

Reduction of paraquat-induced renal cytotoxicity by manganese and copper complexes of EGTA and EHPG

Mohamed Samai^a,
Theresa Hague^b,
Declan P. Naughton^b,
Paul R. Gard^a,
Prabal K. Chatterjee^a,

^a Department of Pharmacology and Therapeutics, University of Brighton, Brighton, East Sussex BN2 4GJ, UK

^b Biomedical and Pharmaceutical Sciences Research Group, Kingston University, Surrey, UK

Abstract

Superoxide anion generation plays an important role in the development of paraquat toxicity. Although superoxide dismutase mimetics (SODm) have provided protection against organ injury involving generation of superoxide anions, they often suffer problems, e.g., regarding their bioavailability or potential pro-oxidant activity. The aim here was to investigate and compare the therapeutic potential of two novel SODm, manganese(II) and copper(II) complexes of the calcium chelator ethylenebis(oxyethylenitrilo)tetraacetic acid (EGTA) and of the contrast agent ethylenebis(hydroxyphenylglycine) (EHPG), against paraquat-induced renal toxicity *in vitro*. Incubation of renal NRK-52E cells with paraquat (1 mM) for 24 h produced submaximal, yet significant, reduction in cellular viability and cell death and produced significant increases in superoxide anion and hydroxyl radical generation. Manganese and copper complexes of EGTA (10–100 μ M) and EHPG (30–100 μ M) reduced paraquat-induced renal cell toxicity and reduced superoxide anion and hydroxyl radical generation significantly. Manganese complexes displayed greater efficacy than copper complexes and, at equivalent concentrations, manganese complexed with EHPG provided the greatest protection. Furthermore, these metal complexes did not interfere with the uptake of [*methyl*- 14 C]paraquat into NRK-52E cells, suggesting that they provided protection against paraquat cytotoxicity via intracellular mechanisms. These complexes did not display cytotoxicity at the concentrations examined. Together, these results suggest that manganese and copper complexes of EGTA and EHPG, and especially the manganese–EHPG complex, could provide benefit against paraquat nephrotoxicity.

Keywords

- Cytotoxicity; Cu(II)-EGTA; Cu(II)-EHPG; Hydroxyl radical; Kidney; Mn(II)-EGTA; Mn(II)-EHPG; NRK-52E; Oxidative stress; Paraquat; Renal; Superoxide anion; Superoxide dismutase mimetics; Free radicals

Introduction

Paraquat (1,1'-dimethyl-4,4'-bipyridium dichloride, also known as methyl viologen) is a widely used broad-spectrum and fast-acting herbicide. However, it is extremely toxic, causing fatalities due to accidental or intentional poisoning, prevalently in developing countries [1] and [2]. Paraquat poisoning causes severe multiple organ failure, with the degree of poisoning dependent on the route of administration, the amount administered, and the duration of exposure. It is rapidly distributed within the body with highest concentrations accumulating within the kidneys, where it produces early and severe nephrotoxicity [3]. Additionally, as it is primarily excreted unchanged via the kidneys, the consequent reduction in renal function increases plasma concentrations by up to fivefold, which contributes to paraquat toxicity in other organs, especially the lungs [4] and [5]. Ultimately, respiratory failure, in the presence of nephrotoxic acute renal failure, is responsible for most deaths caused by paraquat [5], [6] and [7]. Therefore, maintaining renal function in patients suffering from paraquat poisoning remains a therapeutically important treatment strategy.

Generation of reactive oxygen species (ROS), such as superoxide anions, plays a major part in the development of paraquat-induced toxicity [8] and [9] and especially nephrotoxicity [10] and [11]. Current research has therefore focused on the therapeutic potential of antioxidants against paraquat-induced toxicity, especially those that can degrade superoxide anions such as superoxide dismutase (SOD) [12]. Unfortunately, the effectiveness of exogenously administered antioxidant enzymes such as SOD in vivo has been hindered by factors such as its poor bioavailability, low stability, or rapid hydrolysis in the blood and problems regarding its immunogenicity [13], [14] and [15]. Furthermore, SOD itself can have pro-oxidant effects at higher concentrations [16], [17], [18] and [19], for example, via the possibility that Cu^{2+} derived from Cu/ZnSOD may facilitate the generation of oxidative stress in the presence of glutathione [17] or via the ability of superoxide to both initiate and terminate lipid peroxidation [19]. Subsequently, many different types of SOD mimetics (SODm) have been synthesized [15] and [20], including manganese(III) tetrakis(4-benzoic acid) porphyrin (MnTBAP) and M40401 (a manganese-containing SODm), which have been shown to reduce paraquat-induced lung and brain injury in the mouse and rat, respectively [21] and [22]. However, SODm also suffer problems regarding bioavailability and toxicity, e.g., their poor stability in vivo and pro-oxidant activities [23] and [24].

The calcium chelator ethylenebis(oxyethylenitrilo) tetraacetic acid (EGTA) has recently been shown to possess significant SOD activity [25] and has provided benefits in models of multiple sclerosis and Alzheimer disease [26] and [27]. Another chelator, ethylenebis(hydroxyphenylglycine) (EHPG), which is used as a contrast agent in imaging and as a transferrin mimic in the study of manganese transport [28] and [29], also exhibits SODm properties when complexed with manganese (Mn) (II) or copper (Cu) (II) [25]. A major advantage of the potential use of these agents for the treatment of disease is that EGTA and EHPG have already been used therapeutically [26], [27], [28] and [29]. Furthermore, Mn(II) and Cu(II) complexes of EGTA and EHPG are stable in solution and have a good safety profile in that they do not promote pro-oxidant activities [25].

To date, the protection afforded by Mn and Cu complexes of EGTA and EHPG on ROS generation and subsequent injury and death of renal cells has not been investigated. The aims of this study were to: (i) confirm the role of ROS in the development of paraquat-induced renal (NRK-52E) cell cytotoxicity and (ii) investigate and compare the therapeutic potential of EGTA and EHPG and their Mn(II) and Cu(II) complexes against paraquat-induced renal cytotoxicity in vitro. The mechanisms by which metal complexes of EGTA and EHPG could protect NRK-52E cells against paraquat toxicity were investigated, both by assessing the ability of these complexes to reduce ROS generation by paraquat and by examining the ability of the complexes to alter the uptake of paraquat into renal cells.

Materials and methods

Unless otherwise stated, all compounds used in this study were purchased from Sigma–Aldrich Co. Ltd. (Poole, Dorset, UK). Mn(II) and Cu(II) were obtained from Sigma–Aldrich in the form of Mn(II) sulfate and Cu(II) chloride (MnSO₄ and CuCl₂). Mn(II) and Cu(II) complexes of EGTA and EHPG were synthesized as described previously [25].

Culture of NRK-52E cells

NRK-52E cells, which maintain characteristics of renal proximal tubular cells in culture [30], were obtained at passage 24 from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and used between passages 28 and 60. Cells were routinely cultured in 80-cm² Nunc flasks (Fisher Scientific, Loughborough, Leicestershire, UK) and grown in Dulbecco's modified Eagle's medium (DMEM; Cambrex BioScience, Wokingham, Berkshire, UK), supplemented with 10% (v/v) fetal bovine serum (FBS; Biosera, Ringmer, East Sussex, UK), 1% nonessential amino acid solution, 100 U/ml penicillin, and 50 µg/ml streptomycin at 37°C in a humidified 5% carbon dioxide atmosphere. Culture medium was changed every 48 h. NRK-52E cells were subcultured at 90% confluence using a trypsin (0.1% w/v)/versene (0.02% w/v) mixture. For experiments, cells were subcultured and grown on 6- or 24-well Nunc plates (Fisher Scientific) in DMEM as described above except for the substitution of 5% (v/v) FBS. DMEM as described above but containing only 1% (v/v) FBS was used for incubations involving paraquat, metal complexes of EGTA and EHPG, or their individual components.

Measurement of paraquat toxicity

Confluent cultures of NRK-52E cells on 24-well plates were incubated for 24 h with increasing concentrations of paraquat (0.003–1 mM). In detail, culture medium was replaced with incubation medium, which consisted of DMEM containing 1% (v/v) FBS and the required concentration of paraquat. At the end of the incubation period, cellular viability and cell death were determined as described below. From these data, a concentration of 1 mM paraquat was chosen, as this produced a submaximal, but significant, reduction in cellular viability (approximately 90% reduction in mitochondrial respiration compared to untreated controls) and a significant increase in cell death (approximately 80% of the lactate dehydrogenase released by cells treated with Triton X-100).

Effects of Mn(II) and Cu(II) complexes of EGTA and EHPG on paraquat-induced toxicity in NRK-52E cells

To investigate the effects of the metal complexes of EGTA and EHPG on cellular injury and death caused by paraquat, confluent NRK-52E cells were preincubated (30 min at 37°C) with 900 µl DMEM containing 1% (v/v) FBS and increasing concentrations of Mn(II)-EGTA, Cu(II)-EGTA, Mn(II)-EHPG, or Cu(II)-EHPG (0.3–100 µM). After 30 min, 100 µl of a 10 mM stock solution of paraquat or its vehicle (DMEM containing 1% (v/v) FBS) was added to each well to provide a final concentration of paraquat of 1 mM. The concentration range of the complexes used was based on those of similar metal-based SODm which have been shown to provide protection of rat proximal cells against oxidative stress [31] and [32]. It was also assumed, based on previous investigations [25] and on the dosing profile of previous experiments using SODm and renal cells [31] and [32], that the complexes would remain stable throughout the incubation period. In subsequent experiments, the effects of the individual components of the metal complexes of EGTA and EHPG on paraquat cytotoxicity were investigated. Specifically, NRK-52E cells were preincubated with 900 µl of increasing concentrations of EGTA, EHPG, Mn(II), or Cu(II) (0.3–100 µM) prepared in DMEM containing 1% (v/v) FBS, after which 100 µl of 10 mM paraquat or its vehicle (DMEM containing 1% (v/v) FBS) was added to each well. Cells were then incubated for 24 h after which cellular viability and cell death were assessed as described below.

Measurement of cellular viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay)

Cellular viability or injury was determined as described previously via measurement of the mitochondrial-dependent conversion of MTT into a formazan [31], [32] and [33]. Results were calculated as the percentage of the absorbance measured in control (untreated) cells not exposed to paraquat, which was taken as 100% viability and expressed as cellular viability (% untreated control).

Measurement of cytotoxicity (lactate dehydrogenase (LDH) assay)

Cell death was determined by measurement of LDH released into the incubation medium due to the loss of membrane integrity as described previously [31], [32] and [34]. Results were calculated as percentage of the total LDH released from control cells (i.e., those not exposed to paraquat) which were incubated with 1% (w/v) Triton X-100 (TX-100) for 30 min and expressed as LDH release (% TX-100 control).

Effects of Mn(II) and Cu(II) complexes of EGTA and EHPG on paraquat uptake by NRK-52E cells

In order to investigate whether Mn(II) and Cu(II) complexes of EGTA and EHPG could alter the uptake of paraquat into NRK-52E cells, the accumulation of radiolabeled paraquat was assessed as described previously [35]. Briefly, confluent cultures of NRK-52E cells were co-incubated with [*methyl*-¹⁴C]paraquat (0.01 mM) and cimetidine (1 mM), tetraethylammonium (TEA) (1 mM), or 100 µM Mn(II)-EGTA, Cu(II)-EGTA, Mn(II)-EHPG, or Cu(II)-EHPG, prepared in DMEM

containing 1% (v/v) FBS, for 60 min. The incubation time was based on previously published data in which the uptake of paraquat was shown to plateau after 1 h [35]. The concentrations of cimetidine and TEA used (1 mM) were based on those shown to significantly inhibit the uptake of paraquat into a renal cell line (LLC-PK₁) [36]. Concentrations of Mn(II) and Cu(II) complexes of EGTA and EHPG were based on those providing significant reduction of paraquat toxicity based on the data obtained from biochemical assays measuring cellular viability and cell death. Similarly, the concentration of paraquat used in these experiments (0.01 mM) was based on that previously shown to have no toxic effect on confluent NRK-52E cells. The uptake of [*methyl*-¹⁴C]paraquat was expressed as disintegrations per minute per microgram of protein and the results were expressed as percentage of control (i.e., based on cells treated with paraquat only).

Effects of preincubation with Mn(II) complexes of EGTA and EHPG on paraquat-induced toxicity in NRK-52E cells

In a separate set of experiments, the ability of Mn(II)-EGTA and Mn(II)-EHPG to enter and remain within NRK-52E cells was examined in order to investigate whether the Mn(II) complexes were present within cells during the incubation period with paraquat. Confluent cultures of NRK-52E cells were incubated with incubation medium only or with 100 μM Mn(II)-EGTA or Mn(II)-EHPG. After 20 h incubation, for co-incubation studies, incubation medium or paraquat was added to each well to provide a final paraquat concentration of 30 mM. For preincubation studies, cells were rinsed with warm phosphate-buffered saline (PBS; 0.01 M, pH 7.4) and then incubated with either incubation medium or 30 mM paraquat. Earlier studies by the authors have shown that incubation of NRK-52E cells with 30 mM paraquat for 4 h produces a significant reduction in cell viability and cell death in NRK-52E cells (data not shown). After the complete 24-h incubation period, cell viability and death were measured using the MTT and LDH assays, respectively.

Measurement of superoxide anion generation

The generation of superoxide anion was determined using the widely used nitroblue tetrazolium (NBT) assay described previously [37] and [38]. Superoxide anion generation was further confirmed by using hydroxyethidium as previously described [39].

The NBT assay is based on the reduction of NBT by superoxide anion to blue water-insoluble formazan, which can be monitored spectrophotometrically after solubilization of the cells. Briefly, NRK-52E cells grown in six-well plates were incubated for 24 h with DMEM containing 25 μg/ml NBT in the presence or absence of 1 mM paraquat. In addition, NRK-52E cells were co-incubated for 24 h with DMEM with 1% (v/v) FBS containing 25 μg/ml NBT and 1 mM paraquat in the presence or absence of SOD (EC 1.15.1.1; 1000 U/ml), catalase (EC 1.11.1.6; 1000 U/ml), and protective concentrations of Mn(II)-EGTA (100 μM) or Mn(II)-EHPG (100 μM).

Hydroxyethidium has been shown to be relatively specific for superoxide, which is oxidized by superoxide to yield ethidium. Cells were harvested from six-well plates previously incubated in DMEM containing 1% (v/v) FBS with 1 mM paraquat in the presence or absence of SOD (1000 U/ml), catalase (1000 U/ml), Mn(II)-EGTA

(100 μ M), or Mn(II)-EHPG (100 μ M) for 24 h. Cell suspensions were then washed with Hanks' buffered salt solution and then incubated with 10 μ M hydroxyethidium for 15 min at 37°C. Ethidium fluorescence was then measured using a spectrofluorometer set at excitation and emission wavelengths of 473 and 593 nm, respectively.

The concentrations of SOD and catalase were based on previous work by Scheid and colleagues in which these antioxidant enzymes were used to protect renal LLC-PK₁ cells against oxidative stress produced by oxalate [38]. Concentrations of Mn(II)-EGTA and Mn(II)-EHPG were based on concentrations found to provide significant protection against paraquat-induced cellular injury and cytotoxicity in earlier experiments. Results were expressed as superoxide anion generation (NBT abs. at 690 nm or ethidium fluorescence % untreated control).

Measurement of hydroxyl radical generation

The production of hydroxyl radicals by NRK-52E cells in the absence or presence of paraquat was evaluated using the widely used deoxyribose assay, based on the principle that the pentose sugar 2-deoxyribose can be cleaved by hydroxyl radicals to release a substance that reacts, on heating, with thiobarbituric acid (TBA) to produce a pink chromogen [38] and [40]. The chromogen formed in this assay has a spectrum which is identical to that of malondialdehyde (MDA) and, therefore, a standard curve for MDA is constructed from which the TBA content of samples (which is directly proportional to the hydroxyl radicals) can be extrapolated [38]. Briefly, confluent NRK-52E cells grown in six-well plates were incubated for 24 h with DMEM containing 3 mM deoxyribose in the presence of 1 mM paraquat. In addition, NRK-52E cells were co-incubated for a predetermined time period with DMEM containing 3 mM deoxyribose and 1 mM paraquat in the presence of SOD (1000 U/ml), catalase (1000 U/ml), or Mn(II)-EGTA or Mn(II)-EHPG (100 μ M each). At the end of the incubation period, the cell supernatant was removed and assayed for thiobarbiturate acid-reactive substances for MDA as previously described [41]. An aliquot of the cell supernatant was also used for protein quantification by the Bradford assay [42] and the hydroxyl radical content of samples was expressed as MDA formation (nmol/mg protein).

Statistical analysis

Results are expressed as means \pm SEM. Means were obtained from multiple experiments performed in triplicate. Data were analyzed and graphed with a commercially available software (GraphPad Prism, version 3.0; GraphPad Software, San Diego, CA, USA). Differences between mean values within the groups were determined using a Student *t* test or one-way analysis of variance (ANOVA) followed by a Dunnett's test for comparison of multiple means. The level of significance was set at $p < 0.05$.

Results

Effects of paraquat on cellular viability and LDH release by NRK-52E cells

Exposure of confluent NRK-52E cells to paraquat (0.003–1 mM) for 24 h resulted in a significant reduction in cellular viability from 100% for control (untreated) cells to 10% ($p < 0.05$) for cells incubated with 1 mM paraquat (Fig. 1). As also shown in Fig. 1, this was accompanied by a concomitant and significant increase in cell death from a baseline value of approximately 20% for control (untreated) cells to 80% ($p < 0.05$) for cells incubated with 1 mM paraquat.

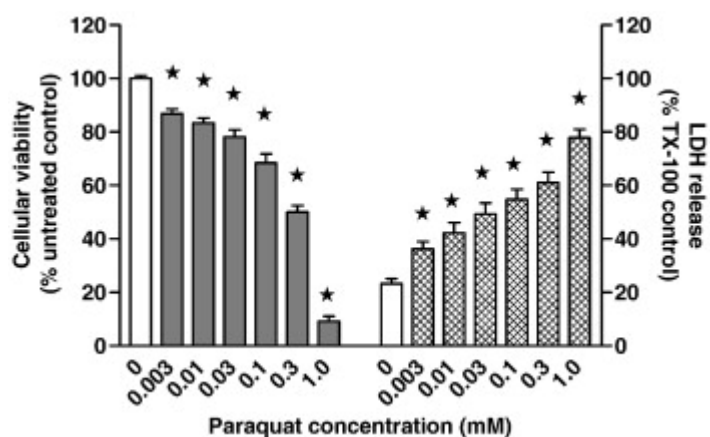


Fig. 1. Effects of paraquat on cellular viability (left) and cell death (right) of NRK-52E cells. Confluent NRK-52E cells were incubated with increasing concentrations of paraquat for 24 h. □ $p < 0.05$ vs 0 mM paraquat (untreated control cells treated with DMEM only) analyzed using one-way ANOVA followed by Dunnett's posttest, $N = 10$.

Effects of Mn(II) and Cu(II) complexes of EGTA and EHPG on paraquat toxicity

Incubation of NRK-52E cells with increasing concentrations of Mn(II)-EGTA (0.3–100 μ M) in the presence of 1 mM paraquat produced a significant improvement of cellular viability and reduced LDH release compared to cells incubated with 1 mM paraquat only (Fig. 2A). Specifically, concentrations of Mn(II)-EGTA greater than 10 μ M increased cellular viability and reduced LDH release significantly ($p < 0.05$, Fig. 2A).

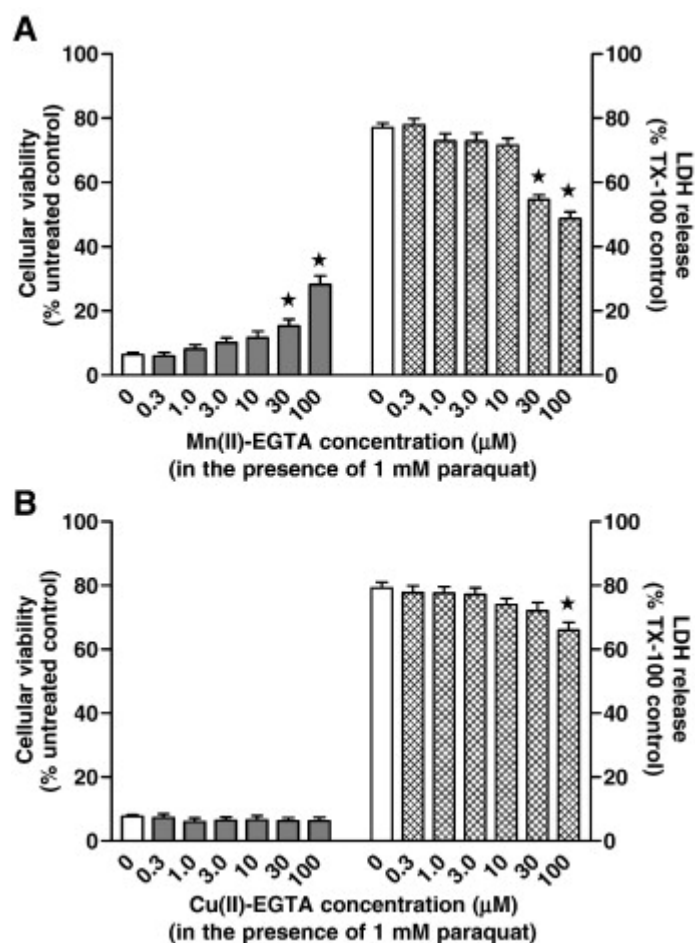


Fig. 2. Effects of (A) Mn(II) and (B) Cu(II) complexes of EGTA on viability (left) and death (right) of NRK-52E cells. Cultures were incubated with increasing concentrations of Mn(II)-EGTA or Cu(II)-EGTA for 24 h in the presence of 1 mM paraquat. \square $p < 0.05$ vs 0 μM Mn(II)-EGTA or Cu(II)-EGTA + 1 mM paraquat analyzed using one-way ANOVA followed by Dunnett's posttest, $N = 10$.

Incubation with increasing concentrations of Mn(II)-EGTA only (0.3–100 μM) produced a modest but significant reduction in cellular viability from 100 (untreated cells) to approximately 90% ($p < 0.05$); however, similar concentrations of Mn(II)-EGTA had no effect on baseline LDH release ($p > 0.05$) (data not shown).

In contrast, incubation of cells with Cu(II)-EGTA did not have any effect on the reduction in cell viability produced by 1 mM paraquat ($p > 0.05$, Fig. 2B). However, the highest concentration of Cu(II)-EGTA produced a modest but significant reduction in LDH release caused by 1 mM paraquat ($p < 0.05$, Fig. 2B). As for Mn(II)-EGTA, Cu(II)-EGTA itself produced a modest yet significant reduction in cellular viability from 100 (untreated cells) to approximately 87% at a concentration range of 1–100 μM ($p < 0.05$). However, and again in common with Mn(II)-EGTA, Cu(II)-EGTA had no effect on baseline LDH release ($p > 0.05$) (data not shown).

Mn(II)-EHPG produced a significant improvement in cellular viability at concentrations greater than 10 μM ($p < 0.05$, Fig. 3A). A similar effect was observed when the effects of Mn(II)-EHPG on paraquat renal cytotoxicity were assessed, with 30 and 100 μM Mn(II)-EHPG significantly reducing LDH release caused by 1 mM

paraquat ($p < 0.05$, Fig. 3A). Mn(II)-EHPG alone did not have any effect on cellular viability or LDH release at the concentration range investigated (0.3–100 μM) ($p > 0.05$) (data not shown).

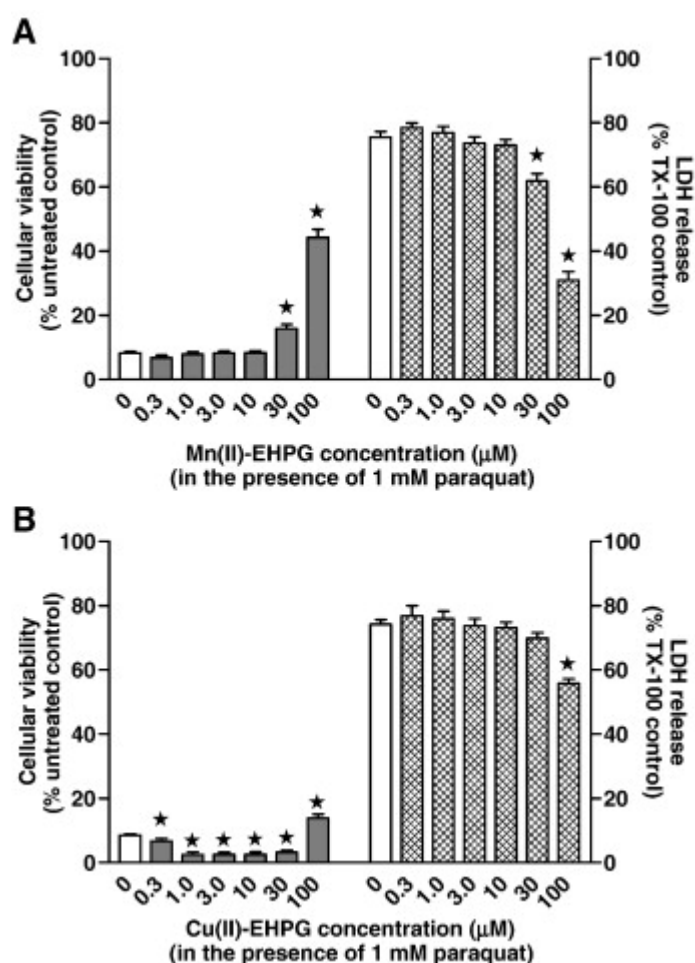


Fig. 3. Effects of (A) Mn(II) and (B) Cu(II) complexes of EHPG on viability (left) and death (right) of NRK-52E cells. Cultures were incubated with increasing concentrations of Mn(II)-EHPG or Cu(II)-EHPG for 24 h in the presence 1 mM paraquat. \square $p < 0.05$ vs 0 μM Mn(II)-EHPG or Cu(II)-EHPG + 1 mM paraquat analyzed using one-way ANOVA followed by Dunnett's posttest, $N = 10$.

Compared to the reduction in cellular viability produced by paraquat, Cu(II)-EHPG significantly reduced cellular viability further at the concentration range 0.3–30 μM ($p < 0.05$, Fig. 3B). However, at the highest concentration examined (100 μM), Cu(II)-EHPG increased cellular viability from 9 (paraquat only) to 14% ($p < 0.05$, Fig. 3B) and reduced paraquat-mediated LDH release significantly from 74 (paraquat only) to 56% (100 μM Cu(II)-EHPG) ($p < 0.05$, Fig. 3B). In contrast to its effects on cellular viability, lower concentrations of Cu(II)-EHPG (0.3–30 μM) had no effect on paraquat-mediated LDH release (Fig. 3B). At the concentrations examined (0.3–100 μM), Cu(II)-EHPG alone had no effect on cellular viability or LDH release ($p > 0.05$) (data not shown).

Comparison of the effects of Mn(II) and Cu(II) complexes of EGTA and EHPG and their individual components on paraquat-induced renal cell injury and cytotoxicity

As shown in [Fig. 4A](#), incubation of NRK-52E cells with 1 mM paraquat for 24 h caused a significant reduction in cellular viability, from 100 (untreated cells) to 7% (paraquat only) ($p < 0.05$, [Fig. 4A](#)). Mn(II)-EHPG, at 100 μ M, afforded the greatest protection against paraquat, increasing cellular viability from 7 (paraquat only) to 44% (Mn(II)-EHPG) ($p < 0.05$, [Fig. 4A](#)) compared to 100 μ M Mn(II)-EGTA and 100 μ M Mn(II), which increased cellular viability to 28 and 24%, respectively. Cu(II)-EHPG, at 100 μ M, also provided protection against the reduction in cellular viability produced by paraquat, increasing it from 7 (paraquat only) to 14% (Cu(II)-EHPG) ($p < 0.05$, [Fig. 4A](#)); however, this was similar to that provided by 100 μ M EHPG alone (11%). As shown in [Fig. 4A](#), Cu(II)-EGTA had no effect on paraquat-induced reduction in cell viability (6%), whereas Cu(II) and EGTA alone actually reduced cell viability further (to approximately 2%, $p < 0.05$).

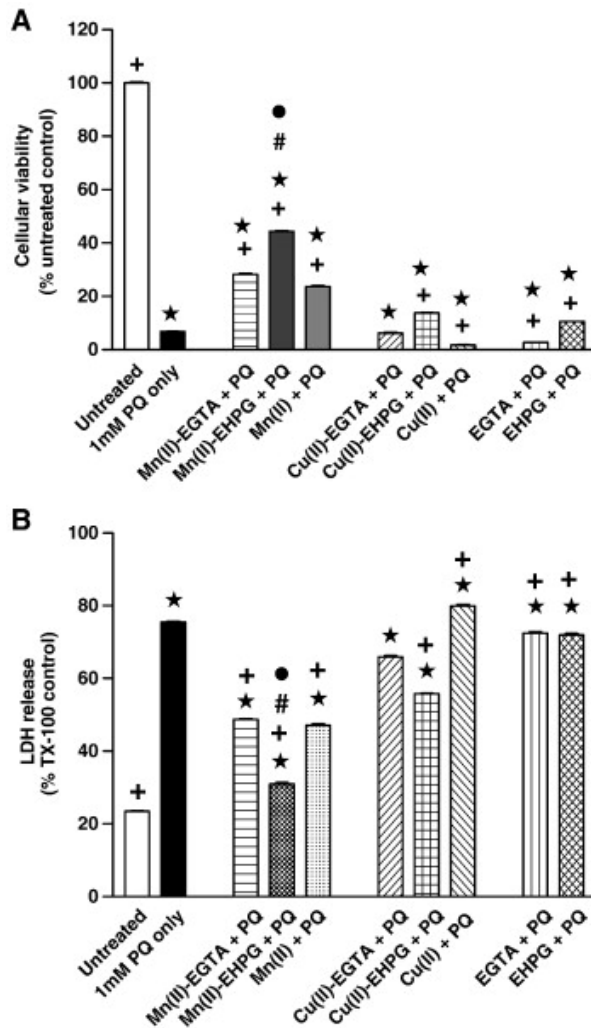


Fig. 4. Summary of the effects of Mn(II) and Cu(II) complexes on (A) cellular viability and (B) cell death in the presence of paraquat (PQ). NRK-52E cells were incubated with 100 μ M Mn(II)-EGTA, Mn(II)-EHPG, Cu(II)-EGTA, or Cu(II)-EHPG or their individual components (100 μ M) in the presence of paraquat for 24 h. \square $p < 0.05$ vs untreated cells (DMEM only); + $p < 0.05$ vs 1 mM PQ only; # $p < 0.05$ vs Mn(II)-EGTA + PQ; \cdot $p < 0.05$ vs Cu(II)-EHPG + PQ, analyzed using one-way ANOVA followed by Dunnett's posttest, $N = 10$.

A similar profile was observed when the effects on the cell death (LDH release) caused by paraquat were assessed (Fig. 4B). Incubation with 1 mM paraquat produced a significant increase in LDH release from 24 (untreated cells) to 76% (paraquat only) ($p < 0.05$, Fig. 4B). Mn(II)-EHPG, at 100 μ M, afforded the greatest protection against paraquat, reducing LDH release from 76 (paraquat only) to 31% (Mn(II)-EHPG) ($p < 0.05$, Fig. 4B). This was a significantly greater protection than that afforded by 100 μ M Mn(II)-EGTA. Cu(II)-EHPG and EHPG, at 100 μ M, reduced LDH release to 56 and 72%, respectively ($p < 0.05$, Fig. 4B). Cu(II)-EHPG, at 100 μ M, also provided protection against paraquat-mediated LDH release, reducing it from 76 (paraquat only) to 56% (Cu(II)-EHPG) ($p < 0.05$, Fig. 4B). In contrast, Cu(II) increased paraquat-mediated LDH release significantly from 76 (paraquat only) to 80% (Cu(II) only) ($p < 0.05$, Fig. 4B).

It should be noted that in the case of the most protective complex examined, i.e., Mn(II)-EHPG, a synergistic degree of protection was obtained when the protection provided by the complex was compared to the additive protection provided by its components, Mn(II) and EHPG, when examined individually. This synergistic activity was apparent in the data obtained from measurement of both cellular viability (Fig. 4A) and LDH release (Fig. 4B).

Effects of cimetidine, TEA, and Mn(II) and Cu(II) complexes of EGTA and EHPG on uptake of paraquat into NRK-52E cells

The uptake of [*methyl*-¹⁴C]paraquat (0.01 mM) into confluent NRK-52E cells was not inhibited by TEA or the Mn(II) and Cu(II) complexes of either EGTA or EHPG, but was significantly inhibited by cimetidine (Fig. 5). Specifically, co-incubation of confluent NRK-52E cells for 60 min with 0.01 mM [*methyl*-¹⁴C]paraquat and 1 mM cimetidine resulted in a significant reduction in the uptake of paraquat by approximately 40% ($p < 0.05$, Fig. 5).

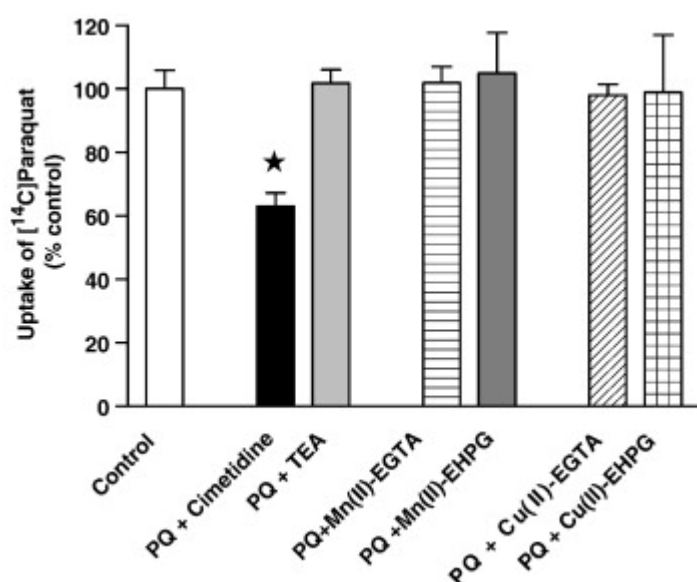


Fig. 5. Effects of cimetidine, TEA, and Mn(II) and Cu(II) complexes of EGTA and EHPG on the uptake of paraquat (PQ) by NRK-52E cells. Confluent NRK-52E cells were co-incubated with 0.01 mM PQ and cimetidine (1 mM), TEA (1 mM), or Mn(II)-EGTA, Cu(II)-EGTA, Mn(II)-EHPG, or Cu(II)-EHPG (100 μ M each) for 60 min. * $p < 0.05$ vs control (cells treated with PQ only), $N = 6$.

Effects of preincubation with Mn(II) complexes of EGTA and EHPG on paraquat-induced toxicity in NRK-52E cells

In order to investigate to what degree the most protective SODm were able to enter and remain within NRK-52E cells, confluent cultures were preincubated with 100 μ M Mn(II)-EGTA or Mn(II)-EHPG followed by a co-incubation with 30 mM paraquat for 4 h or were incubated with 30 mM paraquat only for 4 h. Incubation with 30 mM paraquat for 4 h caused a significant reduction in cell viability (Fig. 6A) and a significant increase in cell death (Fig. 6B). However, if the cells were preincubated with 100 μ M Mn(II)-EGTA or Mn(II)-EHPG for 20 h, followed by co-incubation

with 30 mM paraquat for 4 h, cellular viability increased significantly from approximately 60 to 80% ($p < 0.05$, Fig. 6A). A similar profile of protection was observed for cell death, with co-incubation of the SODm with paraquat reducing cell death from approximately 80 to between 40 and 50% ($p < 0.05$, Fig. 6B). Furthermore, if NRK-52E cells were preincubated with 100 μ M Mn(II)-EGTA or Mn(II)-EHPG for 20 h and then rinsed with warm PBS followed by an incubation with 30 mM paraquat only for 4 h, a similar degree of significant protection against paraquat toxicity was obtained in terms of both cellular viability (Fig. 6A) and cell death (Fig. 6B).

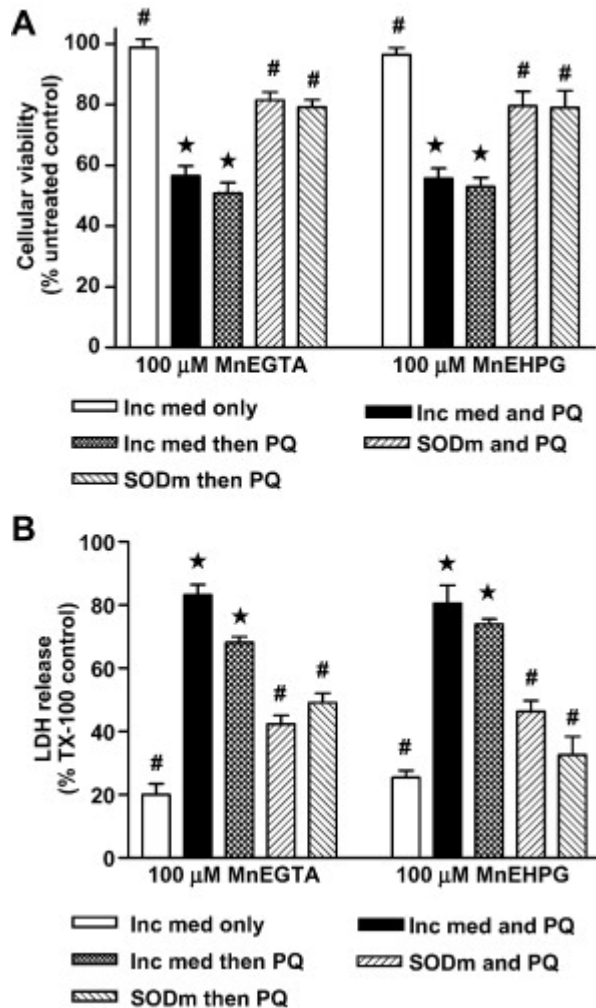


Fig. 6. Effects of preincubation with Mn(II)-EGTA and Mn(II)-EHPG on (A) viability and (B) death of NRK-52E cells incubated with paraquat (PQ). Cultures were incubated with incubation medium only or incubation medium containing 100 μ M Mn(II)-EGTA or Mn(II)-EHPG. After 20 h, paraquat was added to the incubation medium to a final concentration of 30 mM or cells were rinsed and incubated with 30 mM paraquat for 4 h. $\square p < 0.05$ vs 100 μ M Mn(II)-EGTA or Mn(II)-EHPG, $\#p < 0.05$ vs 30 mM paraquat only analyzed using one-way ANOVA followed by Dunnett's posttest, $N = 6$.

Effects of Mn(II) and Cu(II) complexes of EGTA and EHPG on the generation of reactive oxygen species by paraquat

As shown in Figs. 7A and B, incubation of NRK-52E cells with 1 mM paraquat for 24 h produced a significant increase in superoxide anion generation, by 52 and 35% as measured by NBT reduction and ethidium fluorescence, respectively ($p < 0.05$). Paraquat-mediated superoxide anion generation was significantly reduced by 1000 U/ml SOD ($p < 0.05$), but not by 1000 U/ml catalase (Figs. 7A, B). Mn(II)-EGTA (100 μ M) and Mn(II)-EHPG (100 μ M) both reduced superoxide generation by paraquat significantly ($p < 0.05$, Figs. 7A and B). It should be noted that the reductions were not significantly different from the reduction in paraquat-induced superoxide anion generation obtained using SOD (Figs. 7A and B).

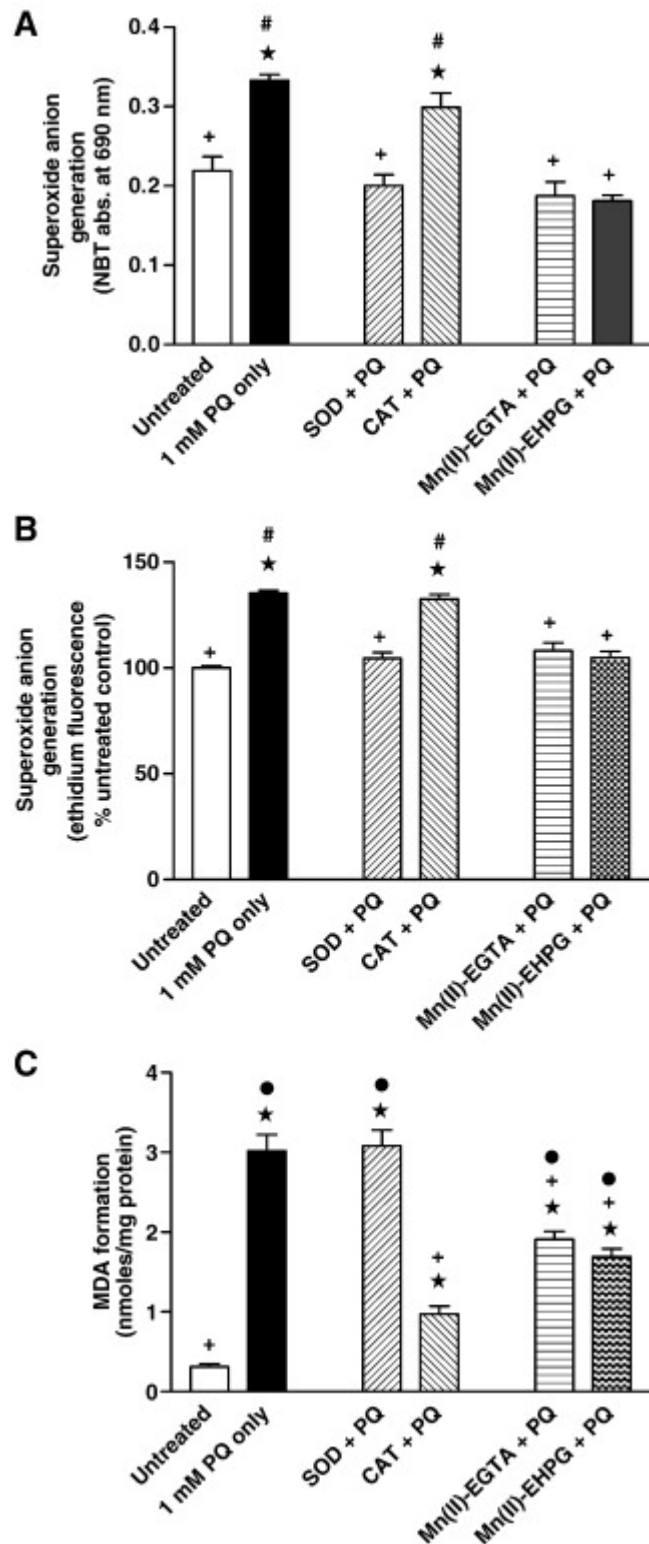


Fig. 7. Effects of Mn(II)-EGTA, Mn(II)-EHPG, and antioxidant enzymes on (A and B) superoxide anion generation and (C) hydroxyl radical production by NRK-52E cells. Cultures were incubated with 1 mM paraquat (PQ) in the presence of 1000 U/ml SOD, 1000 U/ml catalase (CAT), 100 μ M Mn(II)-EGTA, or 100 μ M Mn(II)-EHPG. \square $p < 0.05$ vs untreated cells (DMEM only); + $p < 0.05$ vs 1 mM PQ only; # $p < 0.05$ vs SOD + PQ; \cdot $p < 0.05$ vs CAT + PQ analyzed using one-way ANOVA followed by Dunnett's posttest, $N = 8$.

Incubation of cultures with 1 mM paraquat for 24 h also produced a significant 10-fold increase in hydroxyl radical production ($p < 0.05$, Fig. 7C). In contrast to superoxide generation, 1000 U/ml SOD did not have any effect on hydroxyl radical generation; however, 1000 U/ml catalase reduced paraquat-induced generation of hydroxyl radical significantly by approximately 68% ($p < 0.05$, Fig. 7C). Co-incubation of NRK-52E cells with 1 mM paraquat and 100 μ M of either Mn(II)-EGTA or Mn(II)-EHPG reduced paraquat-induced hydroxyl radical generation from 3.02 (paraquat only) to 1.91 and 1.69 nmol/mg protein, respectively ($p < 0.05$, Fig. 7C). These values were still significantly higher than the reduction in hydroxyl radical generation obtained using catalase ($p < 0.05$, Fig. 7C).

Discussion

The results obtained in this investigation clearly demonstrate (i) the renal toxicity of paraquat, specifically, a concentration-dependent reduction of cellular viability and concomitant increase in cell death, and (ii) the generation of ROS by NRK-52E cells exposed to paraquat, specifically, production of significant quantities of superoxide anions and hydroxyl radicals. The data presented also demonstrate (i) significant protection against paraquat renal cytotoxicity by Mn(II) and Cu(II) complexes of EGTA and EHPG, with Mn(II) complexes displaying higher potency and Mn(II)-EHPG providing the greatest protection, and (ii) the ability of Mn(II)-EGTA and Mn(II)-EHPG to reduce the generation of both superoxide anions and hydroxyl radical. The protection provided by these complexes against paraquat-induced cytotoxicity was obtained at concentrations which were found to be nontoxic to NRK-52E cells. Finally, protective concentrations of Mn(II) and Cu(II) complexes of EGTA and EHPG have been shown not to interfere with the uptake of paraquat into NRK-52E cells.

Superoxide anion generation, and the cellular damage resulting from it, is implicated in the pathophysiology of many conditions, including stroke, ischemia, asthma, atherosclerosis, and several neurodegenerative diseases [43], [44] and [45]. Increased superoxide anion generation due to paraquat and its contribution to paraquat toxicity have been catalogued over many years [8], [9], [46] and [47], and in this investigation, its role in the development of renal cytotoxicity has been confirmed in vitro. The role of superoxide in paraquat toxicity has prompted much interest in the development of safe and effective antioxidants to negate its injurious effects [12]. Unfortunately, native SOD cannot be used effectively due to recognized problems with its short half-life, poor bioavailability, and immunogenicity [13], [14] and [15]. Furthermore, SOD can have pro-oxidant activities at higher concentrations, e.g., Cu/ZnSOD can promote the generation of hydroxyl radicals [16] and [48] and inhibit the ability of superoxide to regulate lipid peroxidation [19] and [49]. This has generated much interest in SODm, leading to the development of agents including macrocyclics, manganese salens, nitroxides, porphyrins, and many other catalytic antioxidants [15], [20], [50], [51], [52], [53], [54] and [55]. However, many of these have also suffered problems ranging from cytotoxicity to poor stability [23] and [56]. Furthermore, in common with native SOD, higher concentrations of some SODm can have pro-oxidant actions [17], [18] and [19].

The recent observation that complexes formed between metal ions and the common chelator sodium edentate or ethylenediaminetetraacetic acid (EDTA) possess

significant SODm and catalase mimetic properties [57] prompted the investigation of the SOD and catalase activities of other commonly used chelators and their metal complexes. Low concentrations of the calcium ion chelator EGTA have been shown to solubilize amyloid plaques in postmortem brain samples from patients suffering from Alzheimer disease and to reduce nitric oxide (NO)-induced calcium influx into endothelial cells and prevent consequent cell death [26] and [27]. Some of these beneficial effects of EGTA have been credited to its ability to bind the excess Cu(II) ions which contribute to disease pathology; however, it is now also clear that complexing EGTA with Cu(II) or Mn(II) promotes significant SOD activity [25]. Recently, the related chelating agent EHPG, which is used as a contrast agent for magnetic resonance imaging and as a transferrin mimic in the study of manganese transport [28] and [29], has also been shown to exhibit significant SOD activity when complexed with Mn(II) or Cu(II) [25]. However, the ability of these complexes to reduce paraquat toxicity, in which superoxide generation plays a major part, is not known and formed the basis of this study.

In this investigation, Mn(II) and Cu(II) complexes of EGTA and EHPG provided significant protection against paraquat cytotoxicity in renal cells, which, in the case of Mn(II)-EHPG, was greater than any protection afforded by the individual components. Mn(II) complexes of EGTA and EHPG provided greater protection than the Cu(II) complexes. The protection afforded by these metal complexes seemed to be related to their SODm activity as suggested by their ability to reduce superoxide generation by paraquat to a degree similar to that obtained using SOD and support the findings of Fisher et al. [25], who demonstrated the SODm activities of Mn(II) and Cu(II) complexes of EGTA and EHPG. The order of protection observed against paraquat renal cytotoxicity was Mn(II)-EHPG > Mn(II)-EGTA > Cu(II)-EHPG > Cu(II)-EGTA. This profile closely reflected the SOD activities of these complexes reported by Fisher and colleagues (Mn(II)-EGTA, 981 U/mg; Mn(II)-EHPG, 879 U/mg; Cu(II)-EHPG, 409 U/mg; and Cu(II)-EGTA, 314 U/mg), by whom measurements were made as units activity per milligram compared to bovine erythrocyte SOD, which itself had an activity of 3730 U/mg [25]. These activities were higher than the activity of Cu(II)-EDTA at 261 U/mg [57] and considerably higher than the activities of Fe(III) complexes of EGTA and EHPG, which were measured as 8.76 and 0 U/mg, respectively [25]. In this investigation, EGTA-based complexes displayed greater potency than EHPG-based complexes as they reduced paraquat-mediated renal cytotoxicity significantly at a concentration of 10 μ M; however, the highest degree of protection was obtained using Mn(II)-EHPG at 100 μ M.

Also of interest was the observation that both Mn(II)-EGTA and Mn(II)-EHPG reduced hydroxyl radical generation significantly, as determined using the TBA reduction assay. Although this was not to the same degree as catalase, it suggests that these Mn(II) complexes may also possess some degree of catalase activity or are able to scavenge hydroxyl radicals; however, further studies are warranted to confirm this. Based solely on the findings of this investigation, it seems that the SODm activity of these complexes predominates.

Paraquat exists as a divalent cation in aqueous solution and is predominantly transported into renal proximal tubular cells via a polyvalent organic cation transporter (OCT) protein. This uptake of paraquat is saturable with time and with

increasing concentration of paraquat. It is also energy-dependent and is inhibited by certain polyvalent cations, such as quinine, cimetidine, and methylglyoxal bis(guanylhidrazine) dihydrochloride [36], [58] and [59]. The inhibitory effect of cimetidine, a divalent ion, on the uptake of paraquat into NRK-52E cells as has been shown in this study indicates the presence of a polyvalent OCT protein on NRK-52E cells and that this OCT is used by paraquat to enter NRK-52E cells. In contrast, TEA, a monovalent ion, did not alter paraquat uptake into NRK-52E cells. The observation that at protective concentrations, the Mn(II) and Cu(II) complexes of EGTA and EHPG did not alter the uptake of paraquat by NRK-52E cells suggests that their beneficial effects were not due to a chelating effect or prevention of paraquat uptake, e.g., via competition for the OCT for cellular uptake. The lack of effect of the Mn(II) and Cu(II) complexes of EGTA and EHPG on paraquat uptake into NRK-52E cells also supports the notion that their protection is mediated via intracellular mechanisms, i.e., due to their SODm activity subsequent to superoxide generation by paraquat within NRK-52E cells.

The Mn(II) and Cu(II) complexes of EGTA and EHPG seemed to maintain their stability in the incubation medium over the 24-h incubation period. As discussed above, the complexes did not alter the entry of paraquat into cells, which accumulates in renal cells to toxic concentrations over a 24-h period. Protection provided by these complexes was observed after 24 h, with the Mn(II) and Cu(II) complexes providing greater protection than their individual components, suggesting that the complexes maintained their structures. In the case of the most protective agent, Mn(II)-EHPG, the complex provided a synergistic level of protection compared to any protection afforded by the individual components Mn(II) and EHPG. Furthermore, the results of the preincubation study suggest that both Mn(II)-EGTA and Mn(II)-EHPG can enter and remain within NRK-52E cells over a 24-h period. Specifically, after 20 h incubation with Mn(II)-EGTA or Mn(II)-EHPG, NRK-52E cells were still protected against a high concentration of paraquat, even after the cells were rinsed with warm PBS. This suggests that these SODm can enter and remain within cells where they provide protection against ROS generated by paraquat. However, further studies, e.g., using high-pressure/performance liquid chromatography, are warranted to investigate whether the SODm remain in an intact state within intact cells or to what degree they may dissociate into their components within cells, which may still provide some degree of protection, e.g., via the individual actions of Mn and EGTA or EHPG.

In this particular study, Mn(II) and Cu(II) complexes of EGTA and EHPG did not display any pro-oxidant activities at higher concentrations tested. Although Mn(II)-EGTA, Cu(II)-EGTA, EGTA, EHPG, Mn(II), and Cu(II) produced a modest yet significant reduction in cellular viability on their own at higher concentrations, and Cu(II)-EHPG, EGTA, and Cu(II) reduced paraquat-mediated reduction in viability even further, none of these effects on cellular viability were reflected by LDH release. This could suggest that these compounds were causing some degree of mitochondrial dysfunction without subsequent cell death; however, it is much more likely that they were interfering directly with the MTT assay. It should be noted here that these complexes were examined at relatively low concentrations (maximum 100 μ M) and at concentrations which were protective against paraquat-induced cytotoxicity; these agents did not produce cytotoxicity themselves. Although it would be interesting to investigate higher concentrations of these complexes to see if greater protection or increasing toxicity could be observed and to compare these with protective and toxic

concentrations of other SODm such as MnTBAP, EUK-8, and M40401, concentrations greater than 100 μ M were not tested due to problems of solubility of these complexes in the incubation medium. However, the relatively safe profile of Mn(II) and Cu(II) complexes of EGTA and EHPG should allow investigations of these complexes in vivo to assess their efficacy against many pathological conditions involving ROS generation and oxidative stress. Specifically, these complexes could be investigated in models of ischemic or nephrotoxic ARF, which involve significant ROS generation and in which other SODm have been shown to provide beneficial effects [31], [32] and [51]. However, although there was no evidence of Cu(II) toxicity in this study, some care may need to be taken when Cu(II) complexes are utilized in view of a report that Cu(II) derived from Cu/ZnSOD may facilitate oxidative stress in the presence of glutathione [17]. Both EGTA and EHPG have the advantage that they have already been used in biological systems for their chelation and imaging properties and therefore their clinical toxicity profiles are already known. Furthermore, SOD and catalase activities of Mn(II), Cu(II), and Fe(III) complexes of another chelating agent, EDTA, have previously been reported [57] and subsequently, EDTA itself has been reported to protect against ischemic renal injury via modulation of endothelial NO synthase and NO production [60]. It is hoped that, based on the findings of this investigation, Mn(II) and Cu(II) complexes of EGTA and EHPG may provide similar benefits.

In conclusion, the novel SODm, Mn(II) and Cu(II) complexes of EGTA and EHPG reduced paraquat renal cytotoxicity significantly via reduction of paraquat-mediated ROS generation, specifically superoxide anions. Mn(II) complexes were generally more potent than the Cu(II) complexes, with the complex Mn(II)-EHPG providing the highest degree of protection. Unlike conventional SODm, which can display pro-oxidant actions at higher concentrations, these complexes at the highest concentration examined were not toxic to the renal NRK-52E cells utilized in this study. These results therefore demonstrate that these novel SODm have the potential to be used for the treatment of paraquat-induced nephrotoxicity and, possibly, for many other pathological conditions involving oxidative stress.

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