

# Effect of the SOD mimetic MnL4 on in vitro and in vivo oxaliplatin toxicity: Possible aid in chemotherapy induced neuropathy

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## Abstract

**Background:** One of the most uncomfortable dose-limiting adverse reactions of effective drugs for the treatment of solid tumors is a peripheral neuropathy which is the main reason for dose reduction and discontinuation of the therapy. We identified oxidative stress as one target of oxaliplatin toxicity in the search of possible adjuvant therapies to prevent neuropathy and alleviate pain. Therefore, we studied an effective SOD mimetic compound, MnL4, as a possible adjuvant treatment in in vitro cellular cultures and in vivo on a rat model of oxaliplatin-induced neuropathy.

**Methods and results:** All rat manipulations were carried out according to the European Community guidelines for animal care. We performed experiments on SH-SY5Y, HT-29 and primary cortical rat astrocytes. Incubation with 100 nM oxaliplatin increased superoxide anion production and caspase 3/7 activity in the neuronal cell line SH-SY5Y and cortical astrocytes. MnL4 (10 nM) significantly reduced the increase in superoxide anion in both cell types, but prevented caspase 3/7 activity only in astrocytes. MnL4 reduced lipid peroxidation induced by oxaliplatin and normalized the intracellular calcium signal evoked by ATP and acetylcholine in astrocytes, preincubated with oxaliplatin. MnL4 did not interfere with the concentration- and time-dependent cytotoxic effects of oxaliplatin on the cancer cell lines HT-29 and LoVo. In vivo MnL4 reduced the response at mechanical noxious and mechanical and thermal non-noxious stimuli in oxaliplatin treated animals. Rat rota-rod performances were improved.

**Conclusion:** Since MnL4 exerts its beneficial effects without interfering with the anticancer activity of oxaliplatin, it could be proposed as adjuvant to prevent and reduce oxaliplatin induced neuropathy.

## 1. Introduction

One of the most uncomfortable dose-limiting adverse reactions of effective drugs for the treatment of solid tumors is a neuropathic syndrome accompanied by persistent pain. The overall incidence of the chemotherapy induced neuropathy (CIN) is estimated to be approximately 38% in patients treated with multiple agents, [1] although this percentage varies depending on chemotherapy regimens, duration of exposure, and assessment methods [2]. Neuropathic pain in chemotherapy-treated patients often begins simultaneously in the hands and feet and it is the main reason for dose reduction and discontinuation of life-saving therapy [3].

Studying CIN in animal models in the search of possible adjuvant therapies to prevent neuropathy and alleviate pain, we identified several targets of oxaliplatin toxicity. In a rat model of oxaliplatin induced neuropathy, markers of oxidative stress (including lipid peroxidation, carbonylated proteins, and DNA oxidation) increased in the systemic circulation, sciatic nerve and lumbar spinal cord [4]. Oxaliplatin mediated damage results in neuronal cell modifications, morphological and molecular alterations in dorsal root ganglia and peripheral nerves [5] and electrophysiological hyperactivity in spinal wide dynamic range neurons [6]. Moreover, oxaliplatin is able to activate glial cells in nervous regions related to pain perception like cortical areas. A noteworthy involvement of astrocytes has been highlighted and strongly related to pain hypersensitivity [7]. A role for oxidative stress in the pathobiology of CIN is supported by multiple in vitro and in vivo studies showing that antioxidants have neuroprotective effects in oxaliplatin toxicity [4,5,8], suggesting a central involvement of mitochondria in CIN degenerative alterations. CIN is associated with a significant increase in the incidence of swollen and vacuolated mitochondria in peripheral nerve axons [9,10]. Oxaliplatin induces significant deficits in Complex I-mediated and Complex II-mediated respiration and significant deficits in ATP production in rat sciatic nerve [11]. Moreover, oxaliplatin promotes apoptosis and a release of cytochrome c from mitochondria to the cytosol in astrocyte cell cultures [12]. The superoxide dismutase enzymes (SOD) can metabolize superoxide ion ( $O_2^{\bullet-}$ ), reduce  $O_2^{\bullet-}$  toxicity and preserve mitochondrial integrity and functions. Decreased MnSOD activity has been described in different CIN, depending on SOD nitration linked to high levels of species with high oxidizing power [13]. Mitochondria are together sources and targets of  $O_2^{\bullet-}$  and other reactive oxygen species (ROS). Their reduced functionality impairs cell metabolic and signaling processes, among which is calcium homeostasis [14]. Calcium dysregulation can induce abnormal spontaneous discharges in neuronal cells, degeneration of nerve fibers and central sensitization promoting allodynia and hyperalgesia [15].

Therefore, we decided to study an efficacious SOD mimetic compound as a possible adjuvant treatment in CIN. The polyamine-polycarboxylate-Mn<sup>II</sup> complex 4,10-dimethyl-1,4,7,10 tetraazacyclododecane-1,7-diacetic acid Mn<sup>II</sup> (Mn<sup>II</sup>Me<sub>2</sub>DO2A, herein indicated as MnL4 [16]) is a small, lipophilic molecule, capable of readily entering into cells and decomposing  $O_2^{\bullet-}$  at cytoplasmic sites of generation. It contains a Mn(II) ion and this characteristic determines a potent SOD-mimetic activity able to reduce ROS toxicity in biological environment. MnL4 shows a high thermodynamic stability, thus reducing the possibility to release the metal in biological fluids [16]. MnL4 ameliorates lung inflammation, oxidative injury and breathing dysfunction induced by exposure to the air-borne allergen in sensitized guinea pigs [17]. The molecule administered both acutely at the dose of 15 mg kg<sup>-1</sup> or by osmotic pump (15 mg kg<sup>-1</sup> die for 14 days, chronic slow

release) largely reduces neuropathic and inflammatory pain in different models of osteoarthritis and rheumatoid arthritis [18]. Moreover, the efficacy of SOD-mimetic compounds was also described in animal models of inflammatory and neuropathic pain [19].

According to our previous work [20], the cytotoxicity of oxaliplatin can be conveniently studied in isolated neuronal cells which might generate preliminary data on the effectiveness of potential adjuvants in CIN treatment and maintenance of oxaliplatin anticancer activity. In isolated astrocytes and in the neuroblastoma cell line SH-SY5Y treated with oxaliplatin, we studied the effect of MnL4, focalizing our research on direct and indirect indexes of  $O_2^{\bullet-}$  toxicity, mitochondrial damage and calcium homeostasis, since  $Ca^{2+}$  signaling may be strongly altered by membrane lipid peroxidation and decrease of mitochondrial energy production. Moreover, in human colorectal adenocarcinoma cell lines (HT-29 and LoVo), we analyzed oxaliplatin cytotoxicity in the absence or presence of MnL4 to evaluate if the antioxidant treatment can interfere with the antitumor activity of the platinum compound. Finally, MnL4 was tested in vivo in a rat model of CIN.

## 2. Methods

### 2.1. Animals

For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy) weighing approximately 200–250 g at the beginning of the experimental procedure were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Four rats were housed per cage (size 26 x 41 cm<sup>2</sup>); animals were fed with standard laboratory diet and tap water ad libitum, and kept at 23/7 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines [21]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2. Materials

All reagents were of the highest chemical purity grade and were obtained from Sigma-Aldrich (Milano, Italy) unless otherwise indicated.

The organic ligand 4,10-dimethyl-1,4,7,10-tetra-azacyclododecane-1,7-diacetic acid (H2L4) was synthesized as previously reported [16]. This compound was isolated as trihydrochloride salt (H2L4 · 3HCl). Its complex, MnL4, was obtained by reaction of MnSO<sub>4</sub> with H2L4 · 3HCl (1 : 1 M ratio) in aqueous solution at neutral pH under nitrogen atmosphere; the MnL4 complex was then isolated as a white solid by precipitation with an ethanol/diethyl ether 2 : 1 mixture, according to a previously described procedure [16]. The complex was further purified by re-crystallization with water/ethanol. The purity of the compound (495%) was ascertained by elemental analysis [16].

### 2.3. Cell cultures

Primary cultures of astrocytes were obtained according to the method described by McCarthy and De Vellis [22]. Briefly, the cerebral cortex of newborn (P1–P3) Sprague Dawley rats (Harlan, Italy) was dissociated in Hanks' balanced salt solution (HBSS) containing 0.5% trypsin/EDTA and 1% DNase for 30 min at 37 °C. The suspension was mechanically homogenized and filtered. Cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with 20% fetal bovine serum (FBS, Gibco, Invitrogen, Italy). Confluent primary glial cultures were used to isolate astrocytes removing microglia and oligodendrocytes by shaking. The purity of astrocyte cultures was determined immunocytochemically by staining for GFAP (Dako, Denmark). Cells were fixed in 4% paraformaldehyde, then incubated with the antibody (1:200) and visualized using Alexafluor conjugated secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). GFAP-positive cells were 95–98% in astrocyte cultures. Experiments were performed 21 days after cell isolation. Astrocytes were cultured in DMEM high glucose with 10% FBS, 2 mM L- glutamine, 1% essential aminoacid mix, 100 IU ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin in 5% CO<sub>2</sub> atmosphere at 37 °C. Human neuroblastoma cell line SH-SY5Y and human colon cancer cell lines HT-29 and LoVo were obtained from the American Type Culture Collection (Rockville, MD). SH-SY5Y were cultured in DMEM/HAM F12 supplemented with 10% FBS. HT-29 were cultured in DMEM high glucose with 10% FBS, LoVo cells were cultured in Ham's F12K supplemented with 10% FBS. Media contained 2mM L-glutamine, 1% essential aminoacid mix, 100 IU ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin. All cell lines were maintained under standard culture conditions (37 °C, 5% CO<sub>2</sub>, 95% air and 100% relative humidity). SH-SY5Y, HT-29 and astrocytes were starved in serum-free DMEM overnight before all treatments. Protein homogenate concentrations were measured by bicinchoninic acid assay. SH-SY5Y were used as such or after treatments to obtain a neuron-like phenotype according to [23]. The method is extensively described in Supplementary Methods and Results and Fig. S1. Briefly, SH-SY5Y cells were treated with retinoic acid 10 mM for 48, 72, and 120 h in starvation medium. Differentiation was analyzed by (A) morphological study, evaluating the neurite out- growth evidenced by the presence of abundant and branched neuritis; (B) immunocytochemistry for the neuronal marker  $\beta$ -tubulin III; and (C) western blot to quantify the expression of Stat-1. The incubation with 10 mM retinoic acid for 72 h was chosen as optimal condition.

For cell growth inhibition studies two representative human colon carcinoma cell lines (HT-29 and LoVo) were used. Cells were incubated with 0.1–100 mM oxaliplatin as indicated for each protocol type (see below) in the absence or presence of 10 mM MnL4. Time intervals and concentrations used in each set of experiments were chosen in respect to the method sensibility and specificity.

### 2.4 SOD-inhibitable superoxide anion production evaluated by cytochrome c assay

SH-SY5Y (undifferentiated or after neuronal differentiation by incubation with 10 mM retinoic acid for 72 h) or astrocytes were plated in 6-well plates (5 x 10<sup>5</sup>/well) and grown until confluence. Cells were then incubated with or without 100 µM

oxaliplatin in serum-free DMEM containing cytochrome c (1 mg/mL) for 4 h at 37 °C, in the absence or presence of 10 mM MnL4. Non-specific cytochrome c reduction was evaluated carrying out tests in the presence of bovine SOD (300 mU/mL). The supernatant of each well was collected, and the optical density was spectrophotometrically measured at 550 nm. After subtracting the non-specific absorbance, the SOD-inhibitable O<sub>2</sub><sup>•-</sup> amount was calculated by using an extinction coefficient of 2.1 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup> and expressed as mM/mg protein/4 h (the method is according to [24]). The 4 h incubation interval was chosen on the basis of preliminary experiments which showed poor reliability for longer cytochrome c exposure to the cellular environment [20].

#### 2.5. Lipid peroxidation (thiobarbituric acid reactive substances, TBARS)

Astrocytes were plated in cell culture Petri dishes (10 cm diameter, 108/dish) and grown until confluence. Thiobarbituric acid reactive substances (TBARS) were quantified as an index of lipid peroxidation after 16 h incubation with 10 mM oxaliplatin in the absence or presence of 10 mM MnL4 with some minor modifications [20]. After incubation, cells were scraped and cell suspension underwent a freeze/thaw cycle. The suspension was added to 4 mL reaction mixture consisting of 36 mM thiobarbituric acid solubilised in 10% CH<sub>3</sub>COOH, 0.2% SDS, pH was adjusted to 4.0 with NaOH. The mixture was heated for 60 min at 100 °C and the reaction was stopped by placing the vials in ice bath for 2 h. After centrifugation (at 1600 x g at 4 °C for 10 min) the absorbance of the supernatant was measured at 532 nm (Perkin-Elmer spectrometer) and TBARS were quantified in mmoles/milligram of total protein using 1,1,3,3-tetramethoxypropane as standard.

#### 2.6. Caspase 3/7 activity

SH-SY5Y cells (undifferentiated or after differentiation in neuron-like phenotype by incubation with 10 mM retinoic acid for 72 h) or astrocytes were plated in 6-well plates (5 x 10<sup>5</sup>/well) and grown until confluence. Cells were incubated with 100 mM oxaliplatin for 4 and 8 h in the absence or presence of 10 mM MnL4. After treatment, cells were scraped in 100 mL lysis buffer as supplied by the manufacturer. Fifty microliters of the supernatants was incubated with 25 mM fluorogenic peptide caspases 3 and 7 substrate rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110; Molecular Probes) at 25 °C for 30 min in duplicate. The amount of cleaved substrate in each sample was measured in a 96-well plate fluorescent spectrometer (Perkin-Elmer; excitation at 496 nm and emission at 520 nm).

#### 2.7 Intracellular Ca<sup>2+</sup> measurement

Intracellular cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was evaluated in fura-2 loaded astrocytes. Briefly, 10<sup>4</sup> cells were plated on round glass coverslips (25 mm diameter), grown for 2–3 days in complete medium and then maintained for 16 h in DMEM alone, DMEM+10 mM MnL4, DMEM +10 mM oxaliplatin or DMEM +10 mM MnL4 +10 mM oxaliplatin. Cells were loaded with 4 mM fura-2AM (Molecular Probes-Invitrogen Life technologies, San Giuliano Milanese, Italy) for 45 min at RT and then washed with HEPES/Tyrode solution of the following composition: (mM): HEPES 5, D-glucose 10, NaCl 140, KCl 5.4, MgCl<sub>2</sub> 1.2, and CaCl<sub>2</sub> 1.8 (pH adjusted to 7.3 with NaOH). Coverslips were mounted in a perfusion chamber and placed on the stage of an epifluorescence microscope (TMD Diaphot, Nikon Co, Tokyo, Japan) equipped with a 75 W Xenon lamp. Fura-2 fluorescence was recorded with an extended ISIS-M camera (Photonic Science, Roberts Bridge, East Sussex, UK) and measured by single cell imaging analysis at 35 °C [25]. Paired experiments were performed for each treatment in at least 3 different astrocyte preparations. Agonists (i.e. 10 mM ACh or 10 mM ATP) were administered soon after recording basal fluorescence (F) and cytosolic [Ca<sup>2+</sup>]<sub>i</sub> dynamic was evaluated in single cells as the increase in fluorescence after subtracting the basal fluorescence (ΔF/F). The area under the curve (AUC) was measured for 300 s after the administration of the different agonist. A signal-to-noise ratio of at least 5 arbitrary fluorescence unit (A.U.) was considered as [Ca<sup>2+</sup>]<sub>i</sub> signal. About 18–20 cells were evaluated blindly for each experimental protocol.

#### 2.8. Cell viability assay

Cell viability was evaluated by the reduction of 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. HT-29 cells were plated into 96 well cell culture plates, grown until confluence and treated for 24 and 48 h in control condition or in the presence of 10 mM MnL4 with different concentrations of oxaliplatin in DMEM (0–100 mM). After extensive washing, 1 mg/mL MTT was added into each well and incubated for 2 h at 37 °C. After washing, the formazan crystals were dissolved in 100 μL dimethyl sulfoxide. The absorbance was measured at 580 nm. Experiments were performed in quadruplicate on at least 3 different cell batches.

#### 2.9. Cell growth inhibition studies

The cytotoxic effects of oxaliplatin against HT-29 and LoVo cell lines, alone or in combination with MnL4 were evaluated according to the sulforhodamine B (SRB) assay described by Skekan et al. [26]. Exponentially growing cells were seeded in 96-well plates in RPMI 1640 supplemented with 10% FCS at a plating density of 4 × 10<sup>3</sup> cells/well. After 24 h, the medium was removed and replaced with oxaliplatin containing medium, in the absence or presence of 10 mM MnL4, for exposure times of 24 h, or 24 h followed by regrowth in drug free medium for additional 24 h, or 48 h. After these exposure times, the assay was terminated by the addition of cold trichloroacetic acid (TCA). Cells were fixed by 10% TCA and stained by SRB solution at 0.4% (w/v) in 1% acetic acid. The SRB fixed to the cells was dissolved in 10 mM Tris-HCl and absorbance was read on an automated plate reader at a wave-length of 540 nm. The IC<sub>50</sub> drug concentration resulting in a 50% reduction in the net protein content (as measured by SRB staining) in drug treated cells as compared to untreated control cells was determined. Each reported IC<sub>50</sub> value represents the mean of three independent experiments.

#### 2.10. Oxaliplatin-induced neuropathic pain model

Rats were treated with 2.4 mg kg<sup>-1</sup> oxaliplatin, administered intraperitoneally (i.p.) for 5 consecutive days every week for three weeks (15 i.p. injections [27]). Oxaliplatin was dissolved in 5% glucose solution. Behavioral and biochemical tests were performed on 21st day. Control animals received an equivalent volume of 5% glucose i.p. Animal body weight was recorded every 2nd day.

#### 2.11. Drug treatments

MnL4 was given by continuous subcutaneous (s.c.) delivery using an osmotic minipump (Alzet 2004, Palo Alto, CA, USA) implanted by surgical procedure under enflurane anesthesia on the back and filled to deliver a daily dose of 15 mg kg<sup>-1</sup> for 21 days [18].

#### 2.12. Paw pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. [28]. Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (25%). For analgesia measures, mechanical pressure application was stopped at 120 g. Experiments were performed blindly.

#### 2.13. Von Frey test

Animals were placed in 20 cm 20 cm plexiglas boxes equipped with a metallic meshed floor, 20 cm above the bench. A habituation of 15 min was allowed before the test. An electronic Von Frey hair unit (Ugo Basile, Varese, Italy) was used: the withdrawal threshold was evaluated by applying force ranging from 0 to 50 g with a 0.2 g accuracy. Punctuate stimulus was delivered to the mid-plantar area of each anterior paw from below the meshed floor through a plastic tip and the withdrawal reflex was automatically displayed on the screen. Paw sensitivity threshold was defined as the minimum pressure required to elicit a robust and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not taken as a withdrawal response. Stimuli were applied on each anterior paw with an interval of 5 s. The measure was repeated 5 times and the final value was obtained by averaging the 5 measures [29].

#### 2.14. Cold plate test

Animals were placed on a stainless box (12 cm 20 cm 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4 °C 71 °C by cold circulating water. Pain-related behaviors (i.e. lifting and licking of the hind paw) were observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 s.

#### 2.15. Rota-rod test

Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed on the basis of walking time and the number of falls from the rod for a maximum of 600 s. After a maximum of 6 falls from the rod, the test was suspended and the time was recorded.

#### 2.16. Statistic analysis

Results were expressed as means 7S.E.M. and the analysis of variance was performed by one way ANOVA. A Bonferroni's significant difference procedure was used as post-hoc comparison. P values of less than 0.05 were considered as significant. Data were analyzed using the "Origins 9.1" software.

### 3 Results

According to our previous results, at least a component of oxaliplatin toxicity is dependent on ROS generation; SH-SY5Y line and freshly isolated astrocytes represent valuable in vitro models to predict the protective effect of adjuvant molecules on oxaliplatin neuropathy [19]. We analyzed several parameters related to ROS toxicity on these cellular models testing the efficacy of MnL4.

#### 3.1 SH-SY5Y cells

As already described [19], the incubation with 100 mM oxaliplatin for 4 h doubled O<sub>2</sub><sup>•-</sup> production in the neuronal cell line SH-SY5Y (Fig. 1A). MnL4 (10 mM) significantly reduced this increase, without influencing basal release. At the same concentration, oxaliplatin incubation for 4 and 8 h significantly increased caspase 3/7 activity (Fig. 1B and C), but the concurrent administration of 10 mM MnL4 was ineffective, indicating that in this neuroblastoma cell line the apoptotic process induced by oxaliplatin could be independent to ROS production. The same experiments were also performed on SH-SY5Y after differentiation in a more mature neuron-like phenotype by retinoic acid treatment (10 mM for 72 h). O<sub>2</sub><sup>•-</sup> concentrations were similar to that obtained in the undifferentiated cells (both for basal and stimulated conditions; data not shown). The incubation with oxaliplatin for 4 h did not modify the activity of caspase 3/7, whereas after 8 h caspase 3/7 activity increased by

six-fold ( $694 \pm 76\%$  in comparison to the basal value  $100 \pm 23\%$ ). MnL4 did not reduce caspase 3/7 activation ( $638 \pm 41\%$ ).

### 3.2 Astrocytes

Since astrocytes participate to the neuropathy induced by the chemotherapeutic agent [7], the effect of MnL4 was evaluated on these glial cells. Astrocytes production of  $O_2^{\bullet-}$  was increased more than 6 times by 100 mM oxaliplatin (4 h). MnL4 strongly reduced this production, while the basal release was only marginally decreased (Fig. 2A). At the same concentration, oxaliplatin significantly increased caspase 3/7 activity at 4 and 8 h (Fig. 2B and C); the contemporaneous administration of 10 mM MnL4 significantly decreased caspase 3/7 activity, suggesting that in these non-neoplastic cells the apoptotic process could be dependent on ROS production. MnL4 reduced lipid peroxidation induced by 10 mM oxaliplatin incubation for 16 h. Indeed, oxaliplatin increased lipid peroxidation more than twice (Table 1) in the controls, while in the presence of 10 mM MnL4, the increase was strongly reduced (Table 1). Then, we measured the kinetics of  $[Ca^{2+}]_i$  increase elicited by ATP and ACh in control and oxaliplatin preincubated astrocytes. As shown, 10 mM ATP induced a sustained, oscillatory calcium increase in astrocytes (Fig. 3). The preincubation with 10 mM oxaliplatin for 16 h amplified the effect of ATP. In cells preincubated with oxaliplatin, the AUC was increased by 60% (Fig. 3, inset). The preincubation with 10 mM MnL4 (16 h) did not modify ATP calcium kinetic in control condition (Fig. 3, inset), but restored ATP signal in cells pretreated with oxaliplatin (contemporaneous preincubation with oxaliplatin and MnL4). Similarly, the calcium transient induced by 10 mM ACh was potentiated by the overnight incubation with 10 mM oxaliplatin (Fig. 4) and MnL4 restored ACh signal as evaluated by the AUC (Fig. 4, inset).

### 3.3 HT-29 cell line

Aimed to evaluate a possible interaction between the anti-oxidant treatment and the therapeutic property of oxaliplatin, we evaluated oxaliplatin-induced cytotoxicity in the presence of MnL4 in a human colon cancer cell line HT-29 sensitive to this drug. Fig. 5 shows the concentration-dependent oxaliplatin (0.1–100 mM) lethal effect after 24 (Fig. 5A) or 48 h (Fig. 5B) incubation in the absence and presence of 10 mM MnL4. MnL4 (10 mM) did not significantly modify oxaliplatin cytotoxicity after 24 h but significantly increased it after 48 h. Antiproliferative effects of oxaliplatin alone or in combination with 10 mM of MnL4 on HT29 cells were also measured after 24 h (followed by regrowth in drug free medium for 24 h) and 48 h with SRB assay. The resulting concentration-response curves are shown in Fig. 6A. As well as in the MTT assay, no significant differences in the cytotoxic effects of oxaliplatin when combined with MnL4 were observed.

### 3.4 LoVo cell line

The cytotoxic effects of oxaliplatin alone or in combination with MnL4 10  $\mu$ M were also evaluated in LoVo cells (human colon cancer cell line intrinsically resistant to oxaliplatin) with SRB assay (Fig. 6B). Similarly as observed for the cell line HT-29 any change in the cytotoxic effects of oxaliplatin when combined with MnL4 was observed.

### 3.5 Oxaliplatin-induced neuropathic pain model

As already described, 5 days/week for 3 weeks of oxaliplatin administration (i.p., 2.4 mg kg<sup>-1</sup> daily) induced a progressive neuropathy characterized by mechanical hypersensitivity and reduced pain threshold to non-noxious stimuli [5]. After 3 weeks, the weight tolerated on the posterior paw decreased by about 50% in oxaliplatin-treated animals (mechanical hypersensitivity to a noxious stimulus, Fig. 7A). As measured with the electronic Von Frey apparatus, control withdrawal threshold to the mechanical non-noxious stimulus was decreased in oxaliplatin treated animals by 30% (Fig. 7B). The threshold decrease of a non-painful thermal stimulus was measured by the licking latency on cold plate. Results showed that oxaliplatin treatment decreased licking latency by 60% (Fig. 7C). The administration of MnL4 by slow, continuous delivery with osmotic pump (15 mg kg<sup>-1</sup> daily) in oxaliplatin treated animals improved neuropathic symptoms as compared to oxaliplatin alone (Fig. 7A–C). Moreover, the walking time and the number of falls on a rotating rod (Rota-rod test) were impaired as a consequence of CIN. Indeed, oxaliplatin-treated rats balanced on the rotating rod less than 1/3rd (Fig. 8A) and failed down 9 times more frequently than control rats (Fig. 8B). MnL4 ameliorated walking performance and decreased the number of falls (Fig. 8A and B) as a consequence of the reduced pain even if it did not recover the highly significant negative effect in body growth induced by oxaliplatin treatment (Fig. 8C). These results suggested that MnL4 reduced neuropathy, although it did not modify the systemic toxicity of oxaliplatin.

## 4 Discussion

Platinum anticancer drugs are effective treatments for several malignancies [30–32]. Oxaliplatin possesses a relative low hematological and gastrointestinal toxicity, but CIN can limit its clinical use [33,34]. Therefore, many efforts have been addressed to find adjuvant strategies to reduce CIN. In our research, we have already characterized ROS as central players of oxaliplatin CIN using in vitro and in vivo models [4,20,35]. The results herein presented show that the SOD mimetic polyaminopolycarboxylic compound MnL4 prevents cellular damage induced by oxaliplatin in vitro, reducing oxidative stress and normalizing calcium dynamic in isolated astrocytes pre-treated with the anticancer drug. Moreover, it reduces CIN in rats when continuously administered by osmotic

pump in the course of oxaliplatin treatment. MnL4 has been already characterized as a membrane permeable, high effective  $O_2^{\bullet-}$  scavenger compound [16], possessing anti-inflammatory properties in allergic asthma and behaving as a potent pain reliever in acute and chronic articular pathologies [18]. In vitro, MnL4 prevents the increase in  $O_2^{\bullet-}$  induced by oxaliplatin incubation without significantly influencing the basal release in the neuroblastoma cell line SH-SY5Y and astrocyte primary cultures. Oxaliplatin induces a six-fold increased  $O_2^{\bullet-}$  production in primary astrocytes, whereas in SH-SY5Y  $O_2^{\bullet-}$  is increased two-fold; to note, the basal control level of  $O_2^{\bullet-}$  production in the neuroblastoma cell line is more than twenty-fold higher than in astrocytes. This different production of  $O_2^{\bullet-}$  in basal control conditions suggests that SH-SY5Y cells are adapted to survive in a ROS rich environment and can be less sensible to the oxidative toxicity. By measuring caspase 3/7 activity a different behavior is observed in astrocytes and SH-SY5Y. In both cell types, caspase 3/7 activity is increased by oxaliplatin incubation in a time-dependent mode. In astrocytes, the concomitant incubation with MnL4 reduces it, whereas in SH-SY5Y, MnL4 is ineffective. An anti-apoptotic effect of antioxidants has been described in SH-SY5Y treated with noxious stimuli like MPP<sup>b</sup> [36], 1,2-diacetylbenzene [37] or hydrogen peroxide [38]. In those reports, an exogenous oxidant stimulus has been employed; on the contrary, as already described [20], oxaliplatin is not a direct oxidant molecule. Its oxidative power is elicited only in whole cells, suggesting the need of a cellular environment to evoke  $O_2^{\bullet-}$  overproduction. We have already described that oxaliplatin might regulate two distinct pathways (mitochondrial-dependent or not) converging to the activation of the final apoptotic marker caspase 3 in the primary, non-neoplastic astrocyte culture and in cancer cell lines [12]. This dissimilar behavior of MnL4 in neuroblastoma cells and astrocytes might reside in a specific tolerance of SH-SY5Y to a high oxidative environment (due to the elevated  $O_2^{\bullet-}$  production in control condition) or to the comparative reduced importance of mitochondrial ATP level in cancer cells as originally suggested by Warburg [39]. Mutations in oncogenes and tumor suppressor genes cause alterations to multiple intracellular signaling pathways that affect tumor cell metabolism [40] inducing a more oxidized redox environment and enhanced resistance to the mitochondrial pathway to apoptosis [41]. Nevertheless, MnL4 did not prevent oxaliplatin-induced apoptosis either in SH-SY5Y differentiated to a more mature neuron-like phenotype (by retinoic acid). On the other hand, despite several morphological and molecular neuronal features of the differentiated cells, the persistence of cancer characteristics cannot be excluded. Among others, a high basal oxidative environment ( $O_2^{\bullet-}$  concentration) is shown in SH-SY5Y before and after differentiation.

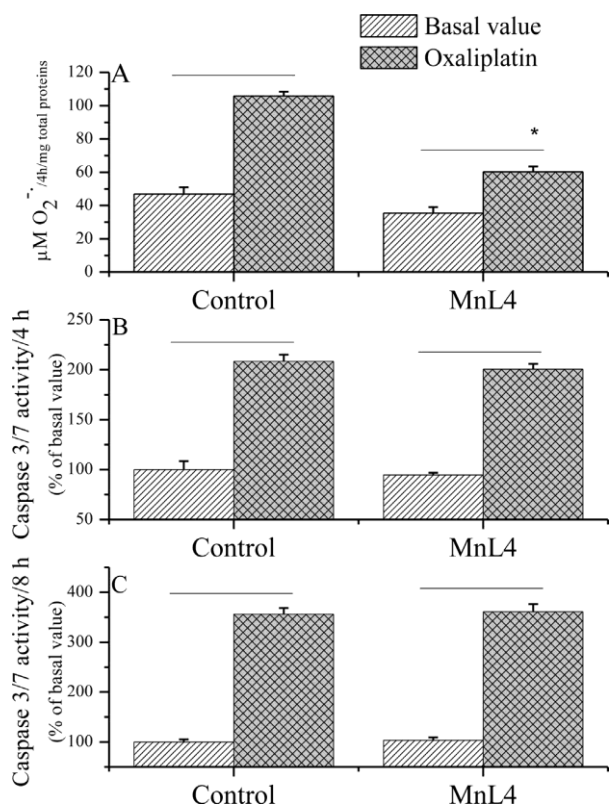


Fig. 1. Effect of 10 mM MnL4 on SOD-inhibitable  $\text{O}_2^{\bullet-}$  concentration (A) and caspase 3/7 activity at 4 (B) and 8 h (C) in SH-SY5Y. Panel A: SH-SY5Y cells were exposed to 100  $\mu\text{M}$  of oxaliplatin for 4 h in the absence or presence of 10 mM MnL4.  $\text{O}_2^{\bullet-}$  concentration was evaluated by cytochrome c assay. The non-specific absorbance was measured for each sample in the presence of SOD (300 mU/mL) and subtracted to the total value. Each value represents the mean  $\pm$  S.E.M. of 4 experiments. Panels B and C: Caspase 3/7 activity in SH-SY5Y. Cells were incubated with oxaliplatin (100  $\mu\text{M}$ ) in the absence or presence of 10 mM MnL4 for 4 (B) or 8 h (C). Caspase 3/7 activity was measured using the fluorescent substrate Z-DEVD-R110. Values are expressed as percent of control caspase 3/7 activity arbitrarily set as 100%. Each value represents the  $\pm$  S.E.M. of 3 experiments. \* $p < 0.01$  versus control. Segments on the top of bars indicate the significance versus basal values.

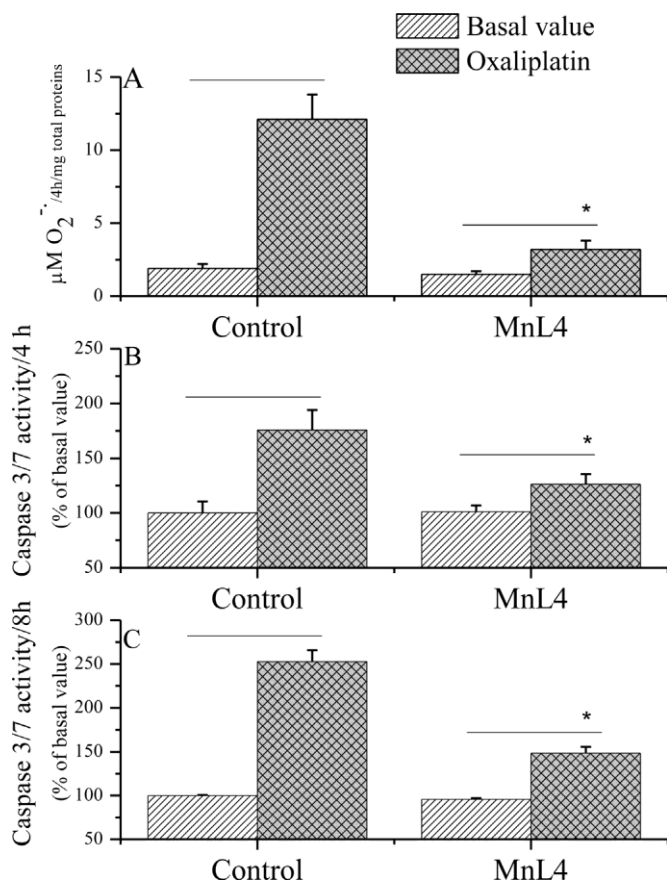


Fig. 2. Effect of 10 mM MnL4 on SOD-inhibitable  $O_2^{\bullet-}$  concentrations (A) and caspase 3/7 activity at 4 (B) and 8 h (C) in astrocytes. Panel A: astrocytes were exposed to 100  $\mu$ M of oxaliplatin for 4 h in the absence or presence of 10 mM MnL4.  $O_2^{\bullet-}$  concentration was evaluated by cytochrome c assay. The non-specific absorbance was measured for each sample in the presence of SOD (300 mU/mL) and subtracted to the total value. Each value represents the mean  $\pm$  S.E.M. of 4 experiments. Panels B and C: Caspase 3/7 activity in astrocytes. Cells were incubated with oxaliplatin (100  $\mu$ M) in the absence or presence of 10 mM MnL4 for 4 (B) or 8 h (C). Caspase 3/7 activity was measured using the fluorescent substrate Z-DEVD-R110. Values are expressed as percent of control caspase 3/7 activity arbitrarily set as 100%. Each value represents the  $\pm$  S.E.M. of 3 experiments. \* $P < 0.01$  versus control. Segments on the top of bars indicate the significance versus basal values.

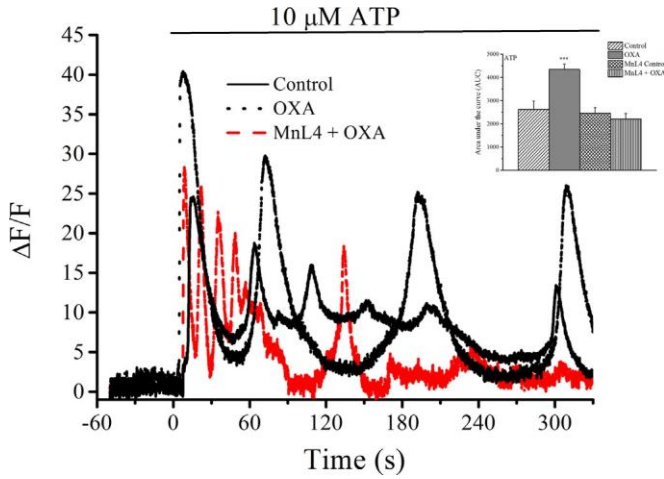


Fig. 3. Effect of ATP on intracellular cytosolic calcium concentration on astrocytes. Typical time-courses of  $[Ca^{2+}]_i$  recorded in control, 10 mM oxaliplatin (16 h pre-treatment) and 10 mM MnL4  $\pm$  10 mM oxaliplatin are shown. ATP (10 mM) was administered at time 0 and maintained throughout the experimental time. For simplicity, the  $[Ca^{2+}]_i$  dynamic obtained in cells preincubated with 10 mM MnL4 alone is omitted. Abscissa: time (s), Ordinate: fluorescence intensity reported as  $\Delta F/F$ . Inset shows the AUC (area under the curve, arbitrary units) calculated in the interval 0-300 s. \*\*\* $P < 0.001$  versus all other groups.

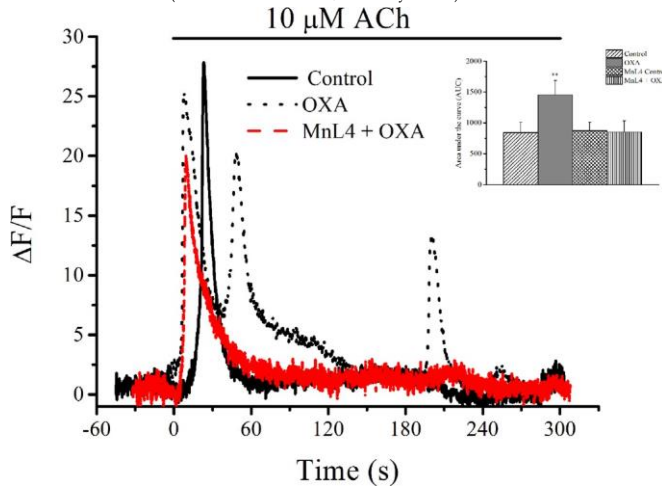
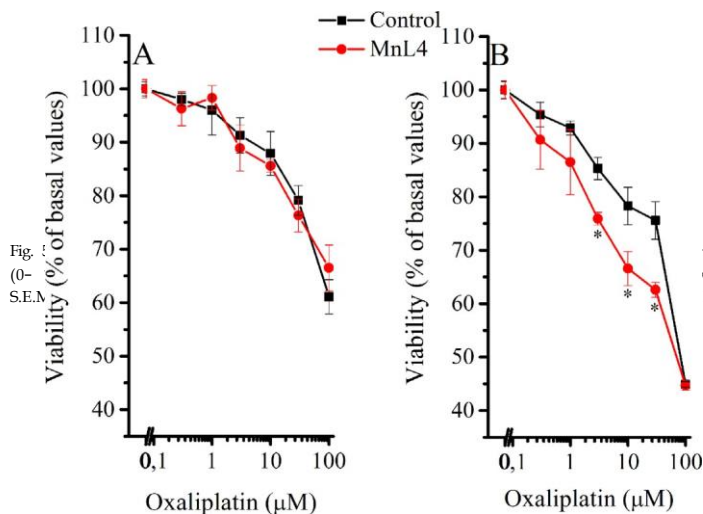


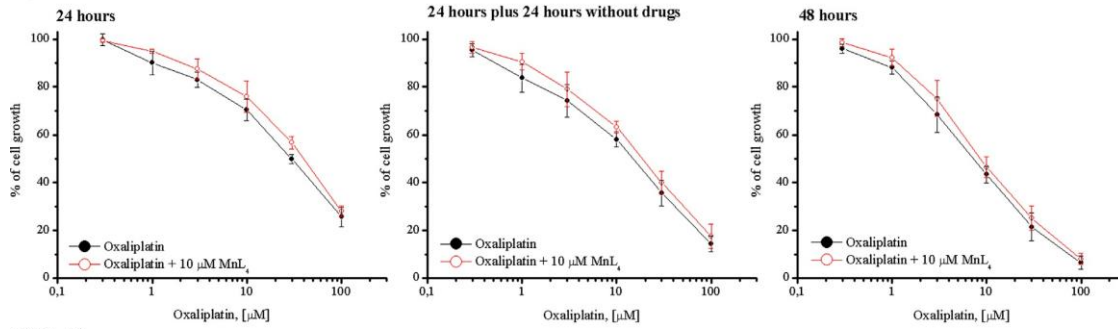
Fig. 4. Effect of acetylcholine (ACh) on intracellular cytosolic calcium concentration on astrocytes. Typical time-courses of  $[Ca^{2+}]_i$  recorded in control, 10 mM oxaliplatin (16 h pre-treatment) and 10 mM MnL4  $\pm$  10 mM oxaliplatin are shown. ACh (10 mM) was administered at time 0 and maintained throughout the experimental time. For simplicity, the  $[Ca^{2+}]_i$  dynamic obtained in cells preincubated with 10 mM MnL4 alone is omitted. Abscissa: time (s), Ordinate: fluorescence intensity reported as  $\Delta F/F$ . Inset shows the AUC (area under the curve, arbitrary units) calculated in the interval 0-300 s. \*\* $P < 0.01$  versus all other groups.



IT-29 cells were quantified by MTT assay after incubation with oxaliplatin. Values are expressed in percentage of control (0  $\mu$ M oxaliplatin) as mean  $\pm$  S.E.M. MnL4 (10 mM) alone did not influence cell viability.



### A) HT-29



### B) LoVo

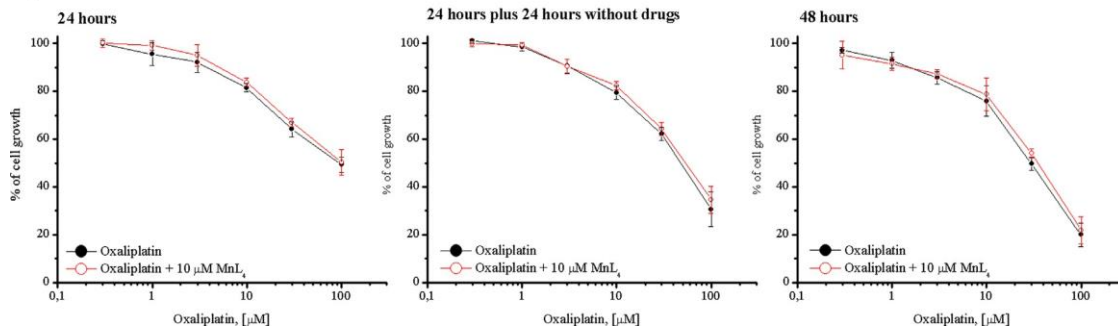


Fig. 6. Effect of 10 mM MnL4 on oxaliplatin toxicity in HT-29 and LoVo. Cell growth inhibition. The antiproliferative activity of oxaliplatin, alone or in combination with 10 mM MnL4, was evaluated in HT29 (A) and LoVo (B) human colorectal carcinoma cell lines. The results (evaluated according to the sulforhodamine B assay) after exposure times of 24 h, or 24 h followed by regrowth in drug free medium for additional 24 h, or 48 h are shown. Absorbance was measured at 540 nm and values were expressed in percentage of control (0 μM oxaliplatin) as mean ± S.E.M. of 3 experiments. Each reported IC<sub>50</sub> value represents the mean of three independent experiments.

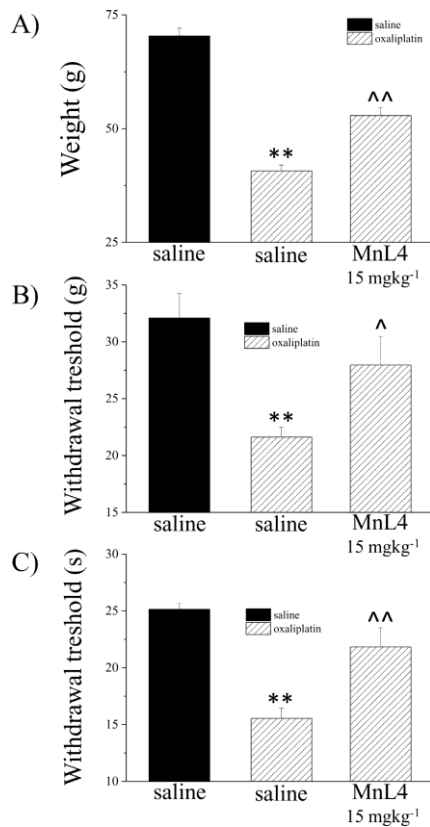


Fig. 7. Effect of MnL4 ( $15 \text{ mg kg}^{-1}$  daily) on mechanical hypersensitivity to a noxious stimulus (Paw-pressure test, A) and reduced pain threshold to non-noxious mechanical (Von Frey test, B) and thermal (Cold plate test, C) stimuli induced by oxaliplatin. Rats were daily intraperitoneally treated with  $2.4 \text{ mg kg}^{-1}$  oxaliplatin (dissolved in 5% glucose), five days/week. Tests were performed at day 21. MnL4 ( $15 \text{ mg kg}^{-1}$  daily, dissolved in saline) was administered by slow, continuous infusion with minipumps (Alzet<sup>®</sup> 2004) for 21 days starting from the first day of oxaliplatin administration. Control animals were treated with vehicles. Each value represents the mean of 6 rats per group. <sup>no</sup> $P < 0.01$  versus controls.  $P \circ < 0.05$  and  $P \blacktriangle < 0.01$  versus oxaliplatin.

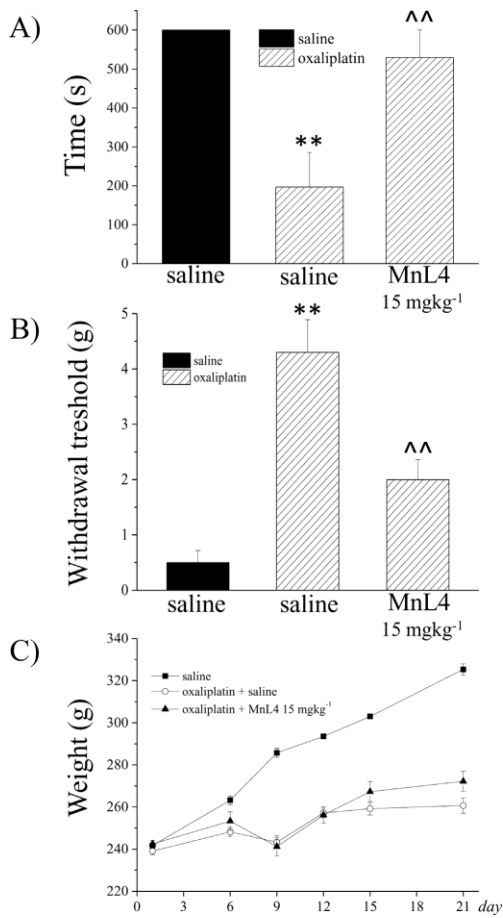


Table 1  
Effect of MnL4 on astrocytes lipoperoxidation to oxaliplatin  
Thiobarbituric reactive substances (mM/mg total proteins)

	Basal value	10 mM Oxaliplatin
Control	15.27 ± 1.2	41.4 ± 1.2 <sup>a</sup>
10 mM MnL4	13.1 ± 2.3	23.6 ± 3.3 <sup>b</sup>

Astrocytes were exposed to  $10 \mu\text{M}$  oxaliplatin for 16 h in the absence or presence of MnL4. TBARS (thiobarbituric acid reactive substances) assay was used to evaluate lipid peroxidation. Calibration curves were performed with malonyl aldehyde (MDA). Values are expressed as the mean  $\pm$  S.E.M. of 5 experiments. <sup>a</sup> $P < 0.01$  versus their respective control and  $P < 0.01$  versus oxaliplatin alone treatment