Toxicology in Vitro 27 (2013) 788–797

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Antioxidant properties of Neu2000 on mitochondrial free radicals and oxidative damage

Nishant P. Visavadiya [a,c], Melanie L. McEwen [a,b,c], Jignesh D. Pandya [b,c], Patrick G. Sullivan [b,c], Byoung Joo Gwag [d], Joe E. Springer [a,b,c,*]

[a] Physical Medicine and Rehabilitation, University of Kentucky, Lexington, KY 40536-0509, United States
[b] Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40536-0509, United States
[c] Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, KY 40536-0509, United States
[d] GNT Pharma, Suwon, Gyeonggido, South Korea

ARTICLE INFO

Article history:
Received 10 September 2012
Accepted 14 December 2012
Available online 23 December 2012

Keywords:
Antioxidant
Reactive oxygen species
Reactive nitrogen species
Lipid peroxidation
Mitochondria
Neu2000

ABSTRACT

Neu2000 [2-hydroxy-5-(2,3,5,6-tetrafluoro-4 trifluoromethylbenzylamino) benzoic acid] is a dual-acting neuroprotective agent that functions both as a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist and a free radical scavenger. In the present study, we investigated the scavenging activity of Neu2000 on various classes of reactive oxygen species and reactive nitrogen species (ROS/RNS) as well as its efficacy for reducing free radicals and oxidative stress/damage induced in spinal cord mitochondrial preparations. Neu2000 exerted scavenging activity against superoxide, nitric oxide, and hydroxyl radicals, and efficiently scavenged peroxynitrite. In the mitochondrial studies, Neu2000 markedly inhibited ROS/RNS and hydrogen peroxide levels following antimycin treatment. In addition, Neu2000 effectively scavenged hydroxyl radicals generated by iron(III)-ascorbate, reduced protein carbonyl formation mediated by hydroxyl radicals and peroxynitrite, and prevented glutathione oxidation caused by *tert*-butyl hydroperoxide in isolated mitochondria. Interestingly, incubation of isolated mitochondria with Neu2000 followed by centrifugation and removal of the supernatant also resulted in a concentration-dependent decrease in lipid peroxidation. This observation suggests that Neu2000 enters mitochondria to target free radicals or indirectly affects mitochondrial function in a manner that promotes antioxidant activity. The results of the present study demonstrate that Neu2000 possesses potent in vitro antioxidant activity due, most likely, to its active phenoxy group.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a major role in a variety of pathological events associated with mutation, carcinogenesis, inflammation, aging, neurodegeneration, and other diseases. Well known examples of ROS and RNS include superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^{\cdot}$), hydroxyl ($NO^{\cdot}$) peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\cdot}$), and alkoxyl ($RO^{\cdot}$). Under physiological conditions, the damaging effects of these free radicals are counterbalanced by the activities of the antioxidant defense pathways. In pathophysiological situations when free radicals are in excess or antioxidant defenses are compromised, ROS and RNS can react with fatty acids, proteins, and nucleic acids and alter their function (Kohen and Nyska, 2002; Stowe and Camara, 2009).

Several studies have demonstrated that oxidative damage and mitochondrial dysfunction are contributing factors to secondary injury following experimental spinal cord injury (SCI) (Hall and Springer, 2004; McEwen et al., 2007; Sullivan et al., 2007; Patel et al., 2009). Recent studies indicate that traumatic SCI results in impaired function of complexes I and II of the electron transport chain (ETC) contributing to increased free radical production and decreased ATP synthesis (McEwen et al., 2007, 2011; Sullivan et al., 2007; Patel et al., 2009). In this context, strategies that limit or counteract free radical-mediated oxidative damage and mitochondrial dysfunction may prove beneficial as therapeutic treatments for SCI.

Neu2000 [2-hydroxy-5-(2,3,5,6-tetrafluoro-4 trifluoromethyl benzylamino)-benzoic acid] is a derivative of acetylsalicylic acid and sulfasalazine, a conjugate of 5-aminosalicylic acid and sulfapyridine (Gwag et al., 2007). Neu2000 has been shown to be neuroprotective against N-methyl-D-aspartate (NMDA)- and iron-mediated neurotoxicity in cortical neuron cultures (Gwag et al., 2007; Cho et al., 2010). Neu2000 also limited the activation of proapoptotic proteins in a mouse model of amyotrophic lateral

* Corresponding author at: Spinal Cord and Brain Injury Research Center, B443 BBSRB, 741 S. Limestone, Lexington, KY 40536-0509, United States. Tel.: +1 859 323 1440; fax: +1 859 257 5737.
E-mail address: jspring@uky.edu (J.E. Springer).

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2012.12.011

sclerosis (Shin et al., 2007). Recently, we demonstrated that Neu2000 reduced mitochondrial ROS levels, promoted tissue sparing, and improved locomotor outcomes in an experimental model of SCI (Springer et al., 2010). Furthermore, Neu2000 attenuated the degeneration of hippocampal CA1 neurons in a rat model of transient forebrain ischemia (Park et al., 2011; Won et al., 2011). However, no studies to date have provided a detailed and comprehensive examination of the antioxidant activity of Neu2000, including identification of scavenging activity on specific free radical species. Further, given the central role of mitochondria in regulating cell function and survival, strategies that limit or counteract free radical-mediated mitochondrial dysfunction have potential clinical significance in the treatment of acute SCI as well as other free radical-mediated pathophysiological conditions. Therefore, the present study was undertaken to elucidate the efficacy of Neu2000 in limiting the actions of various free radical species and suppression of lipid as well as protein oxidation in isolated spinal cord mitochondria, using biochemical and biological in vitro model systems.

2. Materials and methods

2.1. Materials and reagents

Ferrozine [3-(2-Pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine], $FeCl_2$, phenazine methosulfate (PMS), β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), nitrotetrazolium blue chloride (NBT), sodium nitroprusside dehydrate (SNP), sulphanilamide, naphthyl ethelene diamine dihydrochloride, 2-deoxy-D-ribose, thiobarbituric acid (TBA), diethylenetriaminepentaacetic acid (DTPA), dihydrorhodamine-123 (DHR-123), 3-morpholinosydnonimine (SIN-1), mannitol, sucrose, bovine serum albumin (BSA), ethylene glycol tetraacetate (EGTA), ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), potassium phosphate monobasic anhydrous (KH_2PO_4), magnesium chloride ($MgCl_2$), malate, pyruvate, Ficoll, 2,7-dichlorodihydrofluorescin diacetate ($DCFH_2$-DA), Type VI-A horseradish peroxidase (HRP), *tert*-butyl hydroperoxide (*t*BHP) and antimycin A from *Streptomyces* sp. were purchased from Sigma–Aldrich (St. Louis, MO). Amplex Red and monochlorobimane (MCB) were purchased from Molecular Probes (Eugene, OR). BCA protein assay kit was purchased from Pierce (Rockford, IL). All solutions and reagents are listed as final concentrations.

2.2. Anti-oxidant compounds

In the biochemical free radical assays, Neu2000, ascorbic acid, mannitol, and Trolox were used at a concentration range of 0–300 μM. In the biological studies examining spinal cord mitochondria and spinal cord homogenates, Neu2000 was used at a concentration range of 0–125 μM.

2.3. Biochemical model system measurements

2.3.1. Superoxide anion scavenging activity

Superoxide radicals were generated using a PMS-NADH assay, according to previously published methods (Ewing and Janero, 1995) with minor modifications. Neu2000 and ascorbic acid were dissolved in phosphate-buffered saline (PBS), pH = 7.4. The reaction mixture was composed of freshly prepared 20 μM NBT and 98 μM NADH, which was added to the wells of a 96-well plate (Corning, NY). Neu2000, ascorbic acid, or vehicle alone (control) were then added to the wells, and the reaction was initiated by adding 33 μM PMS. After a 10-min incubation at 25 °C, absorbance was measured at 560 nm.

2.3.2. Nitric oxide scavenging activity

Nitric oxide free radical was generated by the SNP reaction, using previously published methods (Ullrich et al., 1997). The reaction mixture was composed of SNP (10 mM) and either Trolox or Neu2000 in PBS, or PBS alone as control. After 2 h at 25 °C under light irradiation, the reaction mixture was reacted with Griess reagent consisting of 1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthyl ethelene diamine dihydrochloride. The absorbance of the chromophore produced after diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthylethelene diamine was measured at 540 nm.

2.3.3. Hydroxyl radical scavenging activity

Hydroxyl radicals were generated using the Fenton reaction to form products that, when coupled with TBA, yield a visible pink chromogen (Halliwell et al., 1987). The final assay reaction was composed of 2.8 mM 2-deoxy-2-ribose, 100 μM $FeCl_3$, 100 μM EDTA, and 1 mM H_2O_2, in the absence (control) or presence of a standard antioxidant (mannitol) or Neu2000 in PBS, or PBS alone as a control. The reaction was initiated by adding 100 μM ascorbate to each of the wells. After 1 h at 37 °C, the samples were reacted with 0.67% TBA followed by heating to 95 °C for 20 min. After cooling to room temperature, the malondialdehyde (MDA)-TBA complex adduct was measured at 532 nm.

2.3.4. Peroxynitrite scavenging activity

Authentic peroxynitrite stock solution was synthesized from sodium nitrite (0.6 M) and acidified hydrogen peroxide (0.6 M H_2O_2 in 0.7 M HCl) and then quenched with NaOH (1.2 M), as previously described (Beckman et al., 1994). The stock solution was kept frozen (−80 °C) until the day of the experiment. The concentration of the stock solution was evaluated immediately before use by measuring the absorbance at 302 nm using the molar extinction coefficient of $\varepsilon = 1670\ M^{-1}\ cm^{1}$. The assay solution contained 100 μM DTPA; increasing concentrations of Neu2000, ascorbic acid, or Trolox; and 5 μM DHR-123 in PBS. PBS alone served as control. Peroxynitrite scavenging activity was determined by the oxidation of DHR-123, as described previously (Kooy et al., 1994), and was initiated by adding either authentic peroxynitrite or SIN-1 to achieve final concentrations of 10 μM or 80 μM, respectively, in the presence or absence of 25 mM $NaHCO_3$. This set of experiments was carried out in the absence or presence of bicarbonate anion, which was included in order to simulate physiological concentrations of carbon dioxide (Whiteman et al., 2002). After 5 min at 37 °C, rhodamine-123 formation (fluorescence) was measured at wavelengths of 485 nm (excitation) and 535 nm (emission) with a Biotek Synergy HT plate reader (Winooski, VT).

2.3.5. Ferrous ion-chelating activity

The ferrous ion-chelating potential of Neu2000 was determined according to a previously published method (Dinis et al., 1994), with minor modifications. Graded concentrations of Neu2000 were mixed with 2 mM $FeCl_2$ in a 96-well microplate, and ion chelation was initiated by adding 5 mM ferrozine to each well. After a 10-min incubation at room temperature, absorbance was measured at 562 nm.

2.4. Biological model system measurements

2.4.1. Mitochondrial preparations and total spinal cord homogenates

A total of 25 adult female Long-Evans rats (Harlan Laboratories, Inc., Indianapolis, IN), weighing 225–250 g, were used for these studies. The rats were allowed *ad libitum* access to water and food. All animal housing conditions and protocols for spinal cord removal and mitochondrial harvesting were conducted according to the University of Kentucky Institutional Animal Care and Use

Committee and the NIH animal care guidelines. Spinal cord mitochondria were isolated from healthy rats according to previously published methods (Patel et al., 2009). Briefly, the entire spinal cord was homogenized in 10 ml of isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA; pH adjusted to 7.2 with KOH) and then centrifuged twice at 1300*g* for 3 min at 4 °C. The supernatant was then centrifuged at 13,000*g* at 4 °C for 10 min to obtain a crude mitochondrial fraction. The crude mitochondrial fraction was subsequently placed into a nitrogen cell disruption chamber (1200 psi, 10 min) to release synaptosomal mitochondria, which were further purified using a discontinuous Ficoll gradient (7.5%, 10%) and centrifugation at 100,000*g* at 4 °C for 30 min. The resulting mitochondrial pellet was washed in isolation buffer without EGTA and centrifuged for 10 min at 10,000*g* at 4 °C. The final mitochondrial pellet was resuspended in 50 μl EGTA-free isolation buffer and the protein concentration measured using the BCA protein assay kit. In one set of experiments, total spinal cord homogenates were prepared using a Potter–Elvehjem homogenizer containing ice-cold 0.1 M PBS, pH = 7.4 (100 mg tissue/ml).

2.4.2. Total ROS/RNS measurement

Formation of mitochondrial ROS/RNS induced by antimycin A was measured using the $DCFH_2$-DA fluorescent probe. The $DCFH_2$ dye is converted to highly fluorescent DCF by interaction with several free radical species including hydroxyl, superoxide, hydroxyl radical, peroxynitrite, carbonate, and nitrogen dioxide radicals (Crow, 1997; Chen et al., 2010; Wrona et al., 2005). Neu2000 was dissolved in 50 mM potassium phosphate buffer (KPB, pH = 7.2). Rat spinal cord mitochondria (0.2 mg/ml) were incubated in KCl-based respiration buffer (125 mM KCl, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 20 mM HEPES, and 0.1% BSA, pH = 7.2) containing the oxidative substrates pyruvate (5 mM) and malate (2.5 mM) (P/M) and various concentrations of Neu2000. The superoxide-producing reaction was initiated by adding antimycin A (10 μM). Subsequently, 10 μM $DCFH_2$-DA and 5 U/ml horseradish peroxidase (HRP) were added, and the changes in $DCFH_2$-DA fluorescence were monitored every minute for 30 min at 37 °C with the Biotek Synergy plate reader, using 485-nm excitation and 520-nm emission filters. Fluorescence of the buffer without mitochondria (background) was subtracted from the results.

2.4.3. Hydrogen peroxide measurement

Production of H_2O_2 by spinal cord mitochondria was assayed using the Amplex Red fluorescent probe, which reacts in a 1:1 stoichiometry with H_2O_2 in the presence of peroxidase to produce resorufin, a red fluorescent oxidation product (Castello et al., 2008; Murphy, 2009). Mitochondria (0.1 mg/ml) were incubated in standard KCl-based respiration buffer containing P/M as oxidative substrates. Subsequently, antimycin A (10 μM) was added in the presence or absence of Neu2000, followed by the addition of 1 μM Amplex Red and 0.25 U/ml HRP at 30 °C. Fluorescence of the oxidized probe was measured every minute over a 10 min period using 530-nm excitation and 590-nm emission filters. Standard curves were obtained by adding known amounts of H_2O_2 to the standard assay in the presence of the reactants (Amplex Red and HRP). Background values (without mitochondria) were subtracted from the results, and the slope for each experiment was calculated.

2.4.4. Lipid peroxidation measurement

Lipid peroxidation was measured using a previously published method with some modifications (Buege and Aust, 1978). Mitochondria (1 mg/ml) were incubated in KCl-based respiration buffer containing P/M and a hydroxyl radical-generating system (50 μM $FeCl_3$ and 100 μM ascorbate), in the presence or absence of Neu2000. The reaction mixture was incubated at 37 °C for 1 h and was stopped by adding 10% trichloroacetic acid. Subsequently, 0.67% TBA was added, and tubes were placed in boiling water for 20 min and then centrifuged at 3000*g* for 10 min. The malondialdehyde (MDA)-TBA complex that formed in the supernatant was measured at 532 nm using the molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Buege and Aust, 1978). In a second set of experiments, the lipid peroxidation reaction was also performed with total spinal cord homogenates, which have higher levels of lipids relative to isolated mitochondria, to evaluate the hydroxyl radical scavenging potency of Neu2000 treatment. Briefly, the reaction mixture contained 50 μl total spinal cord homogenate (100 mg/ml), 50 μl of PBS, Trolox (0–125 μM) or Neu2000 (0–125 μM), and 50 μl of 50 μM $FeCl_3$ and 100 μM ascorbic acid. The reaction mixture was incubated at 37 °C for 1 h and the extent of MDA-TBA formation was measured as described above. In a third set of experiments, isolated mitochondria (1 mg/ml) were first incubated with varying concentrations of Neu2000 (0–25 μM) or Trolox (0–25 μM) for 15 min in 50 μl respiration buffer. The samples were then centrifuged for 8 min at 8000*g* to remove the anti-oxidant mixture. Mitochondrial pellets were resuspended in 50 μl respiration buffer, exposed to the hydroxyl radical-generating reaction (50 μM $FeCl_3$ and 100 μM ascorbate), and the lipid peroxidation assay was performed as described above.

2.4.5. Reduced glutathione (GSH) measurement

Reduced GSH content was measured in isolated mitochondria using the fluorescent probe MCB method described previously (Fernandez-Checa and Kaplowitz, 1990). Mitochondria (2 mg/ml) were incubated for 5 min in 50 mM KPB (pH = 7.2) containing various concentrations of Neu2000 followed by oxidation of GSH by addition of *t*BHP (125 μM) and incubation at 37 °C for 45 min. The mitochondrial samples were then resuspended in 50 mM KPB (pH = 7.2) containing 100 μM MCB and incubated for 30 min at 37 °C in the dark. The samples were then centrifuged at 10,000*g* for 10 min and MCB fluorescence in the supernatant of each sample measured (excitation: 380 nm, emission: 470 nm) using a fluorescence microplate reader. Background fluorescence (buffer without mitochondria) was subtracted from each reading.

2.5. Western blot for protein carbonyls

Energized (P/M treated) mitochondria (1 mg/ml) were pretreated for 5 min with different concentrations of Neu2000 in BSA-depleted respiration media. The hydroxyl radical-generating solution [50 μM $FeCl_3$, 1 mM H_2O_2, and 100 μM ascorbate] or authentic peroxynitrite (400 μM) was subsequently added to mitochondria, and the mixture was incubated for 1 h at 37 °C. The formation of protein carbonyls was then assessed with an OxyBlot protein oxidation detection kit (Millipore, Billerica, MA). Briefly, mitochondrial samples (1 mg/ml) were mixed with 12% SDS and incubated with 2,4-dinitrophenylhydrazine (DNPH) to derivatize the carbonyl groups in the protein side chains to 2,4-dinitrophenylhydrazone (DNP-hydrazone) according to the manufacturer's instructions. After 20 min, the reaction was stopped by adding neutralization solution (2 M Tris in 30% glycerol) and the proteins separated by molecular weight by SDS–PAGE using Criterion 4–20% Tris–HCl gels (Bio-Rad, Hercules, CA). The gels were transblotted onto PVDF membranes and DNP-containing proteins were immunostained using rabbit anti-DNP antibody (1:800) followed by goat anti-rabbit IgG conjugated to HRP (1:1600). The blots were incubated in Enhanced Chemiluminescence-Plus™ solution (GE Healthcare, Piscataway, NJ) and exposed to autoradiography film (Denville Scientific, Metuchen, NJ).

2.6. Statistics

In all assays, a total of three independent and separate experiments were performed to study the different anti-oxidant compounds and the results expressed as the mean ± SEM. Free radical scavenging potency was calculated using the following equation: inhibition (%) = $[(OD_{control} - OD_{sample})/OD_{control}) \times 100]$. The IC_{50} was expressed as the concentration that produced 50% inhibition of free radical levels as calculated from the concentration–response curves using logarithmic regression analysis. The effects of Neu2000 in the mitochondrial studies were evaluated using ANOVAs and Student–Newman–Keuls (S–N–K) post hoc analyses. $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Potency of Neu2000 on biochemical model systems

The first set of experiments was conducted to test the ability of Neu2000 to scavenge various ROS/RNS in a biochemical model system. Our results demonstrate that Neu2000 inhibited the formation of the NBT formazan product, a measure of superoxide radical (Ewing and Janero, 1995), in a concentration-dependent manner (Fig. 1). Neu2000 (IC_{50} = 63.07 ± 1.44 μM) was twice as potent as ascorbic acid in scavenging superoxide radicals (IC_{50} = 149.87 ± 7.88 μM; data not shown). Neu2000 also scavenged SNP-generated nitric oxide radicals, as measured using the Greiss reagent (Tarpey et al., 2004), in a concentration-dependent manner (Fig. 1). The IC_{50} value for Neu2000 in this assay was calculated as 155.8 ± 4.88 μM compared to an IC_{50} value of 307.65 ± 5.72 μM for Trolox (data not shown).

In the deoxyribose degradation assay, Neu2000 sequestered hydroxyl radicals in a concentration-dependent manner (Fig. 1). The calculated IC_{50} value of 58.45 ± 1.74 μM revealed that Neu2000 was approximately five times more potent than mannitol (IC_{50} = 312.61 ± 14.96 μM; data not shown), a well-known hydroxyl radical scavenger (Goldstein and Czapski, 1984).

The peroxynitrite-scavenging potency of Neu2000 was evaluated using authentic peroxynitrite and SIN-1, a peroxynitrite donor. Both compounds lead to the oxidation of DHR-123 to form fluorescent rhodamine-123. The results revealed that Neu2000 was effective in scavenging authentic peroxynitrite (Fig. 2A) as well as SIN-1-derived peroxynitrite (Fig. 2B) in a concentration-dependent manner. However, the concentration–response curve was different between these two conditions and the potency was influenced by the addition of bicarbonate anion. Specifically, Neu2000 more potently scavenged authentic peroxynitrite and SIN-1-derived peroxynitrite when bicarbonate was absent (IC_{50} = 1.59 ± 0.05 and 0.47 ± 0.04 μM, respectively) compared to the presence of bicarbonate and, therefore, physiological concentrations of carbon dioxide (IC_{50} = 4.43 ± 0.39 and 0.94 ± 0.06 μM, respectively). The effectiveness of Neu2000 as a peroxynitrite scavenger was similar to ascorbic acid and Trolox (see Table 1).

A separate set of experiments demonstrated that Neu2000 does not possess any remarkable metal-chelating or pro-oxidant properties, as assessed when the deoxyribose assay was conducted in the absence of EDTA or ascorbic acid, respectively (data not shown). To further confirm the absence of any significant metal-chelating activity, we measured Fe^{2+}-ferrozine complex formation in the presence or absence of Neu2000 or EDTA. Neu2000 showed very weak metal-chelating activity with an IC_{50} value of 14.95 ± 1.11 mM compared to EDTA, which had an IC_{50} value of 194 ± 6 μM (data not shown).

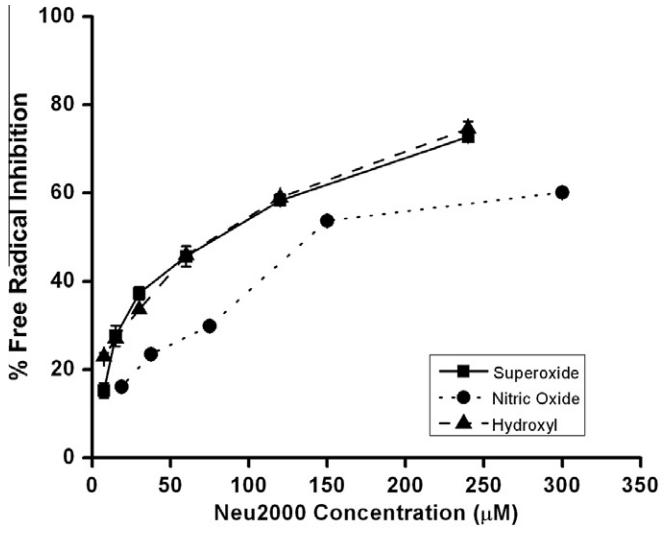

Fig. 1. Concentration-dependent scavenging activity of Neu2000 for superoxide radicals, nitric oxide, and hydroxyl radicals. The results of all three assays are expressed as mean percent (%) inhibition ± SEM (n = 3 independent experiments). The results of these experiments revealed that Neu2000 effectively scavenged superoxide radicals (IC_{50} = 63.07 ± 1.44 μM), nitric oxide (IC_{50} = 155.8 ± 4.88 μM), and hydroxyl radicals (IC_{50} = 58.45 ± 1.74 μM).

3.2. Antioxidant potency of Neu2000 in isolated spinal cord mitochondria

Following the biochemical experiments, we examined the potential antioxidant actions of Neu2000 on various ROS/RNS generated in healthy mitochondria isolated from rat spinal cord. In the first study, antimycin A, an ETC. complex III inhibitor, significantly increased mitochondrial ROS/RNS production over a 30-min incubation period (Fig. 3A). In contrast, Neu2000 decreased, in a dose-dependent manner, the amount of antimycin A-induced ROS/RNS formation with an IC_{50} = 2.21 ± 0.11 μM (Fig. 3A, B).

In addition, we examined the effect of Neu2000 on antimycin A-induced H_2O_2 production in isolated mitochondria. Mitochondrial baseline H_2O_2 levels (control) were significantly increased ($p < 0.05$) from 580.0 ± 28.0 to 1276.0 ± 31.0 pM/min/mg in the presence of antimycin A (Fig. 4A). Neu2000, in a dose-dependent fashion, decreased ($p < 0.05$) the levels of antimycin A-induced H_2O_2 production (Fig. 4A, B). Analysis of the concentration–response curve revealed that Neu2000 inhibited H_2O_2 production with an IC_{50} = 6.04 ± 0.55 μM.

Lipid peroxidation of mitochondrial membranes was induced by the application of $FeCl_3$ and ascorbate and then quantified by measuring MDA formation. As shown in Fig. 5A, treatment with iron and ascorbate produced a fourfold increase (1.62 ± 0.008 nM/mg) in MDA formation ($p < 0.05$) compared to basal levels (0.39 ± 0.019 nM/mg). When mitochondria were pre-incubated with Neu2000, MDA levels significantly ($p < 0.05$) decreased in a concentration-dependent manner (Fig. 5A, B). The calculated IC_{50} for Neu2000 to inhibit MDA formation was 2.72 ± 0.26 μM. We also examined the efficacy of Neu2000 for reducing lipid peroxidation in crude spinal cord homogenates, which contain very high levels of lipids. Fig. 6 shows that Neu2000 effectively reduced iron-ascorbate-induced lipid peroxidation (IC_{50} = 24.56 ± 0.07 μM) and was twice as potent as Trolox (IC_{50} = 57.21 ± 1.13 μM; data not shown), a well-known inhibitor of lipid peroxidation. Additional samples of isolated mitochondria were briefly (15 min) exposed to Neu2000 or Trolox and then washed to remove any excess anti-oxidant prior to application of the Fenton reaction mixture, also showed a concentration-dependent decrease in MDA formation (Fig. 7). Neu2000 significantly reduced MDA formation at concentrations of 3.12 μM and above ($p < 0.05$), whereas

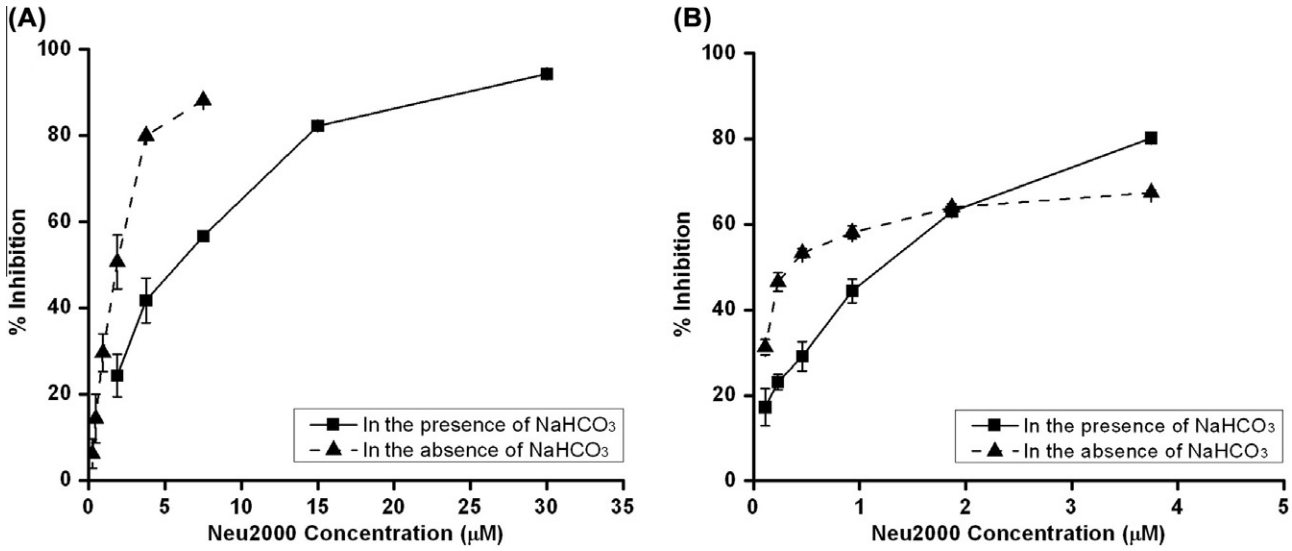

Fig. 2. Neu2000 exhibits potent peroxynitrite scavenging activity. Results are mean percent (%) inhibition ± SEM ($n = 3$ independent experiments). The data show that Neu2000 efficiently scavenged authentic peroxynitrite (A) and SIN-1-derived peroxynitrite (B), both in the presence (IC_{50} = 4.43 ± 0.39 and 0.94 ± 0.06 μM, respectively) and absence (IC_{50} = 1.59 ± 0.05 and 0.47 ± 0.04 μM, respectively) of bicarbonate.

Table 1
The IC_{50} values of Neu2000, ascorbic acid and Trolox for peroxynitrite.

Antioxidants	Authentic peroxynitrite		SIN-1-derived peroxynitrite	
	Absence of $NaHCO_3$	Presence of $NaHCO_3$	Absence of $NaHCO_3$	Presence of $NaHCO_3$
Neu2000 (μM)	1.59 ± 0.05	4.43 ± 0.39	0.47 ± 0.04	0.94 ± 0.06
Ascorbic Acid (μM)	1.97 ± 0.05	6.58 ± 0.51	0.56 ± 0.02	1.06 ± 0.09
Trolox (μM)	1.76 ± 0.07	4.64 ± 0.49	0.62 ± 0.009	0.70 ± 0.02

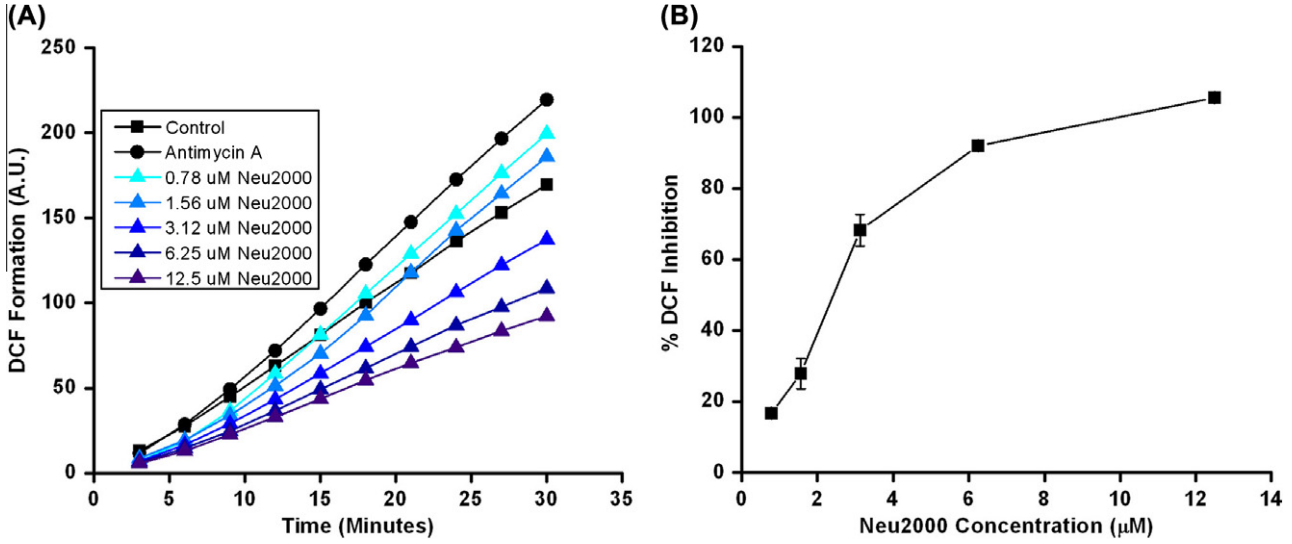

Fig. 3. Effect of Neu2000 on mitochondrial levels of ROS/RNS. (A) Results represent the percent (%) inhibition of DCF formation (means ± SEM, $n = 3$ independent experiments), showing that Neu2000 dose-dependently decreased antimycin A-induced ROS/RNS formation. (B) The IC_{50} value for Neu2000, calculated from the concentration–response curves, was 2.21 ± 0.11 μM.

Trolox was ineffective at all concentrations examined. The calculated IC_{50} for Neu2000 to inhibit MDA formation under these incubation conditions was 14.89 ± 0.23 μM.

We also evaluated Neu2000 antioxidant potential by measuring mitochondrial GSH oxidation. Exposure of intact mitochondria to 125 μM *t*BHP at 37 °C for 45 min resulted in a 44% depletion of mitochondrial GSH content compared to the control (untreated) mitochondria. However, pre-incubation of mitochondria with Neu2000 (0–50 μM) significantly prevented ($p < 0.05$) mitochondrial GSH oxidation compared to *t*BHP alone in a concentration-dependent manner (Fig. 8). Specifically, GSH oxidation was significantly reduced at concentrations of Neu2000 at and above

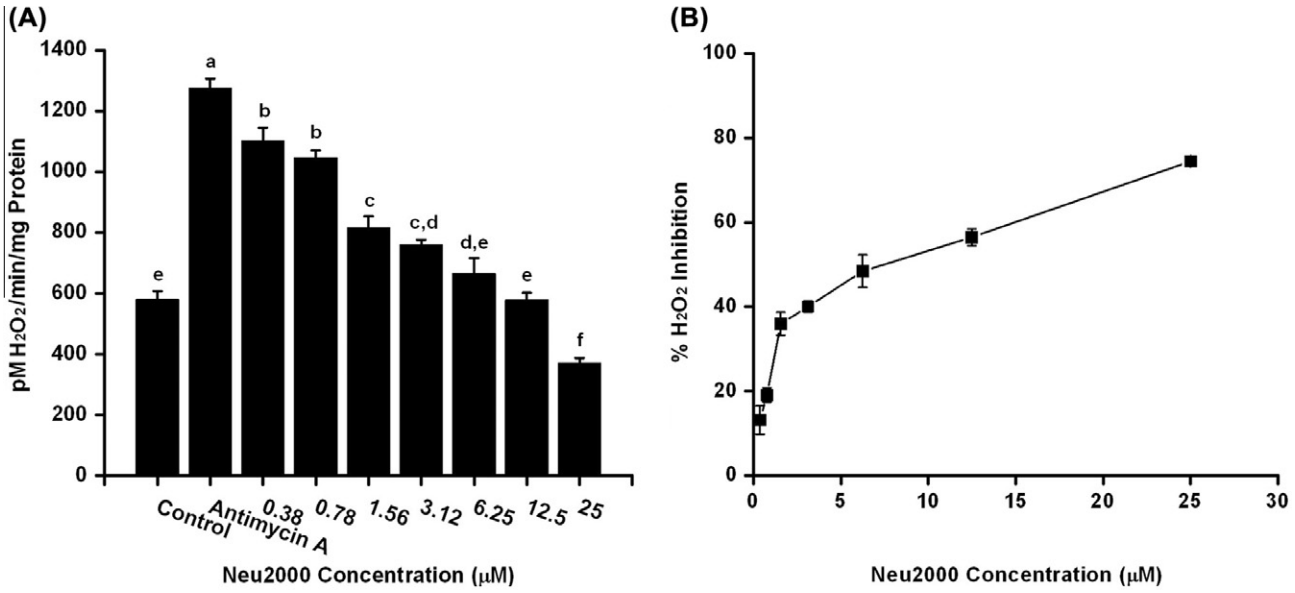

Fig. 4. Effect of Neu2000 on mitochondrial H_2O_2 production. (A) The H_2O_2 production rates are presented as pM H_2O_2/min/mg protein (means ± SEM, $n = 3$ independent experiments). Values with different superscripts (a–f) are significantly different from each other ($p < 0.05$). H_2O_2 levels decreased, compared to antimycin A alone treatment, when mitochondria were also treated with increasing concentrations of Neu2000. The levels of H_2O_2 were similar to control levels (mitochondria alone) after application of 6.25 μM and 12.5 μM Neu2000 and were below control levels after application of the highest concentration of Neu2000. (B) Data are expressed as the percent (%) inhibition of H_2O_2 net production (means ± SEM, $n = 3$ independent experiments). The IC_{50} value calculated from the concentration–response curves was 6.04 ± 0.55 μM.

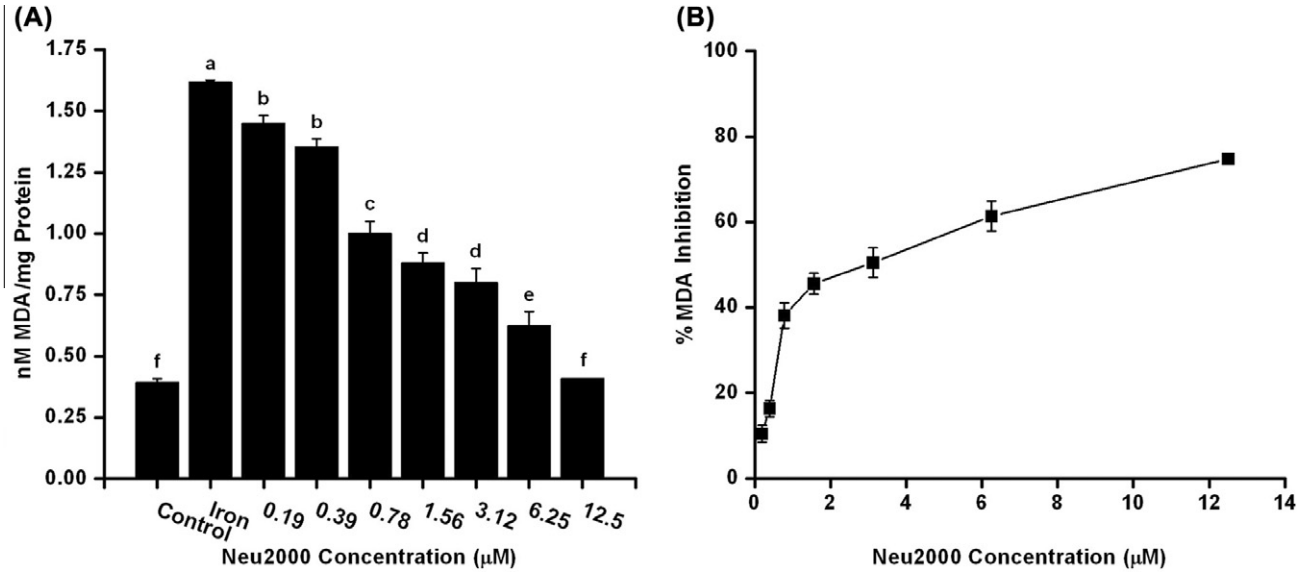

Fig. 5. Effect of Neu2000 on mitochondrial lipid peroxidation. (A) Iron plus ascorbate treatment increased malondialdehyde (MDA) formation over control condition (mitochondria alone) (means ± SEM, $n = 3$ independent experiments). Values with different superscripts (a–f) are significantly different from each other ($p < 0.05$). MDA formation decreased, compared to iron-ascorbate treatment alone, when mitochondria were also treated with increasing concentrations of Neu2000. The levels of MDA formation were similar to control levels (mitochondria alone) after application of the highest concentration of Neu2000 tested (12.5 μM). (B) Results represent the percent (%) inhibition of MDA formation (means ± SEM, $n = 3$ independent experiments). The Neu2000 IC_{50} value was calculated to be 2.72 ± 0.26 μM.

12.5 μM when compared to *t*BHP treatment alone. In addition, 50 μM Neu2000 was no different than control (untreated) mitochondria.

Protein carbonyl formation is a marker of oxidative damage to proteins, and isolated mitochondria were examined for the presence of protein carbonyls after various treatments using the Oxy-Blot system. Protein carbonyl formation was increased several fold in mitochondria incubated with the Fenton reaction or 400 μM authentic peroxynitrite, which was reduced by Neu2000 treatment in a concentration-dependent manner (Fig. 9).

4. Discussion

Neu2000 functions as an NR2B-selective NMDA receptor antagonist with potent cell permeability and spin trapping antioxidant activity. Given its small molecular weight, safety, stability, and amphiphilic properties, Neu2000 has potential as a pharmacological agent for the treatment of nervous system disorders, including stroke, traumatic brain injury (TBI), and SCI. We recently reported that Neu2000 reduced measures of secondary injury following SCI, including a decrease in free radical production by mitochondria

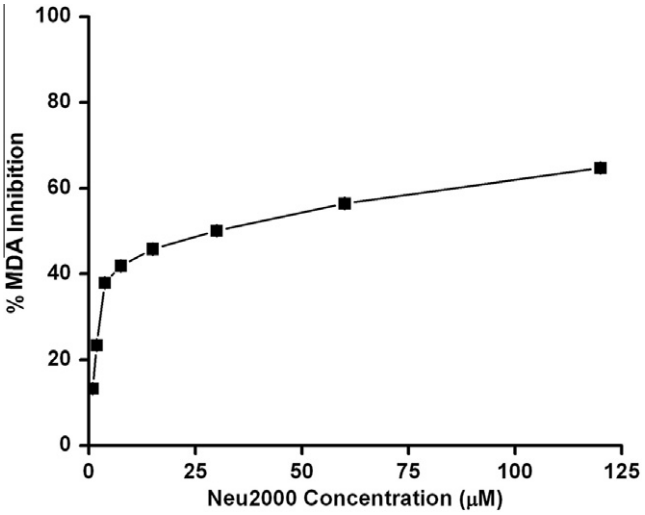

Fig. 6. Effect of Neu2000 on lipid peroxidation in spinal cord homogenates. Results represent the percent (%) inhibition of malondialdehyde (MDA) formation (means ± SEM, $n = 3$ independent experiments). The concentration–response curves revealed that Neu2000 effectively reduced $FeCl_3$-ascorbate-induced lipid peroxidation with an IC_{50} of 24.56 ± 0.07 μM.

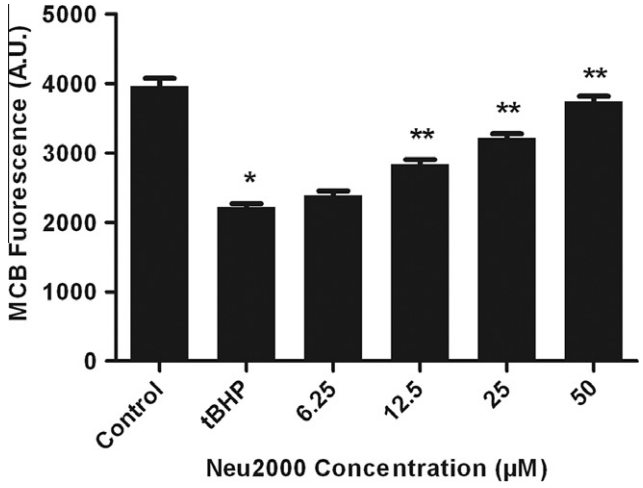

Fig. 8. Effect of Neu2000 on *t*BHP-induced GSH oxidation in isolated mitochondria. Results demonstrate that Neu2000 decreased the levels of oxidized GSH in a concentration-dependent manner (means ± SEM, $n = 3$ independent experiments). $^{*}p < 0.05$ compared to control, $^{**}p < 0.05$ compared to *t*BHP alone.

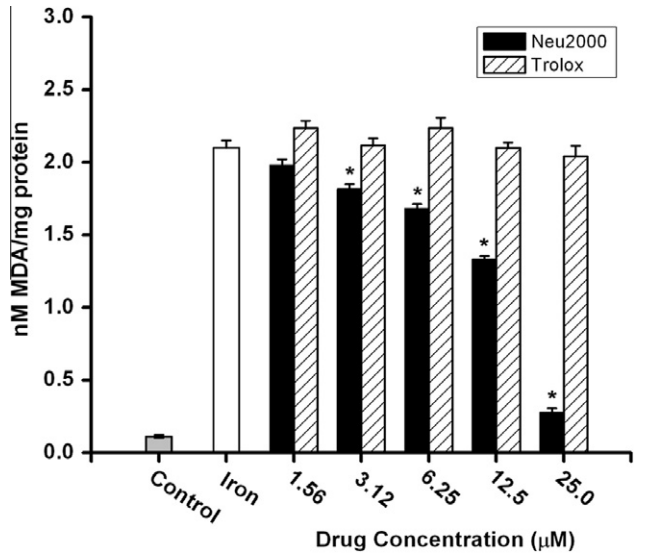

Fig. 7. Effect of short term pre-exposure of mitochondria to Neu2000 or Trolox on lipid peroxidation. Mitochondria were incubated in buffer containing various concentrations of Neu2000 or Trolox, which was then removed prior to application of the Fenton reaction mixture. Results demonstrate that Neu2000 decreased malondialdehyde (MDA) formation in a concentration-dependent fashion. In contrast, there was no effect of Trolox at any concentration tested. Asterisks (*) represent treatment groups that are significantly different from iron-ascorbate treatment alone ($p < 0.05$).

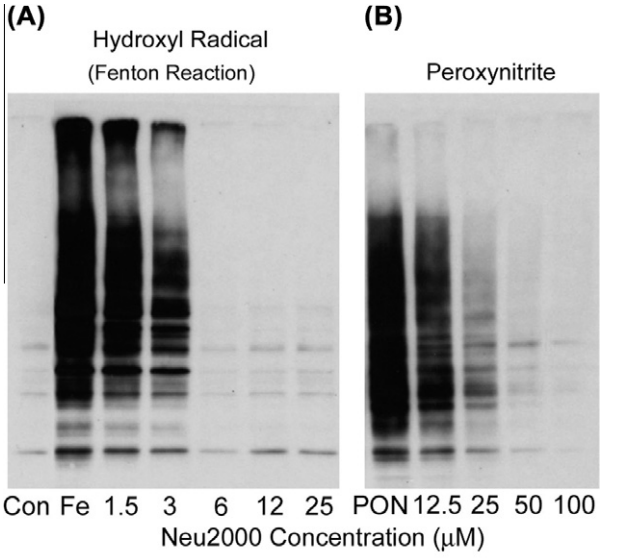

Fig. 9. Effect of Neu2000 on mitochondrial protein carbonyl formation. Neu2000 concentration-dependently reduced protein carbonyl formation in mitochondria treated with the Fenton reaction or 400 μM authentic peroxynitrite compared to iron-ascorbate (Fe) or peroxynitrite (PON) alone. The control (CON) condition refers to mitochondria that were not treated with Fe or PON. The blots are representative of the results obtained from three independent experiments.

isolated from the injured spinal cord (Springer et al., 2010). However, the specific free radical species targeted by Neu2000 treatment were not examined in this or any previous study. Therefore, the present study was designed to more thoroughly investigate the antioxidant actions of Neu2000 in several ROS/RNS pathways using two types of in vitro free radical-generating systems: a biochemical model and a mitochondrial model.

The results of the biochemical assays demonstrate that Neu2000 was a more efficient scavenger of superoxide and nitric oxide radicals relative to several reference antioxidant compounds including ascorbic acid and Trolox. The activation of various inflammatory cell types following CNS injury can generate superoxide radicals, with NADPH oxidase playing an important role (McEwen et al., 2011; Rabchevsky et al., 2011). In addition, nitric oxide can react with superoxide radicals to produce the highly toxic peroxynitrite anion. Nitric oxide is also a mediator of inflammatory processes, such as augmentation of vascular permeability of inflamed tissues, induction of cyclooxygenase, angiogenic, and inflammatory cytokines, activation of matrix metalloprotease, and induction of chondrocyte apoptosis (Miyasaka and Hirata, 1997; Droge, 2002; Kohen and Nyska, 2002). Thus, the scavenging activity of Neu2000 for both superoxide radicals and nitric oxide suggests that it may provide efficacy as an anti-inflammatory agent following CNS injury.

In the Fenton reaction, Neu2000 demonstrated excellent scavenging potency for hydroxyl radicals compared to mannitol, suggesting that Neu2000 may reduce oxidative stress by reducing hydroxyl radical-mediated damage to biomolecules such as nucleic acids, lipids, and proteins. This study also demonstrated that submicromolar concentrations of Neu2000 are capable of scavenging authentic peroxynitrite or SIN-1-derived peroxynitrite. The potency of Neu2000 (in terms of IC_{50} values) was only slightly diminished in the presence of 25 mM bicarbonate. The presence of bicarbonate anions can lead to the generation of peroxynitrite-carbon dioxide intermediate radicals, such as highly the reactive carbonate radical ($CO_3^{\cdot-}$), nitrogen dioxide ($^{\cdot}NO_2$), and hydroxyl radical, all of which have been attributed to lipid oxidation (Radi et al., 2002; Whiteman et al., 2002). Thus, based on the biochemical assays used in the present study, Neu2000 is a potent scavenger of both ROS and RNS.

The results of the biochemical experiments led us to examine the efficacy of Neu2000 as a neuroprotective agent against mitochondria-generated free radicals. Under normal physiological conditions, single electrons leak into the mitochondrial matrix during their transfer along the mitochondrial ETC., resulting in the reduction of molecular oxygen and formation of superoxide anions. Superoxide radicals interact with superoxide dismutase to form H_2O_2, which is membrane permeable and therefore able to leave the mitochondrial matrix and is considered the main precursor for the formation of hydroxyl radicals via the Fenton reaction. Hydroxyl radicals, along with peroxynitrite, can cause oxidative damage to mitochondrial DNA, proteins, and lipids (Turrens, 2003; Murphy, 2009; Stowe and Camara, 2009). Under pathophysiological conditions, such as those observed at acute times following SCI, mitochondrial free radical production is significantly elevated (McEwen et al., 2007; Sullivan et al., 2007; Patel et al., 2009; Springer et al., 2010; McEwen et al., 2011; Rabchevsky et al., 2011). This deleterious condition can be mimicked in vitro by treating healthy isolated mitochondria with inhibitors that target selective sites in the mitochondrial ETC. system, including antimycin A, a potent ETC. complex III inhibitor (Degli Esposti, 2002; Turrens, 2003; Murphy, 2009).

In the present study, Neu2000 decreased both the levels of total ROS/RNS and H_2O_2 induced by antimycin A. Concentrations as low as 1.56 μM Neu2000 normalized the level of mitochondrial ROS/RNS to control values observed in untreated mitochondria, while concentrations as low as 6.25 μM Neu2000 normalized the levels of mitochondrial H_2O_2. These results are in agreement with those of our biochemical assays demonstrating that Neu2000 effectively scavenged various types of ROS/RNS, including superoxide, nitric oxide, and hydroxyl radicals, as well as peroxynitrite.

Iron-ascorbate-catalyzed reactions generate highly reactive hydroxyl radicals, which mediate the degradation of unsaturated fatty acyl side chains leading to alterations of membrane fluidity and lipid composition (Kohen and Nyska, 2002; Repetto et al., 2010). In the present study, mitochondrial samples treated with iron-ascorbate had nearly a fourfold increase in MDA formation compared to control mitochondria. However, MDA levels were significantly reduced when mitochondria were pre-treated with low micromolar concentrations of Neu2000. Neu2000 also reduced MDA formation in total spinal cord homogenates exposed to the same iron-ascorbate-catalyzed hydroxyl radical reaction and was more effective than Trolox. The observed efficacy (IC_{50} value) of Neu2000 on MDA formation was about 10 times more potent in the isolated mitochondria samples (2.72 μM) than the spinal cord homogenates (24.56 μM). The difference is most likely related to fact that the spinal cord homogenates contained a higher proportion of polyunsaturated fatty acid chains associated with myelin compared to the isolated mitochondrial preparations.

Tert-butylhydroperoxide (*t*HBP) generates ROS that contribute to lipid peroxidation, DNA adduct formation, induction of apoptosis and cause GSH oxidation. (Martin et al., 2001; Haidara et al., 2002). Depletion and/or oxidation of the mitochondrial GSH pool has been shown to contribute to a numbers of toxic or pathological events associated with neurodegenerative disorders (Dringen, 2000; Lash, 2006). In present study, incubation of intact mitochondrial samples with *t*BHP resulted in significant GSH oxidation that was reduced by pretreatment with Neu2000. Further, pretreatment with 50 μm Neu2000 normalized GSH level to near control values indicating that direct radical scavenging of *t*BHP, thereby preserving the mitochondrial GSH pool. These findings suggest that the presence of Neu2000 results in a highly reduced intra-mitochondrial environment that preserves the GSH pool and thus decreases the overall susceptibility of the mitochondria against oxidative challenge.

Much focus has been placed on developing compounds that not only exhibit potent antioxidant activity but also target the mitochondrial matrix, which is a major source of free radical production in cells (Murphy and Smith, 2007; Frantz and Wipf, 2010; McEwen et al., 2011). At the present time, it is not known if Neu2000 exhibits any mitochondrial-targeting activity. However, because Neu2000 significantly reduced overall ROS/RNS levels in mitochondria exposed to antimycin A, Neu2000 may be able to enter mitochondria. To test this hypothesis, we temporarily exposed mitochondria to different concentrations of Neu2000 prior to incubation with the Fenton reaction mixture and then measured MDA formation. Even though any excess Neu2000 in solution was removed from the mitochondrial preparation before the Fenton reaction mixture was added, it was still effective in reducing MDA formation, suggesting that Neu2000 entered the mitochondria and inhibited lipid peroxidation in a concentration-dependent manner. In support of this, we did not observe any decreases in MDA formation when mitochondria were temporarily exposed to Trolox prior to addition of the Fenton reaction mixture. Although it is possible that Neu2000 may indirectly increase the mitochondrial antioxidant capacity, the spin trap properties of Neu2000 and the results of the experiments described here raise the possibility that Neu2000 may be able to target intramitochondrial compartments.

In recent years, attention has been directed toward understanding the pathophysiological consequences of protein oxidation, especially with regard to neurodegenerative events associated with aging, chronic inflammation, stroke, TBI, and SCI. The protein backbone and side chains of most amino acids are susceptible to oxidation, the nonenzymatic introduction of aldehyde or ketone functional groups to specific amino acid residues (i.e., carbonylation) (Adams et al., 2001; Bizzozero, 2009; Hall, 2009, 2011; Manolescu et al., 2011). In the present study, metal-catalyzed oxidation (Fenton mediated) and authentic peroxynitrite induced robust protein carbonyl formation in spinal cord mitochondria. Peroxynitrite is a powerful oxidant and reacts with target proteins resulting in nitrotyrosine or carbonyl formation. On the other hand, protein carbonylation by a metal ion-catalyzed process is likely to involve the formation of hydroxyl radicals, which can lead to oxidative modification of threonine, lysine, arginine, and proline residues (Adams et al., 2001; Bizzozero, 2009; Hall, 2009, 2011). In the present study, Neu2000 inhibited the formation of protein carbonyl byproducts caused by Fenton-mediated and authentic peroxynitrite reactions. Based on the results of our biochemical assays, it can be suggested that Neu2000 inhibited mitochondrial protein carbonyl formation by scavenging hydroxyl radicals as well as peroxynitrite and peroxynitrite-carbon dioxide intermediate radicals.

In conclusion, the results of the current study suggest that Neu2000 acts as a potent antioxidant agent by scavenging various ROS/RNS, including superoxide, hydroxyl, nitric oxide, and peroxynitrite radicals. In contrast, Neu2000 demonstrated very weak metal-chelating activity. Neu2000 also inhibited ROS/RNS, H_2O_2,

GSH oxidation and lipid peroxidation as well as Fenton- and peroxynitrite-mediated protein carbonyl formation in isolated spinal cord mitochondria. It could be argued that Neu2000 may reduce mitochondrial free radical production through some unspecified toxic effect. However, mitochondrial respiration studies demonstrated that concentrations of Neu2000 as high as 25 μM have no deleterious effect on state III-dependent ATP production (unpublished observations). Neu2000 is an amphiphilic compound that contains both lipophilic (benzyl and phenol groups) and hydrophilic (—COOH and —OH groups) moieties (Gwag et al., 2007; Cho et al., 2010). As a result, the antioxidant activity of Neu2000 can be attributed to (1) the lipophilic moieties, which may increase its bioavailability through biological membranes, including the mitochondrial phospholipid bilayers and (2) the hydrophilic phenoxy groups, which have been shown to quench the free-radical reactions through proton-donating mechanisms (Mason et al., 2006). Taken together, these studies suggest that Neu2000 has therapeutic potential in the treatment of free radical-mediated disorders.

Conflict of interest statement

Dr. Byoung Joo Gwag reports that a financial conflict of interest exists. All other authors declare that no conflicts of interest exist.

Acknowledgments

This work was supported by a grant from the Kentucky Spinal Cord and Head Injury Research Trust (Grant 7-23 to J.E.S.), the Craig H. Neilsen Foundation (Grant 56879 to M.L.M. and Grant 124814 to J.E.S.), National Institutes of Health (R01-NS062993 to P.G.S.), and an endowment from Cardinal Hill Rehabilitation Hospital (to J.E.S.).

References

Adams, S., Green, P., Claxton, R., Simcox, S., Williams, M.V., Walsh, K., Leeuwenburgh, C., 2001. Reactive carbonyl formation by oxidative and non-oxidative pathways. Front. Biosci. 6, A17–A24.

Beckman, J.S., Chen, J., Ischiropoulos, H., Crow, J.P., 1994. Oxidative chemistry of peroxynitrite. Methods Enzymol. 233, 229–240.

Bizzozero, A.O., 2009. Protein carbonylation in neurodegenerative and demyelinating CNS diseases. In: Lajtha, A., Banik, N., Ray, S.K. (Eds.), Handbook of Neurochemistry and Molecular Neurobiology Brain and Spinal Cord Trauma, third ed. Springer Science, pp. 543–562, Chapter No. 10.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310.

Castello, P.R., Drechsel, D.A., Day, B.J., Patel, M., 2008. Inhibition of mitochondrial hydrogen peroxide production by lipophilic metalloporphyrins. J. Pharmacol. Exp. Ther. 324, 970–976.

Chen, X., Zhong, Z., Xu, Z., Chen, L., Wang, Y., 2010. 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: forty years of application and controversy. Free Radic. Res. 44, 587–604.

Cho, S.I., Park, U.J., Chung, J.M., Gwag, B.J., 2010. Neu2000, an NR2B-selective, moderate NMDA receptor antagonist and potent spin trapping molecule for stroke. Drug News Perspect. 23, 549–556.

Crow, J.P., 1997. Dichlorodihydrofluorescein and dihydrorhodamine 123 are sensitive indicators of peroxynitrite in vitro: implications for intracellular measurement of reactive nitrogen and oxygen species. Nitric Oxide 1, 145–157.

Degli Esposti, M., 2002. Measuring mitochondrial reactive oxygen species. Methods 26, 335–340.

Dinis, T.C., Maderia, V.M., Almeida, L.M., 1994. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch. Biochem. Biophys. 315, 161–169.

Dringen, D., 2000. Glutathione metabolism and oxidative stress in neurodegeneration. Eur. J. Biochem. 267, 4912–4916.

Droge, W., 2002. Free radicals in the physiological control of cell function. Physiol. Rev. 82, 47–95.

Ewing, J.F., Janero, D.R., 1995. Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal. Biochem. 232, 243–248.

Fernandez-Checa, J.C., Kaplowitz, N., 1990. The use of monochlorobimane to determine hepatic GSH levels and synthesis. Anal. Biochem. 190, 212–219.

Frantz, M.C., Wipf, P., 2010. Mitochondria as a target in treatment. Environ. Mol. Mutagen. 51, 462–475.

Goldstein, S., Czapski, G., 1984. Mannitol as an OH· scavenger in aqueous solutions and in biological systems. Int. J. Radiat. Biol. 46, 725–729.

Gwag, B.J., Lee, Y.A., Ko, S.Y., Lee, M.J., Im, D.S., Yun, B.S., Lim, H.R., Park, S.M., Byun, H.Y., Son, S.J., Kwon, H.J., Lee, J.Y., Cho, J.Y., Won, S.J., Kim, K.W., Ahn, Y.M., Moon, H.S., Lee, H.U., Yoon, S.H., Noh, J.H., Chung, J.M., Cho, S.I., 2007. Marked prevention of ischemic brain injury by Neu2000, an NMDA antagonist and antioxidant derived from aspirin and sulfasalazine. J. Cereb. Blood Flow Metab. 27, 1142–1151.

Haidara, K., Morel, I., Abalea, V., Gascon Barre, M., Denizeau, F., 2002. Mechanism of tert-butylhydroperoxide induced apoptosis in rat hepatocytes: involvement of mitochondria and endoplasmic reticulum. Biochim. Biophys. Acta 1542, 173–185.

Hall, E.D., 2011. Antioxidant therapies for acute spinal cord injury. Neurotherapeutics 8, 152–167.

Hall, E.D., 2009. Free radicals and neuroprotection in traumatic brain and spinal cord injury. In: Lajtha, A., Banik, N., Ray, S.K. (Eds.), Handbook of Neurochemistry and Molecular Neurobiology Brain and Spinal Cord Trauma, third ed. Springer Science, pp. 203–228, Chapter No. 10.

Hall, E.D., Springer, J.E., 2004. Neuroprotection and acute spinal cord injury: a reappraisal. NeuroRx 1, 80–100.

Halliwell, B., Gutteridge, J.M., Aruoma, O.I., 1987. The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Anal. Biochem. 165, 215–219.

Kohen, R., Nyska, A., 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol. 30, 620–650.

Kooy, N.W., Royall, J.A., Ischiropoulos, H., Beckman, J.S., 1994. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic. Biol. Med. 16, 149–156.

Lash, L.H., 2006. Mitochondrial glutathione transport: physiological, pathological and toxicological implications. Chem. Biol. Interact. 163, 54–67.

Manolescu, B.N., Berteanu, M., Oprea, E., Chiriac, N., Dumitru, L., Vladoiu, S., Popa, O., Ianas, O., 2011. Dynamic of oxidative and nitrosative stress markers during the convalescent period of stroke patients undergoing rehabilitation. Ann. Clin. Biochem. 48, 338–343.

Martin, C., Martinez, R., Navarro, R., Ruiz-Sanz, J.I., Lacort, M., Ruiz-Larrea, M.B., 2001. Tert-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals. Biochem. Pharmacol. 62, 705–712.

Mason, R.P., Walter, M.F., Day, C.A., Jacob, R.F., 2006. Active metabolite of atorvastatin inhibits membrane cholesterol domain formation by an antioxidant mechanism. J. Biol. Chem. 281, 9337–9345.

McEwen, M.L., Sullivan, P.G., Rabchevsky, A.G., Springer, J.E., 2011. Targeting mitochondrial function for the treatment of acute spinal cord injury. Neurotherapeutics 8, 168–179.

McEwen, M.L., Sullivan, P.G., Springer, J.E., 2007. Pretreatment with the cyclosporin derivative, NIM811, improves the function of synaptic mitochondria following spinal cord contusion in rats. J. Neurotrauma 24, 613–624.

Miyasaka, N., Hirata, Y., 1997. Nitric oxide and inflammatory arthritides. Life Sci. 61, 2073–2081.

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochem. J. 417, 1–13.

Murphy, M.P., Smith, R.A., 2007. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu. Rev. Pharmacol. Toxicol. 47, 629–656.

Park, U.J., Lee, Y.A., Won, S.M., Lee, J.H., Kang, S.H., Springer, J.E., Lee, Y.B., Gwag, B.J., 2011. Blood-derived iron mediates free radical production and neuronal death in the hippocampal CA1 area following transient forebrain ischemia in rat. Acta Neuropathol. 121, 459–473.

Patel, S.P., Sullivan, P.G., Pandya, J.D., Rabchevsky, A.G., 2009. Differential effects of the mitochondrial uncoupling agent, 2,4-dinitrophenol, or the nitroxide antioxidant, tempol, on synaptic or nonsynaptic mitochondria after spinal cord injury. J. Neurosci. Res. 87, 130–140.

Rabchevsky, A.G., Patel, S.P., Springer, J.E., 2011. Pharmacological interventions for spinal cord injury: where do we stand? how might we step forward? Pharmacol. Ther. 132, 15–29.

Radi, R., Cassina, A., Hodara, R., Quijano, C., Castro, L., 2002. Peroxynitrite reactions and formation in mitochondria. Free Radic. Biol. Med. 33, 1451–1464.

Repetto, M.G., Ferrarotti, N.F., Boveris, A., 2010. The involvement of transition metal ions on iron-dependent lipid peroxidation. Arch. Toxicol. 84, 255–262.

Shin, J.H., Cho, S.I., Lim, H.R., Lee, J.K., Lee, Y.A., Noh, J.S., Joo, I.S., Kim, K.W., Gwag, B.J., 2007. Concurrent administration of Neu2000 and lithium produces marked improvement of motor neuron survival, motor function, and mortality in a mouse model of amyotrophic lateral sclerosis. Mol. Pharmacol. 71, 965–975.

Springer, J.E., Rao, R.R., Lim, H.R., Cho, S.I., Moon, G.J., Lee, H.Y., Park, E.J., Noh, J.S., Gwag, B.J., 2010. The functional and neuroprotective actions of Neu2000, a dual-acting pharmacological agent, in the treatment of acute spinal cord injury. J. Neurotrauma 27, 139–149.

Stowe, D.F., Camara, A.K., 2009. Mitochondrial reactive oxygen species production in excitable cells: modulators of mitochondrial and cell function. Antioxid. Redox Signal. 11, 1373–1414.

Sullivan, P.G., Krishnamurthy, S., Patel, S.P., Pandya, J.D., Rabchevsky, A.G., 2007. Temporal characterization of mitochondrial bioenergetics after spinal cord injury. J. Neurotrauma 24, 991–999.

Tarpey, M.M., Wink, D.A., Grisham, M.B., 2004. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R431–R444.

Turrens, J.F., 2003. Mitochondrial formation of reactive oxygen species. J. Physiol. 552, 335–344.
Ullrich, T., Oberle, S., Abate, A., Schroder, H., 1997. Photoactivation of the nitric oxide donor SIN-1. FEBS Lett. 406, 66–68.
Whiteman, M., Ketsawatsakul, U., Halliwell, B., 2002. A reassessment of the peroxynitrite scavenging activity of uric acid. Ann. NY. Acad. Sci. 962, 242–259.
Won, S.M., Lee, J.H., Park, U.J., Gwag, J., Gwag, B.J., Lee, Y.B., 2011. Iron mediates endothelial cell damage and blood–brain barrier opening in the hippocampus after transient forebrain ischemia in rats. Exp. Mol. Med. 43, 121–128.
Wrona, M., Patel, K., Wardman, P., 2005. Reactivity of 2′,7′-dichlorodihydrofluorescein and dihydrorhodamine 123 and their oxidized forms toward carbonate, nitrogen dioxide, and hydroxyl radicals. Free Radic. Biol. Med. 38, 262–270.