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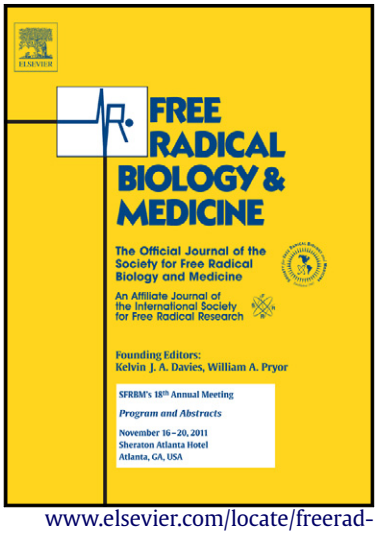

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Iron, copper and manganese complexes with *in vitro* superoxide dismutase and/or catalase activities that keep *Saccharomyces cerevisiae* cells alive under severe oxidative stress

Authors: Thales P. Ribeiro[1], Christiane Fernandes[2], Karen V. Melo[2], Sarah S. Ferreira[2, #], Josane A. Lessa[2, £], Roberto W. A. Franco[3], Gerhard Schenk[4], Marcos D. Pereira[1*], Adolfo Horn Jr.[2*]

1 - Laboratório de Citotoxicidade e Genotoxicidade, Departamento de Bioquímica, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-909, RJ, Brazil.

2 - Laboratório de Ciências Químicas, Universidade Estadual do Norte Fluminense Darcy Ribeiro – Campos dos Goytacazes, 28013-602, RJ, Brazil.

3- Laboratório de Ciência Físicas, Universidade Estadual do Norte Fluminense Darcy Ribeiro – Campos dos Goytacazes, 28013-602, RJ, Brazil.

4- School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, 4072, Australia

* To whom correspondence should be addressed. E-mail: marcosdp@iq.ufrj.br (M. D. Pereira); adolfo@uenf.br (A. Horn Jr.).

Actual address: Instituto Federal Fluminense, Campos dos Goytacazes, 28030-130, RJ, Brazil

£ Actual address: Instituto de Química, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 20559-900, RJ, Brazil

Abstract

Due to their aerobic life style, eukaryotic organisms have evolved different strategies to overcome oxidative stress. The recruitment of some specific metalloenzymes such as superoxide dismutases (SODs) and catalases (CATs) is of great importance to eliminate harmful reactive oxygen species (hydrogen peroxide and superoxide anion). Using the ligand HPClNOL {1-[bis(pyridin-2-ylmethyl)amino]-3-chloropropan-2-ol}, we have synthesized three coordination compounds containing iron(III), copper(II) and manganese(II) ions, which are also present in the active site of the above mentioned metalloenzymes. These compounds were evaluated as SOD and CAT mimetics. The manganese and iron compounds showed both SOD and CAT activities, while copper showed just SOD activity. The copper and manganese *in vitro* SOD activities are very similar (IC_{50} ~ 0.4 μmol dm^{-3}) and about 70-fold higher than the iron one. The manganese compound showed CAT activity higher than the iron species. Analyzing their capacity to protect *Saccharomyces cerevisiae* cells against oxidative stress (H_2O_2 and the $O_2^{\bullet-}$ radical), we observed that all compounds act as antioxidants, increasing the resistance of yeast cells mainly due to a reduction of lipid oxidation. Especially for the iron compound, the data indicate complete protection when wild-type cells were exposed to H_2O_2 or $O_2^{\bullet-}$ species. Interestingly, these compounds also compensate for both superoxide dismutase and catalase deficiencies; their antioxidant activity is metal ion-dependent, in the order iron(III) > copper(II) > manganese(II). The protection mechanism employed by the complexes proved to be independent of the activation of transcription factors (such as Yap1, Hsf1, Msn2/Msn4) and protein synthesis. There is no direct relation between the *in vitro* and *in vivo* antioxidant activities.

INTRODUCTION

Reactive oxygen species (ROS) are chemical species derived from molecular oxygen[1, 2]. Their production has been related to different factors such as a mitochondrial function (*i.e.* incomplete dioxygen reduction), physiological responses (*e.g.* cellular signaling, gene expression, regulation of the immune response and anti-oxidative defense mechanism) and environmental exposure (*e.g.* UV radiation, microbes, allergens, ozone, cigarette smoke, polycyclic aromatic hydrocarbons, photochemical smog, industrial chemicals, metabolism of xenobiotics)[3]. The best characterized and studied forms of ROS are the superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which can generate the highly reactive hydroxyl radical ($HO^{\bullet}$). Despite playing key roles in biological processes such as the immune response, cell signaling and gene expression[4], these oxidants, especially under oxidative stress, also have deleterious effects on cellular components, resulting in lipid, protein and DNA oxidation[1,2,5].

In general, oxidative stress is characterized by a disruption of cellular redox homeostasis due to the inability of the antioxidant defense systems and cellular survival mechanisms to cope with ROS production[5]. Currently, several pathophysiological events such as ischemia, diabetes, cancer, reperfusion-related injuries (*e.g.* heart attack, stroke and organ dysfunction), as well as neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases have been associated with an increase in ROS generation and concomitant oxidative stress[6-8].

As an efficient defense strategy to ameliorate the toxic effects of ROS, aerobic organisms have evolved a number of efficient metalloenzymes, including glutathione peroxidase (GPx), catalases (CATs) and superoxide dismutases (SODs)[6,7]. While GPx and CATs utilize H_2O_2 as substrate, SODs promote superoxide dismutation. GPx is a selenium-containing enzyme[9], while CAT possesses an iron(III) protoporphyrin IX or a

dinuclear manganese active site[10,11]. In SODs, iron, manganese, copper/zinc or nickel are found in the active site[12-15]. SOD is believed to be the main agent to control deleterious effects of the superoxide anion *in vivo*. However, several technical limitations (large size, low cell permeability, short circulating half-life, antigenicity, high manufacturing costs) have limited the use of SOD as a potential clinical drug[16].

An alternative to the use of antioxidant enzymes is the design and development of synthetic low molecular weight compounds which may mimic the effects of the corresponding enzymes[17]. Some biomimetics with the capability to decompose reactive oxygen species generated during oxidative stress have already been reported; examples include systems that use porphyrins, salen, non-aromatic macrocycles or corroles as metal ion ligands[18-23].

Saccharomyces cerevisiae has been reported as an ideal eukaryotic model for oxidative stress studies[24]. Its relevance to human diseases is well established by the conserved genome and cellular biology[25]. Its accessibility to analysis, scalability, short generation time and genetic tractability have led to *S. cerevisiae* being one of the most common eukaryotic model systems used in studies related to cellular processes[25]. *S. cerevisiae* responds to oxidative stress mainly due to regulators that sense the increase of intracellular ROS, for instance Yap1, the Yeast AP-1 transcription factor, a homolog of human AP-1, which ensures the survival of the cell despite its exposure to oxidants[25,26]. This regulator induces the expression of a complex array of antioxidant factors (enzymatic and non-enzymatic), which are responsible for (i) ROS detoxification, (ii) the reduction of the ROS production rate and (iii) the repair of damages in macromolecules[25,26].

Our group has previously described an Fe(III) complex, $[Fe(HPClNOL)Cl_2]NO_3$, with SOD and CAT activity *in vitro*. This compound was also able to reduce the levels

of lipid oxidation *in vivo,* increasing resistance of *S. cerevisiae* strains against the harmful effects of H_2O_2 stress[27]. Here, we investigated this complex's potential to act as a SOD and CAT mimic. In addition, copper(II) and manganese(II) derivatives of this complex were also tested for their both mimetic activity *in vitro* and *in vivo* using the yeast *S. cerevisiae*. We furthermore aimed to clarify the mechanism with which these synthetic antioxidant mimics protect yeast cells during oxidative stress.

METHODS

Syntheses of the compounds

The synthesis of the ligand HPClNOL was previously described by our group[28], as well as its complexes with iron (**1**) and copper (**2**)[27,29].

Synthesis of the [Mn(HPClNOL)$(Cl)_2$] (**3**): An ethanolic solution (5 cm^3) of $Mn(H_2O)_4(Cl)_2$ (0.396 g) was added to 0.583 g of the HPClNOL ligand. A precipitate formed immediately, which was filtered and washed with ethyl ether. The solid was recrystallized in hot methanol, resulting in a microcrystalline solid. Yield: 0.670 g (80 %). Analysis (calcd., found for $MnC_{15}H_{18}N_3OCl_3$): C (43.14, 43.34), H (4.34, 4.54), N (10.06, 9.81); $\Lambda_{M(DMSO)}$ = 38 $cm^2\ \Omega^{-1}\ mol^{-1}$.

Yeast strains and growth conditions

Wild type strains of *S. cerevisiae* BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and EG103 (*MATa; leu2-3, 112; his3Δ1; trp-289a; ura3-52; GAL*$^+$) and their isogenic mutants *sod1*Δ (BY4741 isogenic except *YJR104c::kanMX4*) and *ctt1*Δ (EG103 isogenic except *YGR088w::URA3)* were used in this work to evaluate whether the complexes possess antioxidant SOD or CAT activities, respectively. Strains were acquired from Euroscarf, Frankfurt, Germany (BY4741) and as a kind gift from Dr.

Edith B. Gralla, University of California, Los Angeles, California, USA (EG103). The mutant strain, *yap1Δ*, isogenic to BY4741, was also used to probe the effect of the metal complexes. Stocks of these strains were maintained on solid 2 % YPD (*i.e.* 1 % yeast extract, 2 % glucose, 2 % peptone and 2 % agar) under appropriate conditions to avoid selection of petite (ρ-) mutants or suppressors. For all experiments cells were grown to mid-log phase (0.8 mg dry weight cm^{-3}) in liquid 2 % YPD medium (no agar) or 4 % YPGLY (*i.e.* 1 % yeast extract, 4 % glycerol, 2 % peptone) using an orbital shaker at 28 °C and 160 rpm, with a ratio of flask volume/medium of 5/1.

Evaluation of the cytotoxicity of the metal complexes

In order to evaluate the cytotoxicity of the model systems, *S. cerevisiae* cells cultured under fermentative or respiratory conditions, were exposed to increasing concentrations (25 to 500 μmol dm^{-3}) of each of the complexes **1**, **2**, and **3**, for 60 min at 28 °C and 160 rpm. Subsequently, cell survival was analyzed by plating, after proper dilution (1,000 times) of the cell concentration and in triplicates, on solidified 2 % YPD medium. The plates were incubated at 28 °C for 72 h before colonies were counted. Survival was expressed as the percentage of viable cells. Percentage was determined by the ratio between the average number of colony forming unit (C.F.U) in stressed condition (complex treated or not) and the average of the number of C.F.U in control condition (not stressed).

Oxidative stress conditions

Cells at mid-log phase (0.8 mg dry weight cm^{-3}), either in glucose (2% YPD) or glycerol (4% YPGLY), were directly exposed to severe oxidative stress for 60 min at 28 °C and 160 rpm. In this work, hydrogen peroxide (Reagen, Brazil) and menadione,

2-methyl-1,4-naphtoquinone: vitamin K3 (Sigma, Ref. M5750, batch 116K0749, USA), a superoxide ($O_2^{\bullet -}$) radical generator, were used to induce oxidative stress. In all experimental conditions, the cultures were divided into two groups: one was immediately exposed to stress conditions, while the other was pre-incubated with the complexes. For adaptive treatments, cells harvested at the first exponential phase of growth were treated with 25 μmol dm^{-3} of complex for 60 min at 28 °C and 160 rpm, washed twice with cold distilled water to remove the excess complex from the medium, and then exposed to oxidative stress. While for the wild type BY4741 strain and its isogenic mutant *sod1*Δ menadione was used as oxidative stress inducer (30 mmol dm^{-3} and 20 mmol dm^{-3}, respectively), for the wild type EG103 strain and its mutant *ctt1*Δ hydrogen peroxide was chosen as the stressor agent (40 mmol dm^{-3} and 30 mmol dm^{-3}, respectively). Cell viability was analyzed by plating, after proper dilution of the cell concentration and in triplicates, on solidified 2 % YPD medium. The plates were incubated at 28 °C for 72 h and the colonies were counted. Survival was again expressed as the percentage of viable cells. Cycloheximide at concentrations previously shown to inhibit protein synthesis was also used prior to treatment with the metal complexes[30-32]. Cells at the early exponential phase of growth were exposed to 0.5 mg cm^{-3} of cycloheximide for 2 h, incubated with metal complexes as described above, and then stressed with H_2O_2 (40 mmol dm^{-3}/1 h) or menadione (30 mmol dm^{-3}/1 h). After cycloheximide exposure, cell viability and survival were analyzed as described above.

Detection of Lipid Peroxidation

Lipid peroxidation was determined using the TBARS (thiobarbituric acid reactive species) method, which detects malondialdehyde (MDA) as previously described by Steels[33].

Involvement of metal complex with heat shock response and trehalose accumulation

Severe heat shock was performed by subjecting 5 cm^3 of a culture in the early exponential phase, adapted or not with either mild heat (40 °C/1 h) or metal complexes (25 µmol dm^{-3}/1 h), to 51°C for 8 min. Cell viability and survival were analyzed as described above. Trehalose measurements were performed by first extracting 12 mg (dry weight) of early exponential cells, adapted or not with either heat or metal complexes, with 0.5 mol dm^{-3} of trichloroacetic acid (TCA). Trehalose was then determined using the anthrone method[34].

Measurements of *in vitro* SOD and CAT activities

The SOD activity of the complexes was evaluated employing a modification of the nitrobluetetrazolium (NBT) method[35], using xanthine/xanthine oxidase as source of the superoxide anion. In this method, xanthine oxidase reacts with xanthine, resulting in the production of superoxide anion at a constant rate. The superoxide anion promotes the reduction of NBT to formazan, which can be monitored spectrophotometrically at 560 nm. All the solutions were prepared in phosphate buffer (50 mmol dm^{-3}, pH = 7.8). In a typical experiment, cuvettes containing NBT (56 µmol dm^{-3}, 1 cm^3), xanthine (0.45 mol dm^{-3}, 0.5 cm^{-3}), complex (25-200 mm^3), phosphate buffer (0.5 – 0.7 cm^3) and xanthine oxidase (0.2 U cm^3, 0.1 cm^3) were mixed. The change in the absorption was monitored over 30 min. The absorption reached a maximum after 20 min and the absorption at 25 min was used to calculate the $IC_{50.}$ The experiments were done in triplicates and the IC_{50} was calculated from a linear regression analyses, employing five different complex concentrations[36,37].

The catalase (CAT) activity was determined from the evaluation of the volume of oxygen produced by the reaction of the complexes with H_2O_2 as described previously[38].

EPR studies

The yeast cells, at mid-log phase, were mixed in YPD growth medium with 100 μmol dm^{-3} of each of the compounds or with the corresponding chloride metal salts, for 60 min at 28 °C and 160 rpm. After treatments, 30 mg of cells were harvested and washed twice with distilled sterile water. Then, the pellets were collected on a filter paper, and left to dry at room temperature overnight for drying. Following this procedure, samples were analyzed by electron paramagnetic resonance using a Bruker E500 spectrometer with a high sensitivity cylindrical cavity, operating at X-band (9.7 GHz) at 100 K with 10 mW microwave power, 10 G modulation amplitude, and 60 dB receiver gain. The spectra were recorded for samples containing about 20 ± 2 mg of dried cells.

RESULTS

Syntheses and characterization of the model complexes

Since we have previously shown that the iron(III) complex synthesized with the ligand HPClNOL {1-[bis(pyridin-2-ylmethyl)amino]-3-chloropropan-2-ol} has both *in vitro* SOD and CAT activities, and it is able to protect *S. cerevisiae* cells exposed to H_2O_2, we were interested in investigating whether complexes containing manganese or copper metal ions instead were also able to protect yeast cells from oxidative stress generated by H_2O_2 or $O_2^{\bullet-}$ radicals. In particular, we wanted to evaluate which of these

metal complexes (iron, copper or manganese) would exert the highest degree of protection against oxidative stress.

Among the three complexes employed in this study (Fig. 1) only the manganese compound was isolated as a powder. The molecular structure for the iron and copper complexes were published previously[27,29].

The UV-Vis spectrum of the manganese complex is characterized by the lack of absorption bands in the visible range at concentrations up to $1x10^{-3}$ mol dm^{-3}, suggesting the presence of paramagnetic high-spin d^5 Mn(II) species. The conductivity of a $1x10^{-3}$ mol dm^{-3} solution of the manganese complex suggests a neutral species, indicating that chloride ions are coordinated to the metal center. Cyclic voltammetry of the manganese complex in acetonitrile indicated two *quasi*-reversible redox process at $E_{1/2}$ = 0.292 V and 0.549 V vs. Fc/Fc^+, which are associated with the Mn^{II}/Mn^{III} and the Mn^{III}/Mn^{IV} redox couples, respectively. These redox potentials are close to those observed for a similar manganese complex, $Mn(HPClNOL)(NO_3)_2$[39], which contains nitrate instead of chloro ligands ($E_{1/2}$ = 0.250 and 0.520 V vs. Fc/Fc^+). Since the x-ray structure of the latter complex indicates the presence of a mononuclear species[39] it appears likely that $Mn(HPClNOL)Cl_2$ is also mononuclear, which is in agreement with the CHN elemental analysis result.

Toxicity and *in vivo* antioxidant activity

Initial tests whereby *S. cerevisiae* cells were exposed to increasing concentrations of the three metal ion complexes (up to 500 µmol dm^{-3}) demonstrated the absence of intrinsic cytotoxic effect of these complexes after 60 min as well as after 24 h of incubation.

Prior to inducing oxidative stress, anaerobic or aerobic cultures of *S. cerevisiae* cells were treated with 25 μmol dm^{-3} of each of the three compounds for 60 min. Subsequently, after a washing step, the cells were exposed to H_2O_2 or menadione (source for the O_2^- radical). Figure 2 illustrates that oxidative stress severely affects yeast cells that were not pre-incubated with these complexes, reducing their survival rates to less than 20 % after direct exposure to menadione or H_2O_2. In contrast, *S. cerevisiae* cells pretreated with the complexes exhibit sharply increased resistance to oxidative stress. Remarkably, for the wild type strain BY4741 (Figure 2a). The pretreatment using the Fe(III) complex leads to an increase in cell survival from 10 to 100% upon exposure to menadione. For the Cu(II) and Mn(II) complexes, the survival rates were somewhat lower than that for the Fe(III) complex but still well above untreated cells (*i.e.* 71 % and 57 %, respectively). As a control, treatment of yeast cells with metal salts did not lead to any improvement of the cells' resistance against oxidative stress, thus suggesting that the protective effect on *S. cerevisiae* cells is related with the presence of the complexes.

The residual survival rate of untreated BY4741 cells (Figure 2a) is due to the presence of the enzyme Cu/Zn-SOD (*SOD1*). Removal of the *SOD1* increases the sensitivity towards the effects of the $O_2^{\bullet-}$ radical drastically[40], as illustrated in Figure 2a; the survival rate of the *sod1Δ* mutant strain is essentially zero. However, its survival rates were strongly increased after adaptive treatments with the metal complexes, confirming the antioxidant potential of these compounds (Figure 2a). A similar trend in metal ion efficiency as for wild-type yeast cells was observed (Fe(III)>Cu(II)>Mn(II)).

The above experiments were repeated under aerobic conditions using the *S. cerevisiae* strain EG103 and its catalase-deficient mutant *ctt1*Δ. As oxidative stress inducer H_2O_2 was employed. Again, the survival rates of cells pre-treated with the three

complexes were compared to those that were left untreated (Figure 2b). H_2O_2 stress dramatically decreases the survival rate of the yeast cells, especially that of the CAT-deficient mutant strain. In contrast, the pre-treated cells again displayed a sharp increase in oxidative stress resistance. The iron compound was capable to restore the survival rates of both the wild-type and mutant strains to 100%. The Cu(II) complex was also very efficient in negating the effect of H_2O_2 stress, in particular in wild-type cells, while the Mn(II) compound was the least effective system (but still with remarkable efficiency, increasing the survival rates of wild-type and mutant EG103 to ~85% and ~50%, respectively).

In summary, these above results demonstrate that the metal complexes protect yeast strains against severe oxidative stress, presumably by mimicking SOD and CAT activities, the main protective antioxidants in the cells.

***In vitro* antioxidant activity**

The impressive protective effect of the metal complexes described above maybe due to their *in vitro* intrinsic capability to act as SOD and CAT mimetics. Indeed, with respect to SOD activity (see supplementary material), the manganese and copper complexes are rather efficient catalysts, with IC_{50} values of 0.35 ± 0.01 and 0.43 ± 0.02 μmol dm^{-3}, respectively. A review including related IC_{50} values for a set of manganese complexes has been published recently[23]. Although the most efficient compounds have IC_{50} values varying from 6 to 45 nmol dm^{-3} (manganese-pyridinium-porphyrin family), our manganese compound described here has significantly higher activity than the manganese-salen derivatives (EUK family, IC_{50} ~ 1 μmol dm^{-3}), a well-studied antioxidants SOD mimic. As for the copper complex, its activity is considerably higher than that reported for complexes containing the ligand Br-SAA (5-

bromosalicylideneanthranilic acid; IC_{50} ~ 12-25 μmol dm^{-3}) and only about ten-fold less active than native SOD (0.040 μmol dm^{-3})[41]. The iron complex was the least active in our series with an IC_{50} of 26.8 ± 2.5 μmol dm^{-3}, a value that lies within the range reported for a set of iron complexes containing ligands based on ethylenediame, propanediamine or triazocyclononane backbone (IC_{50} ~ 0.5 - >100 μmol dm^{-3})[42].

With respect to CAT activity, the formation of bubbles was observed when H_2O_2 was added to solutions of the iron and manganese complexes, but not for the copper complex. The time course of dioxygen production is shown in the supplementary material.

In order to determine the rate law of the reaction, the initial rates method was applied. Pseudo-first order kinetic measurements indicated that the rate laws are of the form $v = 2.87x10^{-4}[iron]^{0.84}[H_2O_2]^{1.28}$ and $v = 1.69 \times 10^{3}$ $[manganese]^{2.27}[H_2O_2]$ for the iron and manganese complexes, respectively, suggesting a first order reaction with respect to the metal ion for the iron complex, but a second order reaction for the manganese complex (both reactions are first order with respect to peroxide). This observation indicates that the two metal ion compounds employ distinct mechanisms. While a detailed characterization of these mechanisms is beyond the scope of the current study it is apparent that the CAT activity of the manganese complex is higher than that of the iron compound.

EPR studies

The SOD and CAT activities measured *in vitro* suggest that the Mn(II) complex may be the most reactive, but it affords the least efficient antioxidative protection when compared to its Fe(III) and Cu(II) counterparts (*vide supra*). In an attempt to probe if the complexes may undergo structural changes in an *in vivo* environment we carried out

EPR analyses of *S. cerevisiae* cells incubated with or without the complexes or their corresponding Mn/Cu/Fe-chloride salts (Figure 3). At first glance, it is evident that the cells that underwent treatment with complexes or salts show EPR spectra distinct from that of the cells without treatment; the only exception is observed after treatment with the copper complex that does not lead to any significant change when compared to the untreated cells. Furthermore, the EPR spectra from the cells treated with the complexes are also different from those incubated with the chloride salts.

The EPR spectrum of untreated *S. cerevisiae* shows a broad band (ΔH ~ 2000 G, g = 2.7) and a low intensity signal at g = 4.3, which may be associated with the presence of isolated high spin iron(III) species[43]. The cells treated with the manganese complex and $MnCl_2$ show a well-defined six line signature spectrum at g = 2, which is typical for isolated Mn^{2+} ions[44]. The cells treated with the complex and with the chloride salt also show a broad line at 2.7 (ΔH ~2000 G), whose intensity is considerably large than that in the untreated cell samples. However, with manganese chloride treatment this spectral feature has a more distorted shape than in cells treated with the Mn complex, revealing structural differences *in vivo*.

In the presence of the copper complex, the EPR spectrum of the cells is very similar to that of the untreated cells. No evidence of a Cu^{2+} signal is observed, neither after treatment with the complex nor the $CuCl_2$ salt. A similar observation was reported by Lindahl and co-workers[43], where *S. cerevisiae* cells containing copper concentrations as high as 500 μmol dm^{-3} no EPR signal due to Cu^{2+} was evident, suggesting that the copper ion is either in the reduced form (Cu^{+}) or in a spin-coupled state, which may result in a EPR-silent behavior. Here, in the presence of $CuCl_2$, the cells showed a broad line at g = 2.7, ΔH ~2000 G similar to that observed in the treatment employing the

manganese systems. In the cells treated with the copper complex, this feature is considerably less intense, but due to the similarities observed between the manganese and copper-systems it appears unlikely that this feature is related to the metal ions directly.

With respect the cells treated with the iron complex and iron chloride, an obvious difference is the intensity of the signal associated with iron(III), which is at least seven-fold higher for the latter. This indicates that the uptake of the iron complex and iron chloride may be different or that a proportion of the iron complex underwent reduction to iron(II). A line at g = 2 was observed in both samples, typical of iron systems with cubic symmetry, which has been assigned as iron nanoparticle, whose nature remains unestablished[44]. The spectrum of the cells exposed to iron chloride is dominated by a broad line (g = 2.7, ΔH ~2000 G), indicating the presence of a strong spin-spin interaction. In contrast, the cells treated with the iron complex show prominent signals at g = 4.3 and g = 6, indicating the presence of isolated high spin iron(III) sites with rhombic symmetry[43].

In summary, while it is currently not possible to establish unambiguously if the metal complexes remain structurally unchanged inside the cells, the EPR analyses indicate that the metal ions do not dissociate from the ligands (which would result in spectral data similar to those recorded for the corresponding metal ion salts).

Evaluating if the compounds induces endogenous antioxidant response

While the EPR analysis in the previous section demonstrated that the complexes do not disintegrate in the cellular environment it does not provide insight into the mode

of action of these complexes *in vivo*. Thus, a possible association between the resistance to oxidative stress and endogenous effects induced by the complexes was probed.

During stress conditions such as oxidative stress, the control of cellular redox homeostasis is strongly dependent on the activation of transcription factors such as Yap1, Hsf1 (Heat shock transcription factor 1), Msn2 and Msn4 (Multicopy suppressors of the SNF1 mutation), all of which are involved in the regulation of the expression of protective genes; more than 500 genes are up-regulated in response to H_2O_2 treatment[45].

Therefore, in order to analyze whether the antioxidant activity of the mimics involves the induction of the transcription of protective genes, the response of the wild-type BY4741 strain and its isogenic mutant *yap1Δ* were evaluated under H_2O_2 stress conditions; the latter is deficient in the Yap1 transcription factor. As shown in Figure 4a the absence of Yap1 is critical for cells' survival against H_2O_2 stress; its absence reduces the survival rate of the cells several fold, to less than 3%. However, the pretreatment with antioxidant mimics led again to a significant rise in the survival rates of both wild-type and mutant strains. Interestingly, this effect was approximately three-fold greater in the mutant than in the wild-type cells (Figure 4b). For example, the iron compound increased the resistance to oxidative stress in wild-type cells about five-fold when compared to the control, while the same compound increased the survival rate of the mutant cells 15-fold.

In addition to Yap1, transcription factors such as Hsf1, Msn2 and Msn4 also contribute to the general stress response of an organism, including a response to heat shock, as well as oxidative, ethanol and osmotic stresses[45,46]. The activation of these transcription factors regulates the expression and synthesis of heat shock proteins, trehalose and antioxidant proteins (*e.g.* CAT), which are all involved in cellular protection during stress[5]. In order to test whether the three antioxidant mimics in the

study may activate Hsf1, we tested the capacity of the wild-type EG103 strain, untreated or pretreated with antioxidant mimics, to survive a severe heat shock (51 °C/8 min). As a control, we employed cells heat-treated at 40 °C/1 h, an established condition to induce cell adaptation via Hsf1 activation; under such conditions the synthesis of protective factors such as heat shock proteins, trehalose and CAT is promoted, which increases resistance to a severe heat shock such as an eight-minute exposure at 51 °C[5]. Figure 5a shows that the pre-treatment at 40 °C/1 h indeed induced resistance of EG103 cells to heat shock. However, the complexes failed to confer any protection against heat shock, likely due to the inability to activate Hsf1.

We have further studied the influence of the antioxidant mimics on the induction of trehalose synthesis, which is closely associated with Msn2 and Msn4 activation[47]. Trehalose is a disaccharide involved in the protection mechanism of yeast cells to heat, osmotic and ethanol stresses[48]. Figure 5b shows that the mimetic compounds do not activate the synthesis of trehalose. Together, these experiments indicate that the antioxidant protection of the complexes do not involve the activation of transcription factors (Hsf1, Msn2 and Msn4).

Since the results show that the metal complexes act independently of stress-related transcription factors, we carried out experiments in the presence of cycloheximide, an inhibitor of protein synthesis[30-32], to ascertain that the protective effect of the compounds do not involve the synthesis of any other protection protein. Figure 6 illustrates that indeed the yeasts cells continue to show resistance to oxidative stress even after cycloheximide exposure. To summarize, the combined data above indicate that the acquired tolerance of the *S. cerevisiae* cells to oxidative stress is not an endogenous cellular response to the presence of the coordination compounds.

Effect of the treatment on the cell membrane

During oxidative stress, lipids embedded in cell membranes are the primary targets for ROS (lipid peroxidation), which often leads to a loss of membrane function; for example a loss of selectivity may cause indiscriminate permeation of crucial or undesirable metabolites through the membrane[40,49]. Thus, in order to evaluate whether the metal complexes are able to protect lipids in the cell membrane, we evaluated the levels of lipid peroxidation by measuring the MDA content after oxidative stress in the absence or presence of the mimics. As shown in Figure 7a, in the absence of the complexes, wild-type BY4741 cells display a two-fold increase in lipid peroxidation upon inducing oxidative stress, while in the *sod1Δ* mutant a three-fold increase of the MDA levels is observed. As expected, the SOD-deficient mutant strain is more susceptible to oxidative stress than its wild-type counterpart. In contrast, in presence of the complexes, lipid peroxidation is limited, reaching similar MDA levels as the control samples. Again the Fe(III) complex is the most efficient in warding off oxidative damage.

A similar result was obtained upon inducing stress by adding H_2O_2 (Figure 7b); the main difference is that the peroxidation levels in all systems are higher than under menadione-induced stress (Figure 7a), but the protective effect of the mimics is more pronounced, with the Fe(III) system being again the most effective.

DISCUSSION

Since SOD enzymes utilize Cu, Mn and Fe, and it is known that CATs are efficient with Fe or Mn as cofactors in their active sites, we aimed to compare the properties of corresponding biomimetics containing these metal ions to evaluate the influence of the metal center on the antioxidant activity of these systems.

Studies concerning H_2O_2 and $O_2^{\bullet-}$ stress have attracted increasing attention due to their involvement in the formation of ROS such as $HO^{\bullet}$ and NO_2, and also for their role as causative agents for several diseases[6-8,40]. $O_2^{\bullet-}$ is produced by electron transfer leakage within the mitochondrial respiratory complexes I and III[25]. Spontaneous or enzymatic $O_2^{\bullet-}$ degradation leads to the generation of hydrogen peroxide, which can further react with transition metal ions such as iron(II) in Fenton-type reactions, thus promoting the formation of the highly dangerous $HO^{\bullet}$ and NO_2 radicals[40,50] .

Removal of ROS is a redox-based process controlled by proteinaceous (metalloenzymes) and non-proteinaceous (*e.g.* vitamins A and E, polyphenols) agents. Therefore, a major strategy for the development of effective antioxidants has focused on coordination compounds containing a redox-active metal center[18-23,38,51-53].

In this context, manganese porphyrin derivatives seem to be the most popular class of compounds that present SOD mimetic activity both *in vitro* and *in vivo*[19,23,54,55]. Other manganese compounds synthesized with 1,4,7,10,13-pentazazcyclopentadecane macrocycle[56], H_2salen (EUK family)[24,57] or corrole ligands[21,22] must also be highlighted due to their high SOD activity *in vitro* and their ability to protect organisms exposed to various oxidizing conditions. However, it was shown that a large number of these compounds failed to protect the SOD-deficient mutant *E. coli* and/or *S. cerevisiae* strains (s*od1*Δ) growing under aerobic conditions[58], suggesting that their *in vivo* antioxidant protection is not related to SOD activity[55,58]. However, recently it was reported that an iron-porphyrin system was able to protect an SOD-deficient *E. coli* strain growing in aerobic medium at a 0.1-1μmol dm^{-3}, while a related Mn compound was efficient only at concentrations > 20 μmol dm^{-3} [58]. It is, however, believed that the porphyrin ligand may act only as a carrier to transport the iron inside the cells, since the iron-porphyrin compound degraded after 12 h. Furthermore, the iron-porphyrin was

toxic for the cells at concentrations higher than 1 μmol dm^{-3} [58]. Interesting was the observation that iron(II) citrate shows that same protection effect on the cells, but it is not toxic even at 20 μmol dm^{-3} [58]. This difference in toxicity was attributed to different mechanisms of iron transport into the cells. While the iron salt uptake is mediated by specific compounds (*i.e.* siderophores) and maybe tightly controlled by the cell, the iron porphyrine compound may bypass this regulatory control and accumulate via the heme-uptake system[59].

Our data show that the *in vitro* SOD and CAT activities of the compounds may not be directly related to their *in vivo* activities. The iron compound showed the best protection, which is in contrast to its SOD and CAT activities. On the other hand, the manganese compound presented the best SOD and CAT activities *in vitro*, but the lowest antioxidant protection. These results thus demonstrate that the *in vitro* and *in vivo* antioxidant activities are not directly correlated; this observation may be in agreement with the hypothesis that factors such as compound stability, cellular uptake and mislocalization of the compounds inside the cells can impact on their *in vivo* activity[23,55]. With respect to the chemical behavior of the compounds, we have previously demonstrated, employing potentiometric titrations and mass spectrometry, that the iron compound at pH = 7.0 forms a diiron-μ-oxo species in solution. In the presence of 2´-deoxyadenosine-5´-monophosphate (dAMP) this dinuclear complex is transformed into a mononuclear [Fe^{II}(HPClNOL)(dAMP)]species, thus demonstrating that a new chemical species can readily be formed in the presence of chelating molecules. So, it is possible that inside cell, the coordination environment of the complexes is changings, which may be responsible for the antioxidant activity observed[60]. A similar behavior was observed for the related manganese compound Mn(HPClNOL)$(NO_3)_2$, which, in a buffered solution, also forms a dinuclear manganese

species[38]. Although it is currently not possible to identify unambiguously which factor(s) (*i.e.* coordination environment, redox state and potential, geometry) principally governs the biological activity of this group of compounds, the protective effect exerted by these compounds is remarkable (Figure 2). Our results indicate that the treatments with the metal ion complexes greatly enhanced oxidative stress resistance in both wild-type and mutant cells. Since the mutant cells lack either SOD or CAT activity, the complexes were evidently capable of compensating the lack of these enzymes. While all three metal ion derivatives are efficient in restoring protection against oxidative damage, the Fe(III) complex is the most effective.

Although a number of *in vitro* and *in vivo* studies with synthetic antioxidant mimics have been reported previously[18-23,51-53], only limited knowledge is available about the mechanism of action of how these compounds protect cells from oxidative damage. While the mimics may exert their protective effect on *S. cerevisiae* cells directly via their SOD and CAT activities, the possibility that these compounds operate by regulating/controlling gene expression (*i.e.* indirect protection) must be considered, especially since transcriptional regulation plays a major role in the adaptive response to stress conditions[45]. In *S. cerevisiae*, the transcription factor Yap1, a member of the c-jun family of proteins that display functional homology to mammalian AP-1, regulates a subset of genes associated with the organism's response to oxidative stress[54]. Furthermore, Hsf1 is the major regulator for the expression of genes involved in the heat shock response, which includes CAT[61]. And finally, the transcription factors Msn2 and Msn4, although they also contribute to the heat shock response, their regulatory network is broader, being associated with a global stress response[46].

Our studies demonstrate that the complexes induce strong resistance against oxidative stress in the *yap1Δ* mutant of *S. cerevisiae*, reaching survival rates similar to

that of the wild-type strain (Figure 4). This observation does not only indicate that the synthetic compounds are very efficient in conferring protection against oxidative stress, but also that their activity is not connected to the activation of Yap1. Furthermore, the function of the compounds is also not associated with a heat shock response (which is Hsf1-dependent; Figure 4a), nor a global response to stress (*i.e.* no trehalose is accumulated, which is triggered by transcription factors Msn2 and Msn4; Figure 5b). Thus, these antioxidant compounds act independently of transcriptional regulation that is frequently linked to stress response.

We could also rule out the possibility that the compounds may act by initiating the synthesis of stress response-related proteins. In presence of cycloheximide, an efficient blocker of protein synthesis, the metal compounds remain highly effective, as evidenced by the high survival rates of *S. cerevisiae* cells whose protein synthesis has been blocked by cycloheximide (Figure 6).

Oxidative stress has been related to damages of lipids, proteins and DNA, all of which generally lead to cell death[5]. In particular, cell membranes (via phospholipid peroxidations) are primary targets for ROS attacks[40,46]. Lipid peroxidation is a chain reaction that is strongly related to the loss of membrane selectivity and permeability that culminates in the formation of toxic byproducts such as MDA[33,62]. When *S. cerevisiae* cells were pre-treated with the three complexes, the levels of MDA formation decreased by almost 50% when compared to non-treated cells (Figure 7). This capacity to efficiently reduce lipid peroxidation is even more pronounced in strains lacking the antioxidant enzymes SOD and CAT.

Although there are natural antioxidant metalloenzymes containing iron and copper, it is observed that a large number of synthetic models are Mn-based mimics instead of Fe or Cu ones[19,20,63,64]. These is because the compounds employing the latter

metal ions often show pro-oxidant activity in reactions such as Fenton or Haber-Weiss[49,65-68]. For example, Fe and Cu porphyrins are highly efficient in generating $^{\bullet}OH$ radicals[19,61], accounting for the high toxicity of such complexes. The challenge seems to be the development of an appropriate ligand able in keeping the antioxidant activity higher than the pro-oxidant, as is exemplified by the iron complex containing corrole ligand[21,22]. Therefore, of relevance is the observation that the iron and copper complexes here described, which contain the ligand HPClNOL, showed the best protection, suggesting that this ligand is an interesting platform for the development of antioxidant compounds. Furthermore, it provides a N3O coordination environment for the metal ions, a similar one that is supplied by the protein backbone in the Fe- and Mn-SOD[12,13].

In light of the above discussion our observation that the Fe, Cu and Mn complexes with the HPCINOL ligand possesses efficacy to protect *S. cerevisiae* cells from oxidative damage is significant and may pave the way for the development of potent agents/treatments to lessen the deteriorating health effects of ROS. At present a comparative assessment of the *in vivo* activity of the complexes described here with other known antioxidant biomimetics is hampered due to small number of compounds able in protecting *S. cerevisiae* cells under oxidative stress as a eukaryotic model. However, this simple biological system is an emerging test platform for developing new drugs and study cellular responses[24]. Insofar, the HPCINOL-based complexes may be viewed as potent candidate compounds for future drug development.

Conclusion

In this study we have demonstrated the activity of a group of coordination compounds synthesized with the ligand HPClNOL in protecting *S. cerevisiae* (wild-type

and mutant) cells against oxidative stress generated by H_2O_2 and $O_2^{\cdot-}$. The activity is dependent on the type of metal ion present in the complex, in the order Fe(III)>Cu(II)>Mn(II). Of relevance is the fact that the *in vitro* activity is not directly related to the *in vivo* effect, which suggests that the active species inside the cells may be different from that present *in vitro*.

The mode of action of the compounds involves the protection of the lipid membrane but does not induce the activation of transcription factors such as Yap1, Hsf1 or Msn2/Msn4, all of which are related to the induction of the synthesis of protective factors such as heat shock proteins, trehalose and antioxidant proteins. Therefore, the results are a strong indication that the compounds act as antioxidant mimics with dual activity (*i.e.* SOD and CAT activities), removing the excess of $O_2^{\bullet-}$ and H_2O_2 due to their intrinsic catalytic activity. However, until now it was not possible to determine the nature of the active specie inside the cell.

Our work established a new group of compounds with a strong potential to be suitable and useful agents in biological systems, where they may act as synthetic protector molecules against oxidative stress. The encouraging *in vivo* studies reported here are anticipated to prompt further studies in animal models to develop new treatments against a plethora of diseases associated with ROS formation.

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Figures and Captions

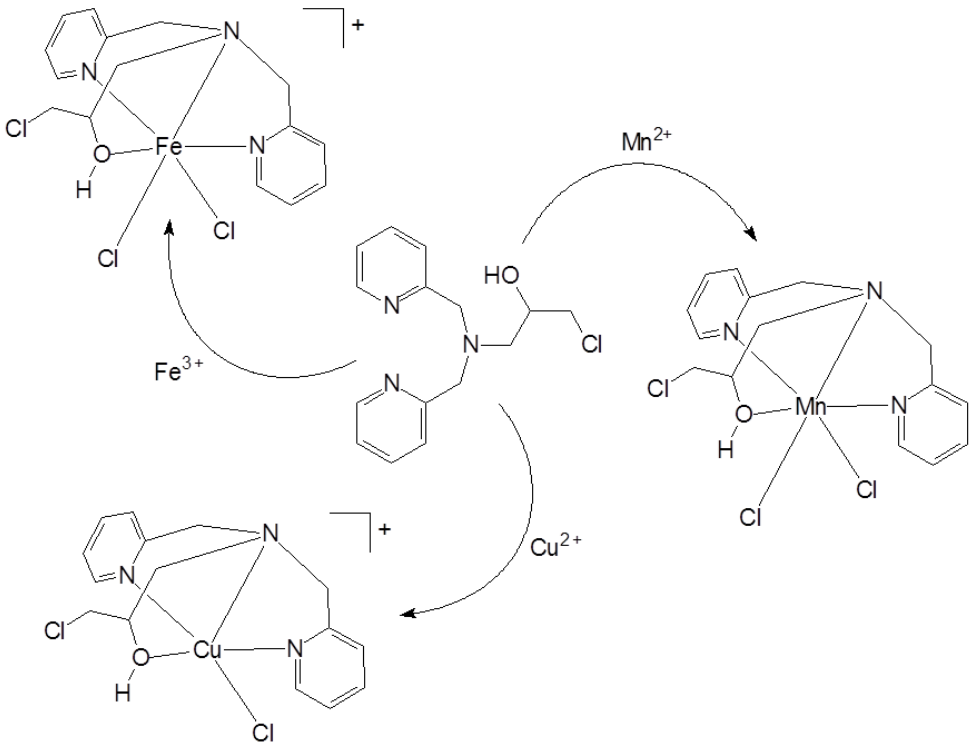

Figure 1: Scheme illustrating the synthesis of the iron, copper and manganese complexes obtained with the ligand HPClNOL. The drawing of the iron and copper complexes are based on the molecular structure solved by x-ray diffraction.

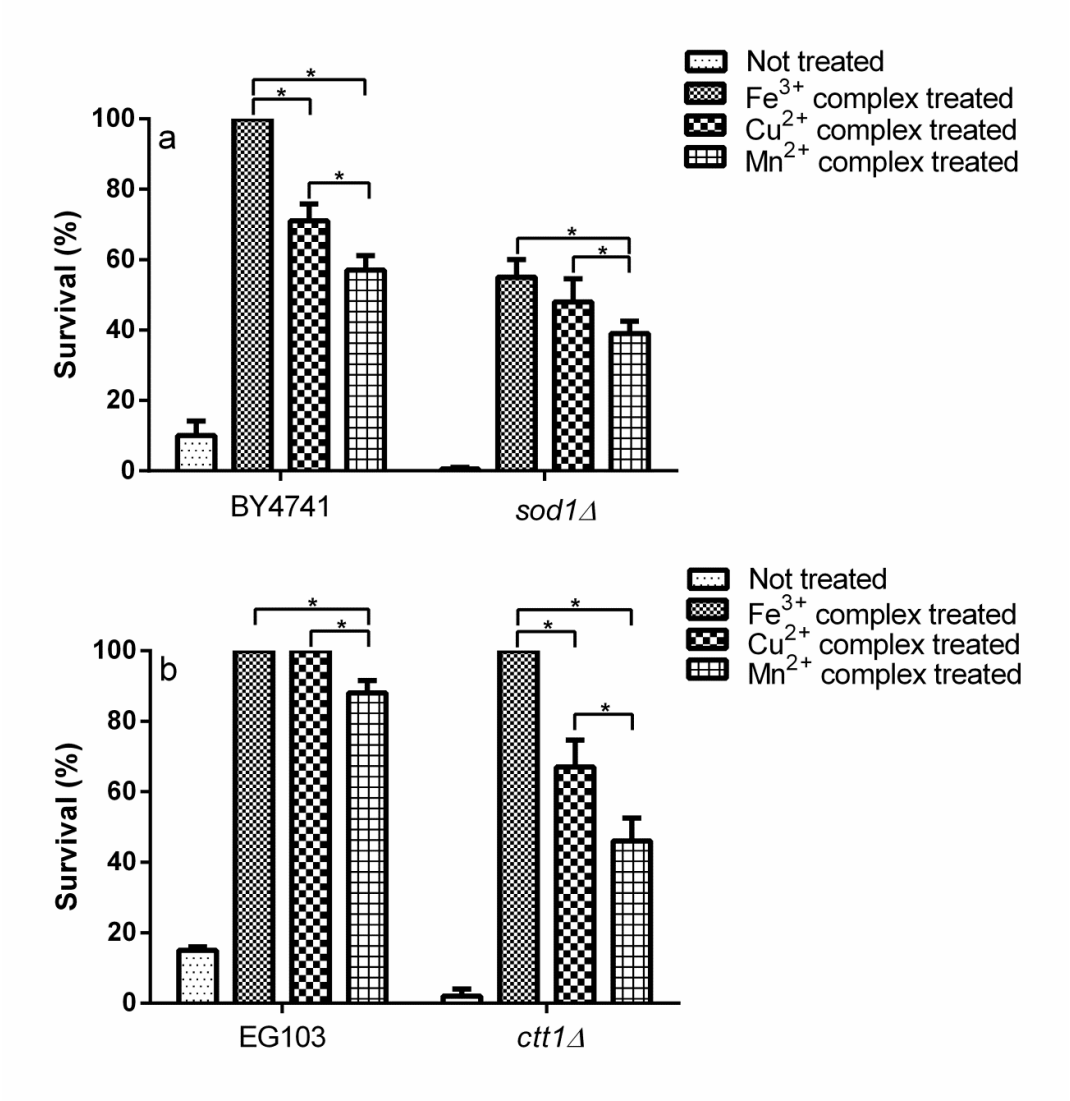

Figure 2: Effect of the biomimetics on the survival rates of *S. cerevisiae* cells exposed to oxidative stress. *S. cerevisiae* cells were pre-incubated with each of the complexes (25 µM/ 1 h) and then exposed to oxidative stress caused by menadione (a) or H_2O_2 (b). H_2O_2- and menadione-stressed cells were cultured in 4% YPGly and 2% YPD medium, respectively. Cellular viability was measured before and after exposure to oxidative stress by plating cells on 2% YPD medium and expressed as percentage of survival. The results represent the mean ± standard deviation of at least three independent experiments. As a control, cells that were not pre-incubated with the complexes are also included. Statistical differences between complex treatments were tested using a t-student test which denotes homogeneity between experimental groups at $P<0.05$ (* represents statistically different results).

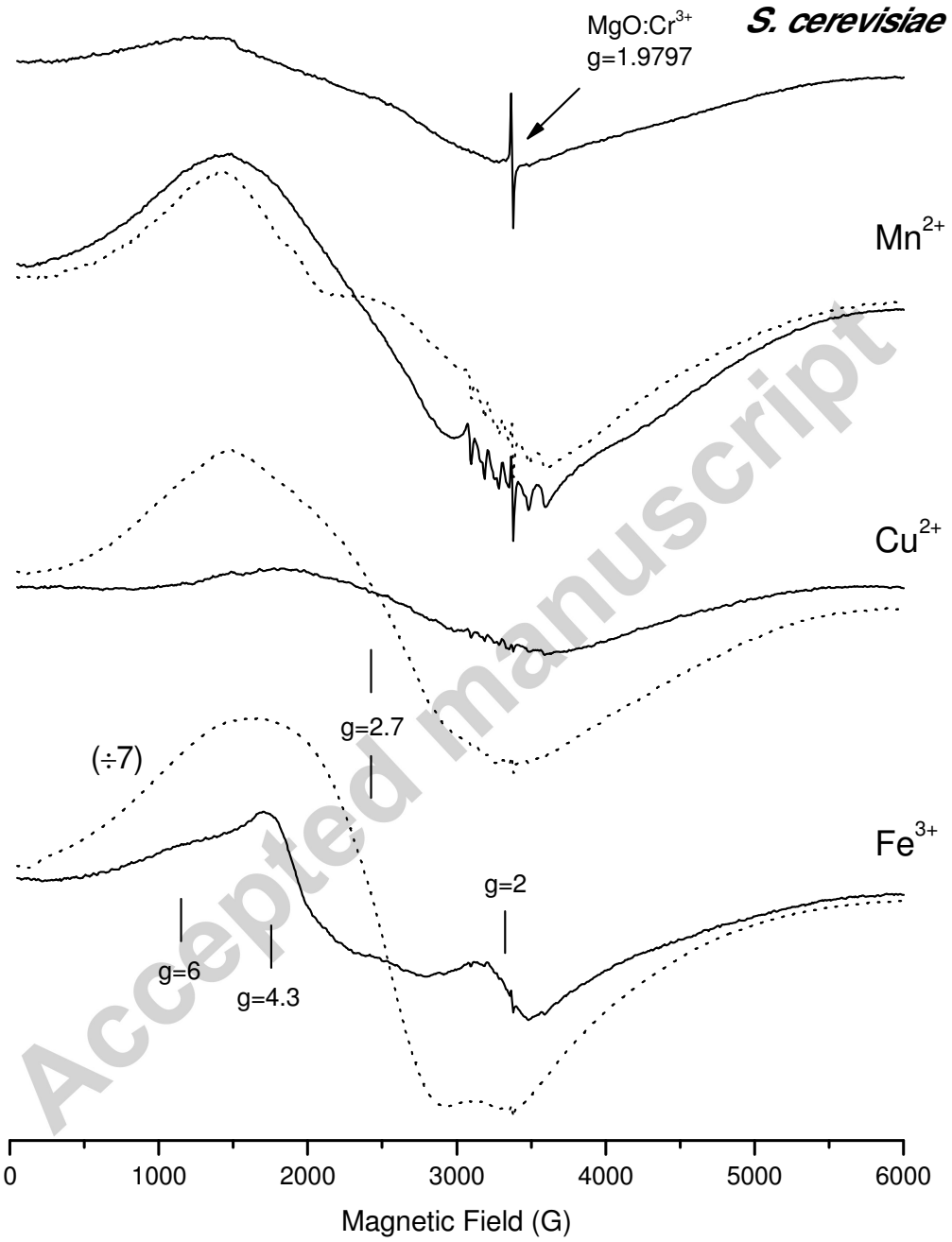

Figure 3. EPR spectra for *S. cerevisiae* samples that were (i) not incubated and (ii) incubated with the iron, copper and manganese complexes (solid lines) or with iron, copper and manganese chloride salts (dashed lines).

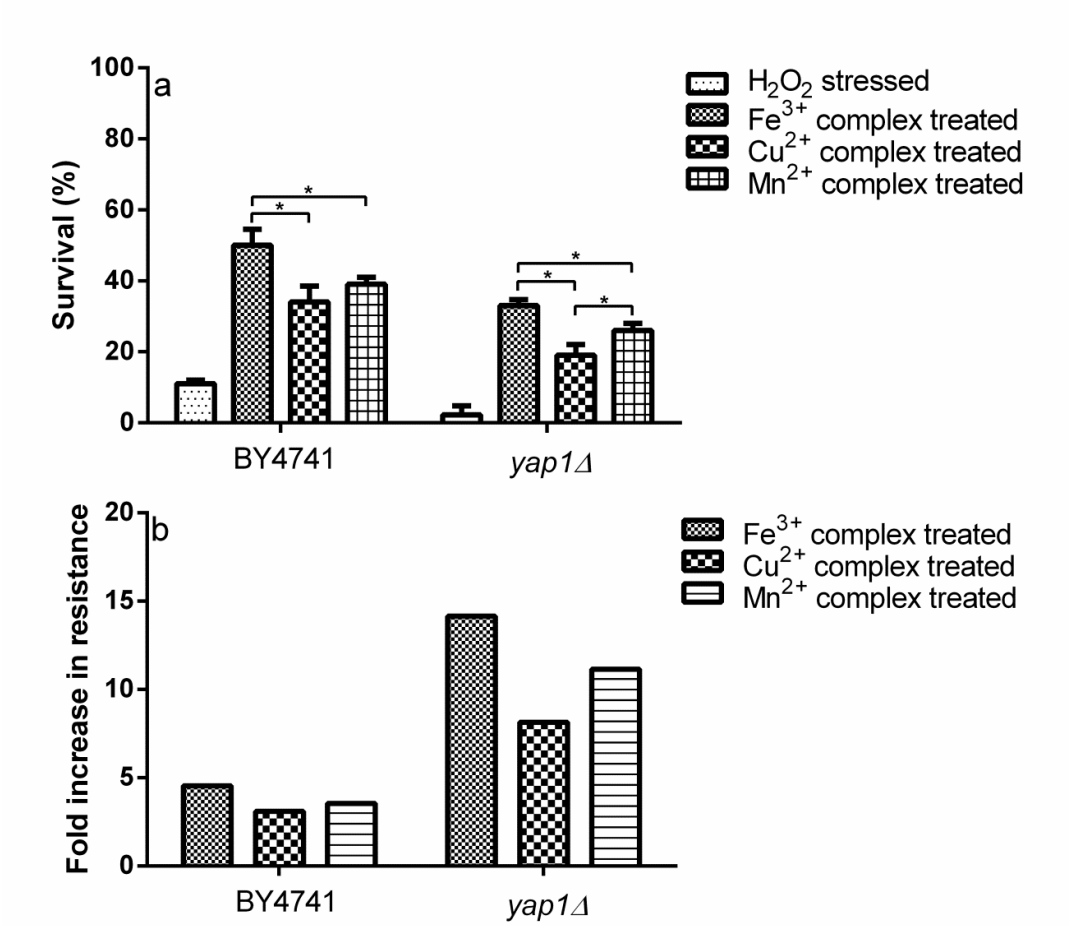

Figure 4: Survival rates of *S. cerevisiae* cells after treatment with the complexes and exposure to H_2O_2 stress. BY4741 and its isogenic mutant, *yap1*Δ, were cultured in 2% YPD medium and then pretreated with the complexes (25 µmol dm^{-3}/1 h), before adding 2.5 mmol dm^{-3} H_2O_2 for 1 h. Cells that were not pre-incubated with the complexes were included as negative control (a). Cellular viability was measured before and after exposure to H_2O_2 stress by plating cells on 2% YPD medium and expressed as percentage of survival. Fold increase in resistance were determined as the ratio between the survival rates (%) of cells that were treated with the complexes and the negative control (b). The results represent the mean ± standard deviation of at least three independent experiments. Statistical differences between complex treatments were tested using a t-student test which denotes homogeneity between experimental groups at $P<0.05$ (* represents statistically different results).

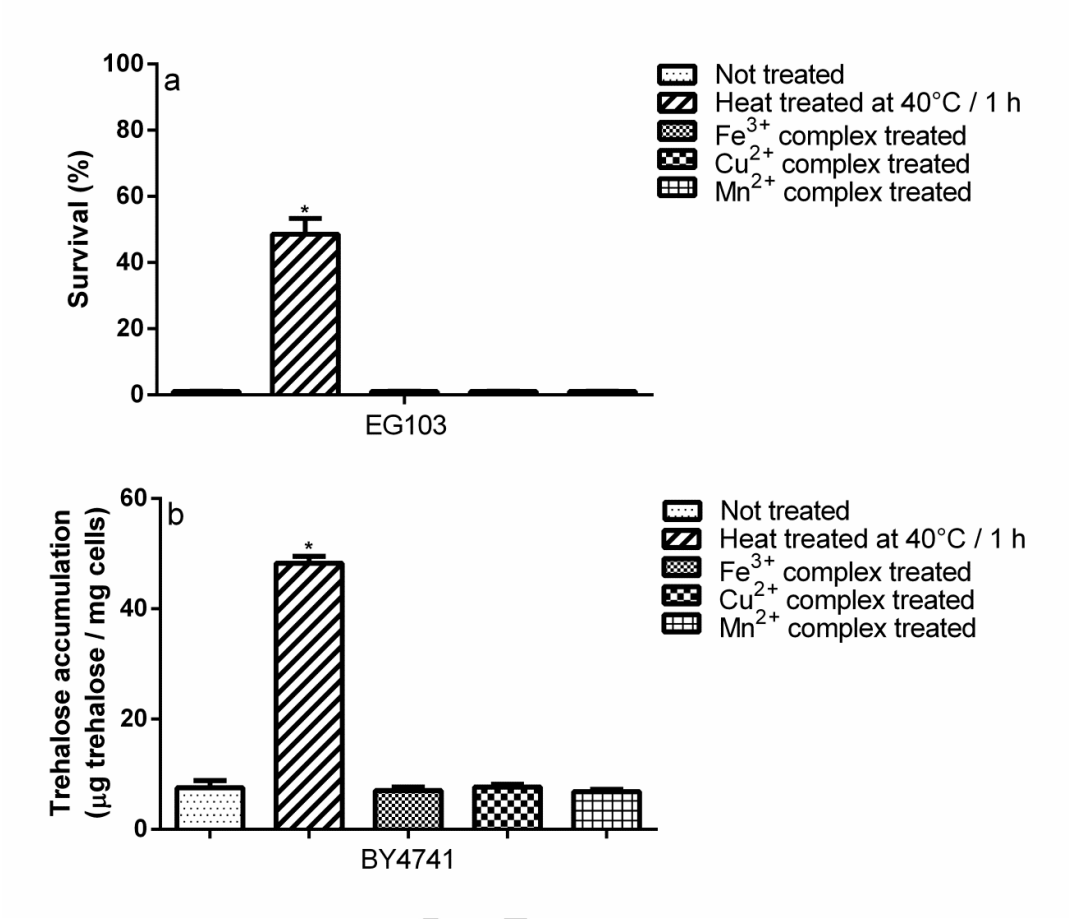

Figure 5: Effect of the complexes on the heat shock response (a) and trehalose accumulation (b) in *S. cerevisiae*. *S. cerevisiae* cells treated with complexes (25 µmol dm^{-3}/1 h) were exposed to severe heat shock (51°C/8 min). Negative control cells were exposed to heat shock without pre-incubation with the complexes (a). As a positive control, cells were previously treated with mild heat shock at 40°C for 1 h and then exposed to heat stress. Intracellular levels of trehalose were determined in BY4741 cells treated with mild heat shock at 40°C for 1 h or with the complexes (25 µmol dm^{-3}/1 h) (b). Trehalose was determined by the antrone method and expressed as µg trehalose/mg cells. The results represent the mean ± standard deviation of three independent experiments. Statistical differences between mild heat and complex treatments were tested using a t-student test which denotes homogeneity between experimental groups at $P<0.05$ (* represents statistically different results).

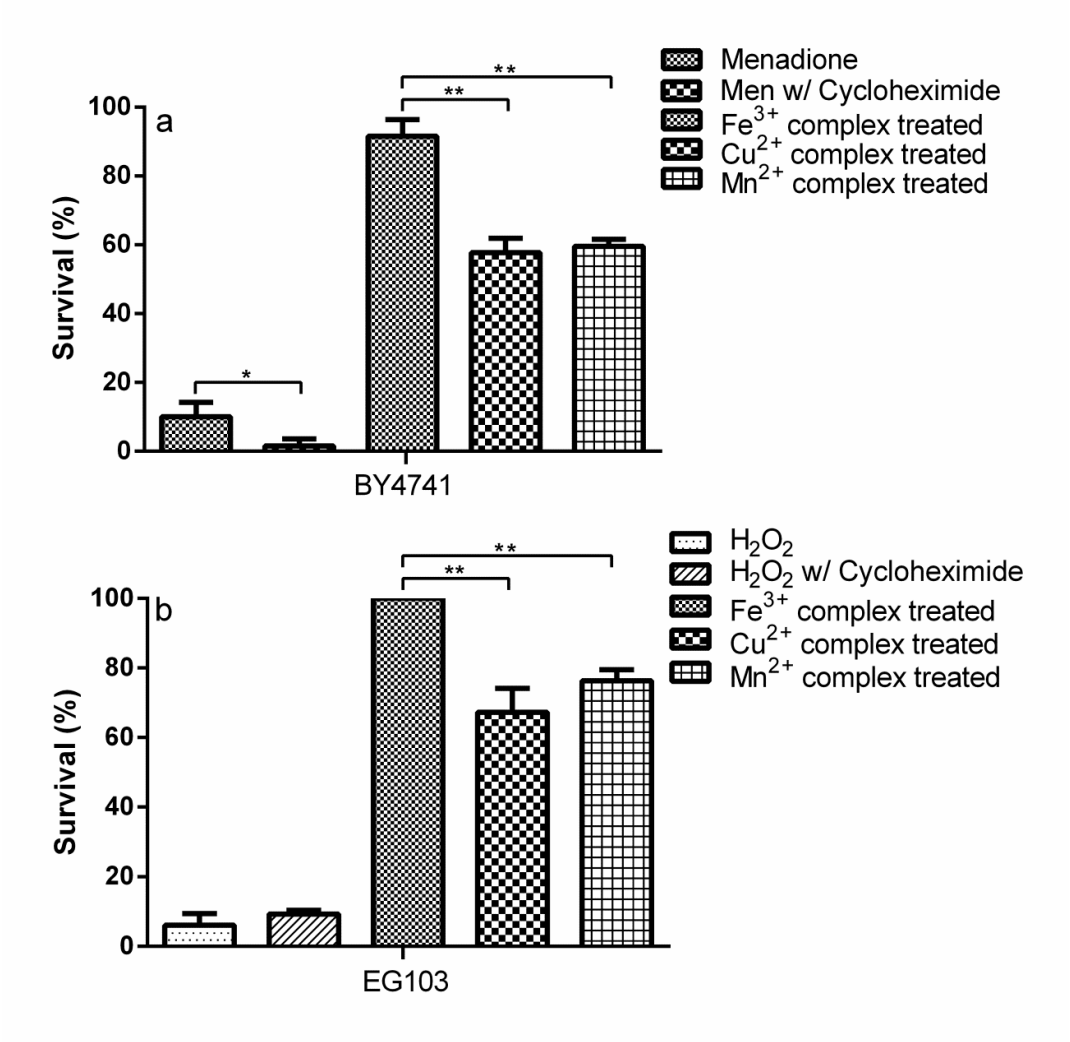

Figure 6: Effect of cycloheximide on the survival rates of *S. cerevisiae* cells treated with the complexes and exposed to oxidative stress. *S. cerevisiae* cells submitted to cycloheximide (0.5 mg cm^{-3}) for 2 h prior to treatment with the complexes (25 µmol dm^{-3}/1 h) were exposed to oxidative stress caused by menadione (a) or H_2O_2 (b) stress. For H_2O_2 and menadione stress cells were cultured in 4% YPGly and 2% YPD medium, respectively. Cellular viability was measured before and after exposure to stresses by plating cells on 2% YPD medium and expressed as percentage of survival. The results represent the mean ± standard deviation of at least three independent experiments. Statistical were tested using a t-student test which denotes homogeneity between experimental groups at $P<0.05$. * represents statistically different results between cycloheximide treated and non-treated cells. ** represents different results between complex treatments.

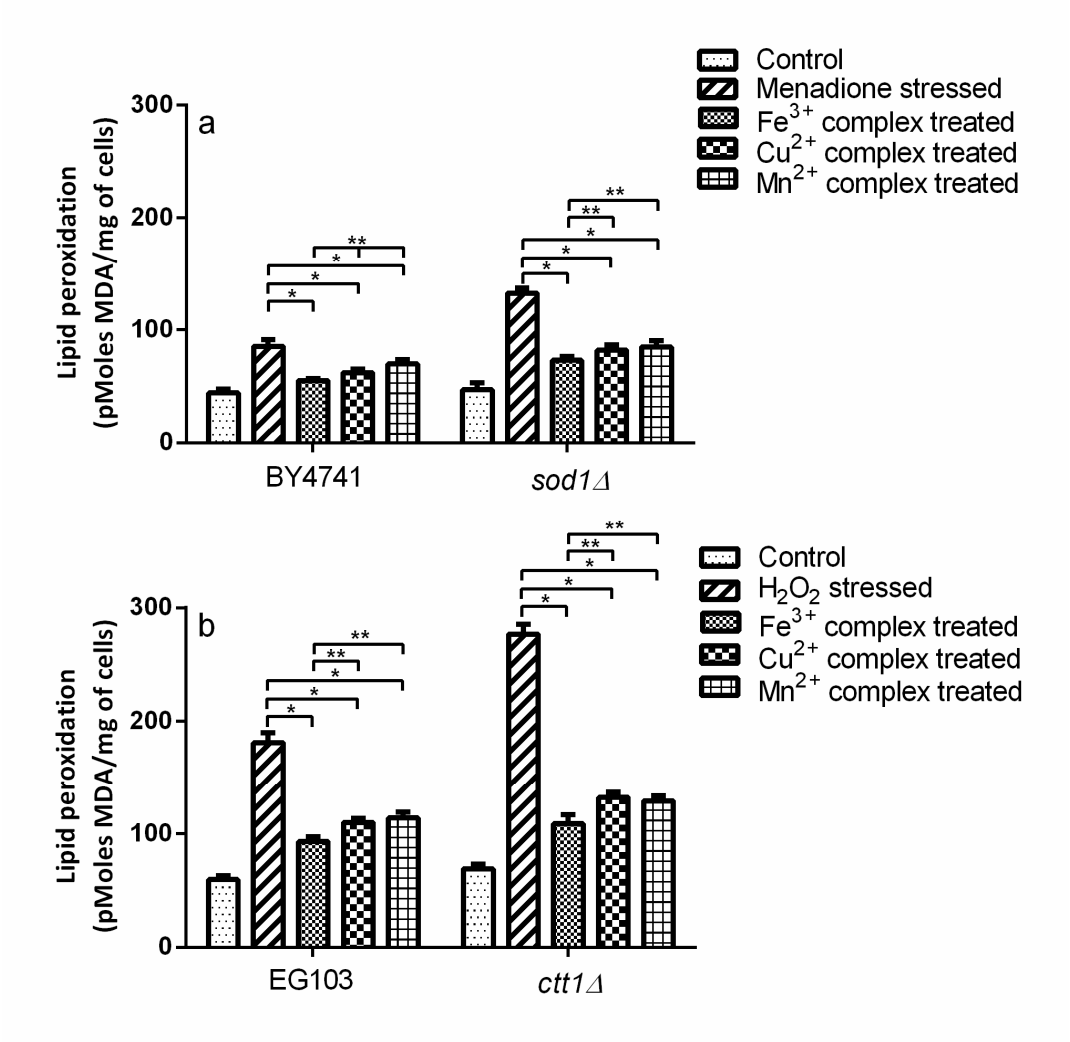

Figure 7: Lipid peroxidation levels of *S. cerevisiae* cells after treatment with the complexes and exposed to oxidative stress. Lipid peroxidation was measured in *S. cerevisiae* cells treated with the complexes (25 µmol dm^{-3} /1 h) and further exposed to oxidative stress caused by menadione (a) or H_2O_2 (b) (as a negative control cells that were not treated with the complexes were included). As an additional control, the levels of MDA in non-stressed cells were also determined. The levels of lipid peroxidation were determined by the TBARS method. Lipid peroxidation was expressed as pmol of MDA/mg cell. The results represent the mean ± standard deviation of at least three independent experiments. Statistical were tested using a t-student test which denotes homogeneity between experimental groups at $P<0.05$. * represents statistically different results between stressed and complex treated/stressed cells. ** represents different results between complex treatments.

- Iron, copper and manganese complexes showed protective antioxidant effects.
- The iron complex was the most effective in protecting *Saccharomyces cerevisiae.*
- The complexes may be considered SOD and CAT mimetics.
- The complexes decreased the level of lipid peroxidation in the cells.