Novel 1-hydroxypyridin-2-one metal chelators prevent and rescue ubiquitin proteasomal-related neuronal injury in an in vitro model of Parkinson’s disease

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IN VITRO SYSTEMS

Abstract
Ubiquitin proteasome system (UPS) impairment, excessive cellular oxidative stress, and iron dyshomeostasis are key to substantia nigra dopaminergic neuronal degeneration in Parkinson’s disease (PD); however, a link between these features remains unconfirmed. Using the proteasome inhibitor lactacystin we confirm that nigral injury via UPS impairment disrupts iron homeostasis, in turn increasing oxidative stress and promoting protein aggregation. We demonstrate the neuroprotective potential of two novel 1-hydroxy-2(1H)-pyridinone (1,2-HOPO) iron chelators, compounds C6 and C9, against lactacystin-induced cell death. We demonstrate that this cellular preservation relates to the compounds’ iron chelating capabilities and subsequent reduced capacity of iron to form reactive oxygen species (ROS), where we also show that the ligands act as antioxidant agents. Our results also demonstrate the ability of C6 and C9 to reduce intracellular lactacystin-induced α-synuclein burden. Stability constant measurements confirmed a high affinity of C6 and C9 for Fe³⁺ and display a 3:1 HOPO:Fe³⁺ complex formation at physiological pH. Reducing iron reactivity could prevent the demise of nigral dopaminergic neurons. We provide evidence that the lactacystin model presents with several neuropathological hallmarks of PD related to iron dyshomeostasis and that the novel chelating compounds C6 and C9 can protect against lactacystin-related neurotoxicity.

Keywords Alpha-synuclein · Dopaminergic · Iron chelators · Lactacystin · Parkinson’s disease · Reactive oxygen species

Abbreviations
Aβ Amyloid β-peptide
α-SYN α-Synuclein
C Compound
DFP Deferiprone
DDA Deoxyribose degradation assay
DFO Desferoxamine
DHR Dihydrorhodamine
DMSO Dimethyl sulfoxide
DPH 2,2-Diphenyl-1-picrylhydrazyl
DMT1 Divalent metal transporter 1
DAergic Dopaminergic
DMEM Dulbecco’s Modified Eagle’s medium
UV Ultraviolet
EDTA Ethylenediaminetetra-acetic acid
eROS Extracellular ROS
FT Ferritin
FPN Ferroportin
TfR Transferrin
FBS Foetal bovine serum
GLEE Glass electrode evaluation
Hepc  Hepatocyte-secreted hormone
HEPES  4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HOPO  Hydroxypyridinone
1,2-HOPO  1-Hydroxypyridin-2-one
HRP  Horseradish peroxidase
ICP-MS  Inductively-coupled-plasma mass spectroscopy
iROS  Intracellular ROS
IRP2  Iron regulatory protein 2
LBs  Lewy bodies
LMCT  Ligand to metal charge transfer band
MS  Mass spectrometry
MFI  Mean fluorescence intensity
MTS  (3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Mfrn2  Mitoferrin-2
NMR  Nuclear magnetic resonance
PD  Parkinson’s disease
ppm  Parts per million
PBS  Phosphate-buffered saline
PAGE  Polyacrylamide gel electrophoresis
PVDF  Polyvinylidene difluoride
RIPA  Radioimmunoprecipitation assay
ROS  Reactive oxygen species
RT  Room temperature
RPM  Rotations per minute
6-OHDA  6-Hydroxydopamine
SDS  Sodium dodecyl sulfate
SD  Standard deviation
SN  Substantia nigra
SNpc  Substantia nigra pars compacta
TBA  Thiobarbituric acid
UPS  Ubiquitin proteasome system
UV  Ultraviolet

Introduction

Parkinson’s disease (PD) associates with the progressive degeneration of nigro-striatal dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc), resulting in the development of motor symptoms such as resting tremor, muscle rigidity, and bradykinesia (Poewe and Mahlknecht 2009). Although the exact cause of idiopathic PD remains unknown, excessively high levels of localized iron concentrations within the brain has been deemed contributory to progressive PD (Sian-Hülsmann et al. 2011). Iron cytotoxicity ensues when iron exists in a labile state and elicits a cascade of cellular deleterious events, driven by participation in redox reactions i.e., the Haber–Weiss cycle (Halliwell and Gutteridge 1984). The pathogenic mechanism underlying iron-mediated cellular damage and eventual neuronal death seen in PD-affected brains likely involves excessive generation of intracellular free radicals [e.g., reactive oxygen species (ROS)], which eventually overwhelms the cell’s antioxidant- or radical scavenging defense systems. This includes reducing levels of glutathione, a prominent brain anti-oxidant, resulting in oxidative damage to lipids, proteins, DNA, and mitochondria (Dixon and Stockwell 2014). SNpc DAergic neurons are particularly vulnerable to oxidative stress. Mechanisms underlying such selective vulnerability include nigral DAergic neurons’ high iron levels and dopamine (DA) molecules’ high rate of oxygen utilization (Sayre et al. 2005). The extensive SNpc DAergic damage seen in PD-affected brains contrasts with normal ageing, during which iron is more likely to be deposited in a different portion of the SN, the pars reticulata part (Mas troberardino et al. 2009).

The ubiquitin proteasome system (UPS), a critical regulator of protein homeostasis in eukaryotic cells is deemed essential for maintaining iron homeostasis (Thompson and Bruick 2012). Under non-pathological conditions, the UPS keeps the cellular environment free of misfolded and defective proteins; however, several neurodegenerative diseases, including PD, is characterised by proteasomal dysfunction, resulting in accumulation of aggregation-prone proteins and thus aberrant iron homeostasis. In particular, α-synuclein (α-SYN), an abundantly expressed 140-amino acid protein encoded by SNCA, involved in various cellular processes including DA release and -reuptake (Abeliovich et al. 2000; Braak et al. 2003), undergoes sequential aggregation during progressive PD, to form so-called intracellular Lewy bodies (LBs) and Lewy neurites, which underlies PD pathology. Experimental evidence supports the notion that abnormal α-SYN aggregates spread from brainstem to neocortex, propagating from cell-to-cell in a prion-like manner (Watts et al. 2013).

The realisation that protein fibrillisation comprises a fundamental pathological hallmark of PD-affected brains has resulted in increasing use of proteasome inhibitors for modeling PD both in vitro and in vivo, thereby serving as a test bed for novel therapeutic strategies. The microbial metabolite lactacystin induces a PD-like motor phenotype in rodents, concomitant with loss of DAergic cell bodies in the SNpc and DAergic terminals in the striatum, striatal DA depletion, as well as α-SYN accumulation in remaining SN DAergic neurons (McNaught et al. 2002; Lorenc-Koci et al. 2011; Pienaar et al. 2015a, b; Savolainen et al. 2017). For instance, mice intracerebrally microinjected with lactacystin revealed increased iron deposition in the SN, accompanied by nigral DAergic neurodegeneration (Zhu et al. 2010). Although the exact mechanism by which iron deposition associates with lactacystin neurotoxicity remains unknown, this study suggested that this may relate to abnormal regulation of iron-related...
proteins, including iron regulatory protein 2 (IRP2) and divalent metal transporter 1 (DMT1) (Zhu et al. 2010). Further evidence that iron plays an important role in PD neuropathology induced by UPS impairment stems from reports that pharmacological iron chelators such as desferoxamine (DFO) and 5-[4-(2-hydroxyethyl) piperazin-1-yl (methyl)-8-hydroxyquinoline (VK-28) prevented iron deposition and DAergic neurodegeneration in lactacystin-injected mice (Zhang et al. 2005; Zhu et al. 2007).

Hence, due to the putative interplay between lactacystin-induced UPS impairment and iron misregulation, this toxin serves as a useful tool for exploring how an interplay between the iron-regulating and UPS pathways result in the DAergic neuronal degeneration and abnormal intracellular α-SYN-containing intracellular aggregates seen in PD. In addition, it serves as a translational cell model for assessing the disease-modifying potential of chelators of labile iron. The therapeutic use of iron chelators for removing excessive amounts of iron and/or redox-silencing in PD patients is receiving considerable research and clinical attention (Ward et al. 2015). Previous work performed on rats showed that brain-permeable iron chelators can alleviate lactacystin-induced DAergic neuronal degeneration, restore proteasome activity and reduce the presence of ubiquitin-positive intracellular inclusions in the SN (Zhang et al. 2005; Zhu et al. 2007).

Here we provide physicochemical information on two novel 1,2-HOPO-based iron chelators, compounds C6 and C9 to quantify their chelating power towards Fe³⁺, the protonated species distribution and their complex formation with Fe³⁺ in relation to pH. We previously studied C6 and C9 in SH-SY5Y human neuroblastoma cells exposed to 6-hydroxydopamine (6-OHDA) neurotoxic insult (Workman et al. 2015), which reproduce some features of PD-related neuropathology. These include increased ROS production, mitochondria functional inhibition and iron liberation from storage proteins such as ferritin (FT), resulting in DAergic neuronal death thereby impairing DA homeostasis (Alberio et al. 2012). The current study provides further characterization of the antioxidant, neuroprotective and neurorescuing properties as well as the ability by the novel iron chelators to reduce α-SYN intracellular load in SH-SY5Y cells treated with lactacystin. We compared the compounds’ effects to the clinically approved 3-hydroxy-4(1H)-pyridinone chelator, deferiprone (DFP), which was shown to improve PD patients’ motor and non-motor symptoms (Moreau et al. 2018).

**Material and methods**

**Materials**

Distilled water was purified by passing through a mixed bed of ion-exchanger (R3 type; Thermo Fisher Scientific, Waltham, MA, USA) and activated carbon (the Oxygen Release Compound type; Thermo Fisher Scientific). Metal cation solutions were prepared from their perchlorate salts. Their concentrations were determined via colorimetric titrations with ethylenediaminetetraacetic acid (EDTA) solution (Titriplex III, Merck Millipore, Watford, UK), in accordance with standard procedures (Fish 1988; Skoog et al. 2000). Sodium hydroxide and perchloric acid were used to adjust pH during titrations. The ionic strength of all solutions was fixed at 0.1 M using sodium perchlorate.

**Potentiometry**

The protonated forms of the ligands C6 and C9 (10⁻³ M) and the stability constants (log β) of their complexes with Cu²⁺ ([L]/[Cu²⁺] ≈ 2.2, L = C6 or C9) were characterized by potentiometric titrations in water in accordance with a previously published procedure (Gillet et al. 2017) using an automatic titrator system (904 DMS Titrino, Metrohm Limited, Switzerland) with a combined glass electrode (6.0234.100; Long Life, Metrohm Limited, Switzerland) filled with 0.1 M NaCl. The Fe³⁺ complexes were already fully formed at pH 2 and could therefore not be studied via this technique.

The potentiometric data were refined with the Hyperquad 2008 program (Gans et al. 1996), which uses non-linear least-squares methods, considering the formation of metal hydroxide species. The titration of each system was repeated in triplicate. The distribution curves, as a function of the pH of the protonated forms of C6, C9 and their Cu²⁺ complexes, were calculated using the Hyperquad Simulation and Speciation (HySS 2009) software program (Alderighi et al. 1999).

**Spectrophotometry**

The protonation constant (log K) of a molecule is a key parameter that influences several of its drug-like properties, such as its lipophilicity, half-life, oral bioavailability, and the ability to cross cell membranes and also the blood–brain barrier. The log K values of the ligands C6 and C9 and their stability constants with Cu²⁺ and Fe³⁺ were determined by ultraviolet (UV)-visible spectrophotometric titrations versus pH, by simultaneously recording pH and UV–visible spectra. Between pH = − 0.5 and pH = 2 (brackets indicate that the pH value was calculated) the batch technique was used, following a previously published procedure (Gillet et al. 2017).

Direct titrations were carried out between pH 2 and 12.5. For the protonation studies, an aliquot of 30 ml of ligand C6 or C9 solution was introduced into a thermostat-jacketed cell (25.0(2) °C). A known volume of perchloric acid solution was added to adjust the pH to 2 and a dynamic titration was carried out by adding known volumes of NaOH solution using a Metrohm 904 Titrino.
equipped with a Dosino 800 burette driven by the version 2.5 TiAmo software. The titrator was interfaced with an Agilent Cary 60 UV–visible spectrophotometer which automatically recorded a spectrum after an equilibration time of 2 min, following each addition of base. The complexity studies of C6 and C9 with Fe³⁺ and Cu²⁺ were carried out in the same way, on L:M stoichiometric ratios ([L]/[Fe³⁺] = 3.2 and [L]/[Cu²⁺] = 2.2).

Competition experiments with EDTA in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.4) were also carried out using the batch technique. Each batch sample contained an equivalent concentration of FeL₃ complex ([L]/[Fe³⁺] = 3.2, L = C6 or C9) and increasing amounts of EDTA (0 < [EDTA]/[Fe³⁺] < 1). The sample was left to equilibrate overnight before a UV–visible spectrum and the same was recorded. All the spectrophotometric data were fitted with HypSpec software (https://www.hyperquad.co.uk) (Gans et al. 1996), for calculating the protonation constants of the ligands, the stability constants (log β) of the formed species and the coordination model of the studied systems.

Distribution coefficients

The lipophilicity of the ligands C6 and C9, along with their corresponding Fe³⁺ complexes, were determined by calculating their respective distribution coefficient (log D₃,4) values using the Shake Flask method, by referring to the published method of Ma and colleagues (Ma et al. 2012). The method rests on the principle that the solute’s distribution is determined as a ratio of concentrations of the test compound in a solution mixture, consisting of two immiscible phases, namely n-octanol and aqueous HEPES buffer (pH 7.4).

2,2-Diphenyl-1-picrylhydrazyl assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed with C6 and C9, in accordance with previously published methods (Blois 1958). For this, 50 µl of different concentrations of C6, C9 and DFP (0.01 to 0.1 mg/ml in methanol) was dissolved in 2 ml of DPPH methanolic solution (the concentration was adapted to achieve an absorbance of ~1). The samples were shaken vigorously and were allowed to stand until the reaction reached steady-state. The absorbance of the samples was then measured at 515 nm using a UV–visible absorption spectrophotometer (UV-2401 PC; Shimadzu Corporation, Kyoto, Japan). Scavenging of DPPH free radicals was calculated as: DPPH scavenging activity (%) = [(Ac − At)/Ac] × 100 where, Ac is the absorbance of the control tube (containing all reagents except the test compound), while At is the absorbance of the test tube.

Deoxyribose degradation assay

To investigate the origin of the antioxidant properties of the ligands (direct OH scavenging activity or indirect inhibition of the Fenton reaction through Fe chelation), we applied the deoxyribose degradation assay (DDA) with FeCl₃ or FeEDTA as the Fe³⁺ source, as previously described (Chaves et al. 2012). Briefly, a mixture of ascorbic acid, H₂O₂ and FeEDTA (or FeCl₃) complex generated hydroxyl radicals via the Fenton reaction. These radicals subsequently react with deoxyribose to generate malondialdehyde, which in turn reacts with thiobarbituric acid (TBA) to form a pink chromogen. Each assay consisted of 20 mM KH₂PO₄/ K₂HPO₄ buffer (pH 7.4), 2.8 mM deoxyribose, 0.1 mM FeCl₃, 0.1 mM EDTA, different concentrations of the ligand (0.6–3.0 mM), 0.2 mM H₂O₂ and 0.1 mM ascorbic acid, in a final volume of 1.0 ml. Solutions of FeCl₃–EDTA, H₂O₂ and TBA, were made up immediately before use, with ascorbic acid that was added to initiate the reaction. In the presence of OH, deoxyribose is degraded to a product which, when heated with TBA at acidic pH forms a pink chromogen. After incubating the solutions for 1 h at 37 °C, the color reaction was developed by adding 1 ml of 2.8% (w/v) trichloroacetic acid and 1 ml of 1% (w/v) TBA to 0.05 M NaOH, followed by heating the reaction mixture for 30 min at 100 °C. The solutions were cooled, after which absorbance was measured spectrophotometrically at a wavelength of 532 nm (λmax). Controls containing no deoxyribose in the reaction mixture were included for all ligand concentrations.

Cell culture, drug and neurotoxin treatment paradigm

Undifferentiated, adherent human neuroblastoma SH-SY5Y cells (cat #: ECACC 94030304) were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in complete growth medium (Dulbecco’s Modified Eagle’s medium (DMEM) with Ham’s F12, 10% foetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 10,000 units/ml penicillin (Life Technologies, Carlsbad, CA, USA) and 10,000 µg/ml streptomycin (Life Technologies) at 37 °C in 5% CO₂. Cells were plated in 96-well plates for 48 h at 4000 cells/100 µl DMEM with 10% FBS for determining cell viability (determined by hemocytometer cell count) with a similar density used for Western immunoblotting, whilst 20,000 cells/100 µl DMEM with 10% FBS was plated for measuring ROS levels. For assessing the ability of C6 and C9 to provide cytoprotection against proteasome inhibition via lactacystin, either C6, C9 or DFP, all at a dose of 100 µM (dissolved in media), was added to the cell cultures for 24 h, followed by addition of the irreversible proteasome inhibitor, lactacystin (5 µM, Enzo Life Sciences, UK) for a
further 24 h. We also determined the cell death rescue effects of each of the compounds of interest compared to DFP, by treating the cell cultures for 24 h with lactacystin, followed by 100 µM of C6, C9 or DFP. Stock solutions of lactacystin were prepared in fresh medium containing 100% dimethyl sulfoxide (DMSO), which was diluted in fresh medium, to a final DMSO concentration of 0.05%. Control conditions were included for all biochemical assays, i.e., for the cytoprotection assay, the control cells were incubated with the compound of interest for 24 h, followed by treatment with the toxin’s vehicle (0.05% DMSO, dissolved in media) for another 24 h, whilst for the rescue assay, the compound-toxin vehicle treatment protocols were reversed.

**Inductively-coupled-plasma mass spectroscopy**

Iron, copper and zinc levels were measured via inductively-coupled-plasma mass spectroscopy (ICP-MS), similar to a previously published protocol (Duce et al. 2010). In brief, 150 µg of total protein was lyophilized before resuspension in 100 µl Aristar-grade nitric acid (69% v/v), overnight at RT. Samples were then heated at 90 °C for 1 h, before adding an equivalent volume of 30% hydrogen peroxide (H2O2; Merck Millipore). Samples were then incubated for 15 min at RT before heating for 30 min at 70 °C. To evaluate metal content against calibration standards (IMS-102; Ultra Scientific, North Kingston, RI, USA) samples were diluted in double-distilled water until within quantifiable parameters using a NexION 350X ICP-MS (PerkinElmer, Waltham, MA, USA). Each sample was measured in triplicate and normalized to its total protein concentration.

**Cell viability assay**

Cell viability was assessed via a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, which utilizes the ability of mitochondria to reduce a substrate (MTS) into a soluble formazan product. For the assay, the CellTiter 96 AQueous One Solution cell proliferation colorimetric assay (Promega, Madison, WI, USA). The MTS assay was performed using 96-well plates containing the treated cells. Following the treatment paradigms, 20 µl MTS reagent was added to each well, and the plates incubated at 37 °C for 3 h. The absorbance of the solution was read at 490 nm using a FLUOstar Omega spectrophotometer (BMG LabTech, Germany). Experiments were performed in triplicate. Results were expressed as a percentage of control. The MTS assay-derived data were validated by means of traditional trypan blue dye exclusion. For this, an aliquot of cell suspension in PBS was mixed with an equal volume of trypan blue solution (0.4% in PBS). In total, 10 µl of cells were added to a Neubauer haemocytometer chamber and the average number of cells was counted by an observer blind to treatment protocol. Cell death was expressed as percentage trypan blue positive cells per total population of stained and unstained cells.

**Intracellular and extracellular ROS level measurements**

Production of intracellular ROS (iROS) and also extracellular ROS (eROS) was determined using the substrate dihydrorhodamine (DHR)-123 (Sigma-Aldrich), a non-fluorescent laser dye that becomes oxidized by ROS to the fluorescent Rhodamine 123 (Kirchner et al. 2012). For measuring iROS levels using the DHR-123 assay, we followed published guidelines (Wang et al. 2016; Das et al. 2015), as well as the manufacturer’s guidelines, available online (https://www.sigmaaldrich.com/catalog/product/sial/d1054). Briefly, SH-SY5Y cells (20,000 cells/100 µl DMEM for each well) were plated and cultured in 96-well plates for 48 h. Lactacystin (5 µM, Enzo Life Sciences) was then applied for 24 h, followed by treatment with either C6, C9 or DFP (all at 100 µM, dissolved in medium) for a further 24 h, without removal of the lactacystin solution. After the 48 h period of incubating the cells with the toxin and drugs, using typical incubator conditions (5% CO2, 95% air at 37 °C), the medium was discarded, and the cells were incubated in the dark with 100 µl of 10 µM DHR-123 solution and supplement-free growth medium, at typical incubator conditions for 30 min, similar to the DHR-123 incubation period proposed in several published protocols (e.g., Das et al. 2015; Meng et al. 2016; Wang et al. 2016). Subsequently, the medium was removed and the cells were washed with PBS (x3) before immediate analysis of the fluorescence intensity of each well using a microplate reader (FLUOstar Omega), with an excitation filter of 485 nm and an emission filter of 520 nm.

For determining eROS levels, the procedure was repeated until after the cells had been incubated with DHR-123 solution, for the same period of time and dose as was used for determining iROS. The culture media was then transferred (without detaching the adherent cells) to a fresh 96-well microplate for measuring extracellular fluorescence intensity, as described above.

**Cell lysis, protein extraction and protein estimation**

For preparing whole cell lysates, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested and then lysed in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate; Sigma-Aldrich), also containing a protease inhibitor...
cocktail consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride (2 mM), aprotinin (0.3 μM), bestatin (116 μM), E-64 (14 μM), leupeptin (1 μM) and EDTA (1 mM). The suspension was homogenized in a fresh Eppendorf tube by shaking for 15 min on ice. Samples were clarified to remove denatured nucleic acid by centrifugation at 14,000×g for 15 min at 4 °C. The protein containing the supernatant was then transferred to new tubes before total protein concentration was determined in each sample via a Bradford assay (Bradford Reagent, Sigma-Aldrich).

**Western blots**

SH-SY5Y cells (4000 cells/100 μl DMEM with 10% FBS) were cultured in 96-well plates for 48 h before lactacystin (5 μM, Enzo Life Sciences) treatment for 24 h, followed by the specific compound treatment protocols for a further 24 h (resulting in cells being exposed to lactacystin for 48 h). To prepare whole cell lysates, the cells were washed in ice-cold PBS, harvested and then lysed with RIPA buffer containing 4-2-aminoethyl benzenesulfonyl fluoride (2 mM), aprotinin (0.3 μM), bestatin (116 μM), E-64 (14 μM), leupeptin (1 μM) and EDTA (1 mM). The lysates were centrifuged at 13,000 rotations per minute for 15 min at 4 °C, with the supernatant used for analysis. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories). Prior to immunoblotting, protein extracts were separated on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) using an electrophoretic transfer cell (Mini Trans-Blot®, Bio-Rad Laboratories) and by applying constant current at 200 mA for 1.5 h. The blots were blocked with 5% w/v dried milk in PBS for 90 min before incubating for 4 h in a solution containing rabbit anti-α-SYN primary antibody (1:1,000 dilution; Cell Signalling Technology, UK) on a shaker at 4 °C. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (polyclonal goat anti-rabbit, 1:3,000 dilution, Abcam, Cambridge, UK) for 2 h at room temperature (RT), immunoreactive proteins were visualized by enhanced chemiluminescence detection kit (Pierce™ ECL plus Western Blotting Substrate, Thermo Fisher Scientific). The light-emitting bands were detected with X-ray film. An anti-GAPDH (glycer-aldehyde-3-phosphate dehydrogenase) antibody (polyclonal rabbit; 1:1,000 dilution, Chemicon International, Inc., Billerica, MA, USA) was used as a control. The band intensities were quantified by densitometric analysis using ImageJ software (v1.45 software, Wayne Rasband, National Institute of Health, USA).

**Nuclear magnetic resonance spectroscopy**

The potential trapping of the reactive lactone formed by lactacystin (clasto-lactacystin β-lactone) by C6 was studied via 1H Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS) in two deuterated solvent systems, namely DMSO-d6 and 10% DMSO-d6 in D2O. Lactacystin (200 μg, 5.313 × 10⁻⁷ mol) was dissolved in 0.2 ml DMSO-d6 with 0.1 ml of this solution that was removed and diluted to a total volume of 0.5 ml with DMSO-d6. This solution was placed in an NMR tube and the 1H NMR spectrum recorded on a JEOL ECS400FT Delta spectrometer (399.78 MHz for 1H) to obtain a reference (in DMSO-d6). The remaining 0.1 ml of lactacystin solution was diluted to a volume of 1 ml with D2O; the spectrum was recorded similarly (in 10% DMSO-d6 in D2O). Reference NMR spectra of C6 (1.328 μM) in each of these solvent systems were obtained. For each solvent system, 0.2 ml (1 molar equivalent) of C6 stock solution was added to the NMR tube containing lactacystin. The NMR tube was then inverted to mix several times, before the 1H NMR spectrum was recorded. A total of three additions (3 molar equivalents) of C6 stock solution were added to each NMR tube and the NMR spectra were recorded each time. PBS (0.2 ml, pH = 7.4) was then added to each NMR tube to raise the pH from 7.0 to 7.4 and the NMR spectra were re-recorded. Aliquots from each NMR tube were then analysed by MS using electrospray ionization (ThermoFinnigan LCQ Advantage Max mass spectrometer, ThermoFinnigan Corporation, San Jose, CA, USA).

**Statistical analyses**

Statistical significance was calculated by means of a one-way ANOVA followed by a Dunnett’s post-hoc test to assess for differences between the groups. Statistical tests were performed using GraphPad Prism version 7 (GraphPad Software, Inc., CA, USA). A p value ≤ 0.05 was considered statistically significant. Values are expressed as the mean ± standard deviation (SD) of triplicate observations.

**Results**

**Physicochemical characterization**

A detailed physicochemical study was carried out in aqueous solution on ligands C6 and C9 to assess their coordination model, affinity for Fe³⁺ and Cu²⁺ and selectivity. The lipophilicity and antioxidant ability of the ligands and their Fe³⁺ complexes were also evaluated via log D₂₄, as well as DPPH and DDA assay determination, respectively.
Protonation properties versus pH

The protonation properties of ligands C6 and C9 were studied through both potentiometric titrations and UV–Vis absorption spectrophotometric titrations versus pH (Fig. 1a). The acido-basic properties of ligands C6 (Fig. 1b) and C9 (Fig. 1c, d) as well as their metal complexes were also studied in strongly acidic conditions between pH = 0.5 and 2 by means of spectrophotometric titrations versus pH (brackets indicate that the pH value was calculated). As such low pH cannot be measured with an electrode, the batch titration technique was used and the pH of the solutions was fixed by adding known volumes of standardized HClO₄ (see material and methods section for details). The ionic strength was not fixed below pH 1 in the batch titrations and no decomposition of the ligands was observed, even under...
highly acidic conditions. For C6, a log $K_2^\text{H}$ was determined from the spectral variations in the batch titration between $p\text{H} = -0.5$ and 2 (Fig. 1b) and may be attributed to the protonation of the 2-oxo group, similarly to 1,2-HOPO (Gardner and Katritzky 1957). Similar titration of C9 showed no evidence of protonation in this $p\text{H}$ range (Fig. 1c), suggesting that the additional intra-molecular hydrogen bond between the carboxylic acid and the 2-oxo group precludes protonation of this group.

The average protonation constant (log $K$) values ($\pm$ SD) are shown in Table 1; those of DFP and 1,2-HOPO are given for comparison. Log $K_1^\text{H}$ was attributed to the N–OH proton for both ligands and log $K_2^\text{H}$ corresponds to the carboxylic acid proton in the case of C9. Measurement of protonation constants of C6 and C9 allowed us to calculate the electronic spectra of their protonated species (C6, C9, Fig. 1e; C9, Fig. 1g), as well as their distribution diagrams (C6, Fig. 1f; C9, Fig. 1h). These showed that under physiological pH conditions (pH 7.4), C6 exists primarily (96%) in its totally deprotonated form (C6$^-$), in accordance with a log $D_{7.4}$ value of $-1.71(7)$, while under similar conditions, DFP is primarily in its neutral form and has a log $D_{7.4}$ value of $-0.77(2)$ (Table 1). At this pH, C9 existed as a mix of its totally deprotonated form (C9$^{2-}$, 80%) and monoprotonated form (C9H$^-$, 20%) with the hydroxide acid group being protonated. These low protonation constants suggest that C6 and C9 would have a poor ability to cross cell membranes via passive diffusion.

However, despite their relatively low protonation constants, both C6 and C9 clearly reduced intracellular Fe$^{3+}$ in the SH-SY5Y cells, as was revealed by our results that labile iron was reduced which resulted in downstream beneficial effects, as discussed later.

### Complex formation with Fe$^{3+}$ and Cu$^{2+}$ versus pH

Similar to the protonation study, spectrophotometric batch ($-0.5 < p\text{H} < 2$) and direct ($2 < p\text{H} < 12$) titrations were then carried out on the Fe$^{3+}$ complexes of ligands C6 (Fig. 2a, b) and C9 (Fig. 2c, d). The titrations showed the appearance of a ligand to metal charge transfer band (LMCT) centered around 480 nm for both ligands, characteristic of the complexation of Fe$^{3+}$. This band undergoes a hyper- and hypsochromic shift to 410 nm as pH increases, sign of the successive coordination of one, two, and three bidentate ligands to the Fe$^{3+}$ cation to achieve a hexacoordinate complex. When the pH continues to increase, a decrease of the charge transfer band was observed suggesting the substitution of ligands in the Fe$^{3+}$ coordination sphere by hydroxide anions until the total release of Fe$^{3+}$ and its precipitation as Fe(OH)$_3$, as suggested by the total loss of the LMCT band and a general increase in noise and baseline in the titration of the complexes above pH 9.8 (Fig. 2b, d).

For both ligands, the presence of a hydroxylated complex, FeL$_2$(OH)$_2$ (L = C6, C9), was observed before precipitation occurred. For ligand C9, that exhibited a non-coordinating carboxylic acid function, the additional formation of the protonated Fe(C9)H species at a very acidic pH was also observed. The overall stability constants (log $\beta$) of the observed species are shown in Table 2, which also shows that the pFe values of both C6 and C9 are very similar, and around one order of magnitude lower than that of DFP.

To chelate labile Fe$^{3+}$ and prevent oxidative damage, a therapeutic iron chelator must be able to form a reasonably stable FeL$_3$ complex (L = chelator) in which the inner coordination sphere of Fe$^{3+}$ is fully saturated. However, the stability of the complex need not be so high as to result in total removal of Fe$^{3+}$ from either the cell or from Fe$^{3+}$-saturated transferrin. Determination of the stability constants of the C6 (Fig. 2e) and C9 (Fig. 2g) Fe$^{3+}$ complexes allowed us to calculate the electronic spectra of their protonated species and compile their respective distribution diagrams (C6, Fig. 2f; C9, Fig. 2h), to show the major complex species present at physiological pH.

### Competition experiments

Competition experiments were carried out with EDTA in HEPES buffer at pH 7.4 to verify our complexation model and confirm the FeL$_3$ stability constants of C6 (Fig. 3a) and C9 (Fig. 3b), where FeL$_3$ should be the only species present (L = C6 or C9). The fitting of the data confirmed the presence of a single FeL$_3$ species for both ligands. The conditional stability constants of the FeL$_3$ complexes were determined and converted to overall stability constants (Ringbom 1963) which were found to be in good agreement with those obtained from the spectrophotometric titrations versus pH (Table 2).

Since Cu$^{2+}$ is also redox-active and implicated in PD pathogenesis, we also studied the Cu$^{2+}$ binding affinity of C6 and C9. In the case of Cu$^{2+}$ complexation, spectrophotometric titrations versus pH showed the successive formation of CuL and CuL$_2$ complexes for both C6 and C9 ligands (Fig. 4a–d), followed by decomplexation and precipitation of Cu(OH)$_2$ above pH 9 (Fig. 4e, f). Similar to Fe$^{3+}$ complexation, ligand C9 showed the additional formation of the protonated Cu(C9)H species. The affinity of C6 and C9 (Table 2) for Cu$^{2+}$ was ca. 7 orders of
magnitude lower than their affinity for $\text{Fe}^{3+}$, suggesting that both compounds have excellent selectivity for $\text{Fe}^{3+}$ over $\text{Cu}^{2+}$ (Table 2). This may be due to $\text{Cu}^{2+}$ being a softer Lewis acid than $\text{Fe}^{3+}$ and therefore having a lower affinity for hydroxypyridinones (HOPOs), which are hard Lewis bases, according to Pearson’s hard-soft acid base theory (Pearson 1963).

**Distribution coefficients**

The distribution coefficients ($\log D_{7.4}$) between an n-octanol phase and a HEPES-buffered aqueous phase at pH 7.4 were determined for ligand C6 ($\log D_{7.4} = -1.71(7)$) and its $\text{Fe}^{3+}$ complex ($\log D_{7.4} = -0.18(2)$) (Table 2). In the case of C9 and its $\text{Fe}^{3+}$ complex, no significant decrease in the
absorbance values of the aqueous phase were observed in our experimental conditions, suggesting that this ligand and its complex are too hydrophilic to provoke substantial migration to the n-octanol phase at this pH. As with the protonation constants, the low distribution coefficients of C6 and C9 suggest these compounds have a relatively low ability to cross cell membranes by passive diffusion. However, the possibility that the compounds could cross cell membranes by means of an active transport mechanism cannot be ruled out completely.

**DPPH radical scavenging assay**

To evaluate the antioxidant ability of C6 and C9, a DPPH free radical scavenging assay was carried out at pH 7.4. The kinetics of reaction suggested that these ligands can be classified as being slow (Mishra 2012). C6 showed a slower reaction rate than DFP, but reached a percentage of inhibition of DPPH of 84 ± 1%, similar to that of DFP, while C9 reached a maximum of only 32 ± 5% (Fig. 5).

**Mechanistic evaluation of the compounds’ antioxidant properties**

To gain further insight into the inherent antioxidant properties of ligands C6 and C9 (at various concentrations), DDAs were performed. The results obtained for C6 (Fig. 6a) and C9 (Fig. 6b) were superimposed with the distribution curves of FeL3, which was obtained from the complexation titration experiment previously described. In the presence of FeCl3, the strong inhibition of deoxyribose degradation observed for both C6 and C9 was primarily due to their ability to strongly bind Fe3+ at physiological pH \( \log \beta (\text{Fe(C6)3}) = 19.5, \log \beta (\text{Fe(C9)3}) = 18.9 \) at pH 7.4, thus preventing the formation of OH. through the Fenton reaction. These results suggest that the tris-bidentate complexes formed at this pH do not participate in the generation of OH.

In the presence of Fe-EDTA, the level of inhibition tended to follow the formation of the FeL3 complex \( (L = \text{C6}, \text{C9}) \), in accordance with the high stability of the Fe5+ complexes at this particular pH. This suggests that, under these experimental conditions, the antioxidant effect of the ligands
would be mainly due to the strong Fe$^{3+}$ chelation ability of ligands C6 and C9 that inhibits the Fenton reaction, rather than from direct scavenging of OH• radicals. Furthermore, calculation of the IC$^{50}$ values suggested strongly that the 1,2-HOPO based ligands C6 (IC$^{50} = 0.74(1)$ mM) and C9 (IC$^{50} = 0.61(1)$ mM) function at least as efficiently as antioxidants such as DFP (IC$^{50} = 0.63(9)$ mM).

C6 and C9 treatment induces changes in neuronal labile metal content in lactacystin-treated SH-SY5Y cells

The post-test analysis of total intracellular iron levels measured by ICP-MS revealed that the application of lactacystin to the cells produced a significant increase of 27.6 ± 0.05% (p = 0.047) compared to untreated cells (Fig. 7a). However, lactacystin-treated cells, co-incubated with compound, restored iron levels to control levels. This was the case for

![Diagram](image_url)
Fig. 6 Percentage inhibition of deoxyribose degradation by the ligands a C6 and b C9 in the presence of FeCl₃ (×) or FeEDTA (filled triangle) and distribution curves of FeL₃ complexes versus ligand concentrations. Solvent: 20 mM phosphate buffer pH 7.4, [Fe] = [EDTA] = 0.1 mM, 0 < [C6], [C9] < 3 mM

Fig. 7 a Iron levels, as measured by ICP-MS, following treatment of SH-SY5Y cells with lactacystin, were greatly increased; in contrast, all tested compounds reduced such levels to a range comparable to the non-toxin treated control cells. b Such iron chelation associated with neuroprotective activity shown by C6 and C9 against lactacystin-induced degeneration of SHSY-5Y cells. In the absence of any of the compounds, incubation with lactacystin (5 μM) for 24 h decreased cell viability by > 75% (a, b). The compounds (100 μM) protected against neurotoxin-mediated cell death (cell viability was increased by C6: 54.3%, C9: 38.9% and DFP: 32.8%, relative to the toxin-only condition), where treatment with C6 provided most protection to the cells (**p = 0.0002). c Addition of C6 and C9, as well as DFP, also significantly rescued against such toxin-associated cell death. Cellular incubation with the two novel 1,2-HOPO derivatives or DFP (100 μM per compound) also rescued against lactacystin-induced cell death (% cell viability increases compared to toxin-only treatment were: C6: 37.2%, C9: 24% and DFP: 28%). The level of viable cells was measured at OD 490 nm using the CellTiter 96® AQeous One Solution Cell Proliferation Assay (MTS, Promega). Experiments were carried out × 3 with each experiment done in triplicate. Data represent the mean ± SD.
both C6 (40.9 ± 0.04%, $p = 0.003$) and C9 (61.1 ± 0.03%, $p = 0.0001$), when compared to cells treated with lactacystin only. At the compounds’ concentration used in this study (100 µM), only C9 (and not C6) showed a greater capability than DFP for reducing accumulated iron in the toxin-treated cells, where C9 reduced the lactacystin-induced high iron levels by a further 26.5 ± 0.03% ($p = 0.0028$) compared to DFP treatment.

To eliminate the possibility that lactacystin indirectly altered iron through modulation of total metal homeostasis and to assess whether these compounds had a general chelating effect on other divalent metals, levels of copper and zinc were also measured in the same conditions (data not shown). Exposure to lactacystin decreased the cellular stores of copper (46.5 ± 0.004%) and zinc (28.3 ± 0.05%), but this change did not reach statistical significance. No statistical significance was observed upon treatment with either C6 (copper decreased by 1.3 ± 0.003%; zinc increased by 29 ± 0.07%) or C9 (copper decreased by 26.8 ± 0.003%; zinc 3.7 ± 0.05%) of the lactacystin-treated cells. This was comparable to data obtained for DFP (copper increased by 3.9 ± 0.004%; zinc 30 ± 0.03%).

**Compound rescue and protection against lactacystin toxicity**

The potential of C6 and C9 to rescue and protect against lactacystin-induced neurotoxicity was compared to DFP in SH-SY5Y cell cultures. Rescue was evaluated by the addition of the compound (for 24 h) after lactacystin exposure for 24 h, whereas protection was tested by the application of compound (for 24 h) prior to lactacystin (also for 24 h). In each condition the negative control comprised of compound-vehicle (dissolved in media) only. Lactacystin (in the absence of compound) reduced cell numbers compared to the untreated control cells, as seen in both the ‘protection’—(by 75.8 ± 6%, $p < 0.0001$) (Fig. 7b) and the ‘rescue’ assay (by 77.9 ± 12%, $p < 0.0001$) (Fig. 7c). In lactacystin exposed cells, treatment with C6 significantly increased cell survival when administered either prior (by 54.3 ± 20.3%, $p = 0.0002$) (Fig. 7b) or following (by 37.2 ± 12.8%, $p < 0.0001$) (Fig. 7c) neurotoxin exposure. C9 also revealed significant protective (by 38.9 ± 19.1%, $p = 0.0206$) (Fig. 7b) and rescue effects (by 24 ± 9.3%, $p = 0.001$) (Fig. 7c) against lactacystin-induced cell death. A similar neuroprotection (by 32.8 ± 22%, $p = 0.004$) (Fig. 7b) and neurorescue profile (28 ± 9.1%, $p = 0.0002$) (Fig. 7c) as the two test compounds was observed with assessment of the known clinical chelating compound, DFP.

**C6 and C9 reduce lactacystin-induced production of intracellular and extracellular ROS**

Levels of ROS produced intracellularly (iROS) or extracellularly (eROS) were measured upon lactacystin treatment, followed by treatment with a compound of interest. Without addition of the compound, lactacystin-treated cells had a significant increase in levels of iROS (139 ± 20.5%, $p < 0.0001$) (Fig. 8a) and eROS (106 ± 27.3%, $p < 0.0001$) (Fig. 8b), compared to untreated control cells. The addition of C6 to the toxin model produced a significant reduction in iROS (121.2 ± 43.5%, $p = 0.0002$) (Fig. 8a) and eROS (97.9 ± 34%, $p = 0.0102$) (Fig. 8b). This rescue effect was less so with addition of C9 [iROS: 74 ± 19%, $p = 0.0002$ (Fig. 8a); eROS: 78 ± 60.6%, $p = 0.0102$] (Fig. 8b)]. Intriguingly, the clinically available compound DFP was unable to exert...
a significant reducing effect on either iROS (19 ± 16.7%, $p = 0.2894$) (Fig. 8a) or eROS levels (8 ± 34.5%, $p = 0.7637$) (Fig. 8b) that were increased by treatment with lactacystin.

**C6 and C9 reduce lactacystin-induced α-SYN overexpression levels**

Endogenously expressed α-SYN in SH-SY5Y cells was upregulated by incubation with lactacystin for 24 h (17.8 ± 0.18%, $p < 0.0001$) (Fig. 9). This response was negated by post-treatment with the compounds of interest, producing a significant response with C6 (13.5 ± 0.3%, $p = 0.0163$) and DFP (25.8 ± 0.03%, $p < 0.0001$), while C9 produced the most striking reduction in lactacystin-induced α-SYN protein levels (43.5 ± 0.08%, $p < 0.0001$) (Fig. 9).

**NMR spectroscopy**

The interaction of lactacystin with C6 was studied in both DMSO-$d_6$ and 10% DMSO-$d_6$ in $D_2$O in the absence and presence of PBS (with osmolarity, ion concentration and a pH of 7.4). This buffer was added to replicate the more physiologically relevant environment of the SH-SY5Y cells. Reference spectra of both C6 and lactacystin were first obtained in each of these solvent systems. Resonances due to lactacystin were assigned by comparing values to previously published $^1$H NMR data (Hayes et al. 2008).

Upon addition of 1 equivalent of C6 to a solution of lactacystin in DMSO-$d_6$, no spectral changes were observed, suggesting there was no formation of the lactacystin-C6 adduct. Further additions of C6 (up to a total of 3 equivalents) again did not lead to the appearance of any new resonances for the lactacystin-C6 adduct, with only the resonances for lactacystin and C6 being observed. When the NMR experiments were conducted in 10% DMSO-$d_6$ in $D_2$O, no spectral changes were observed. Similarly, addition of PBS to raise the pH also did not lead to any spectral changes and there was no evidence for formation of either the clasto-lactacystin β-lactone or the lactacystin-C6 adduct. Finally, no new resonances were observed upon addition of three equivalents of C6 to lactacystin, and this was also the case in the presence of phosphate buffer. MS analysis of the solutions from both solvent systems (data not shown) did not show the expected mass peaks due to the lactacystin-C6 adduct, confirming that there was no interaction between lactacystin and C6 under these conditions.

Should the lactacystin-C6 adduct have been formed (assuming that the hydroxymethyl group is the most nucleophilic group in C6, see the discussion section), a new set of resonances for the three aromatic protons, and a new set of doublets for the methyl groups would be expected. In addition, the diastereotopic methylene protons would be expected to appear as a pair of doublets centered at ca. 4.5 ppm (parts per million).

**Discussion**

The aim of the present study was to combine physicochemical characterization and cellular investigation of the treatment effects of two novel 1,2-HOPOs, C6 and C9, that were produced in our lab, in a lactacystin cellular model of PD, with this model replicating key changes seen in both neurotoxin animal models of the disease and also post-mortem PD brains. As a first-in class inhibitor of the UPS, the mechanism that controls cell turnover and regulates protein catabolism in the mammalian cytosol and nucleus, lactacystin exposure has been demonstrated as an experimentally useful tool for mimicking aspects of neurodegenerative diseases that display deficits in clearing misfolded proteins, to lead to intracellular protein aggregation, cytotoxicity, and
eventual cell death (Konieczny et al. 2014; Harrison et al. 2015; Fenteany et al. 1995).

We compared the results obtained from testing C6 and C9 to the clinically available oral iron chelator, DFP. This clinically licensed bidentate ferric chelator has been successfully used for over 2 decades against iron storage pathologies, including recent promising results achieved in a clinical phase-I trial conducted on a small cohort of PD patients, to reveal beneficial effect on patients’ motor symptoms (Moreau et al. 2018). Our results reveal that C6 and C9 are capable of protecting and rescuing against lactacystin-induced neuronal death, with our results implying strongly that the mechanism relates to the compounds’ ability to reduce levels of labile iron, which leads to reduced levels of ROS, as well as the ability to reduce abnormally high levels of $\alpha$-SYN that the compounds mediated in the SH-SY5Y lactacystin cell model of PD.

Here we provide several lines of evidence for the ability by lactacystin to reduce the intracellular labile iron pool, and also demonstrate the ability of the novel compounds C6 and C9 to interact with Fe$^{3+}$ and thereby restore iron levels. This includes measuring intracellular levels of Fe-56 (the most common isotope of iron) via ICP-MS, which revealed that application of lactacystin to the cells significantly increased iron content compared to untreated cells. In contrast, the same measures showed that all the tested compounds reduced the toxin-induced iron accumulation in cells. Moreover, general agreement exists that a build-up of intracellular iron stimulates oxidative stress, and hence that iron accumulation, followed by oxidative stress typically precedes all other cellular changes, including changes in iron storage proteins (Devos et al. 2014). In this regard our results also show elevated levels of both iROS and eROS upon lactacystin treatment compared to toxin untreated control cells, while administration of both novel compounds, but particularly C6, exerted a significant iROS and eROS reducing effect. The association between lactacystin and the molecular mechanisms responsible for absorption of iron and maintenance of iron homeostasis, as well as how our novel compounds might interact with these factors to restore iron homeostasis in this toxin model of PD, will require further investigation. Several iron response proteins have been identified, including ferritin (FT), a protein for binding and carrying iron mainly in the blood serum; mitoferrin-2 (Mfrn2), for importing iron into mitochondria, the iron exporter ferroportin (FPN) and also hepatocyte-secreted hormone (Hepc), an iron absorption inhibitor mainly produced by the liver (Finberg 2013; Wallace 2016). Previously published work provided insight into the interactions between lactacystin and some of these molecular iron regulators. In differentiated SH-SY5Y cells exposed to lactacystin, Carroll and others (2011) reported no change in protein expression levels of either TfR or FT, but saw upregulation for FPN and Mfrn2 protein, but without concomitant changes in associated mRNA levels. This result, showing largely a lack of change in several known iron regulatory proteins, and inconsistency between changes at the transcription versus translation level for regulators that were altered, suggests that other factors such as increased labile iron and accumulation of oxidatively damaged proteins may rather account for lactacystin-induced cell death. Greater insight to better understand the nature of the molecular interactions between this UPS toxin and the elaborate mechanisms that have evolved to sense iron levels and adjust iron absorption and recycling to maintain iron homeostasis could deliver new diagnostic and therapeutic possibilities for iron-related disorders. However, such further studies may be best placed by utilizing an in vivo system, since the balance of iron homeostasis is maintained through a dynamic equilibrium between systemic tissues, and hence an animal system will allow for a comparison between various body compartments, while molecular iron regulators identified so far have been shown to operate in man as well as rodents (Wallace 2016). In this regard, our previous work showed that rats that had received a unilateral nigral stereotoxic injection of lactacystin accurately mirror the behavioural and neuropathological deficits shown by PD patients (Pienaar et al. 2015a, b; Sharma et al. 2020).

Also, in a previous study (Workman et al. 2015) we showed that in SH-SY5Y cells pre-incubated with 50 μM of iron, subsequent exposure to either C6 or DFP changed protein expression levels of FT and TfR to levels seen in cells not treated with iron, while a non-iron binding control compound left the FT and TfR levels unaltered. Future experiments are warranted to identify all genes/protein products involved in the complex pathway devoted to iron regulation that C6, C9 (and clinically available DFP) can alter downstream of their capacity to bind iron and restore toxin-induced ROS, with most insight that may come from screens conducted in animals with targeted mutations in iron regulatory genes.

Although it remains unclear whether changes in iron regulatory proteins translates into an actual change in cellular iron content, such mechanistic studies will further understanding as to the downstream implications that follow a UPS impairment as a stimulus for iron overload as seen in PD-affected brains that may contribute to neuronal death. On the other hand, detailing the iron protein response network following exposure to a 1,2-HOPO iron chelator such as C6 or C9, will further enlighten on the mechanistic basis for the protective response to lactacystin exposure that our data demonstrates.

The ligands C6, C9 and DFP differed in their respective metal coordination abilities and in their relative acidities, resulting in different metal-complex stability constants and hence this line of investigation was unable to afford a rationale for their pro-survival effects seen in this neurotoxin
cell model. We thus calculated the respective pM values to represent the amount of free metal in solution at physiological pH, allowing us to compare the chelation power for various metals bound by the different ligands assessed in this study. The substituents present in C6 and C9 induced an increase of their affinity towards Fe$^{3+}$ by one order of magnitude compared to DFP. Both pFe values lay below those observed for transferrin (pFe = 23.62) so that, similar to DFP, C6 and C9 would therefore be expected to donate chelated iron to unsaturated transferrin, thus avoiding a loss of total iron and favoring its repositioning to other sites of use. This is similar to DFP, which was demonstrated as chelating excess intracellular labile iron and then redistribute it to extracellular apotransferrin (Sohn et al. 2008, 2011). This finding could suggest that, despite their relatively low protonation constants, C6 and C9 exerted their neuroprotective and neurorescue effects in the lactacystin cellular model of PD by entering into SH-SY5Y cells, and then chelating intracellular labile Fe$^{3+}$, a suggestion that is in agreement with our previous study which showed that both C6 and C9 reduced intracellular Fe$^{3+}$ levels in similar cells in the 6-OHDA in vitro model of PD (Workman et al. 2015).

However, it is also possible that C6 and C9 exerted their neuroprotective and neurorescue effects simply by chelating extracellular labile Fe$^{3+}$ instead. This depletion in extracellular labile Fe$^{3+}$ would re-adjust the equilibrium between extracellular and intracellular labile Fe$^{3+}$ and would reduce apotransferrin-mediated influx of Fe$^{3+}$ into the cell, leading to a net reduction in the intracellular labile Fe$^{3+}$. In addition, whilst the low protonation constants of C6 and C9 suggest that neither compound is significantly capable of crossing into the SH-SY5Y cells through passive diffusion, we cannot rule out the possibility that these compounds could enter the cells as the neutral complex of other metals such as Mg$^{2+}$ or Zn$^{2+}$ or via an active transport mechanism (e.g., via a glucose transporter protein anchored in the cell membrane).

Distribution diagrams show that at physiological pH, C6 exist as neutral Fe(C6)$_3$, in accordance with log $D_{7,4}$ values of $-0.18(2)$, suggesting a less hydrophilic complex than DFP at a log $D_{7,4}$ of $-2.60(5)$ (Hayes et al. 2008). At similar conditions, ligand C9 existed as Fe(C9)$_3$ and did not show significant migration to the n-octanol phase in our partition experiments, thus precluding the determination of its log $D_{7,4}$ value. This finding indicates its highly hydrophilic nature and suggests that the neutral, relatively hydrophobic Fe(C6)$_3$ complex would be able to leave the cell via passive diffusion and underscores that redistribution of Fe$^{3+}$ between cells is likely. On the other hand, the charged Fe(C9)$_3$ complex would be highly unlikely to do so.

The DAergic cell death that characterizes PD pathology is underpinned by oxidative stress, concomitant with cellular metabolic impairment (Halliwell and Gutteridge 1984). Amongst other cell death mechanisms, lactacystin instigates a marked increase in oxidative stress and metabolic failure, thereby modeling cellular effects seen in PD, with one study reporting that lactacystin treatment increased ROS production, which subsequently induced mitochondrial cytochrome c release indirectly, via translocation of the Bax protein, ultimately resulting in cell death (Perez-Alvarez et al. 2009). Iron is a potent generator of free-radicals via the Haber–Weiss reactions. In this regard, it is well documented that UPS impairment, as instigated by application of lactacystin, results in excessive iron, which in turn increases oxidative stress via overproduction of ROS (Halliwell and Gutteridge 1984; Dixon and Stockwell 2014). In turn, these events impair proteasomal function, thus promoting protein aggregates that result in eventual cell death (Rhodes and Ritz 2008).

Our data reveals that C6 treatment produced a greater reduction in iROS and eROS in lactacystin-treated cells than when cells were treated with C9. In addition, evaluation of the ability of C6 and C9 to act as radical traps using the DPPH assay revealed that C6 reached a much better efficiency than C9, with C6 being nearly as efficient as DFP. It was found that 3-hydroxy-4(1H)-pyridinone derivatives, including DFP, are poor radical scavengers (Rhodes and Ritz 2008; Kayyali et al. 1998). Their ability to protect cells against oxidative damage is therefore not thought to be linked to the trapping of ROS. In fact, it was found that such metal chelators are effective at preventing Fe$^{3+}$ from taking part in Haber–Weiss reactions, but also that the inhibition is stoichiometry dependent, requiring an FeL$_3$ complex for inactivation (L = ligand) (El-Jammal and Templeton 1996; Merkofer et al. 2004, 2006; Devanur et al. 2008; Chaves et al. 2012). Their mode of action therefore appears to rather be linked to redox silencing of metals. It is also known that catechol groups (present in DA and l-DOPA) are pro-oxidants in the presence of metals, with DFP that was found capable of preventing the pro-oxidant effect of this combination (Sun et al. 2018), thereby indicating multiple beneficial mechanisms of action.

Lactacystin’s neurotoxic mechanism of action relates to the covalent bond it forms with specific catalytic subunits of the 20S catalytic core of the proteasome, believed to regulate critical aspects for recognising and unfolding ubiquitinated proteins. This binding inhibits multiple peptidase activities of the enzyme complex, resulting in lack of proteasomal activity (Tomoda and Omura 2000). Based on this known mode of action of lactacystin, we postulated that besides iron chelation and an anti-ROS effect, C6 could potentially play an alternative role in protecting SH-SY5Y cells from lactacystin-induced neuronal damage, ultimately resulting in cell death, similar to what is seen in SNpc DAergic neurons in PD-affected human brains. In this regard we reasoned that the primary hydroxyl group of C6 could trap the reactive clasto-lactacystin β-lactone intermediate intracellularly, to
form the far less reactive lactacystin-C6 adduct, which is unable to acylate the N-terminal threonine residue in the 20S subunit of the proteasome. A similar nucleophilic attack by the N-hydroxyl group of C6 would only form an activated ester that would maintain the reactivity and therefore biological action of clasto-lactacystin β-lactone (Finkelstein et al. 2017). Thus, in addition to chelating and passivating intracellular iron, C6 could potentially act as a sacrificial compound that inactivates the reactive clasto-lactacystin β-lactone intermediate formed by lactacystin.

Accordingly, we assessed via 1H NMR whether the neuronal protection and rescue effect we observed here in lactacystin-treated cell cultures co-incubated with the novel iron chelators was due to the formation of an adduct between C6 and lactacystin. In view of the novel compounds offering a potential treatment of PD, it was necessary to demonstrate that C6 is capable of preventing oxidative damage to neurons regardless of which toxin in vitro model is used for generating excessive levels of ROS (e.g., 6-OHDA, MPTP or lactacystin). In due regard, the NMR and MS experimental data indicates that C6 is not capable of chemically degrading lactacystin in this manner. This suggests that in the presence of C6, lactacystin is not deactivated and remains capable of producing ROS and that C6 protects neurons against oxidative stress-mediated neurodegeneration, regardless of the PD toxin used to generate excessive ROS for modeling PD-like cells. Since it appears that C6 does not degrade lactacystin, a similar NMR study on the interaction of lactacystin with C9 was not carried out in the present report.

Due to the interplay previously shown to exist between iron and α-SYN accumulation and aggregation (Golts et al. 2002), iron chelators have been deemed a promising therapeutic strategy for reducing levels of α-SYN pathology seen in PD-affected brains. The current results showed that C6 could normalise α-SYN levels that had been increased due to lactacystin treatment, while DFP and C9 were capable of reducing α-SYN levels substantially below the normal state, with C9 showing the most potent effect in this regard.

Our results show the therapeutic potential of the novel iron chelators, where it is well established that α-SYN aggregation, as seen in PD brains, can be promoted by excess iron (Xiao et al. 2018). The central role that iron plays in LB promotion was further underlined by work reporting iron co-localization within LBs in the brains of PD patients (Castellani et al. 2000). The ability of metal-chelators such as PBT434, a novel quinazolinone compound bearing a metal-binding motif that holds moderate affinity, to prevent iron-mediated α-SYN toxicity, had previously been demonstrated (Finkelstein et al. 2017). In this study, Finkelstein and others also revealed effective reduction of insoluble α-SYN levels in brain lysates taken from mice harboring the A53T α-SYN mutation that had been treated with the iron chelator clioquinol, compared to placebo-treated control mice.

In the current study we demonstrate the ability of DFP to reduce abnormally high levels of α-SYN in the SH-SY5Y lactacystin cell model of PD. This result adds to recent data showing that DFP is capable of reducing α-SYN aggregation in the hippocampi and cortices of transgenic mice bearing the A53T α-SYN mutation, with such mice that had been fed iron for exacerbating the motor phenotype (Carboni et al. 2017). However, the protein reduction effect was weak, with significant variance detected between the mice.

Future investigations to identify the toxic nature of α-SYN induced by lactacystin, and to determine at what stage the test compounds, relative to DFP, may interfere in organized fibril assembly, sequencing as the formation of dimers, oligomers, protofibrillar structures and eventual mature linear fibers, may further enlighten on the therapeutic mechanism by which the presently tested novel iron chelators showed ability to clear such protein abundance. Such additional characterisations, for example by utilizing immunoelectron microscopy, will strengthen the view proposed by the current data, that α-SYN accumulation is a target for C9 especially. In addition, studies to determine the capacity of C6 and C9, relative to DFP, to reduce other PD-relevant, aggregate-prone proteins such as ubiquitin, where lactacystin had been shown to induce ubiquitinated protein aggregation in vitro (Yang et al. 2005), will give further insights as to how these novel iron chelators might act as agents inducing deubiquitination or for degrading aggregated intracellular proteins.

Work to determine the sequence of steps by which lactacystin exposure produces toxic forms of intracellular protein also promises to enlighten on the mechanism of protein accumulation, where such insight will help develop this in vitro PD model for reflecting aspects of PD. In this regard, its currently unknown whether lactacystin induces a primary event that leads to aggregation of α-SYN due to its proteasome inhibitor effect, or whether this post-translational modification might rather occur firstly as a result of the reaction of lactacystin-induced excessive ROS production with α-SYN.

Conclusions

For improving our understanding of the therapeutic mechanisms of two novel 1,2-HOPO iron chelators (C6 and C9), we reveal, for the first time, their ability to both partially prevent and rescue against cell death due to lactacystin-induced UPS inhibition, as was determined in an in vitro model of PD. We further revealed that such mitigating effects on the toxin-treated cells are likely due to the ability of the compounds to chelate and passivate intracellular labile iron, thereby reducing ROS production (accumulating both intracellularly and extracellularly) and prevent protein...
aggregation, both of which are neuropathological hallmarks of the cellular dysfunction seen in PD. Direct trapping of the reactive clasto-lactacystin β-lactone intermediate by C6 was not observed by NMR spectroscopy. Measurement of the stability constant values for Fe$^{3+}$ and Cu$^{2+}$ complexes confirmed that each of the compounds have a good affinity and selectivity for Fe$^{3+}$. Our results provide further evidence in support of labile iron as a viable therapeutic target and justifies further development of iron chelators as potential therapies for treating PD. Thus, our results suggest that 1,2-HOPO chelators are a valuable class to explore further as potential disease-modifying agents against PD, in addition to 3-hydroxy-4(1H)-pyridinones.

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Author contributions ISP and FWL conceived the original experimental research idea and designed the lactacystin-based experiments using the compounds C6 and C9 designed and previously published by DT. FWL, SF, JB, MS, JLE and ISP performed all experiments, provided funding for the experimental work and analysed the data. All authors wrote and reviewed the final version of the manuscript.

Compliance with ethical standards
Conflict of interest The authors declare that they have no conflict of interest.

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References
Abeliovich A et al (2000) Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron 25:239–252
Alberio T, Lopiano L, Fasano M (2012) Cellular models to investigate biochemical pathways in Parkinson’s disease. FEBS J 279:1146–1155
Alderighi L, Gans P, Lenco A, Peters D, Sabatini A, Vacca A (1999) HyperQuadr simulation and speciation (HySS): a utility program for the investigation of equilibria involving soluble and partially soluble species. Coord Chem Rev 184:311–318
Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181:1199–1200
Braak H, Del Tredici K, Rüb U, de vos RA, Jansen Steur EN, Braak E (2003) Staging of brain pathology related to sporadic Parkinson’s disease. Neurobiol Aging 24:197–211
Carboni E et al (2017) Deferiprone rescues behavioral deficits induced by mild iron exposure in a mouse model of alpha-synuclein aggregation. Neuronomol Med 19:309–321
Carroll CB, Zeissler M-L, Chadborn N, Gibson K, Williams G, Zajicek JP, Morrison KE, Hanemann CO (2011) Changes in iron-regulatory gene expression occur in human cell culture models of Parkinson’s disease. Neurochem Int 59:76–80
Castellani RJ, Siedlak SL, Perry G, Smith MA (2000) Sequestration of iron by Lewy bodies in Parkinson’s disease. Acta Neuropath 100:111–114
Chaves S, Canário S, Carrasco MP, Mira L, Amélia Santos M (2012) Hydroxy(thio)pyrrole and hydroxy(thio)pyridine iron chelators: physico-chemical properties and anti-oxidant activity. J Inorg Biochem 114:38–46
Das M, Yi DK. An SSA (2015) Analyses of protein corona on bare and silica-coated gold nanorods against four mammalian cells. Int J Nanomed 10:1521–1545
Devanur LD, Neubert H, Hider RC (2008) The Fenton activity of iron(III) in the presence of deferiprone. J Pharm Sci 97:1454–1467
Devos D et al (2014) Targeting chelatable iron as a therapeutic modality in Parkinson’s disease. Antiox Redox Signal 21:195–210
Dixon SJ, Stockwell BR (2014) The role of iron and reactive oxygen species in cell death. Nat Chem Biol 10:9–17
Duce JA et al (2010) Iron-export ferroxidase activity of β-amyloid precursor protein is inhibited by zinc in Alzheimer’s disease. Cell 142:857–867
El-Jammal A, Templeton DM (1996) Iron-hydroxypyridine redox chemistry: kinetic and thermodynamic limitations to Fenton activity. Inorg Chim Acta 245:199–207
Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 268:726–731
Finberg KE (2013) Regulation of systemic iron homeostasis. Curr Opin Hematol 20:208–214
Finkelstein DI et al (2017) The novel compound PBT434 prevents iron mediated neurodegeneration and alpha-synuclein toxicity in multiple models of Parkinson’s disease. Acta Neuropathol Commun 5:53
Fish WW (1988) Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. Methods Enzymol 158:357–364
Gans P, Sabatini A, Macca A (1996) Investigation of equilibria in solution. Determination of equilibrium constants with the HYPER-QUAD suite of programs. Talanta 43:1739–1753
Gardner JN, Katritzky AR (1957) N-oxides and related compounds. Part V. The tautomorism of 2- and 4-amino- and -hydroxy-pyridine 1-oxide. J Chem Soc. https://doi.org/10.1039/JR9570004375
Gillett R et al (2017) A bispidol chelator with a phosphonate pendant arm: synthesis, Cu(II) complexation, and 64Cu labeling. Inorg Chem 56:11738–11752
Golts N, Snyder H, Frasier M, Theisler C, Choi P, Wolozin B (2002) Magnesium inhibits spontaneous and iron-induced aggregation of alpha-synuclein. J Biol Chem 277:16116–16123
Halliwell B, Gutteridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1–14
Harrison IF, Crum WR, Vernon AC, Dexter DT (2015) Neurorestoration induced by the HDAC inhibitor sodium valproate in the lactacystin model of Parkinson’s is associated with histone acetylation and up-regulation of neurotrophic factors. Br J Pharmacol 172:4200–4215
Hayes CJ et al (2008) Enantioselective total synthesis of omuralide, 7-epi-omuralide, and (+)-lactacystin. J Org Chem 73:2041–2051
Savolainen MH, Albert K, Airavaara M, Myöhänen TT (2017) Nigral
Ringbom A (1963) Complexation in analytical chemistry. A guide for
Rhodes SL, Ritz B (2008) Genetics of iron regulation and the possible
Pienaar IS et al (2015b) Pharmacogenetic stimulation of cholinergic
Pienaar IS et al (2015a) An animal model mimicking pedunculopontine
Perez-Alvarez S, Solesio ME, Manzanares J, Jordan J, Gallindo MF
Pearson RG (1963) Hard and soft acids and bases. J Am Chem Soc
Moreau C, the FAIRPARK-II study group. et al (2018) Iron as a thera-
Mishra K, Ojha H, Chaudhury NK (2012) Estimation of antiradical
Merkofer M, Kissner R, Hider RC, Brunk UT, Koppenol WH (2006)
Merkofer M, Kissner R, Hider RC, Brunk UT, Koppenol WH (2004) Redox
Meng QX et al (2016) Solanine-induced reactive oxygen species inhibit
Ma Y, Roy S, Kong X, Chen Y, Liu D, Hider RC (2012) Design and
Mastroberardino PG et al (2009) A novel transferrin/TfR2-mediated
McNaught KS, Björklund LM, Belizaire R, Isacson O, Jenner P,
Olanow CW (2002) Proteasome inhibition causes nigral degen-
eration with inclusion bodies in rats. NeuroReport 13:1437–1441
Meng QX et al (2016) Solanine-induced reactive oxygen species inhibit
the growth of human hepatocellular carcinoma HepG2 cells.
OncoLett 11:2145–2151
Merköfer M, Kissner R, Hider RC, Koppenol WH (2004) Redox
properties of the iron complexes of orally active chelators CP20,
CP502, CP509, and ICL670. Hely Chim Acta 87:3021–3033
Merköfer M, Kissner R, Hider RC, Brunk UT, Koppenol WH (2006)
Fenton chemistry and iron chelation under physiologically rele-
vant conditions: electrochemistry and kinetics. Chem Res Toxicol
19:1263–1269
Misha K, Ojha J, Chaudhury NK (2012) Estimation of antiradical
properties of antioxidants using DPPH assay: a critical review
and results. Food Chem 130:1036–1043
Moreau C, the FAIRPARK-II study group. et al (2018) Iron as a thera-
peutic target for Parkinson’s disease. Mov Disord 33:568–574
Pearson RG (1963) Hard and soft acids and bases. J Am Chem Soc
85:3533–3539
Perez-Alvarez S, Solesio ME, Manzanares J, Jordan J, Gallindo MF
(2009) Lactacystin requires reactive oxygen species and Bax
redistribution to induce mitochondria-mediated cell death. Br J
Pharmacol 158:1121–1130
Pienaar IS et al (2015a) An animal model mimicking pedunculopontine
nucleus cholinergic degeneration in Parkinson’s disease. Brain
Struct Funct 220:479–500
Pienaar IS et al (2015b) Pharmacogenetic stimulation of cholinergic
pedunculopontine neurons reverses motor deficits in a rat model
of Parkinson’s disease. Mol Neurodegener 10:47
Poepe W, Mählknecht P (2009) The clinical progression of Parkinson’s
disease. Parkinsonism Relat Disord 15:S28–32
Rhodes SL, Ritz B (2008) Genetics of iron regulation and the possible
role of iron in Parkinson’s disease. Neurobiol Dis 32:183–195
Ringbom A (1963) Complexation in analytical chemistry. A guide for
the critical selection of analytical methods based on complexation
reactions. In: Elving PJ, Kolthoff IM (eds) Chemical Analysis: a
series of monographs on analytical chemistry and its applications,
16th edn. Wiley, New York
Savolainen MH, Albert K, Airavaara M, Myöhänen TT (2017) Nigral
injection of a proteasomal inhibitor, lactacystin, induces wide-
spread glial cell activation and shows various phenotypes of
Parkinson’s disease in young and adult mouse. Exp Brain Res
235:2189–2202
Sayre LM, Moreira PI, Smith MA, Perry G (2005) Metal ions and
oxidative protein modification in neurological disease. Ann Inst
Super Sanita 41:143–164
Sharma P et al (2020) DREADD activation of pedunculopontine cho-
linergic neurons reverses motor deficits and restores striatal dopa-
mine signaling in parkinsonian rats. Neurotherapeutics. https://
doi.org/10.1007/s13311-019-00830-4 (in press)
Sian-Hülsmann J, Mandel S, Youdim MBH, Riederer P (2011) The
relevance of iron in the pathogenesis of Parkinson’s disease. J
Neurochem 118:939–957
Skog DA, West DM, Holler FJ, Crouch SR (2000) Analytical chemis-
ty: an introduction, chapter 15, 7th edn. Saunders College Divi-
sion, Rochester
Sohn YS, Breuer W, Munnich A, Cabantchik ZI (2008) Redistribution
of accumulated cell iron: a modality of chelation with therapeutic
implications. Blood 111:1690–1699
Sohn YS, Mitterstiller AM, Breuer W, Weiss G, Cabantchik ZI (2011)
Rescuing iron-overloaded macrophages by conservative relocation
of the accumulated metal. Br J Pharmacol 164:406–418
Sun Y, Pham AN, Waite TD (2018) Mechanism underlying the effect-
iveness of deferiprone in alleviating Parkinson’s disease symp-
toms. ACS Chem Neurosci 9:1118–1127
Thompson JW, Bruick RK (2012) Protein degradation and iron homo-
eostasis. Biochim Biophys Acta 1823:1484–1490
Tomoda H, Omura S (2000) Lactacystin, a proteasome inhibitor:
discovery and its application in cell biology. Yakugaku Zassi
120:935–949
Wallace DF (2016) The regulation of iron absorption and homeostasis.
Clin Biochem Rev 37:51–62
Wang Y et al (2016) Discovery of piperlongumine as a potential novel
lead for the development of senolytic agents. Aging (Albany NY)
8:2915–2926
Ward RJ, Dexter DT, Crichton RR (2015) Neurodegenerative diseases
and therapeutic strategies using iron chelators. J Trace Elem Med
Biol 31:267–273
Watts JC et al (2013) Transmission of multiple system atrophy prions
to transgenic mice. Proc Natl Acad Sci USA 110:19555–19560
Workman DG et al (2015) Protection from neurodegeneration in the
6-hydroxydopamine (6-OHDA) model of Parkinson’s with novel
1-hydroxypyridin-2-one metal chelators. Metallomics 7:867–876
Xiao Y et al (2018) Iron promotes α-synuclein aggregation and trans-
mision by inhibiting TFE-β-mediatedautophagosome-lysosome
fusion. J Neurochem 145:34–50
Yang H et al (2005) The block of ubiquitin-proteasome pathway induces
cell death and the formation of ubiquitin-immuneactive inclu-
sions in PC12 cells. Zhonghua Shenjing Ke Zazhi 38:430–433
Zhang X, Xie W, Qu S, Pan T, Wang X, Le W (2005) Neuroprotec-
tion by iron chelator against proteasome inhibitor-induced nigral
degeneration. Biochem Biophys Res Commun 333:544–549
Zhu W et al (2007) Prevention and restoration of lactacystin-induced
nigrostriatal dopamine neuron degeneration by novel brain-perme-
able iron chelators. J Trace Elem Exp Med 21:3835–3844
Zhu W et al (2010) Genetic iron chelation protects against proteasome
inhibition-induced dopamine neuron degeneration. Neurobiol Dis
37:307–313

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