as well as the superoxide dismutase (SOD)-like activity. Cu₂PO had an antioxidant capacity (0.1 g eq TROLOX mol⁻¹) within the order of magnitude of ascorbic acid, and both, Cu₂PO and Cu₂PC were nontoxic to cultured peripheral mononuclear blood cells. The SOD-like activity was evaluated using the nitroblue tetrazolium assay, and both compounds presented an excellent activity: for Cu₂PO, the IC₅₀ was 52 nM and for Cu₂PC an IC₅₀ of 0.5 μM was obtained comparable to CuZn SOD IC₅₀ 17 nM (Fernandes et al., *J Inorg Biochem* 2007;101:849–858). These results suggest that synthetic binuclear macrocycles are good candidates to be used as synthetic bioactive molecules with applications in biomedicine. © 2010 Wiley Periodicals, Inc. *J Biochem Mol Toxicol* 24:379–383, 2010; View this article online at wileyonlinelibrary.com. DOI 10.1002/jbt.20350

KEYWORDS: Cyclophane; Macrocylic; Copper; Antioxidant capacity; TEAC

INTRODUCTION

Supramolecular chemistry deals with the construction of molecular scaffolds for including atoms or molecules by noncovalent interactions [1]. Macrocycles and their metal complexes are considered promising agents for diagnosis and treatment of different diseases [2]. In addition, some macrocyclic complexes have been suggested as a potential class of superoxide dismutase (SOD) mimetics, mainly because of their high thermodynamic stability. The stability of a metal complex is critical for its use as a pharmaceutical. The possibility that a potentially harmful redox-active metal ion could be liberated from a complex in an inappropriate biological compartment is clearly a risk to be avoided. The overall chemical and metabolic stability are critical factors for pharmaceutical applications. Many antioxidants have a redox-active metal center, although only superoxide dismutase catalyzes the O₂^{•-} dismutation to hydrogen peroxide. Copper(II) aqueous ion is a superoxide scavenger, although serum albumin has a high-affinity site for this metal. Hence, free Cu(II) ions cannot work as an SOD mimetics [3].

In addition to high stability, a copper(II) complex that possesses SOD mimetic activity should have a flexible arrangement of the ligands around the metal ion to allow the reduction to copper(I). We studied macrocycles that bind metals with high affinity and with a coordination similar to the bi-copper containing proteins, such as antioxidant superoxide dismutases [4]. Novel macrocycles with bioactive properties have been reported; in particular manganese(II)-substituted macrocyclics that were not cytotoxic and performed well *in vivo* on injured mice [5] and, more recently, copper(II) closed complexes with good SOD-like activity [3].

Cyclophanes PO and PC at pH ≤ 7 yield neutral Cu²⁺ complexes [Cu₂L]⁰ [4]. The resulting metal

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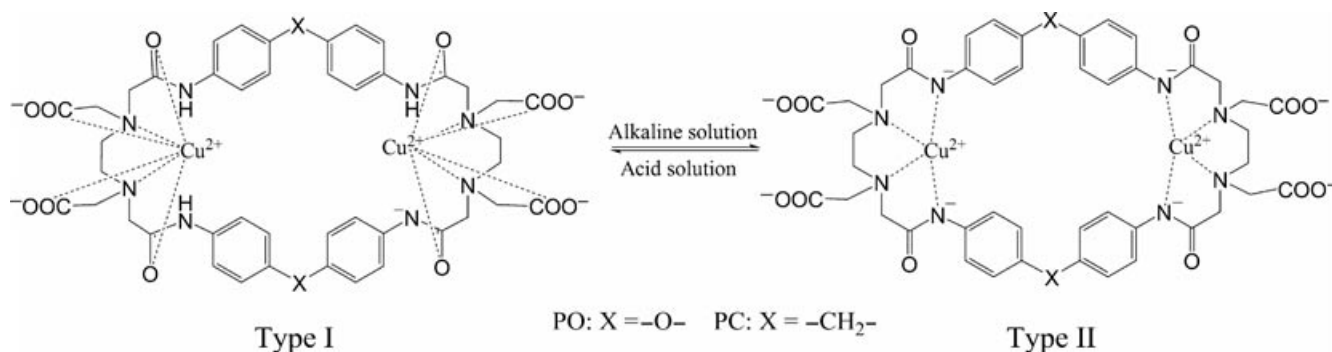


FIGURE 1. Chemical structure of the octahedral (type I) and square-planar (type II) forms of PO and PC cyclophane copper complexes.

chelates have a type I octahedral structure where each Cu atom is coordinated to two carboxylate oxygens, two amine nitrogens, and two amide oxygen atoms; one of the amide oxygen atoms may be replaced by a water oxygen atom (Figure 1). In alkaline solution, the complexes change to the charged and square-planar geometry type II $[\text{Cu}_2\text{LH}_4]^{4-}$, and Cu atom is bonded to two deprotonated amide nitrogens and two amino nitrogens. In the present work, we present evidence that Cu-bound cyclophanes have antioxidant properties, have SOD-like activity, and are not toxic to cells *in vitro*. These results are encouraging to further investigate their potential biomedical applications.

MATERIALS AND METHODS

Cyclophanes

The cyclophanes were synthesized by a reaction between ethylenediaminetetraacetic dianhydride and bis(4-aminophenyl) ether as reported previously [6]. The lithium salt of the cyclophane was recrystallized repeatedly from water until a colorless solid was obtained and was converted to the corresponding acid with diluted HCl at pH \sim 2. The purity was checked with ^1H NMR. The starting materials were purchased from Aldrich (Toluca, México). These receptors were complexed with copper by dissolving in water with a minimal amount of solid Na_2CO_3 , until pH \leq 7. Then, a solution containing $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ was added to form a precipitate in a quantitative yield [4].

Antioxidant Capacity

The TROLOX equivalent antioxidant capacity (TEAC) assay is based on determination of TROLOX-equivalents, which is a water-soluble analog of vitamin E. TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and was pur-

chased from Aldrich (Milwaukee, WI). ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) and ascorbic acid (vitamin C) were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade or better. PO and PC copper complexes were evaluated at a 33- μM concentration using a modified TEAC method [7] to determine their antioxidant activity. The TEAC method measures the antioxidant capacity to scavenge the blue-green $\text{ABTS}^{\bullet+}$ radical cation vs. the scavenging capacity of TROLOX [8]. The $\text{ABTS}^{\bullet+}$ radical cation was obtained by mixing 5 mL of a 7 mM ABTS solution and 88 μL of a 140 mM $\text{K}_2\text{S}_2\text{O}_8$ solution. One milliliter of the $\text{ABTS}^{\bullet+}$ was dissolved in 88 mL of PBS. In a quartz cell, the reaction contained 3900 μL of $\text{ABTS}^{\bullet+}$ and 100 μL of each test sample or dilutions of the TROLOX standard. The absorbance was read during 30 min after the initial mixing. Calculations were made correlating the scavenging capacity of TROLOX vs. each sample [7,8]. The antioxidant capacity was expressed as grams equivalent TROLOX per mol of test sample.

Cellular Toxicity Assays

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and separated by a density gradient centrifugation using Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden). The cell concentration was adjusted to 2×10^6 cells in RPMI-1640 containing 10% heat-inactivated fetal calf serum, 50 mM 2-mercaptoethanol, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1 $\mu\text{g}/\text{mL}$ amphotericin B (Sigma, Toluca, México). PBMC (1.5×10^5 cells per well) were seeded onto 96-well tissue culture plates and stimulated with phytohemagglutinin (PHA, 5 $\mu\text{g}/\text{mL}^{-1}$) in the presence of different concentrations of Cu_2PO (0, 0.04, 0.4, 4, and 40 μM). Cells were incubated at 37°C in 5% CO_2 , and at 6, 18, 24, and 48 h posttreatment; cell viability was evaluated by the trypan blue dye exclusion assay.

Superoxide Dismutase-Like Activity of Cu₂PO and Cu₂PC Cyclophanes

SOD activity was determined according to Beyer and Fridovich [9], modified as follows: The working solution consisted of 27 mL bidistilled water, 1.5 mL of L-methionine solution (30 mg mL⁻¹), 1 mL nitroblue tetrazolium (1.41 mg mL⁻¹), and 0.75 mL of 1% Triton X-100.

The reaction contained 0.03 mL of riboflavin (4.4 mg 100 mL⁻¹), 0.4 mL of Cu₂PO or Cu₂PC stock solution (for a final concentration of 0.015, 0.030, 0.10, 0.20, 0.30, or 0.50 μM), and 3 mL of working solution. The reaction mixture was stirred and then exposed to fluorescent light (λ = 375 nm) emitted by two lamps of 20 W for 15 min. Then, the quartz cell was mounted into a UV-vis spectrophotometer (Varian), and the absorbance was measured at 560 nm. The reaction velocity was determined as absorbance increment due to nitroblue tetrazolium formazan formation per unit of time. One unit of SOD was defined as the concentration that inhibits 50% of nitroblue tetrazolium formazan formation (IC₅₀). Assays were performed at room temperature (24–26°C).

RESULTS AND DISCUSSION

Cyclophanes PO and PC are able to switch between an octahedral to a square planar geometry, which is consistent with the antioxidant and dismutase activity.

Antioxidant Capacity

We decided to start characterizing these novel cyclophanes by measuring the antioxidant activity since this is an important characteristic for biomedical applications. The Cu₂PO (0.10 g eq TROLOX mol⁻¹) had more antioxidant capacity than Cu₂PC cyclophane and within the range of ascorbic acid (0.15 g eq TROLOX mol⁻¹) or reduced glutathione [10] (Figure 2). This property is important when using these types of molecules during controlled release or in hypoxia or inflammation therapy [11]. Based on our results, cyclophanes copper complexes have antioxidant capacity comparable to phenolic acids, although lower than flavonoids and vitamins C and E [10,12]. Differences between the antioxidant capacity of Cu₂PO and Cu₂PC may be related to their ability to stabilize the square-planar metal conformation involved in the dismutation reaction.

Cellular Toxicity

To evaluate the cytotoxicity of Cu₂-cyclophanes, different concentrations of Cu₂PO and Cu₂PC were

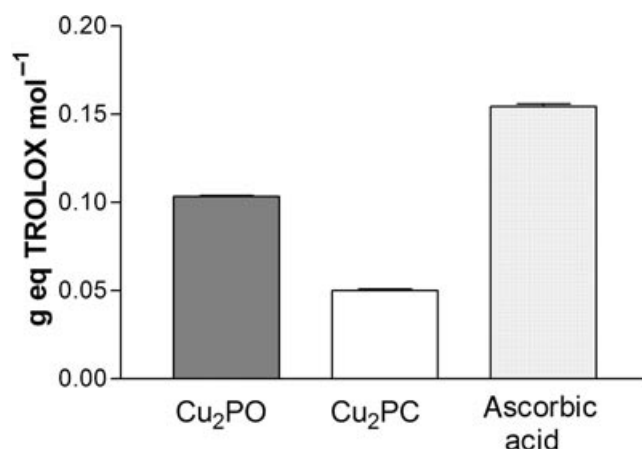


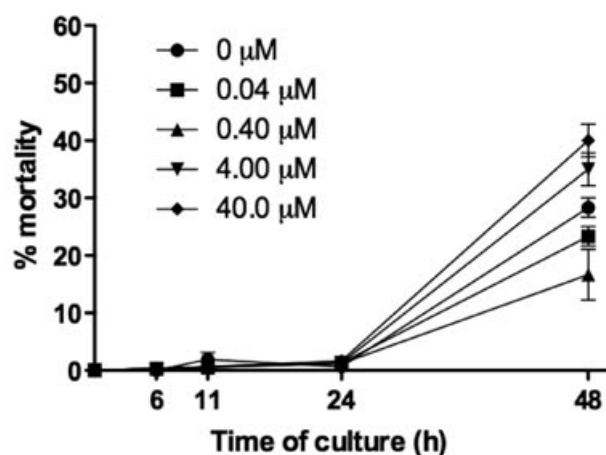
FIGURE 2. Antioxidant capacity of PO and PC cyclophane copper complexes by the TEAC method. SEM is drawn for each bar.

incubated with PBMCs cells and the percentage of mortality was evaluated by the trypan blue dye exclusion assay. Figure 3A shows that Cu₂PO induced a marginal mortality in the cells at 6, 11, or 24 h, when either low (0.04 μM) or high (40 μM) concentrations of Cu₂PO were used. At 48 h of culture, mortality increased to 35% in control untreated cells; however, low doses of Cu₂PO appeared to have a protective effect, since mortality was less than 29%. These results indicate that Cu₂PO was not cytotoxic to the cells in the first 24 h of culture even when high doses were used. The cytotoxic effects of Cu₂PC were also evaluated on PBMCs, finding a similar trend compared to Cu₂PO, this is that after 24 h mortality increased because lack of nutrients and not because cyclophane toxicity occurred.

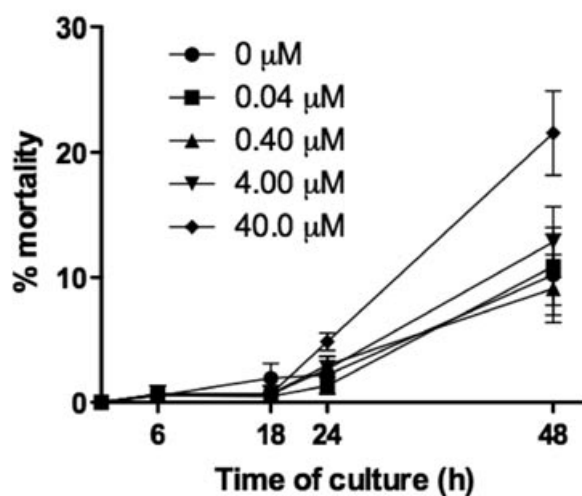
Superoxide Dismutase-Like Activity of Cu₂PO and Cu₂PC Cyclophanes

The SOD-like activity was measured using the nitroblue tetrazolium (NBT) formazan assay, with modification from the Beyer and Fridovich assay [9]. Since SOD or the compound tested should inhibit NBT oxidation, data are represented as an inhibition or IC₅₀, as the concentration of the complex that inhibits 50% of NBT formazan formation (Figure 4). Cu₂PO IC₅₀ 52 nM had a significant SOD-like activity similar CuZn SOD enzyme (IC₅₀ 17 nM). Meanwhile, for Cu₂PC IC₅₀ 0.5 μM is comparable to values reported for copper(II) closed oxo-aza cyclophanes (IC₅₀ 6–30 μM) reported by Fernandes [3].

In previous work, we have determined that Cu₂PO octahedral geometry changes to the square-planar



(A)



(B)

FIGURE 3. Cellular toxicity was studied in mononuclear cells (PBMNC) at different posttreatment times with four concentrations of Cu_2PO (panel A) or Cu_2PC (panel B). The graph represents the mean \pm SEM of a representative experiment of two representative experiments performed.

configuration at $\text{pH} \geq 6.4$, whereas the Cu_2PC change begins at $\text{pH} \geq 7.1$ [4]. Thus, at $\text{pH} 7.4$, Cu_2PO equilibrium is displaced toward the square-planar structure compared to Cu_2PC , and this could be one factor for the differences in SOD-like activity. Another important feature is the solubility of the compounds. Some of the SOD mimetics that have been developed have not reached the market, mainly due to poor biopharmaceutical properties, in which low aqueous solubility is included [13]. Cu_2PO and Cu_2PC were soluble in the mM range and presented antioxidant and SOD-like activity at concentrations in the nM to μM range at near physiological conditions ($\text{pH} 7.4$).

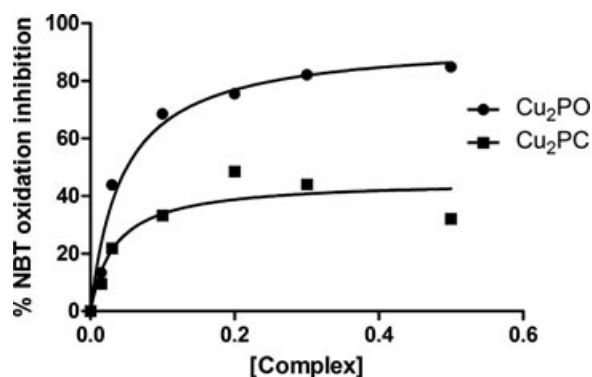


FIGURE 4. SOD-like activity of Cu_2PO and Cu_2PC macrocycles, as inhibitors of the nitroblue tetrazolium formazan formation.

CONCLUSIONS

Macrocyclic complexes have been shown to capture superoxide ions, in a superoxide dismutase fashion [5] and to have a potential as pharmaceuticals. Such molecules have been subjected to *in vivo* testing and demonstrated to potentiate interleukin-2 during treatment of metastatic renal cell carcinoma and malignant melanoma [11]. Copper(II), in complex with simple dipeptides or proteins, has also an antioxidant effect on photosensitized liposomes, protecting against peroxidation [14]. Cu_2PO and Cu_2PC presented a significant SOD-like activity with a good antioxidant capacity and no harmful effect on cells *in vitro*.

The SOD-like activity depends on structural features, namely the conformation of the metal binding site. Copper(II) has a preferred square-planar configuration and occasionally one or two additional weakly bonded axial ligands, which, upon reduction, lead to a copper(I) closed d^{10} shell structure. For the latter, the stable configuration corresponds to a tetrahedral four-coordinated or trigonal three-coordinated geometry. Thus, the macrocycle structure should slightly stabilize Cu(II) over Cu(I), since excessive stabilization toward square-planar geometries may not be favorable for the O_2^- dismutation [1]. The type of donor groups coordinated to the metal center also affect the stability of Cu(I) over Cu(II). For example, the placement of soft ligands in the coordination sphere increases the Cu(I)/Cu(II) reduction potential [1].

In Cu_2PO and Cu_2PC square-planar type complexes, copper atoms are bound to amide deprotonated groups [4]. Since the lone pair of the amide group is linked to an aromatic group, the electron density around Cu atoms is low and the reduction potential of Cu(II) may be decreased, so that switching between Cu(II) and Cu(I) could be easier than with other copper complexes. To further obtain insights into the

structure relation of these molecules, the SOD-like activity of other cyclophanes with an amide-coordinating group bound to an aliphatic backbone should be studied.

Since cyclophanes were found to be noncytotoxic and have a considerable antioxidant capacity and SOD-like activity with an IC_{50} in nanomolar range for Cu_2PO , further studies to deeply understand the mechanisms, as well as their properties, in an animal model system for inflammation should be pursued.

ACKNOWLEDGMENTS

We acknowledge support from Universidad de Sonora and the seed grant "BIONANO" from Centro de Investigación en Alimentación y Desarrollo A.C. We thank Dr. Gloria Yepiz-Plascencia for critical reading and helpful suggestions, and Monica Villegas and Monica Resendiz from CIAD for technical assistance.

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