A Water-Soluble Manganese(II) Octanedioate/Phenanthroline Complex Acts as an Antioxidant and Attenuates Alpha-Synuclein Toxicity

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Abstract

The overproduction of reactive oxygen species (ROS) induces oxidative stress, a well-known process associated with aging and several human pathologies, such as cancer and neurodegenerative diseases. A large number of synthetic compounds have been described as antioxidant enzyme mimics, capable of eliminating ROS and/or reducing oxidative damage. In this study, we investigated the antioxidant activity of a water-soluble 1,10-phenantroline-octanedioate Mn^{2+} -complex on cells under oxidative stress, and assessed its capacity to attenuate alpha-synuclein (aSyn) toxicity and aggregation, a process associated with increased oxidative stress. This Mn^{2+} -complex exhibited a significant antioxidant potential, reducing intracellular oxidation and increasing oxidative stress resistance in *S. cerevisiae* cells and *in vivo*, in *G. mellonella*, increasing the activity of the intracellular antioxidant enzymes superoxide dismutase and catalase. Strikingly, the Mn^{2+} -complex reduced both aSyn oligomerization and aggregation in human cell cultures and, using NMR and DFT/molecular docking we confirmed its interaction with the C-terminal region of aSyn. In conclusion, the Mn^{2+} -complex appears as an excellent lead for the design of new phenanthroline derivatives as alternative compounds for preventing oxidative damages and oxidative stress - related diseases.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 7 million people worldwide aged 60 years or older [1, 2]. The typical motor features of PD include bradykinesia (slow movement), resting tremor, akinesia, and rigidity, which are attributed to the irreversible loss of dopaminergic neurons of the substantia nigra (SN) [1, 2]. PD is also characterized by the misfolded protein alpha-synuclein (aSyn), whereas the formation of inclusions known as Lewy bodies (LB) is the main component of the pathological hallmark of the disease [3]. Although the physiological function of aSyn is still under debate, it has been reported its interaction with negatively charged lipids at the presynaptic terminal, which could be crucial for its association in vesicle trafficking [4, 5]. PD is associated with alterations in basic mechanisms of the aging process, which is due, at least in part, to an interaction of genetic and non-genetic risk factors (e.g., cell senescence, inflammation, gut and gut microbiome dysregulation, mitochondrial dysfunction, oxidative stress, alteration of proteostasis and the ubiquitin-proteasomal and autophagy systems) [6].

aSyn is a monomeric intrinsically-disordered 14-kDa protein consisting of 140 amino acids. In addition, aSyn has metalloprotein properties through its three metal-binding sites: two of each are located at the N-terminal domain and the third is located at the C-terminal domain [7, 8]. Among these three metal-binding sites, those located near residues 49–52 and 110–140 are well-known divalent metals (e.g. Mn^{2+}) binding sites [7–9]. Although the molecular events of aSyn aggregation are still under investigation, several multibiological events, either environmental or genetics have been described to accelerate or facilitate the aggregation and fibrillation of aSyn. Oxidative stress is an established factor related to this process, since the increase in free radicals can break the α -helical structure and may induce covalent

bonds and stabilization of aSyn filaments, consequently leading to the formation of protofibrils and fibrils [10].

With the aim of reducing oxidative damage and increasing cellular tolerance to oxidative stress, several antioxidant molecules have been investigated, in both experimental and clinical studies, for preventing the onset and progression of the PD [11–14]. It has been reported that some synthetic antioxidants with high antifibrillogenic capacities destabilize the pre-existing fibrils caused by oxidative conditions [15]. Amongst these reports, it has been claimed that the manganese (II) penta-azamacrocyclic complex M40403, which is a well known superoxide dismutase (SOD) mimetic, exhibits beneficial effects in human SH-SY5Y neuroblastoma cells and *Drosophila Melanogaster* against the oxidative damage induced by paraquat, which is commonly used to trigger PD symptoms in animal models [16]. Moreover, M40403 has also been used to reverse PD symptoms associated with early-onset forms of PD in different models [16–18].

In this study, we used *S. cerevisiae* and *Galleria melonella* to assess the antioxidant potential of the Mn^{2+} -complex salt $\{[Mn_2(\mu_2\text{-oda})(phen)_4(oda)_2]^{2-}[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2]^{2+}\}$, which has previously been reported to be a SOD and catalase (CAT) mimic [19]. We also investigated the capacity of this compound to attenuate the toxicity of human aSyn in yeast. Additionally, we evaluated whether the Mn^{2+} -complex could be capable of reducing aSyn oligomerization and aggregation *in vitro* and in the human neuroglioma cell line expressing aSyn. Finally, NMR and a combined DFT/molecular docking study confirmed that the Mn^{2+} -complex can interact with aSyn at the C-terminus.

Materials And Methods

Synthesis of the Mn^{2+} -complex

Synthesis of $\{[Mn_2(\mu_2\text{-oda})(phen)_4(oda)_2]^{2-}[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2]^{2+}\}$ (where $odaH_2$ = octanedioic acid; phen = 1,10-phenanthroline) was performed as previously reported with the solid-state structure shown in **Figure 1A** [20]. Conductivity measurements suggest that $\{[Mn_2(\mu_2\text{-oda})(phen)_4(oda)_2]^{2-}[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2]^{2+}\}$ dissociates extensively in aqueous solution yielding the biologically active dication $[Mn_2(oda)(phen)_4(H_2O)_2]^{2+}$ (herein referred to as the Mn^{2+} -complex) and free inactive oda^{2-} [20]. The stock solution of Mn^{2+} -complex was prepared in milliQ water at 5.0 mM final concentration.

Cell models and growth conditions

The *S. cerevisiae* wild-type strain, BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), was used to evaluate the antioxidant activity of the Mn^{2+} -complex. BY4741 was acquired from Euroscarf, Frankfurt, Germany. Stocks were maintained on solid YPD 2% (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/mL) in liquid YPD 2% medium with a ratio of flask volume/medium of 5/1 and incubated in shaker at 28°C and 160 rpm. Human neuroglioma (H4)

cells were maintained in Opti-MEM I Reduced Serum Medium (Gibco, Carlsbad, Germany) supplemented with 10% Fetal Bovine Serum Gold (FBS) (PAA, Cölbe, Germany) and 1% penicillin-streptomycin (PAN, Aidenbach, Germany). Cells were grown at 37°C in a 5% CO₂ atmosphere.

Toxicity of Mn²⁺-complex

Mid-exponential fermentative *S. cerevisiae* cells (0.8 mg/mL) were exposed to increasing concentrations (0.1 to 100.0 µM) of the Mn²⁺-complex for 1 h at 28°C and 160 rpm. Subsequently, cell survival was analyzed by plating on solidified YPD 2% medium after proper dilution (1.000 x) of the cell concentration. 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 (CFU) of complex-treated cells and the average number of CFU of the untreated cells. To determine the Mn²⁺-complex cytotoxicity in human cells, H4 cells were treated with concentrations between 0.1 and 100 µM of the Mn²⁺-complex for 24 h. Two controls were performed: one negative, using only the OPTI-MEM culture medium, and one positive, adding 2% (v/v) Triton X-100 to the cell culture and incubating for 20 minutes. After treatment, the culture medium was collected for analysis of lactate dehydrogenase levels using the LDH detection kit (Cytotoxicity Detection Kit, Roche, Penzberg, Germany). The assays were prepared in 96-well microplates (Corning Costar, New York, USA), according to the manufacturer's instructions and the plates were subsequently analyzed in an Infinite M200 PRO microplate reader (TECAN, Mannedorf, SWI) at a wavelength adjusted to 492 nm. Toxicity was expressed as a percentage relative to negative and positive controls, the mean absorbance values were subtracted with the mean absorbance value obtained in the background control according to the manufacturer. Percentage cytotoxicity = 100 × (experimental LDH release - spontaneous LDH release)/(maximal LDH release – spontaneous LDH release).

Oxidative stress and Mn²⁺-complex treatment

Cells at mid-exponential phase (0.8 mg/mL) on glucose (YPD 2%) were directly exposed to severe oxidative stress condition caused by 2 mM H₂O₂ for 1 h at 28°C. Under all experimental conditions, half of the culture was immediately exposed to stress conditions, whilst the other was pre-incubated with 0.1 to 10.0 µM of Mn²⁺-complex for 1 h at 28°C and 160rpm, washed with cold distilled water to remove the excess of complex from the medium, and then submitted to oxidative stress condition. Cell survival was analyzed as previously described.

Intracellular oxidation and mitochondrial superoxide determination

Intracellular reactive oxygen species (ROS) production in *S. cerevisiae* was determined using the oxidant sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Sigma-Aldrich). This cell-permeant dye is non-fluorescent in a reduced state and exhibits bright green fluorescence (with excitation/emission at 504/524 nm) upon oxidation mediated by ROS. The ROS levels was detected before and after treatment with Mn²⁺-complex (1.0 and 10.0 µM) and incubated with agitation for 1 h at 28°C and 160

rpm. Cells were then stained for 15 min at 28°C and 160 rpm with H₂DCFDA, (10.0 µM, final concentration). Then, 50 mg of each sample were cooled on ice, harvested by centrifugation and washed twice with 50 mM phosphate buffer, pH 6.0. The pellets were resuspended in 500 µL of the same buffer, and 1.5 g of glass beads were added. The samples were disrupted from cycles of 1 min agitation on a vortex mixer followed by 1 min on ice, repeated three times. The supernatant solutions were acquired after centrifugation at 13.000 rpm for 5 min, diluted sixfold with water destiled and then, fluorescence was measured. Intracellular ROS produced by H4 cells were examined by determining mitochondrial superoxide radicals using MitoSOX Red (Life Technologies, Carlsbad, EUA). This probe selectively reacts with superoxide radicals within mitochondria and undergoes oxidation to form a stable fluorescent compound. Briefly, H4 cells were plated at the initial concentration of 1.5×10^4 , in 96-well plates and maintained at 37°C, 5% CO₂. After 24 h of plating, the cells were treated with 0.1, 1.0 and 10.0 µM Mn²⁺-complex for 24 h. After treatment, the medium was removed, and the cells washed with PBS twice. Then, H4 cells were stained with 5.0 µM MitoSOX Red reagent for 30 min at 37°C. After the end of the incubation time, the cells were washed with PBS, and incubated with 200.0 mM H₂O₂, for 30 minutes. The experiments were carried out in triplicate and fluorescence intensity values analyzed by Infinite M200 PRO modular microplate reader (TECAN, Mannerdörf, SWI) (absorption/emission = 510/580 nm). The results were expressed in arbitrary units, normalized from the gross values of red fluorescence intensity emitted by the complex formed between MitoSOX bind to superoxide radical.

Catalase and superoxide dismutase activity

Extracts for enzymatic of sod and catalase resolutions were made from the rupture of yeast cells (50 mg) by 3 cycles of 1 min vortexing and 1 min on ice with 1.5 g of glass beads in 50 mM sodium phosphate buffer pH 7.8, holding a protease inhibitor mixture (cOmplete, mini, EDTA-free protease mixture inhibitor tablets; Sigma-Aldrich Merck) [21]. Protein concentration was obtained according to Stickland [22]. Sod1 activity was valued *in situ* after native polyacrylamide gel electrophoresis with 60 µg of protein in the presence of riboflavin and nitroblue tetrazolium (NBT) [23,24]. The activity was resolved based on the ability of superoxide dismutase to inhibit the reduction of NBT by superoxide radical. Then, polyacrylamide gel electrophoresis was scanned on EC3 imaging system (UVP Bioimaging Systems), and Sod1 bands were analyzed, taking into consideration the area density by using ImageJ software. Activity was expressed as a fold increase in Sod1 activity between treated (1.0 and 10.0 µM of Mn²⁺-complex) and non-treated cells. The catalase activity of soluble protein extracts was performed by enzymatic kinetics in cells treated or not with the Mn²⁺-complex. The H₂O₂ decomposition was monitored at 240 nm on a Beckman Coulter DU 700 model spectrophotometer for 3 minutes, with readings taken every 10 seconds [25]. One catalase unit is expressed as the amount of the enzyme that catalyzes the degradation of 1.0 µmol H₂O₂ per minute.

Evaluation of Mn²⁺-complex antioxidant activity in the model organism *Galleria mellonella*

Maintenance of *Galleria mellonella* larvae were carried out at the insectarium of Biochemistry Department, Chemistry Institute from UFRJ, Brazil, accordingly [26]. The larvae were selected by weight (0.2 - 0.3 g), characteristic of larvae from the 7th instar, and absence of pigmentation. For oxidative stress exposure a group of 10 larvae were exposed to oxidative stress condition by injection of 10.0 μ L of 5 M H_2O_2 directly into the haemocoel through the last left pro-leg of the larvae using a Hamilton syringe (Hamilton, USA). Determination of Mn^{2+} -complex antioxidant activity was carried out by pretreating a group of 10 larvae with an injection of 10.0 μ L of Mn^{2+} -complex suspension (0.5 and 1.0 mg/mL, resulting in final test concentrations of 5 and 10 μ g/larvae) 3 h before the exposure to oxidative stress condition. After injections, larvae were placed in Petri dishes and incubated without food at 37°C for up to 7 days in the dark. Larvae survival was monitored daily by visual inspection of the color (brown-dark brown) and by the lack of movement after physical stimulus with clamp; larvae were considered dead when no reaction to stimulus was observed. As a physical injury control, the same volume of sterile H_2O , the solvent for H_2O_2 and Mn^{2+} -complex solutions, was also injected into *G. mellonella* larvae. Survival was expressed as the percentage of living larvae along the experimental time accordingly [27].

Cell transformation and cell transfection

Strains of *S. cerevisiae* containing the sequence of the *SNCA* gene, responsible for coding the human aSyn protein in its original form (wild type) were used in this study to evaluate the toxicity associated with the aSyn expression. The genetic modification was carried out by inserting the empty plasmid p426GAL and its derivatives p426GAL-aSynWT-EGFP or p426GAL-aSynWT [28] into BY4741 background. The aSynWT plasmids were tagged with or without EGFP, both under the control of the yeast GAL1 promoter. The P426GAL [29] is a multicopy plasmid that contains *URA3* and *AMP^r* as selective markers in yeast and bacteria, respectively [28,29]. These plasmids were kindly provided by Dr. Tiago Fleming Outeiro from the Göttingen Medical University (UMG), Göttingen, Germany. The standard lithium acetate heat shock procedure was used for the transformation of yeast cells. Transformed cells were selected by plating on SD 2% (2% glucose, 0.67% nitrogenous base without amino acids and 0.02% of leucine, methionine and histidine) without uracil [30]. H4 cells (5.0×10^4 cells/mL) were plated in 12-well plates (Costar, Corning, New York, USA) 24 h before the transfection. Transfection was performed with metafectene (Biotex, Munich, Germany) according to the manufacturer's instructions. In brief, transfection was carried out by using equimolar amounts of aSyn-VC and VN-aSyn [31] was added to a solution containing metafectene leading to a final DNA:metafectene ratio of 1:3. Additionally, H4 cells (5.0×10^4 cells/mL) were plated in 12-well plates (Costar, Corning, New York, EUA) in fresh Opti-MEM 24 h before transfection. Six hours prior to transfection medium was replaced with fresh one. Five (5.0) μ L of a 2.5 M $CaCl_2$ solution was added to 45 μ L of a solution containing equal amount of the plasmids encoding SynT and Synphilin1-V5 [31]. 2xHBS calcium phosphate buffer [50 mM N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) pH 7.05, 280 mM NaCl, 1.5 mM $Na_2PO_4 \times 2 H_2O$], pH 7.05 was added drop wise to the solution and vigorously mixed. The mix was incubated for 20 min and added dropwise to the cells while the plate was gently rocked.

Toxicity of aSyn in yeast cell model

Cytotoxicity was evaluated in *S. cerevisiae* cells expressing aSyn using the p426GAL-aSynWT vector. As a control, we used *S. cerevisiae* cells transformed with the empty vector, p426GAL. Cells in exponential phase (0.8 mg/mL) of growth in liquid SD 2% (aSyn OFF) were harvested (10 mg), washed twice with cold-distilled water and transferred to 10 mL SGal 4% (4% galactose, 0,67% nitrogenous base without amino acids and 0,02% of leucine, methionine and histidine) (aSyn ON). Then, the cell culture was incubated at 28°C/160 rpm and after 4, 6, 12 and 24 h aSyn toxicity was monitored by plating cells in solid SD 2%. Cell survival was analyzed as previously described. Attenuation of aSyn toxicity was performed in cells treated with Mn²⁺-complex (0.1, 1.0 and 10.0 µM) in SGal 4%.

Lipid peroxidation

The lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS assay), which detects malondialdehyde (MDA), one of the main lipid peroxidation byproducts [32]. After induction and aSyn-expression in yeast cells as previously described, 30 mg of cells were harvested by centrifugation and washed twice with 20 mM Tris-HCl buffer, pH 7.4. Next, the pellets were resuspended in the same buffer containing 10% trichloroacetic acid, and then, 1.5 g of glass beads were added. The cell lysates were obtained by agitation on a vortex by 6 cycles of 20 seconds each, alternating between agitation and ice bath. After centrifugation at 2.500 g for 5 min, supernatants were mixed with EDTA 0.1 M and 1% (w/v) thiobarbituric acid in NaOH 0.05 M. The reaction mixture was heated at 100°C for 15 min in a water bath, and after ice cold the absorbance at 532 nm was measured in a DU spectrophotometer.

Mn²⁺-complex's treatment in H4 Cell

Twenty-four hours after transfection, H4 cells were reinoculated into fresh medium and treated with Mn²⁺-complex at final concentrations of 0.1, 1.0 and 10.0 µM up to 4 h before the experiments. At the end of the treatment, cells were washed with PBS to remove residual complex outside of the cells and were subjected to immunocytochemistry for studying aSyn inclusions 36 h after the transfection.

Immunocytochemistry

After treatment with Mn²⁺-complex, H4 cells were washed with PBS after transfection and fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature, followed by a permeabilization step with 0.5% Triton X-100 (Sigma- Aldrich, St. Louis, MO, USA) for 20 minutes at room temperature. After blocking in 1.5% BSA (NZeTech, Lisbon, Portugal) for 1 h, cells were incubated with primary antibody mouse anti-aSyn (1:1000, BD Transduction Laboratory, New Jersey, USA) overnight at 4°C and then cells were washed with PBS and incubated with secondary antibody (Alexa Fluor 488 donkey anti-mouse IgG (1:2000, Life Technologies-Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. Finally, cells were stained with DAPI (Life Technologies- Invitrogen, Carlsbad, CA, USA) (1:5000 in DPBS) for 5 min and then maintained in PBS for fluorescence microscopy. Microscopy images for determination of aSyn inclusions

were captured with a 40x objective on a Leica DMI 6000B microscope with a Leica DFC 350 FX R2 camera.

Western blot analyses

H4 cells were lysed with Radioimmunoprecipitation Assay (RIPA) lysis buffer (50.0 mM Tris pH 8.0, 0.15 M NaCl, 0.1% SDS, 1% NP40, 0.5% Na-Deoxycholate), 2.0 mM EDTA and a Protease Inhibitor Cocktail (1 tablet/10 mL) (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using the Bradford assay (BioRad Laboratories, Hercules, CA, USA) and the gels were loaded with 40 mg of protein after denaturation for 10 min at 100°C in protein sample buffer (125.0 mM of 1.0 M Tris HCl pH 6.8, 4% SDS 0.5% Bromophenol blue, 4.0 mM EDTA 20% Glycerol 10% b-Mercaptoethanol). The samples were separated on 12% SDS-polyacrylamide gels (SDS-PAGE) with a constant voltage of 110 V using Tris-Glycine SDS 0.5% running buffer (250.0 mM Tris, 200.0 mM Glycin, 1% SDS, pH 8.3) for 1 h. Samples transference to nitrocellulose membrane (Protran, Schleicher and Schuell, Whatman GmbH, Dassel, Germany) was carried out during 1:30 h with constant current at 0.3 A using Tris-Glycine transfer buffer. Membranes were blocked with 5% (w/v) BSA (Fluka, Sigma-Aldrich, St. Louis, MO, USA) in 1x TBS-Tween (50.0 mM Tris, 150.0 mM NaCl, 0.05% Tween, pH 7.5) for 1 h. Membranes were further incubated with the primary antibody, either 1:1000 mouse anti-aSyn (BD Biosciences, San Jose, CA, USA) and 1:1000 mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA) in 3% Bovine Serum Albumin Fraction V (BSA)/TBS-Tween (NZYTech, Lisbon, Portugal), overnight at 4°C. After washing step (three times) in TBS-Tween for 5 min, the membranes were incubated for 1 h with secondary antibody, anti-mouse IgG, (GE Healthcare, Bucks, UK) at 1:10,000 in 3% BSA/TBS-Tween. Detection was performed using luminol reagent with peroxide solution (Millipore, Billerica, MA, USA) and applied to the membrane 1 min. before scanning in AlphaMager FluoroChem software (AlphaInnotech). Protein levels were quantified using ImageJ and normalized to the β-actin levels.

Thioflavin T (ThT) fibrilization assays

The *in vitro* assay for detecting aSyn aggregation were performed in the absence and in the presence of the Mn²⁺-complex. aSyn monomers were purified and lyophilized as described previously by Fernandes *et al.*, 2017 [26]. For kinetics experiments, the samples containing aSyn (100.0 μM), Mn²⁺-complex (2.0, 25.0 or 50.0 μM) and ThT (10.0 μM) were incubated at 37°C under continuous shaking (350 rpm) in a 96-well plate (Costar # 3631) together with Teflon spheres, 0.3 cm diameter. At appropriate time points, the fluorescence (excitation at 450 nm, emission at 477 nm) was recorded using a SpectraMax Paradigm Multi-Mode Microplate Reader. The kinetics of aSyn aggregation was determined by monitoring the fluorescence of Thioflavin-T binding to aSyn for 7 days.

Dynamic Light Scattering

Dynamic light scattering measurements were performed using Quasi-Elastic Light Scattering in a 90Plus/BI-MAS Multi Angle Particle Sizing analyzer (Brookhaven Instruments, Holtsville, NY) operating at λ = 633 nm. The protein samples were prepared at 100.0 μM (aSyn monomer equivalents) and

buffered in MOPS (pH 7.4) and incubated at 37°C for time points. The effect of Mn²⁺-complex on the aggregation of aSyn was evaluated adding a freshly prepared solution of the standardized Mn²⁺-complex to obtain aSyn:Mn²⁺-complex 1:2 or aSyn:Mn²⁺-complex 1:25 and/or aSyn:Mn²⁺-complex 1:50 molar ratios. Multimodal size distribution analysis of proteins was calculated from the values of intensity weighted sizes obtained from the non-negatively constrained least squared (NNLS) algorithm [33]. All samples were measured 5-10 times and reported results are the averages of 5 different measurements.

Nuclear Magnetic Resonance spectroscopy

NMR spectra were collected at 15°C on a Bruker Avance III 800 MHz spectrometer (National Center for Nuclear Magnetic Resonance Jiri Jonas, IBqM-UFRJ) equipped with an inverse-detection 5 mm TXI HCN z-gradient probe. 100 µM ¹⁵N-aSyn in 10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 10% (v/v) ²H₂O was used for NMR measurements. 2D [¹H, ¹⁵N] HSQC spectra were recorded in the absence (control) and presence of 10 µM Mn²⁺-complex. ¹H and ¹⁵N resonance assignments were performed according to previously reported chemical shifts for intrinsically disordered aSyn (Rao *et al.* 2009). Spectral intensities were measured in the absence (I₀) and presence (I) of the Mn²⁺-complex and intensity ratios (I/I₀) were used to monitor the interaction and determine aSyn residues that directly bind the complex. Spectra were collected and processed with TopSpin 3.1 (Bruker Biospin), and analyzed with CcpNmr Analysis [34].

Computational Study

Firstly, the structure of the Mn²⁺-complex was drawn in MarvinSketch v21.11 (Chemaxon Ltd; <https://chemaxon.com>) enabling correct assignment of coordinate bonding. The resulting structure was 'cleaned' in 3D and saved in .gjf format for input into the Gaussian 16 revC.01 package (Gaussian Inc. 340 Quinipiac st., Bldg. 40, Wallingford CT 06492, USA). The molecular structure of the Mn²⁺-complex was subsequently optimized in water as a solvent via the hybrid B3LYP (Becke-3 Parameter-Lee-Yang-Parr) [35] DFT (Density Functional Theory) method in GEN mode to enable assignment in the calculation of the 6-31G** and LANL2DZ basis sets, to atoms H, O, C, N and Mn respectively. Gaussview 6 (Gaussian Inc. 340 Quinipiac st., Bldg. 40, Wallingford CT 06492, USA) was next used to examine the structures of all converged structures and the final optimized structure was exported in .pdb file format.

The Mn²⁺-complex was subsequently imported into MOE 2020.09 (Chemical Computing Group ULC; <https://www.chemcomp.com/>) and docked to PDB entry 1XQ8 with triangle matching placement and scoring with the London dG scoring function. Subsequent refinement of all docked poses using the GBVI/WSA dG scoring function with induced fit was undertaken. Finally, the top docked pose was manipulated and visualized also using MOE 2020.09 (Chemical Computing Group ULC; <https://www.chemcomp.com/>).

Statistical Analysis

For all experiments data were analyzed using Graph Pad Prism 8 (San Diego, California, USA) software. All values were expressed as mean values $\pm$ standard deviation (SD) of at least three independent experiments and were analyzed using Student's t test which denotes homogeneity between experimental groups at $P \leq 0.05$.

Results

Mn²⁺-complex increases oxidative stress resistance by increasing the activity of antioxidant enzymes

Initially, to evaluate the toxicity of the Mn²⁺-complex, the wild-type strain (BY4741) of *S. cerevisiae* was directly exposed to increasing concentrations (0.1 to 100.0 μ M) of the complex. According to our results we have not observed any cytotoxic effect at lower (up to 25.0 μ M) Mn²⁺-complex dosage (**Figure 1B**). However, a significant cytotoxicity was observed after the treatment with the Mn²⁺-complex at higher concentrations, above 50.0 μ M (**Figure 1B**). Since the Mn²⁺-complex was not toxic at lower concentrations, we evaluate whether this complex would be able to reduce the susceptibility of *S. cerevisiae* to oxidative stress. According to **Figure 1C** it can be observed that under oxidative stress conditions, the survival of cells is drastically reduced by approximately 10% after H₂O₂ stress. However, after treating *S. cerevisiae* with of Mn²⁺-complex (0.1, 1.0 and 10.0 μ M) for 1 h the survival levels increased to approximately 20%, 30% and 50%, respectively. Moreover, using the oxidant sensitive probe 2',7'-dichlorodihydrofluorescein diacetate, which is a well-known tool to measure intracellular ROS, we also observed that the pre-treatment with the Mn²⁺-complex was able to reduce the high levels of ROS generated after 2.0 mM H₂O₂ stress (**Figure 1D**). Interestingly, in the presence of H₂O₂ we observed that the fluorescent intensity increased about 8 times in comparison to the untreated cells, whilst after treating with Mn²⁺-complex (1.0 and 10.0 μ M) followed by H₂O₂ stress, the levels of fluorescence intensity significantly dropped (almost 4-fold decrease) to the levels of untreated cells fluorescence (**Figure 1D**). As expected, the single treatment with the Mn²⁺-complex has not induced a great production of ROS (**Figure 1D**). Overall, we can conclude that the Mn²⁺-complex was able to decrease the oxidative events caused by H₂O₂ stress in the intracellular environment. At this point, by observing that the Mn²⁺-complex can protect yeast cells against an oxidative stress and decreases the levels of ROS intracellularly, we investigated whether the increase of the activity of antioxidant enzymes, such as superoxide dismutase (Sod) and catalase (Cat), could be related to the acquisition of oxidative stress response. As expected, a very low catalase activity, less than 1.0 U/mg of protein, was observed for cells under fermentative conditions. However, after the treatment with the Mn²⁺-complex, we can observe an almost 2-fold increase in catalase activity (**Figure 1E**). Regarding Sod activity, we observed that Mn²⁺-complex treatment (1.0 and 10.0 μ M) increased the relative Sod activity approximately to 1.2 and 1.5-times in relation to the untreated cells, respectively (**Figure 1F**). These results indicate that the Mn²⁺-complex protected yeast cells by increasing intracellular Cat and Sod activity, which together seems to be related to the reduction of ROS accumulation under H₂O₂ stress.

Mn²⁺-complex reduces H₂O₂-induced toxicity in *Galleria mellonella*

Given the antioxidant activity of the Mn²⁺-complex on yeast cell model, we investigated the efficacy of the Mn²⁺-complex on H₂O₂ toxicity in an *in vivo* model using the larvae of the invertebrate, *G. mellonella*. Our results showed that *G. mellonella* was susceptible to H₂O₂ injection since after the first day of injection larvae appeared melanized (**Figure 2A**). The process of melanization was rapidly followed by the death of larvae since only 30% of *G. mellonella* survived after the 3rd day of H₂O₂ injection, reaching to 10% of survival after the 7th day (**Figure 2B**). Relevant to the antioxidant activity of the Mn²⁺-complex, we can observe that the Mn²⁺-complex was well tolerated by *G. mellonella*. No signal of melanization and death were observed in the larvae treated with the Mn²⁺-complex (**Figure 2A and B**). Moreover, a significant reduction in larvae melanization was observed after the first day of treatment with the Mn²⁺-complex followed by H₂O₂ injection (**Figure 2A**). Indeed, the Mn²⁺-complex attenuated the toxic effect of H₂O₂ injection since it was observed (after 48 h) the beneficial effects of Mn²⁺-complex for the *G. mellonella* larvae. In a comparative perspective, we can observe that after 72 h and 168 h of injection with 5.0 µg/larvae of the Mn²⁺-complex, an increase in survival of 85% and 50%, respectively, whilst in the case of larvae injected with 5.0 M H₂O₂, the survival was, approximately, 30% and 10%, respectively. The treatment with 10.0 µg/larvae of the Mn²⁺-complex increased the survival in 60% and 50% at 72 h and 168 h, respectively (**Figure 2B**).

Mn²⁺-complex protects yeast cells against aSyn-induced toxicity

Using the yeast model for aSyn expression we evaluated whether the Mn²⁺-complex could modulate the pattern of aSyn toxicity. The Mn²⁺-complex concentrations tested were selected based on our previous analysis, which showed that the treatments with 0.1, 1.0 and 10.0 µM of the Mn²⁺-complex were non-toxic and increased yeast survival under oxidative stress. As expected, aSyn expression drastically affects cell viability (**Figure 3A**). The results demonstrate a discrete reduction in cell survival after 4 and 6 h of the induction of aSyn expression. However, after 12 h of the aSyn expression, an increase in aSyn toxicity was observed, reducing the survival of cells to 40% and after 24 h under aSyn expression, the yeast cell survival decreased drastically to 15%, confirming that aSyn expression was toxic for *S. cerevisiae* (**Figure 3A**). As a control, cells containing the empty vector showed no changes in survival over the timecourse in SGal medium (**Figure 3A**). This result proves that the survival of cells without aSyn expression is not affected by the growth in SGal. Regarding the effect of the Mn²⁺-complex on survival of yeast cells expressing aSyn, we observed that all treatments with the complex reduced aSyn toxicity. At lower dose treatment (0.1 µM), although the complex did not protect yeast cells after 24 h of aSyn expression, it was possible to observe that yeast cells were protected by the complex during short time aSyn expression (**Figure 3A**). Survival of cells expressing aSyn increased after treatment with 1.0 and 10.0 µM of the complex. Treating yeast cells with 1.0 µM of the Mn²⁺-complex we observed an increase in cell survival to 90% and 55% after 12 and 24 h of aSyn expression, respectively, whilst at 10.0 µM of the complex treatment cells were totally tolerant to aSyn toxicity, presenting similar survival rates from those

observed in the control cells (with empty plasmid) (**Figure 3A**). To the best of our knowledge, the toxicity of aSyn is associated with the formation of cytosolic inclusions [28]. Thereby, we then assessed whether the enhancement in cell viability could be a consequence of changes in the formation of aSyn inclusion. Indeed, treatment with the Mn^{2+} -complex reduced the formation of aSyn inclusions (**Figure 3B**).

To assess the induction of oxidative stress upon aSyn expression, we determined the levels of lipid peroxidation in cells containing the empty vector (BYp426), as well as in yeast cells expressing human aSyn (BYaSyn). Cells harvested after 0, 12 and 24 h of growth in SGal medium for aSyn-expression were assayed for determination of lipid peroxidation using, as reference, malondialdehyde (MDA), a final product of lipid oxidation. Control cells, containing the empty vector showed a two-fold increase in the levels of lipid peroxidation after 12 and 24h of growth in SGal medium (**Figure 3C**). Regarding to lipid peroxidation in aSyn expressing cells, a 4 and 5-fold increase in the level of lipid peroxidation can be observed after 12 and 24 h of the induction of aSyn-expression, respectively. After 24 h of aSyn-expression, the increase in lipid peroxidation is even more pronounced, with MDA levels increasing more than 2-times compared to the values observed in the control strain. These results showed that the Mn^{2+} -complex alleviates aSyn-associated oxidative stress.

Mn^{2+} -complex protects human H4 cells from H_2O_2 -induced toxicity and reduces aSyn oligomerization and inclusion formation

Cytotoxicity of Mn^{2+} -complex was evaluated in H4 cells, and as expected the treatments with the low complex dosage (0.1, 1.0 and 10.0 μM) presented a similar LDH release, close to 10%, to the untreated cells indicating that H4 cells well-tolerate Mn^{2+} -complex. However, after the treatment of cells with 50.0 and 100.0 μM we observed an increase in the LDH release, 25% and 30%, respectively (**Figure 4A**). Next, the antioxidant activity of the Mn^{2+} -complex was determined in H_2O_2 -stressed H4 cells. As expected, we observed a 2.5-fold increase in ROS formation in H_2O_2 -stressed cells in comparison to the untreated cells. However, when cells were treated with different concentrations of the Mn^{2+} -complex, we observed that all concentrations reduced the ROS formation compared to the condition with H_2O_2 . These results show that the Mn^{2+} -complex also displayed an antioxidant activity in the H4 cells, corroborating the previous results described for the yeast and *G. mellonella* models (**Figure 4B**).

Next, we used the bimolecular fluorescence complementation assay (BiFC) to assess whether the Mn^{2+} -complex might modulate aSyn oligomerization in H4 cells [31]. The BiFC assay is based on reconstitution of an intact fluorescent protein when two complementary non-fluorescent fragments are brought together by a pair of interacting proteins. Thus, the interaction of two aSyn monomers, forming dimers or oligomers, reconstitute the N-terminal and C-terminal portions of venus protein, which emits a green fluorescence (**Figure 5A**). Our results showed that the oligomerization of aSyn was stronger in untreated cells. However, treating cells with Mn^{2+} -complex resulted in a reduction in fluorescence. This appears to be due to the greater diffusion of venus fluorescent protein caused by the Mn^{2+} -complex treatment (**Figure 5B**). By analyzing the fluorescence intensity determined by flow cytometry, the H4 cells treated

with the Mn^{2+} -complex (0.1, 1.0 and 10.0 μM) presented lower fluorescence compared to the untreated cells (**Figure 5C**).

To assess the effect of Mn^{2+} -complex treatment on the formation of mature aSyn inclusions, we used the SynT (SynT and Synphilin-1) model in H4 cells [31,36]. Immunoblotting analysis showed that 1.0 μM concentration did not change the levels of aSyn, since the quantification of aSyn expression showed similar levels to the control (**Figure 6A and B**). However, after the treatment of H4 cells with 10.0 μM of the Mn^{2+} -complex, the expression of SynT was significantly reduced (**Figure 6A and B**). We subsequently investigated the effect of the Mn^{2+} -complex on aSyn aggregation (**Figure 6D**). We observed that the Mn^{2+} -complex treatment increased the percentage of cells without aSyn inclusions. Treating H4 cells with both Mn^{2+} -complex dosage we observed an almost 2-fold increase in the number of transfected cells without aSyn inclusions (**Figure 6D**).

Mn^{2+} -complex inhibits aSyn fibrillation via binding at the C-terminus

The fibrillation process in the presence of the Mn^{2+} -complex showed an unaltered lag phase, but the fluorescence intensity was significantly lower compared to the system without the addition of the Mn^{2+} -complex (**Figure 7A**). The incubation of aSyn with the Mn^{2+} -complex revealed that the compound modulated aSyn aggregation, reducing the formation of ThT positive structures at the end of the reaction by an almost 20-30% (**Figure 7A**). This reduction indicated the inhibition of aSyn fibrillation by the Mn^{2+} -complex. In parallel, we also determined the size of aSyn aggregates in the presence and absence of the Mn^{2+} -complex, which the hydrodynamic diameter of the protein at the early stages of aggregation were estimated by dynamic light scattering (DLS) (**Figure 7B**). The distribution of the diameter with respect to the particle volume in solution were recorded at the same initial timepoints of those for aSyn fibrillation. At early incubation times (before the beginning of the fibril formation detected by ThT fluorescence intensity), DLS confirmed the existence of aSyn in a monomeric state (mean diameter = 8.2 ± 0.06 nm) as reported in literature. As expected, the hydrodynamic diameter of aSyn increased exponentially, in the absence of the Mn^{2+} -complex, indicating the occurrence of aggregates (mean diameter = 2042.5 ± 98.08 nm at 72 h). Under the presence of 2.0 μM of the Mn^{2+} -complex, the size distribution of aSyn remains essentially unchanged. When aSyn was incubated with 25 and 50 μM of the Mn^{2+} -complex, the formation of large aggregates was significantly inhibited from 24 h to 72 h of timescale. The hydrodynamic diameter of aSyn significantly decreased over time, with observed values of 1019.3 ± 41.08 with 25 μM and 884.8 ± 16.47 with 50 μM at 72 h. However, DLS measurements suggest that the observed reduction in ThT fluorescence corresponding to an effective decrease in the levels of aSyn fibers was well correlated with the decrease in the dispersion of light in the presence of the Mn^{2+} -complex after 24, 48 and 72 h. These results suggest that the Mn^{2+} -complex interacts with aSyn reducing its aggregation kinetics and the size of aSyn amyloid fibers. To resolve the potential interaction between the Mn^{2+} -complex and aSyn we investigated the details of the Mn^{2+} -complex binding to aSyn by NMR spectroscopy. To study this interaction, 2D [^1H , ^{15}N] HSQC spectra were recorded in the absence and

presence of the Mn^{2+} -complex (1:0.1 molar ratio). Addition of the Mn^{2+} -complex caused broadening and a decrease in intensity of specific amide resonances, due to paramagnetic relaxation effects induced by the Mn^{2+} center, indicating that the Mn^{2+} -complex directly binds monomeric, soluble aSyn (**Figure 7C**). Residues Asp115, Asp119, Asp121, Asn122, Asp135, and Glu137, located at the highly acidic C-terminal region, exhibited the strongest intensity reduction (**Figure 7D**), thus confirming the binding features of the Mn^{2+} -complex salt $\{[Mn_2(\mu_2\text{-oda})(phen)_4(oda)_2]^{2-}[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2]^{2+}\}$ and showing the role played by carboxylic side chains at the C-terminal region of aSyn as anchoring moieties.

Interestingly, our combined DFT analysis and molecular docking study (**Figure 8**) also indicated that the Mn^{2+} -complex interacts with those keys negatively charged acidic residues identified by NMR (e.g. Asp135) and located at the C-terminal of aSyn monomers. All docked poses of the Mn^{2+} -complex were found at the C-terminal and it is thus likely that fibril formation is impeded via shielding of the charge at the C-terminal by the Mn^{2+} -complex through both hydrogen bonding and π -stacking.

Discussion

Although the exact function of aSyn is still unclear, it is well-known that it can interact and affect the functioning of several proteins. Due to this aSyn-protein interactive network, several functions (e.g. stabilization of cell structure and protein trafficking, transmembrane transport, formation of synaptic vesicles and regulation of synaptic functions) have been addressed for aSyn. However, as intrinsically disordered protein, aSyn can adopt abnormal structures gaining toxic functions, which disturbs mitochondrial function, mainly due to the induction of ROS formation, increasing the occurrence of oxidative stress [37, 38]. Moreover, it has been shown that yeast expressing high levels of aSyn (aSyn HiTox) showed mitochondrial dysfunction and oxidative stress caused by aSyn toxicity [39]. In addition, it has been reported that lipid peroxidation, protein and DNA oxidation have also been found to be systematically elevated in post-mortem tissues of PD patients. The phosphorylation at serine 129 (pSer aSyn) in the C-terminal domain, which is the most relevant PTMs associated with the aSyn toxic gain of function, disrupts mitochondrial function leading to mitochondrial senescence and an induction of ROS formation [38, 40, 41]. All of these aSyn toxic functions are likely to contribute to the establishment of oxidative stress [40, 42, 43].

In this study, we showed the antioxidant potential of a cationic Mn^{2+} -complex, $[Mn_2(\mu_2\text{-oda})(phen)_4(H_2O)_2]^{2+}$, in *S. cerevisiae* and *G. mellonella* models of study. Since oxidation is one of the key features of aSyn abnormal function, we also proved that the antioxidant activity of the Mn^{2+} -complex attenuated the proteotoxicity of aSyn. The Mn^{2+} -complex is a dinuclear Mn^{2+} , cationic, bis-phenanthroline octanedioate molecule that besides being considered a remarkable cytotoxic agent against human cancer cells, also showed reduced toxicity (e.g. *in vitro* and *in vivo*) and a potent Sod and Cat mimetic activity (e.g. *in vitro*), demonstrating an exceptional superoxide radical and H_2O_2 elimination rate [19]. Indeed, the aromatic N,N-ligand 1,10-phenanthroline (phen), and its extensive derivatives have been extensively studied due to their versatility and diverse biological properties [44–48]. Regarding the

antioxidant capability of phen-based complexes, it was first reported by our group the antioxidant activity of Ag⁺ dicarboxylate complexes containing 1,10 phenanthroline in *S. cerevisiae* cell model [48]. Here, a new biological property for the Mn²⁺-complex containing the phen ligand is reported since it was capable of promoting an antioxidant protection against oxidative stress in different organisms (e.g. *S. cerevisiae*, *G. mellonella* and H4 cells).

Treatment of yeast cells with the Mn²⁺-complex increased the tolerance against H₂O₂ presumably by increasing the activity of Sod and Cat antioxidant enzymes, which reduce intracellular ROS accumulation. Regarding the protective effect exerted by the Mn²⁺-complex in *G. mellonella*, the 5-fold increase in larval survival after Mn²⁺-complex treatment following H₂O₂ injection consolidates the antioxidant capacity observed in *S. cerevisiae*, whose increasing survival varied from 2 to 5 times according to the doses of the Mn²⁺-complex used in the treatments. To the best of our knowledge this is the first time that *G. mellonella* was used to evaluate the antioxidant property of a compound against oxidative stress generated by H₂O₂ administration. Indeed, larvae of *G. mellonella* have been used recently as a convenient and inexpensive *in vivo* screening model to assess the therapeutic potential of new drugs producing results that are compatible to those obtained with mammalian models [49]. H4 cells also contributed to show the antioxidant protective behaviour of the Mn²⁺-complex since after treatment of cells with this complex reduced ROS production in the mitochondria. Moreover, observing the antioxidant activity of a distinct metal-based complex containing the 1,10 phenanthroline ligand, we suggest that this chemical group could be an interesting platform for the development of novel antioxidant compounds.

Here, we showed that aSyn-toxicity was related to lipid oxidation and impairment of cell survival, given the significant increase in the levels of malondialdehyde upon aSyn expression. It has already been reported that lipid peroxidation end products, even at low molar excess, can induce aSyn oligomers formation, showing once again the straight relationship between aSyn toxicity and oxidative stress [50]. In contrast, treating aSyn expressing cells with antioxidant compounds (e.g. ascorbic acid) reduces aSyn inclusion formation and protects cells from aSyn-induced toxicity [51]. Interestingly, the Mn²⁺-complex treatment reversed the proteotoxicity of aSyn since yeast growth was improved under aSyn expression. Moreover, this complex also interferes in the formation of aSyn inclusions in yeast cells, which suggests a new function for complexes containing the 1,10-phenanthroline ligand.

Several coordination compounds have been synthesized and its antioxidant activity studied to establish new alternative strategies, capable of reducing or avoiding the consequences of oxidative stress and related diseases, such as cardiovascular, neurodegenerative and inflammatory disorders [52]. The development of synthetic antioxidants is mostly based on the redox activity of metal-based complexes [52–54]. In this context, although manganese at a certain dosage can act as a neurotoxin [55], for the synthesis of antioxidants catalysts, this metal was established as the preferred choice over the most toxic transition metals, such as iron and copper [56]. Herein, we showed a promising antioxidant activity together with a great capacity to reduce aSyn proteotoxicity for a Mn²⁺-complex.

To the best of our knowledge, there are three main structural classes of manganese-containing catalytic antioxidants (e.g. metalloporphyrins, macrocyclics and salen complexes) that have been extensively studied as antioxidant agents [57]. The EUK compounds, a class of salen complexes, demonstrated great capacity as superoxide and hydrogen peroxide scavenger, and can react with lipid peroxides. Amongst salen complexes, the EUK-134 showed to reduce the oxidative damages in rats [58]. Regarding metalloporphyrins derivatives, it has been reported that AEOL 11203, AEOL 11207 and AEOL 11114, would act as potential candidates for further clinical therapy for PD [14]. The manganese cyclic polyamine, M40403, has been shown to compensate the loss of either Sod1 or Sod2 in *Drosophila melanogaster* and provided protection against oxidative damage induced by paraquat treatment [16]. Although these compounds have shown a promising effect against oxidative stress and related diseases, they all have serious undesirable effects (e.g. toxicity in low concentrations, low solubility in water, soluble in toxic organic solvents) that hinder their use in clinical trials [59–63].

In contrast, the Mn^{2+} -complex presented herein is water-soluble and is not toxic in *S. cerevisiae* and *in vivo*, in *G. mellonella*, over a wide range of concentrations. Moreover, the protective antioxidant activity of the Mn^{2+} -complex against oxidative stress seems to be related to the complex's ability to control the formation of ROS, either by acting as Sod and Cat mimics or activating these intracellular antioxidant enzymes. Interestingly, the ability of the Mn^{2+} -complex to modulate the size and aggregation of aSyn was also observed *in vitro* and in a human cell-based model of synucleinopathy, demonstrating that the inhibitory mechanism exerted by this complex on amyloid assembly can be a direct consequence of its interaction with aSyn, as shown by NMR analysis and supported by computational analysis. NMR-based biophysical studies have shown that aSyn presents a poor affinity for Mn^{2+} ion, in the range of 1.0 mM, and the binding site of Mn^{2+} is located at the C-terminal region of aSyn, specifically at residues Asp121, Asn122, and Glu123 [8]. Structural information on the binding of Mn^{2+} to aSyn by native top-down and ion mobility mass spectrometry, revealed that Mn^{2+} binds at two locations at the acidic C-terminal region of aSyn, $^{119}\text{DPDNEAYE}^{126}$ and $^{132}\text{GYQDY}^{136}$, which constitute the primary and secondary binding sites, respectively [64]. In agreement with this, both our NMR and computational data suggest the Mn^{2+} complex interacts via hydrogen-bonding of two Mn coordinated water molecules to acidic residues at the C-terminus of aSyn. Additional pi-stacking interactions are made between the phenanthroline moiety and aromatic residues of aSyn and the binding of the Mn^{2+} complex leads to disruption of aSyn oligomerization and/or aggregation. In agreement, has been previously described modifications to the C-terminal that resulted in alteration to the charge at the C-terminal which played an important role in preventing early fibril formation [65].

Taken together, our results point out the potential use of the Mn^{2+} -complex as an alternative to circumvent the harmful effects of oxidative stress, which can be used as antioxidant therapy with promising activity to attenuate aSyn toxicity.

Declarations

Consent for publication:

All authors have agreed with the content of this study, expliciting consent to submit. In addition, previously to submission all authors have obtained the consent from the responsible authorities at the research institute/organization where this study was carried out.

Data Availability:

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests:

The authors have declared no competing interests (financial or non-financial) that are relevant to the content of this article.

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Author Contributions:

Daniela Dias Queiroz, Thales de Paula Ribeiro, Tiago Fleming Outeiro and Marcos Dias Pereira contributed to the study conception and design. Material preparation, data collection and analysis were performed by Daniela Dias Queiroz, Thales de Paula Ribeiro, Julliana Muniz Gonçalves, Larissa Maura de Melo Mattos, Júlia Freitas, Fernando Lucas Palhano, Anderson Sá Pinheiro, Andrew Knox and Marcos Dias Pereira. All authors have contributed for data interpretation. The first draft of the manuscript was written by Daniela Dias Queiroz. Anderson Sá Pinheiro, Susana Frases, Michael Devereux, Andrew Knox, Tiago Fleming Outeiro and Marcos Dias Pereira carried out the writing-review and editing the original draft. All authors have commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Mn²⁺-complex protects *S. cerevisiae* from H₂O₂ stress. (A) Structure of the Mn²⁺-complex; (B) Yeast cells were incubated with different concentrations of the Mn²⁺-complex (0.1 to 100.0 µM/1 h) at 28°C and 160 rpm. Tolerance against Mn²⁺-complex was expressed as percentage of survival. (C) Yeast cells cultured in YPD 2% medium were pretreated or not with the Mn²⁺-complex (0.1 to 10.0 µM/1 h) before being subjected to 2.0 mM H₂O₂/1 h. Survival was measured before and after exposure to oxidative stress and expressed as percentage of survival; (D) Intracellular oxidation was measured in yeast cells exposed to single dose of the Mn²⁺-complex (1.0 or 10.0 µM/1 h) and oxidative stress as well as to pre-treatments with the Mn²⁺-complex (1.0 and 10.0 µM/1 h) followed by oxidative stress. ROS levels were measured as fluorescence intensity of oxidized dichlorofluorescein (H₂DCFDA); (E) Catalase and (F) superoxide dismutase activity was determined in mild-exponential yeast cells subjected or not to Mn²⁺-complex (1.0 and 10.0 µM/1h) treatment. Cat activity were expressed as specific activity (U/mg ptn) and Sod activity was expressed as fold increase measured by the ratio of Sod activity between treated and untreated yeast cells.

A

B

Figure 2

Mn²⁺-complex increases the survival of *G. mellonella* larvae after the H₂O₂-induced toxicity (A) Representative visual appearance of *G. mellonella* larvae treated with: the Mn²⁺-complex, H₂O₂ stress and/or treated with the Mn²⁺-complex and then stressed with H₂O₂; (B) Larvae of *G. mellonella* (10 per group) were subjected to the toxic effects of H₂O₂ by direct injection into the larvae haemocoel, and the larvae survivability was followed up for 7 consecutive days. In parallel, the larvae were treated with the

Mn2+-complex to evaluate its capacity to prevent the toxic effects of H2O2. Thus, larvae were pretreated with 5.0 or 10.0 µg/larvae of Mn2+-complex for 4 h and then submitted to H2O2 stress. The larvae survival was determined accordingly. All experiments were performed at least three times and maintained at 28°C in the dark.

Figure 3

Mn2+-complex protects *S. cerevisiae* from aSyn-induced toxicity. (A) The effect of treatment with the Mn2+-complex on viability of BY4741 cells subjected to the aSyn expression. Transformed cells were treated or not with the Mn2+-complex up to 24 h in SGal medium (aSyn ON). (B) Representative confocal microscopy images of yeast positive aSyn cells and treated with 10.0 μM of the Mn2+-complex for 24 h (Scale bar: 10.0 μm). (C) Effect of aSyn expression on the lipid peroxidation in *S. cerevisiae*. The cells were incubated in synthetic galactose medium (aSyn ON) for 0 (light gray), 12 (dark gray) and 24 h (black). Lipid peroxidation was expressed in picomoles of MDA per milligram of cells. Values represent the mean ± SD of three independent experiments.

Figure 4

Mn2+-complex is not toxic and reduces the levels of mitochondrial ROS formation in H4 cells. (A) Cytotoxicity of Mn2+-complex in human H4 cells measured by LDH release. (B) Levels of mitochondrial ROS formation in H4 cells detected by MitoSOX fluorescence. The results represent the mean ± SD of three independent experiments.

Figure 5

aSyn oligomerization is attenuated by Mn²⁺-complex. (A) Schematic representation of the BiFC assay. (B) A representative fluorescence microscopy image of H4 cells expressing aSyn (Scale bar: 10.0 μm). The column MERGE indicates a superposition between venus and the nucleus of the cell. Venus fluoresces green whilst the nucleus appears blue due to DAPI staining. (C) The intensity of venus fluorescence was determined by flow cytometry, and values represent the mean ± SD of three independent experiments.

Figure 6

Mn²⁺-complex modifies the number of aSyn inclusions. (A) Immunoblotting of aSyn profile in H4 cells transfected with SynT and treated or not with the Mn²⁺-complex; (B) Quantification of the immunoblots normalized to β-actin levels that was used as loading control; (C) Representative image of cells expressing SynT. H4 cells expressing SynT constructs were treated with Mn²⁺-complex for 4 h and analyzed by fluorescence microscopy. Scale bar 10 μm. (D) Cells subjected to immunofluorescence labeling were assessed for determination of the number of cells, control and Mn²⁺-complex treated, with or without the presence of aSyn inclusions.

Figure 7

Mn $^{2+}$ -complex inhibits aSyn aggregation in vitro by its interaction with specific amino acids at the C-terminal domain. (A) Aggregation kinetics was monitored with 100.0 μ M of aSyn alone (filled circle) or in the presence of 2.0 μ M (filled triangle) 25.0 μ M (square triangle) and 50.0 μ M of the Mn $^{2+}$ -complex (filled lozange). Intensity of ThT fluorescence is plotted as a function of time. (B) Dynamic light scattering of the aSyn WT in solution (black bars) and the decrease in diameter upon incubation with 2.0 μ M (light gray bars) 25.0 μ M (medium gray bars) and 50.0 μ M (dark gray bars) of the Mn $^{2+}$ -complex indicating disaggregation of aSyn fibers. (C) Overlay of the 2D [^1H , ^{15}N] HSQC spectra obtained for 100.0 μ M aSyn in the absence (black) and presence (blue) of 10.0 μ M of the Mn $^{2+}$ -complex. Amide resonances that disappear from the spectrum upon binding to the Mn $^{2+}$ -complex are labeled. (D) Intensity ratio (I/I_0) calculated from the amide resonance intensities obtained in the presence (I) and absence (I_0) of the Mn $^{2+}$ -complex shown as a function of aSyn residue number. Residues that show the lowest intensity values (below 0.15) are marked with asterisks and labeled accordingly.

Figure 8

DFT/molecular docking study confirmed that the Mn²⁺-complex interact with aSyn at the C-terminus. (A) The predicted binding mode of the Mn²⁺-complex and (B) those specific residues of involved in hydrogen bonding and π-stacking, with the C-terminus of human aSyn (PDB entry 1XQ8).