

Cellular Redox State Modifications Induced by Bioactive Fe(III)-Cyclophane Complexes Approaching to Selective Therapy Drug Design

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Abstract

Previously we reported the capacity of the bio-inspired Fe(III) complexes, Fe₂PO and Fe₂PC, to mimic the activity of superoxide dismutase and peroxidase enzymes, as well as their capacity to reduce the cytotoxicity generated by superoxide radicals in human peripheral blood mononuclear cells, under stress conditions. Based on those findings, we decided to evaluate the cytotoxicity, antioxidant and redox state modulation capacity of Fe₂PO and Fe₂PC complexes, pondering them as drug candidates against free radicals unbalance disorders. Fe₂PO and Fe₂PC deactivated the ABTS synthetic radical with an IC₅₀ value of 38.4 ± 0.9 μM and 28.9 ± 0.2 μM, respectively, while against the DPPH radical, the IC₅₀ value was over the higher concentration tested (>200 μM) for both complexes. As desirable for any drug candidate, none of the metallic complexes (at 25, 50 and 100 μM) induced cytotoxicity on M12.C3.F6 cells (a murine B-cell lymphoma model), but differences in the redox state modulation were observed on the basis of fluorescence detection of a 2',7'-dichlorofluorescein probe by flow cytometry. Cells under normal conditions and preincubated with Fe₂PO and Fe₂PC complexes slightly augmented the reactive oxygen species concentration, meanwhile, cells under stress condition preincubated with H₂O₂ and metallic complexes, showed a higher augmentation in the reactive oxygen species concentration, in comparison to the controls. Finally, a cellular internalisation assay was performed, showing that Fe₂PO and Fe₂PC exert those effects from the outside of the cells. All these results suggest the ability of Fe₂PO and Fe₂PC complexes to selectively increase the reactive oxygen species concentration in cells with a free radical unbalance, without inducing mortality in cells under normal conditions.

Keywords: Fe(III) complexes; Biomimetic; Antioxidant; Cytotoxicity; Cellular redox state; Selective therapy drug

Introduction

The catalytic activity of metalloenzymes is generally associated with the presence of transition metals ions (e.g. Mn, Fe, Co, Ni, Cu and Zn) incorporated into protein structures [1-3]. Metalloenzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (PO), represent the first line of defence against reactive oxygen species (ROS) in the cellular environment. SOD catalyses the conversion of superoxide (O₂^{•-}) to hydrogen peroxide (H₂O₂) and O₂, while the other two enzymes participate in the removal of H₂O₂ from the cellular environment [4,5].

Despite living organisms, under normal conditions, continuously generate ROS as a consequence of a large number of biological processes, ROS can overcome the cellular defence and modulation systems, affecting the redox homeostasis and given as a result oxidative stress [4]. Biological systems achieve these processes by using iron, due to its variable redox potential easily tuned by the coordinating ligand selected. The generation of O₂^{•-} and H₂O₂ as a consequence of aerobic respiration is widely reported, as well as how iron catalyses a cascade of one-electron transfer processes to reduced them [6].

A series of metallic complexes of Fe(III) and Cu(II) [7,8], based on cyclophane macrocycles as scaffolds [9], has been previously synthesised and reported by our research group. Given the structural resemblance of the synthesised Fe(III) and Cu(II) binuclear macrocycle complexes to the active site of some metalloenzymes, we demonstrated the ability of these complexes to act as mimickers of SOD and PO enzymes [7,8]. In addition, these Fe(III) and Cu(II) complexes provided protection to human peripheral blood mononuclear cells (PBMC) under stress conditions by acting as superoxide scavengers [7,8].

On this basis, it is natural to think in these metallic complexes as possible drugs, candidates to treat free radical unbalance disorders. A reason why, the present study aims to get a deeper knowledge regarding the bioactive properties of iron complexes synthesised in our group, by evaluating how they affect the cellular redox state of a murine B-cell lymphoma (M12.C3.F6) cell line model, considering cell integrity after H₂O₂ exposure. In addition to the redox state evaluation, chemical antioxidant assays, cellular toxicity and the ability of the complexes to cross the cell membrane were evaluated.

Materials and Methods

Chemicals

Dimethyl sulfoxide, Dulbecco's modified Eagle's medium (DMEM), sodium bicarbonate (≥ 99.5%), L-asparagine (98%), L-arginine monohydrochloride (≥ 98%), L-glutamine (200 mM solution), sodium pyruvate (100 mM solution), penicillin-streptomycin (1000 units of penicillin and 1 unit of streptomycin per mL), ascorbic acid, methanol, ethanol, L-glutathione reduced (GSH), 2,2-diphenyl-

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1-picryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH DA; $\geq 97\%$) and propidium iodide were purchased from Sigma Chemicals (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) solution (30%) was purchased from J. T. Baker Chemicals. Fetal bovine serum was purchased from Gibco. Caffeic acid phenethyl ester (CAPE) was synthesised in our lab based on the procedure of Grunberger et al. [10], as reported by Hernandez et al. [11].

Synthesis of binuclear iron(III) complexes

To obtain the Fe_2PO and Fe_2PC binuclear iron(III) complexes, we previously synthesised 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, named as PO and PC receptors, respectively. The methodological procedure used was previously reported by Inoue et al. [9]. The purity was confirmed by 1H -NMR ($D_2O/NaCO_3$, pD=9.5, 400 MHz, DSS).

As we reported previously, Fe_2PO and Fe_2PC complexes were obtained by dissolving the PO and PC receptors in water with a minimum amount of solid Na_2CO_3 and mixing both solutions separately with an aqueous solution of $FeCl_3 \cdot 6H_2O$ in a molar ratio of 1:2 [7]. Immediately, the solution derived from the PO receptor turned reddish as an indicator of the complex formation, meanwhile, the solution derived from the PC receptor turned yellowish as the complexation reaction starts. The melting/decomposition point, elemental analysis and high-resolution electrospray ionisation mass spectra were obtained to corroborate the purity of the Fe_2PO and Fe_2PC metallic complexes.

Chemical antioxidant assays

The chemical antioxidant capacity was determined based on the original procedures of ABTS [12] and DPPH [13] assays, with slight modifications. A dose-response curve was constructed employing different concentrations of Fe_2PO and Fe_2PC , and the half-maximal inhibitory concentration (IC_{50}) was reported. An ABTS ethanolic solution was adjusted to 0.7 ± 0.02 absorbance at 754 nm and a DPPH methanolic solution was also adjusted to 0.7 ± 0.02 absorbance at 515 nm. An ABTS radical solution (245 μL) was placed in a 96-well microplate (Costar, USA) and 5 μL sample solutions (12.5, 25, 50, 100 and 200 μM) were added. After 5 min of incubation, the samples were read at 754 nm. Otherwise, 280 μL of DPPH radical was placed in each well of a microplate and samples of 20 μL (12.5, 25, 50, 100 and 200 μM) were also added per well. After 30 min of incubation, the samples were read at 515 nm. In both cases, an Omega spectrophotometer (BMG Labtech Inc., Ortenberf, Germany) was used.

Cellular viability assay

To evaluate the effect of the Fe(III) complexes (Fe_2PO and Fe_2PC) on the M12.C3.F6 cell line (murine B-cell lymphoma), cell proliferation was determined using an MTT assay [14] with some modifications [11]. Briefly, cells (1×10^4 per well, 50 μL) were placed in each well of a 96-well plate (Costar, USA). After 24 h of incubation at 37°C in a 5% CO_2 atmosphere to allow cells attachment, aliquots (50 μL) of the medium containing different concentrations of Fe(III) complexes were added and the cell cultures were incubated for 48 h. Previously, iron complexes were dissolved in Milli-Q water and filtered through a Millipore 0.22- μm filter, and subsequently diluted in the culture medium. We used the cytotoxic drug CAPE as a positive control in

the cellular viability assay. In the last 4 h of the cell culture, 10 μL of an MTT solution (5 mg/mL) was added to each well. The cell viability was assessed by the ability of metabolically active cells to reduce tetrazolium salt to coloured formazan compounds. The formazan crystals were dissolved with acidic isopropyl alcohol. The sample absorbance was measured on a microplate reader (Multiskan EX, ThermoLabSystem) using a test wavelength of 570 nm and a reference wavelength 630 nm. The effect over cell viability of iron complexes was reported as a proliferation percentage to each concentration evaluated.

Cellular redox state evaluation

To evaluate the effect of Fe_2PO and Fe_2PC metallic complexes on the cellular redox state, we used the B-cell lymphoma M12.C3.F6 cell line as a model, due to its oxidative stress sensibility reflected on its cellular morphology [15]. We evaluated the amount of intracellular ROS in M12.C3.F6 cell line after incubation treatment with Fe_2PO and Fe_2PC metallic complexes, in addition to stress induction with H_2O_2 , based on the methodology reported by Wang and Joseph [16], modified [17] and adapted to cell cytometry as reported by Eruslanov and Kusmartsev [4]. M12.C3.F6 cells were seeded in a 12-well plate (Costar, USA) at 2×10^5 cell/mL, and after 24 h of incubation at 37°C in a 5% CO_2 atmosphere, Fe_2PO or Fe_2PC were added. Followed to a 1 h incubation at 37°C in a 5% CO_2 , cells were harvested and washed with cold PBS (pH 7.2) ($575 \times g$, 7 min, 4°C), which were then resuspended in 1 μM of DCFH-DA in cold PBS (pH 7.2). Cells were incubated in the dark for 30 min at 37°C in a 5% CO_2 atmosphere. Afterward, the intracellular oxidative stress was induced using H_2O_2 (1 mM) and incubating cells for 5 min at room temperature. Then, cells were washed with cold PBS (1700 rpm, 7 min, 4°C) and incubated with propidium iodide (1 $\mu g/mL$) for 10 min at room temperature in the dark. Finally, cells were washed with cold PBS (1700 rpm, 7 min, 4°C) and resuspended in cold PBS for analysis, considering the fluorescence of an oxidised DCF probe, according to propidium iodide cell exclusion by flow cytometry (FACS Canto II, Becton Dickinson, CA, USA).

Cellular internalisation assay

In order to determine whether the metallic complexes used in the CAA assays are internalised by M12.C3.F6, we proceeded to detect where both complexes remained after the treatment described here: M12.C3.F6 cells were seeded in a 12-well plate (Costar, USA) at 2×10^5 cells/mL, and after 24 h of incubation at 37°C in a 5% CO_2 atmosphere, Fe_2PO or Fe_2PC complexes were added in 25, 50 and 100 μM concentrations to finally incubate for 1 and 3 h, at 37°C in a 5% CO_2 atmosphere. After incubation, cells were harvested and centrifuged (1700 rpm, 7 min, 4°C). Supernatants were separated and reserved for further analysis. Cell pellets were washed with PBS and resuspended with 1 mL of absolute ethanol, and to achieve complete lysis, 5 min of vortexing was applied. Cell lysates were centrifuged to separate soluble material from both the membrane and proteinaceous aggregates. Finally, supernatant from the culture medium and ethanolic lysed cells solutions were analysed by UV-Vis spectroscopy, to detect where the metallic complexes remained after incubation treatments.

Results and Discussion

Chemical antioxidant assays

To determine the antioxidant effectiveness of Fe_2PO and Fe_2PC , both metallic complexes and antioxidant standards (ascorbic acid, GSH and CAPE) were evaluated by ABTS and DPPH radical assays. For the ABTS assay, both metallic complexes were capable of reaching 50% radical deactivation. Fe_2PC was slightly more effective at

deactivating ABTS radicals in comparison to Fe₂PO, which required higher concentration to achieve the IC₅₀ value (Table 1). Otherwise, by the DPPH assay, an IC₅₀ value of both metallic complexes was not achieved at the tested concentrations. Similar activity behaviour was exhibited by GSH in both assays, with IC₅₀ values consistent to those reported by other authors [18,19]. Ascorbic acid and CAPE were both capable of deactivating ABTS and DPPH radicals in a similar manner, however CAPE showed the highest antioxidant activity with an IC₅₀ value under 12.5 μM in both assays, with the results in agreement with previous reports [20,21].

Antioxidants can deactivate radicals by two mechanisms, hydrogen atom transfer (HAT) and single electron transfer (SET). ABTS and DPPH radicals may be neutralised by either [22,23]. Fe₂PO and Fe₂PC were able to deactivate ABTS radicals in a similar manner, however, DPPH radical deactivation was not efficient enough to inhibit 50% at the tested concentrations. Since Fe₂PO and Fe₂PC deactivate radicals by donating electrons, the IC₅₀ values obtained in the ABTS and DPPH assays represent exclusively the portion of these radicals deactivated by electron transfer. The DPPH radical colour can be dimmed via the HAT and/or SET mechanisms, and the steric accessibility is determinant for its occurrence. Thus, small molecules, like CAPE, have better access to the radical site, showing apparently, a higher antioxidant activity in this assay [24], in comparison to Fe₂PO and Fe₂PC, which are larger and stiffer complexes. Despite GSH being a small molecule, the fact that a second GSH molecule is required to act as an antioxidant, could be the reason for its reduced efficiency in deactivating the DPPH radical.

Cellular viability assay

In order to evaluate whether Fe₂PO and Fe₂PC exert changes over the redox state of M12.C3.F6 cells, we prior determined the appropriate

	Half-maximal inhibitory concentration (IC ₅₀ , μM)	
	ABTS	DPPH
Fe ₂ PO	38.4 ± 0.9	>200.0
Fe ₂ PC	28.9 ± 0.2	>200.0
GSH	<12.5	>200.0
Ascorbic Acid	14.9 ± 0.9	17.7 ± 0.9
CAPE	<12.5	<12.5

Data is expressed as mean of three independent experiments performed by triplicate

Table 1: Chemical antioxidant activity of metallic complexes.

doses of both metallic complexes without achieving cytotoxicity to the cell culture. We evaluated the effect of Fe₂PO and Fe₂PC on cell viability by MTT assay after 48 h of treatment (Figure 1). We found that none of the metallic complexes significantly affected cell viability at any tested concentration (0, 12.5, 25, 50 and 100 μM). At the highest concentration assessed (100 μM), cell culture viability remained over 80% after both complexes treatments. The control, CAPE, induced a strong antiproliferative effect over M12.C3.F6 cells, even at the lowest tested concentration (0.63 μM), reducing cell proliferation by approximately 40%.

These results are in agreement with previous reports of our group, where the cytotoxicity of Fe₂PO and Fe₂PC was evaluated in PBMC, obtaining mortality percentages under 1% after 24 h of incubation at similar concentrations tested here [7]. These slight differences observed in proliferation percentages could be due to a higher sensibility of M12.C3.F6 cells, in comparison to PBMC.

CAPE, an esterified derivative of caffeic acid, was used as an antiproliferative control compound, since it is a previously reported antiproliferative compound, as well as an effective antioxidant [11,15]. CAPE showed a reduction of M12.C3.F6 cell proliferation in 50% at a concentration of ~1 μM, suggesting for these data of CAPE, the low antiproliferative effect of Fe₂PO and Fe₂PC at 100 μM.

Cellular redox state evaluation

With the aim of assessing whether Fe₂PO and Fe₂PC complexes induce changes over the M12.C3.F6 cells redox state, we incubated them with different concentrations of metallic complexes, followed or not by oxidative stress induction (H₂O₂). Cells under normal conditions (with no H₂O₂ added) and treated with Fe₂PO and Fe₂PC (25, 50 and 100 μM) showed a slight increase in intracellular ROS amount, according to the MFI values, in comparison to basal control. A dose-response relationship was not observed for Fe₂PO treatment, however, as the concentration increased for Fe₂PC treatment, so did the intracellular ROS concentration. Meanwhile, M12.C3.F6 cells pre-treated with cyclophane complexes and then stressed with H₂O₂, exhibited a significant increase in intracellular ROS concentration in all tested concentrations, in comparison with basal M12.C3.F6 cells, H₂O₂-stressed cells, as well as with cells pre-treated only with Fe₂PO and Fe₂PC (Table 2). CAPE and water were used as reference and dissolvent controls, respectively [15].

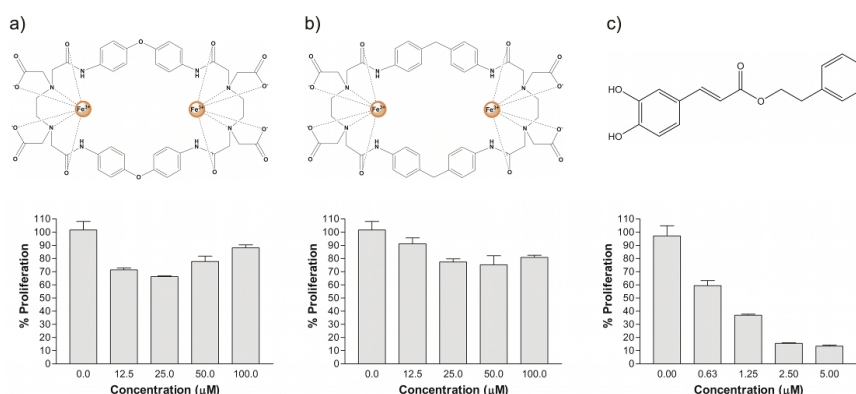


Figure 1: Proliferation percentages of M12.C3.F6 cells cultured with a) Fe₂PO, b) Fe₂PC and c) CAPE as a control, at a different concentrations.

Modifications in the M12.C3.F6 cells redox state, due to the presence of Fe₂PO and Fe₂PC resulted interesting, specifically, in those cells that were pre-treated with metallic complexes, followed by oxidative stress induction with H₂O₂. These changes in the cellular redox state could be related to that observed in some studies with cancer cells [25], where systems with similar characteristics, exhibit a major susceptibility under stress conditions to accumulate ROS, ROS-promoters and/or compounds capable of modifying the redox state of the system, such as Fe₂PO and Fe₂PC.

It is important to note that at the time of the oxidative stress evaluation, a remarkable cellular viability was observed through a propidium iodide staining assay in all experimental conditions tested. Fe₂PC showed a dose-response relationship, increasing the MFI value with increasing concentration, while Fe₂PO did not. Differences in MFI values between Fe₂PO and Fe₂PC could be attributable to the higher structural flexibility of Fe₂PO, facilitating the interaction between Fe(III) centres with the medium. Similar results were previously obtained by evaluating the immune response of human embryonic kidney (HEK) 293T cells treated with Fe₂PO and Fe₂PC. After 8 h of incubation, cells augmented the expression of genes related to oxidative stress and inflammation, but after 24 h of incubation, cells regulated themselves by expressing anti-inflammatory genes, resulting in non-cytotoxic treatment.

Cellular internalisation assay

Since metallic complexes, especially Fe₂PO, modify the redox basal state of M12.C3.C6 cells, it was necessary to establish where this effect was taking place, inside or outside the cells. Supernatant culture media after Fe₂PO and Fe₂PC metallic complexes treatment were collected by centrifugation, therefore, cell pellets were lysed to obtain ethanolic solutions. UV-Vis spectra of supernatants and cell lysates were recorded. Supernatant culture media of both complexes at all tested conditions (25, 50 and 100 μM, after 1 and 3 h incubation), described a similar electronic spectrum to those obtained by Fe₂PO and Fe₂PC reference solutions (25, 50 and 100 μM), indicating that most of the Fe₂PO and Fe₂PC metallic complexes remained in extracellular milieu and were not internalised (Figure 2). In contrast, for ethanolic lysed cell solutions, none of the recorded spectra described the spectra observed for the references, at any of the tested conditions (Figure S1).

Considering the structural features, rigidity and charges of Fe₂PO and Fe₂PC metallic complexes, in addition to the relatively short period treatment, a limited or none cell internalisation capacity was expected for both complexes. The UV-Vis spectra showed that Fe₂PO and Fe₂PC remained in culture media after incubation treatment and did not in ethanolic cell lysates, concluding that, even when both metallic complexes do not cross the cell membrane, they keep its capacity to modify the cellular redox state of M12.C3.C6 cells, under conditions here tested.

Sample		MFI	
Basal ^a		1315 ± 166	
H ₂ O ₂ ^b		2743 ± 237	
Fe ₂ PO	no H ₂ O ₂		
	25 μM	1711 ± 228	3478 ± 44
	50 μM	2276 ± 174	3443 ± 19
Fe ₂ PC	100 μM	1955 ± 103	5417 ± 73
	25 μM	1078 ± 10	3573 ± 8
	50 μM	1151 ± 27	3723 ± 163
	100 μM	1579 ± 91	2470 ± 165

^aBasal represent cells with no stressor or metallic complex treatment. ^bH₂O₂ represents cells with H₂O₂ 1 mM and no metallic complex treatment. Incubation time, 1 h. CAPE 5 μM treatment was employed as antioxidant positive control (MFI of 449). Water was used as dissolvent control (treatment v/v %) with an MFI value of 883. Mean fluorescence intensity (MFI) is expressed in fluorescence intensity arbitrary units (FIAU). Fluorochromes employed: dichloro-dihydro-fluorescein-diacetate (DCFH-DA) and propidium iodide (PI).

Table 2: Cellular redox state evaluation of Fe₂PO and Fe₂PC iron complexes on M12.C3.F6 cells.

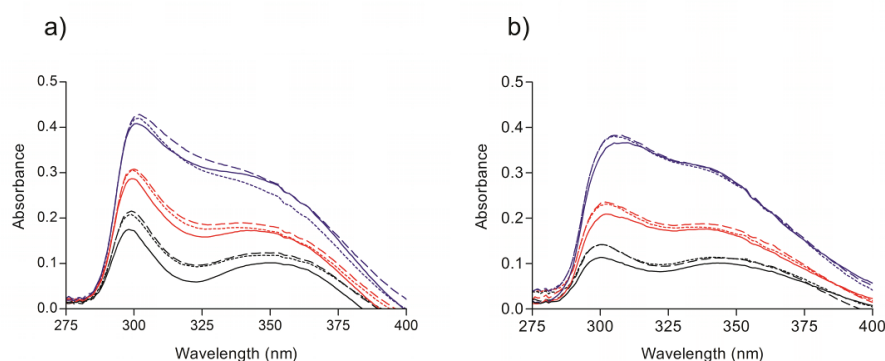


Figure 2: Cellular internalisation assay. Supernatant culture medium (DMEM) after metallic complex treatment. Panel a) Fe₂PO and panel b) Fe₂PC. Reference solutions (metallic complex dissolved in culture media) are described by solid lines, while dashed and dotted lines represent 1 and 3 h incubation treatments with metallic complexes, respectively. Black spectra represent a concentration of 25 μM metallic complex treatment; red spectra a concentration of 50 μM and blue spectra a concentration of 100 μM.

Conclusions

Based on the structural and electrochemical features of Fe₂PO and Fe₂PC, the ABTS antioxidant assay was more appropriate for determining their radical scavenger capacity in comparison with the DPPH antioxidant assay. Nevertheless, chemical assays do not reflect what really occurs in a cell system, concerning the redox state. Even when both metallic complexes remained outside M12.C3.F6 cells, intracellular changes in redox state were observed, selectively increasing the reactive oxygen species concentration in cells under induced oxidative stress, but without cytotoxicity effects over cell under normal conditions.

The free radical scavenger capacity comprises a wide range of applications in different industries. The fact that Fe₂PO and Fe₂PC complexes exert changes in cellular redox state without inducing cytotoxicity, place them as drug candidates in a new field of investigation to be explored. The selective therapy against cells under inflammatory processes is a fast growth area, with opportunities for complexes with a variety of mechanisms of action, including not only elimination of ROS but also its generation as well, since it is just the biological features of ROS what gives them the capacity to be used as tumour cells killers.

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