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Pre-clinical Therapeutic Development of a Series of Metalloporphyrins for Parkinson's disease

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Abstract

Reactive oxygen species are a well-defined therapeutic target for Parkinson's disease (PD) and pharmacological agents that catalytically scavenge reactive species are promising neuroprotective strategies for treatment. Metalloporphyrins are synthetic catalytic antioxidants that mimic the body's own antioxidant enzymes i.e. superoxide dismutases and catalase. The goal of this study was to determine if newly designed metalloporphyrins have enhanced pharmacodynamics including oral bioavailability, longer plasma elimination half-lives, penetrate the blood brain barrier, and show promise for PD treatment. Three metalloporphyrins (AEOL 11216, AEOL 11203 and AEOL 11114) were identified in this study as potential candidates for further pre-clinical development. Each of these compounds demonstrated blood brain barrier permeability by the i.p. route and two of three compounds (AEOL 11203 and AEOL 11114) were orally bioavailable. All of these compounds protected against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity, including dopamine depletion in the striatum, dopaminergic neuronal loss in the substantia nigra, and increased oxidative/nitrative stress indices (glutathione disulfide and 3-nitrotyrosine) in the ventral midbrain of the mice without inhibiting MPTP metabolism. Daily therapeutic dosing of these metalloporphyrins were well tolerated without accumulation of brain manganese levels or behavioral alterations assessed by open field and rotarod tests. The study identified two orally active metalloporphyrins and one injectable metalloporphyrin as clinical candidates for further development in PD.

Keywords

antioxidants; Pharmacokinetic Analysis; Dopamine; Drug development; Oxidative stress; MPTP

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Conflict of interest statements:

Dr. Patel is a consultant for Aeolus Pharmaceuticals which develops catalytic antioxidants for human diseases including the compounds used in this work. Dr. Day is a consultant for and holds equity in Aeolus Pharmaceuticals. Dr. Day is an inventor on a number of issued US patents on metalloporphyrins that National Jewish Health has licensed to Aeolus Pharmaceuticals.

INTRODUCTION

Numerous studies have suggested that oxidative stress is an important mediator contributing to the pathogenesis of Parkinson's disease (PD). Oxidative stress can occur as a consequence of dopamine oxidation, mitochondrial dysfunction, protein aggregation, inflammation, heavy metal overload, exposure to pesticides and autophagy (Beal, 2001; Beal, 2003; Jenner, 2003; Przedborski *et al.*, 2003; Janda *et al.*, 2012; Hauser and Hastings, 2013). Restoration of dopaminergic neuronal function by using L-DOPA, dopamine agonists, and inhibitors of monoamine metabolism are the current major therapeutic strategies in PD. However, these therapeutic approaches are often associated with significant adverse effects and fail to provide long-term control of this progressive disease. Antioxidant therapy is a potentially promising neuroprotective strategy for PD treatment. Vitamin E, Vitamin C and creatine supplementation were partially successful in cell and animal models of PD but did not show any significant clinical beneficial effects in human PD studies (Jin *et al.*, 2013). Relatively high dose CoQ10 only slowed progression at early stages of the disease (Shults *et al.*, 2002). Although overexpression of superoxide dismutases (SODs) has been shown to exert neuroprotection in PD mouse models (Przedborski *et al.*, 1992; Klivenyi *et al.*, 1998), the molecular size of these antioxidant enzymes limits their blood-brain barrier (BBB) permeability, oral absorption, and each have short circulating half-lives that hinders their use clinically.

Metalloporphyrins are a class of synthetic catalytic antioxidants that mimic the body's own antioxidant enzymes i.e. SODs and catalase (Day, 2004) and are capable of detoxifying a wide range of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), peroxynitrite, and lipid peroxyl radicals (Patel and Day, 1999; Patel, 2016). These compounds overcome many limitations of endogenous SOD/catalase enzymes such as large size, short circulation half-life, antigenicity and expense. Some water-soluble metalloporphyrin compounds, including manganese (III) meso-tetrakis (4-benzoic acid) porphyrin (MnTBAP) and manganese(III) meso-tetrakis (N,N'-diethylimidazolium-2-yl) porphyrin (AEOL10150) have been shown to be efficacious in animal models of central nervous system disorders, including status epilepticus (Liang *et al.*, 2000) and stroke (Mackensen *et al.*, 2001) by direct injection of the compounds into the brain. However, major drawbacks of the water-soluble porphyrins are their poor oral bioavailability and modest BBB permeability. To overcome these issues, a glyoxylate series of metalloporphyrins were developed that are more lipid soluble, which allows them to be more bioavailable, better penetrate the BBB, and active *in vivo* (Gauuan *et al.*, 2002; Trova *et al.*, 2003). Our previous study demonstrated that oral administration of AEOL 11207, a glyoxylate metalloporphyrin, penetrates the BBB and inhibited MPTP-induced neurotoxicity in mice (Liang *et al.*, 2007). To further optimize glyoxylate metalloporphyrins antioxidant properties, we have analyzed more than 20 glyoxylate metalloporphyrin compounds for their ability to scavenge endogenously generated H_2O_2 production in respiring brain mitochondria (Castello *et al.*, 2008). This study identified a number of glyoxylate metalloporphyrins, including AEOL 11207, AEOL 11209, AEOL 11216 and AEOL 11215, as potent H_2O_2 scavengers. Together these data suggest the potential utility of glyoxylate metalloporphyrins such as AEOL11207 as therapeutic agents in PD.

The goal of this study was to determine the pharmacokinetics, oral bioavailability and BBB permeability of a selected series of glyoxylate metalloporphyrins in adult mice, optimize the dosing regimen, and examine potential neuroprotective effects of these compounds on MPTP-induced dopamine depletion, oxidative stress and dopaminergic neuronal loss.

MATERIALS AND METHODS

Reagents

All reagents, including MPTP were purchased from Sigma Aldrich. AEOL 11203, AEOL 11209, AEOL 11215, AEOL 11216 and AEOL 11114 were obtained from Aeolus Pharmaceuticals (Mission Viejo, CA).

Animals

Animal studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). All procedures were approved by the Institute Animal Care and Use Committee (IACUC) of University of Colorado Denver (UCD), which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care. C57BL/6 mice (male, ~25g weight, ~10 weeks old) were purchased from Jackson laboratories (Bar Harbor, ME) and used for all experiments. Upon arrival, mice were group housed on a 12/12 light/dark cycle with ad libitum access to both food and filtered water. Mice were treated with saline, AEOLs and/or MPTP after one week of acclimation.

Metalloporphyrin Pharmacokinetic Analysis

Metalloporphyrin administrations—Mice were administered a single bolus dose (15 mg/kg) of each metalloporphyrin by the p.o. or i.p. route and sacrificed at various times (1, 3, 6, 12, 24, 48 and 72 h) for pharmacokinetic analyses. Metalloporphyrins were synthesized or supplied by Aeolus Pharmaceuticals as previously reported (Trova *et al.*, 2003) and all were at least 95% pure (Figure 1). AEOL 11216 and AEOL 11215 were dissolved in 5% or 10% ethanol, respectively. AEOL 11203, AEOL 11114 or AEOL 11209 were dissolved in 1%, 5% or 30% dimethyl sulfoxide (DMSO), respectively. Metalloporphyrins were first dissolved in a small volume of 100% ethanol or 100% DMSO and then diluted with sterilized phosphate buffered saline (PBS) to achieve the desired final concentration. The control animals were treated with corresponding vehicles. Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at various times after AEOLs treatment and terminal blood samples (~0.2 mL) were obtained by cardiac puncture. Animals were then perfused with ~60 mL PBS containing 0.1 units heparin/mL to eliminate blood contamination in the brain. The forebrains were collected and frozen on dry ice immediately. Blood samples were centrifuged at 13,000 rpm for 10 min. A 50 µL aliquot of plasma was transferred to new tubes, frozen on dry ice, and then stored at -80°C until assayed.

Metalloporphyrin assays—Metalloporphyrin levels were measured using HPLC with spectrophotometric detection (Elite LaChrom System; Hitachi, Japan) with a YMC-Pack ODS-A column (4.6 × 150 mm, 3 µm, 120 Å; Waters, Milford, MA, USA). Mobile phase A contained 20 mM triethylamine and 20 mM trifluoroacetic acid, and mobile phase B

consisted of 100% acetonitrile. Flow rate was 1 mL/min. Plasma and brain samples were extracted and protein precipitated with 0.05 N perchloric acid in 50 % methanol. Samples were centrifuged at 16,000 g for 20 min. The resulting supernatant was filtered through a 0.22- μ m filter. An aliquot of supernatant (10 μ L) was injected into the HPLC equipped with a UV detector set at 446 or 450 nm as previously described (Kachadourian *et al.*, 2002). Recovery of metalloporphyrins from plasma samples was determined to be 96–99%.

Antioxidant Properties of Metalloporphyrins

SOD activity assay—The SOD activity of metalloporphyrins was measured following the method as previous described (Misra and Fridovich, 1972). The autoxidation of 0.3 mM epinephrine in 50 mM sodium carbonate buffer, pH10.2 at 30°C releases superoxide and produces adrenochrome which can be measured at 480 nm. A unit of SOD activity is defined as the amount of compound that inhibits one-half the formation of adrenochrome at pH 10.2.

H₂O₂ scavenging assay—H₂O₂ scavenging was measured using the horseradish peroxidase (HRP)-linked fluorometric assay following the commercial protocol (Amplex Ultra Red; Invitrogen, Carlsbad, CA). H₂O₂ (50 μ M) was added as 100% non-inhibition and the percentages of H₂O₂ consumption over 1 minute from different concentrations of the compound were used to determine IC₅₀ values obtained by fitting the data from a sigmoidal curve with nonlinear regression analysis (Prism 5, GraphPad Software, San Diego, CA) for each compound.

Thiobarbituric acid reactive species (TBARS) assay—TBARS formation was used as an index of lipid peroxidation and measured following the method as previous described (Day *et al.*, 1999). Rat brain homogenates (~2 mg protein) were incubated with varying concentrations of AEOLs at 37°C for 15 min. Oxidation of the rat brain homogenate was initiated by the addition of a freshly prepared anaerobic stock solution containing iron(II) chloride (0.25 mM) and ascorbate (1 mM). Samples were placed in a shaking water bath at 37°C for 30 min. The reactions were stopped by the addition of 0.1 ml of a stock butylated hydroxytoluene (60 mM) solution in ethanol. The oxidized rat brain homogenates (200 μ L) were acidified with 200 μ L of 0.2 M phosphoric acid. The color reaction was initiated by the addition of 25 μ L of a 0.11 M thiobarbituric acid solution and samples were placed in a 90°C heating block for 45 min. TBARS were extracted with 0.5 ml of *n*-butanol by vortexing samples for 3 min and chilling on ice for 1 min. The samples were then centrifuged at 16,000 $\times$ g for 5 min. Aliquots (150 μ L) of the *n*-butanol phase were placed in each well of a 96-well plate and read at 535 nm in a Spectromax platereader (Molecular Devices, Sunnyvale, CA, USA) at 25°C. The concentration of metalloporphyrin required to inhibit 50% TBARS formation in rat brain homogenates was used as an index for lipid peroxidation.

Metalloporphyrins Prevent MPTP Neurotoxicity

MPTP and metalloporphyrin administration—Mice were administered MPTP 15 mg/kg, subcutaneous (s.c.), daily for 3 days alone or in combination with metalloporphyrin (15 mg/kg by p.o. or i.p. treatment; 1h prior to MPTP and daily thereafter for a total of 3 days, once daily). MPTP was dissolved in sterilized saline. The control animals were treated

with corresponding vehicles. Mice were sacrificed 4 days or 7 days post first injection of MPTP and the striata and ventral midbrain regions were dissected as previously described (Andreassen *et al.*, 2001) for DA assay, oxidative stress or dopaminergic neuron count. The animal carcasses, needles and MPTP waste were disposed according to UCDHSC hazardous chemical disposal guidelines and as recommended in the literature (Przedborski *et al.*, 2001).

Dopamine assay—Striatal dopamine levels were determined using a previously described method by HPLC equipped with an electrochemical detector (Liang and Patel, 2004). In brief, mouse striatum was dissected out and frozen immediately in liquid nitrogen, sonicated in ice-cold 0.2 M perchloric acid and centrifuged at 16,000 g for 15 min, 4 °C. An aliquot (20 µL) of the supernatant solution was analyzed by HPLC equipped with an electrochemical detector (CoulArray system ESA Model 5600; ESA, Boston, MA). Samples were injected onto a catecholamine column (3 µm, 100×4.6 mm, Waters, Milford, MA, USA) with an automatic injector (ESA Model 540). The mobile phase was composed of 100 mM citric acid, 2% methanol, 1 mM EDTA and 5 mg/L sodium octyl sulfate (pH 3.0). Electrochemical detector potentials were set at 0/50/200/300 mV (vs. Pd).

Tyrosine hydroxylase (TH) immunohistochemistry and stereological analysis

—TH immunohistochemistry and stereological analysis were determined using previously described methods (West, 1999; Liang *et al.*, 2007). Briefly, brain blocks containing the midbrain (~4 mm thick) were fixed in 4% paraformaldehyde dissolved in 0.1 M PBS, pH 7.4. The midbrain blocks were frozen and cut into 40µm sections using a cryostat that included the whole SN pars compacta (SNpc) from bregma −2.60 to −3.80 mm. Every fifth section from the whole midbrain region was immunostained with a rabbit antibody to TH 1:500 (cat# AB152, Chemicon, Temecula, CA) using the ABC method (ABC Elite Kit, Vector Laboratories, Burlingame, CA). Sections were counterstained with cresyl violet following TH staining. Stereological neuronal counts were performed by using a computer-assisted image analysis system consisting of a Nikon Optiphot-2 80i microscope (Nikon Inc., Melville, NY) equipped with a MC-XYZ-LC (Applied Scientific Instrumentation, Eugene, OR) computer-controlled motorized stage. The substantia nigra pars compacta was delineated on each section at low magnification (4× magnification), and then systematically sampled at 60× magnification from a random start position using unbiased stereological analysis performed with the optical fractionator from StereoInvestigator (MicroBrightfield, Williston, VT, USA). To avoid counting TH positive dendrites and axons (TH positive expression) with unusual shapes, TH positively stained cells were counted only when their nuclei were optimally visualized in one focal plane. The numbers of TH positive neurons in the SNpc were counted by an investigator (R.F.) who was blinded to the treatments.

Glutathione (GSH), glutathione disulfide (GSSG), tyrosine, and 3-nitrotyrosine (3-NT) assays

—GSH, GSSG, tyrosine and 3-NT assays were performed with an ESA (Chelmsford, MA) 5600 CoulArray HPLC equipped with eight electrochemical cells in series as previously described (Beal *et al.*, 1990; Hensley *et al.*, 1998; Liang *et al.*, 2007). The potentials of the electrochemical cells were set at 0/150/300/450/570/690/810/860 mV. Analyte separation was conducted on a TOSOHAAS (Montgomeryville, PA) reverse-phase ODS 80-TM C-18 analytical column (4.6 mm×250 cm; 5 µm particle size). A two-

component gradient elution system was used with component A consisting of 50 mM NaH₂PO₄ pH 3.2, and component B composed of 50mM NaH₂PO₄ and 50% methanol pH 3.2. A gradient elution profile was used as follows: 0–25 min, 100% A; 25–45 min, linear ramp to 50% B; 45–50 min, isocratic 50% B; 50–55 min, linear ramp to 100% A; 55–60 min, isocratic 100% A at a flow rate of 0.6 mL/min. The brain tissue samples were sonicated in 10 % (w/v) ice cold 0.1 M perchloric acid and centrifuged at 16,000 g for 15 min, 4 °C. An aliquot (40 µl) of the supernatant was analyzed by HPLC. The level of 3-NT was expressed as a ratio of 3-NT to tyrosine. The retention time of GSH, tyrosine, GSSG and 3-NT was ~6.50, 16.20, 24.55 and 35.80 minutes and the standard curve ranged from 20 to 300, 3 to 50, 0.1 to 2 and 0.01 to 1 nmol, respectively.

1-Methyl-4-phenylpyridinium ion (MPP⁺) assay—Striatal MPP⁺ levels were determined as previously described (Liang and Patel, 2004). MPP⁺ was analyzed by HPLC equipped with a UV detector set at 295 nm. The mobile phase (30% acetonitrile, 0.02 M potassium phosphate; adjusted to pH 2.5 with H₃PO₄) was delivered at a flow rate of 0.5 mL/min on a YMC ODS column 3 µm, 150×4.6mm (Waters, Milford MA). MPP⁺ retention time was ~5.87 min. The injection volume was 20 µl and a standard curve ranged from 50 to 1000 ng/mL.

Manganese Toxicity Assessments

Mice were divided into 5 groups and treated with MnCl₂, AEOL 11114, AEOL 11207, AEOL 11203 (15mg/kg i.p) or vehicle alone daily for a period of 7 days, respectively. Behavioral functions were assessed at the end of the treatment. Mice were deeply anesthetized by sodium pentobarbital (50 mg/kg, i.p.) 3–4h after the last injection. The forebrains were frozen immediately with liquid nitrogen and stored at –80°C for manganese assay after perfusion with 60 ml PBS with 0.1 unit/mL heparin. The levels of manganese in the forebrains were determined by inductively coupled-plasma mass spectrometry (ICPMS) (Exova Group, Santa Fe Springs, CA), which is the most sensitive and definitive technique with the lowest detection limits for manganese (0.01ug/L in nitric acid).

Behavioral assessments for manganese accumulation—Neurobehavioral function was evaluated using the open field test and the accelerated rotarod test following treatment with metalloporphyrins. Open field test was conducted in a square arena mounted within specially designed sound-proof plexiglass shells. The open field testing apparatus consisted of two identical behavioral arenas constructed of expanded PVC (40 cm × 40 cm; wall height = 30.5 cm). The mouse was placed in the center of the brightly lit open field arena and allowed to move freely for 5 minutes. The total distance traveled and velocity was measured and quantified by an automated tracking system (Topscan, Clever Sys, Inc., Reston, VA). The rotarod test was performed utilizing an Accelerated Rota-Rod model 47650 for mice (UGO Basile, Italy) with a rod diameter of 3 cm according to the manufacturer's instructions and protocol (Kaur *et al.*, 2003). The mice were allowed to acclimatize for 20 s at the lowest 5 rpm speed, and then the rotor was accelerated to 40 rpm during a period of 2.5 min. The time from acceleration to fall of the animal from the rod was recorded for behavioral assessment.

Statistical Analysis

All data are expressed as mean $\pm$ S.E.M. Statistical differences were analyzed by one-way ANOVA with post hoc multiple comparison test corrected for multiple comparisons (Tukey test). To evaluate the overall effects of metalloporphyrins dosing routes on MPTP treatment groups, two-way ANOVAs with Bonferroni posttest analyses were also performed. P values less than 0.05 were considered statistically significant. All analyses were performed using Prism 5 software (Prism 5. GraphPad Software, San Diego, CA). Pharmacokinetic data was obtained using a one compartment model with first order input and first order output (PK analyst, model #3, MicroMath, Salt Lake City, UT).

RESULTS

Pharmacokinetic Analysis of Metalloporphyrins

Previous work in our laboratory has determined that AEOL 11209, 11215, and 11216 are potent scavengers of H₂O₂ released from isolated mitochondria (Castello *et al.*, 2008). Therefore, these compounds were initially selected for pharmacokinetic analysis in mice with both i.p. (parenteral) and p.o. (oral) routes of administration to estimate oral bioavailability.

The plasma concentrations of AEOL 11209 were moderate after i.p. administration but relatively low after oral administration (Figure 2A). The brain concentration of AEOL 11209 after i.p. or p.o. administration was variable and relatively low (Figure 2B). These results indicate that this compound has low gastrointestinal absorption and poor blood-brain barrier (BBB) permeability. The concentrations of AEOL 11215 achieved moderate levels in the plasma but it was not detectable in the brain which indicates it has poor BBB permeability after i.p. administration (Figure 2C). AEOL 11215 was not reliably detected in plasma after p.o. administration at any time point suggesting that it lacked gastrointestinal absorption (data not shown). AEOL 11216 achieved relatively high levels in the plasma with prolonged retention at more than 100 nM in the brain for 24 h after i.p. administration but this was not achieved by oral administration (Figure 2D & E) suggesting that it was poorly absorbed by the gastrointestinal tract.

Based on the above results, a targeted synthesis was performed to incorporate trifluoromethyl groups to add electron-withdrawing activity from the porphyrin ring and increase lipophilicity creating a new compound AEOL 11114. The pharmacokinetic analysis of AEOL 11203 and AEOL 11114 was also performed. AEOL 11203 achieved high plasma concentrations (20 μ M) after i.p. administration and relatively high plasma concentrations (5 μ M) after oral administration (Figure 3A). It penetrated the BBB and remained stable (>100 nM) in the brain for 24 h after i.p. administration and its levels were ~50 nM in the brain for 24 h after oral administration (Figure 3B). AEOL 11114 achieved a very high concentration in plasma after i.p. (~25 μ M) and oral administration (~7 μ M) (Figure 3C). AEOL 11114 concentrations in the brain remained stable (>200 nM) for 24 h regardless of the route of administration (Figure 3D). The results suggest both AEOL 11203 and AEOL 11114 were absorbed by the gastrointestinal tract and penetrated the BBB.

Parenteral and oral AEOL 11114 and AEOL 11203 concentration-time data best fit a one compartment PK model with first-order input and output (Table 1). AEOL11203 and AEOL 11114 elimination plasma half-lives ($T_{1/2}$) for the oral route are estimated to be 5.6 h and 13 h, respectively. AEOL 11203 and AEOL 11114 time to maximum plasma concentration (T_{max}) for the oral route are estimated to be 6 h and 20 h, respectively. AEOL 11203 and AEOL 11114 area under the time curves (AUC) by oral route are estimated to be 130 $\mu\text{mol h/L}$ and 430 $\mu\text{mol h/L}$ in plasma, respectively. The oral bioavailability studies via oral and intraperitoneal routes (calculated with $\text{AUC}_{\text{oral}}/\text{AUC}_{\text{ip}} \%$) of AEOL 11203 and AEOL 11114 are estimated to be 30% and 33%, respectively from a single bolus 15 mg/kg dose. The dosing schemes for both AEOL 11203 and AEOL 11114 were chosen from pharmacokinetic model analyses that would achieve a plasma C_{max} of $\sim 8 \mu\text{M}$ and brain C_{max} in the range of 50–230 nM with a 15mg/kg daily oral dosing route (Table 2).

Before testing protective effects against MPTP-induced toxicity *in vivo* with AEOL 11114 and AEOL 11203, the *in vitro* antioxidant properties of both compounds were determined and compared with AEOL 11207 which has already been demonstrated to be effective against MPTP-induced toxicity *in vivo* (Liang *et al.*, 2007). These metalloporphyrins were assessed for their SOD and catalase-like activities and ability to inhibit lipid peroxidation. In comparison with AEOL 11207, AEOL 11114 has relatively higher SOD activity (three times higher), similar lipid peroxidation inhibition activity and H_2O_2 scavenging; and AEOL 11203 showed similar SOD activity and lipid peroxidation inhibition activity, although its H_2O_2 scavenging was relatively weaker. AEOL 11209, AEOL 11215 and AEOL 11216 showed higher H_2O_2 scavenging activities but they were not absorbed by the gastrointestinal tract and/or failed to penetrate the BBB (Table 3).

Assessment of Manganese Accumulation and Metalloporphyrin Toxicity

Metalloporphyrins have manganese as a metal center for catalyzing redox reactions. This property could potential induce neurotoxicity resulting from the release of manganese from the porphyrin ring. To evaluate for this possibility, manganese levels were measured by inductively coupled-plasma mass spectrometry (ICPMS) in the brains of mice after administration of AEOL 11114, AEOL 11207, AEOL 11203 or MnCl_2 as a positive control. This study revealed that while treatment with MnCl_2 increased brain manganese levels significantly, none of the metalloporphyrins produced any significant change in brain manganese levels compared to vehicle-treated mice (Figure 4).

To further rule out potential manganese neurotoxicity following treatment with metalloporphyrins, mice were evaluated by two neurobehavioral tests i.e. the open field test and the accelerated rotarod test 24 h and 48 h respectively, after the final dose of metalloporphyrins. Both tests provide simple assessment for general levels of movement activity in mice. No significant behavioral changes were observed between mice treated with vehicle or any of the metalloporphyrins. However, a significant deficit in movement was observed by rotarod test in MnCl_2 treated mice compared to vehicle controls (Figure 5).

Metalloporphyrins Attenuate MPTP Neurotoxicity

Metalloporphyrins protect against MPTP-induced striatal dopamine depletion

—Based on pharmacokinetic results, AEOL 11114 and AEOL 11203 were selected to determine their protective effects against MPTP-induced toxicity. The dosing regimen (15mg/kg, daily for three days) of AEOL 11203 and AEOL 11114 was selected due to the prior dose-response studies with AEOL 11207 that revealed a significant protective effect against MPTP-induced striatal dopamine depletion (Liang *et al.*, 2007). MPTP-treated mice showed an 81 % loss of DA levels in striata compared to vehicle-treated mice 4 days after the first dose of MPTP. AEOL 11114 showed statistically significant protection against MPTP-induced striatal dopamine depletion by i.p. (14.1%) and p.o. (13.6%) administration (Figure 6A). AEOL 11203 also showed statistically significant (13.0%) protection against MPTP-induced striatal dopamine depletion by i.p. administration but only 10.0% without significant protection by p.o. administration (Figure 6B). The levels of DA in the striatum of the mouse had no any significant change with AEOL 11114 or AEOL 11203 alone treatment.

Metalloporphyrins protect against MPTP-induced loss of TH-positive SNpc cells

—To further examine whether AEOL 11114 and AEOL 11203 attenuate MPTP-induced toxicity, stereological analysis of the number of TH-positive cells in defined SNpc of the mouse was performed. MPTP-treated mice showed a 50.6 % loss of TH-positive dopaminergic neurons in the SNpc compared to vehicle-treated mice 7 days after the first dose of MPTP. Compare to vehicle, administration of AEOL 11114 and AEOL 11203 produced significant protection of 40.7 % and 31.9% by i.p. administration and 34.0% and 29.3% by p.o. administration, respectively against the loss of SNpc dopaminergic neurons following MPTP treatment (Figure 7A and 7B). The protection against MPTP-induced loss of SNpc dopaminergic neurons was achieved from both metalloporphyrins by both administration routes. The numbers of TH-positive dopaminergic neurons in the SNpc of the mouse showed no significant change with AEOL 11114 or AEOL 11203 treatment alone.

Metalloporphyrins protect against MPTP-induced oxidative stress—The effect of AEOL 11114 and AEOL 11203 on GSH, GSSG and 3-NT levels was determined in the mouse ventral midbrain to verify whether these neuroprotective effects were associated with their antioxidant properties. Following MPTP treatment, the levels of GSH were modestly depleted (~20%); whereas the corresponding levels of GSSG were significant increased to more than ~300% of control and the resulting GSH/GSSG ratio decreased to 25% of control mice. 3-NT level in the mouse ventral midbrain region was also measured to further determine the peroxynitrite scavenging capability of AEOL 11114 and AEOL 11203. The free 3-NT concentrations increased more than 3 fold with MPTP treatment in comparison to vehicle-treated mice. This result is consistent with previously reported measurements of free 3-NT by HPLC (Pennathur *et al.*, 1999) or protein bound 3-NT by mass spectrometry (Pennathur *et al.*, 1999). Administration of AEOL 11114 via i.p. or oral routes significantly attenuated MPTP-induced increased GSSG and 3-NT (Figure 8A). Administration of AEOL 11203 via i.p. or oral routes also significantly attenuated MPTP-induced increased GSSG and 3-NT as well (Figure 8B).

Metalloporphyrins did not alter striatal MPP⁺ levels—MPTP neurotoxicity to the nigrostriatal system is dependent on conversion to its active metabolite, MPP⁺ in the striatum (Giovanni *et al.*, 1991). The levels of MPP⁺ was determined in the striatum 3h after metalloporphyrin administration to eliminate the possibility that metalloporphyrins interfere with striatal MPTP metabolism (Liang *et al.*, 2007). No differences were observed in striatal MPP⁺ concentrations measured 3 h after a single dose of MPTP (15 mg/kg, s.c.) alone or 1h after 15mg/kg, i.p. dose of AEOL 11207, 11203 or 11114 (MPTP+vehicle: 14.30 ± 2.74 , (6); MPTP+AEOL 11207: 14.95 ± 1.98 , (4); MPTP+AEOL 11203: 14.63 ± 3.36 , (4) and MPTP+AEOL 11114: 14.79 ± 2.81 , (4) $\mu\text{g/g}$ tissue; values are expressed as mean+S.E.M. (N)) This data suggests that the protective effects of metalloporphyrins on MPTP neurotoxicity were not due to altered MPTP metabolism and/or bioavailability.

DISCUSSION

In this study, we identified several metalloporphyrins (AEOL 11203, AEOL 11209, AEOL 11215, AEOL 11216 and AEOL 11114) with improved pharmacodynamics profiles. This series of glyoxylate metalloporphyrins were designed to have greater lipid solubility, oral bioavailability, and ability to cross the BBB (Trova *et al.*, 2003). The results indicate that three of the compounds (AEOL 11203, AEOL 11216 and AEOL 11114) are BBB permeable by the i.p. route and two of them (AEOL 11203 and AEOL 11114) are orally active. The data suggest that AEOL 11203 and AEOL 11114, which are similar to AEOL 11207 (Liang *et al.*, 2007), have relatively long plasma half-lives, rapid absorption by the gastrointestinal tract, ability to penetrate the BBB, and can achieve therapeutically active concentrations in the brain. Our pharmacokinetic data demonstrate a 33 % and 30% oral bioavailability ($\text{AUC}_{\text{oral}}/\text{AUC}_{\text{ip}} \%$) and AUC_{oral} of $9.46 \mu\text{M/h}$ and $3.10 \mu\text{M/h}$ in the brain for AEOL 11114 and AEOL 11203, respectively. AEOL 11114 was more effective orally than AEOL 11203 at inhibiting markers of oxidative stress and MPTP neurotoxicity in our animal model. This is likely due to the higher oral availability and higher AUC of AEOL 11114 compared to AEOL 11203.

Previous data suggest that metalloporphyrins are extremely stable and retain their metal even in the presence of EDTA (Kachadourian *et al.*, 2004). Several of these compounds have been found to be safe and efficacious when used in both *in vitro* and *in vivo* models (Day and Crapo, 1996; Patel *et al.*, 1996). In our study, we did not detect alterations in manganese levels in brain or neurobehavioral abnormality reminiscent of manganese toxicity following daily dosing of AEOL 11207, AEOL 11114, or AEOL 11203. In contrast, the manganese levels increased ~10 fold accompanied by a significant rotarod deficit in mice treated daily with an equivalent dose of MnCl_2 . These results indicate that the glyoxylate series of metalloporphyrins lack signs of neurotoxicity and do not result in brain manganese accumulation. AEOL 11114 and AEOL 11203 are highly lipophilic compounds and were dissolved in 5% or 1 % DMSO, respectively. No significant change in body weight or behavior was found in the mice with 5% or 1% DMSO vehicle administration, which are the same volumes and doses as AEOL 11114 and AEOL 11203 injection ($1\text{mL/kg/day} \times 3$, i.p. or p.o.), comparison with saline controls. Taken together, our data suggests that AEOL 11114 and AEOL 11203 are excellent candidates for clinical therapeutic development of neurological diseases. MPTP is a neurotoxicant producing clinical, biochemical, and

neuropathological changes in both human and experimental animals that are analogous to those observed in idiopathic Parkinson's disease (PD) (Beal, 2001; Przedborski and Vila, 2003). MPTP treatment induces significant DA depletion in the striatum and DA neurons loss in the SNpc of C57BL6 mice which has been considered a well-established mouse model of PD for 30 years (Sonsalla and Heikkila, 1986; Liberatore *et al.*, 1999; Przedborski and Vila, 2003; Liang *et al.*, 2007). The stereological method we employed to estimate the number of TH-positive cell in SNpc as well as HPLC measurement of DA in the striatum have been shown to be reliable techniques for evaluating dopaminergic system function. This function was found to be protected by metalloporphyrin treatment in the MPTP model.

One of the major mechanisms of MPTP neurotoxicity *in vivo* is thought to arise from oxidative stress due to superoxide ($O_2^{\cdot-}$) production from mitochondrial complex I inhibition and impaired energetics. MPTP neurotoxicity is also thought to result in excessive nitric oxide ($\bullet NO$) production by microglial activation via inflammatory mechanisms (Przedborski and Jackson-Lewis, 1998). NO can react with $O_2^{\cdot-}$ to form the highly reactive nitrogen species peroxynitrite ($ONOO^-$), which causes nitration of tyrosine residues forming 3-NT in various proteins, including TH and α -synuclein, and results in protein dysfunction (Ara *et al.*, 1998; Blanchard-Fillion *et al.*, 2001). Increased 3-NT is considered an important biomarker of oxidative/nitrative protein damage in MPTP-induced Parkinsonism (Przedborski *et al.*, 1996; Pennathur *et al.*, 1999; Wu *et al.*, 2002). The levels of GSH, GSSG and the ratio of this redox couple (GSH/GSSG) serve as a major intracellular indicator of redox status and oxidative stress in cells and tissues (Valko *et al.*, 2007). A depletion of GSH levels has been observed in postmortem samples from the SN of PD patients and in the ventral midbrain region of MPTP-treated rodents (Perry *et al.*, 1982; Bannon *et al.*, 1984; Ferraro *et al.*, 1986; Sian *et al.*, 1994). In fact, GSH depletion is thought to play a primary pathogenic role in the etiology of PD (Jenner *et al.*, 1992). Our results show that a MPTP treatment decreased the GSH/GSSG ratio and increased 3-NT formation, which correlate with the occurrence of dopamine depletion and dopaminergic cell loss. The ability of metalloporphyrins to attenuate MPTP-induced alterations of GSH/GSSG ratios and 3NT correlates with their antioxidant activities and their C_{max} in the brain. For example, AEOL 11203, which has a lower catalase activity and C_{max} (~50 nM) was less effective against MPTP-induced oxidative stress and DA depletion compared to AEOL 11114 which has a C_{max} of ~230 nM in the brain via the oral route. Better clinically relevant oxidative stress biomarkers are needed to bridge pre-clinical animal study results with optimizing safe and efficacious doses in potential PD clinical trials.

The data collectively suggest that orally active metalloporphyrin catalytic antioxidants that readily penetrate the BBB may be potential therapeutic agents for chronic neurologic diseases such as PD. Two glyoxylate metalloporphyrins catalytic antioxidants identified in this study (AEOL 11114 and AEOL 11203) and one glyoxylate metalloporphyrin catalytic antioxidant (AEOL 11207) identified in a previous study (Liang *et al.*, 2007) may be potential candidates for further clinical development therapy for PD.

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Highlights

A series of metalloporphyrins were optimized in a mouse model of parkinsonism

Two novel orally active, brain permeable antioxidant metalloporphyrins were identified

The identified metalloporphyrins were well tolerated

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Figure 1. Structures of Metalloporphyrins

Figure 1. Structures of Metalloporphyrins

Structures of AEOL 11203, AEOL 11215, AEOL 11216, AEOL 11114, AEOL 11209 and AEOL 11207.

Figure 2: AEOL 11209, AEOL 11215 and AEOL 11216 Pharmacokinetic Analysis

Figure 2. AEOL 11209, AEOL 11215 and AEOL 11216 Pharmacokinetic Analysis

The concentration of AEOL 11209 in plasma (A) and brain (B), AEOL 11215 in plasma (C) and AEOL 11216 in plasma (D) and brain (E) of C57BL/6 mice at different times points after a single dose of 15mg/kg administered by i.p or p.o. route. Points represent mean + S.E.M. Each point is the average of 3–4 animals.

Figure 3: AEOL 11203 and AEOL 11114 Pharmacokinetic Analysis

Figure 3. AEOL 11203 and AEOL 11114 Pharmacokinetic Analysis

The concentrations of AEOL 11203 in plasma (A) and brain (B), AEOL 11114 in plasma (C) and brain (D) of the C57BL/6 mice at different times points after a single dose (15mg/kg) administered by the i.p. and p.o. route. Points represent mean + S.E.M. Each point is the average of 3–4 animals.

Daily therapeutic dosing of the metalloporphyrins without accumulation of brain manganese levels

Figure 4. Daily therapeutic dosing of the metalloporphyrins without accumulation of brain manganese levels

Manganese levels in brains of mice at 7 days after injection of vehicle or 15 mg/kg, i.p. daily injections of MnCl₂, AEOL 11114, AEOL 11207 or AEOL 11203. Bars represent mean + S.E.M., * $p < 0.01$ vs. control. One-way, ANOVA, $n=6$ mice per group.

Figure 5: Daily therapeutic dosing of the metalloporphyrins without behavioral alterations

Figure 5. Daily therapeutic dosing of the metalloporphyrins without behavioral alterations

Total moving distance (A), moving velocity (B) in 5 minutes measured by open field test and latency (seconds) from acceleration to fall in the rotarod (C) in mice 7 days of vehicle injection or 15 mg/kg, i.p. daily injections of MnCl₂, AEOL 11114, AEOL 11207 or AEOL 11203 i.p. (15mg/kg, daily). Bars represent mean + S.E.M. *p< 0.01 vs. control. One-way, ANOVA, n=6–8 mice per group.

The metalloporphyrins attenuated MPTP-induced dopamine depletion

Figure 6. The metalloporphyrins attenuated MPTP-induced dopamine depletion

Dopamine levels in the striata of C57BL/6 mice 3 days after injection of MPTP (15mg/kg×3, s.c., 24h intervals) alone or in the presence of AEOL 11114 (A) or AEOL 11203 (B) 15 mg/kg i.p or p.o. (1 h prior to MPTP and daily thereafter×3). Bars represent mean + S.E.M, n=3–7 mice per group. Statistical analysis: Two-way, ANOVA, Figure (A): interaction [p=0.200], Bonferroni posttests, #p<0.05 MPTP+vehicle versus MPTP+AEOL 11114 i.p and p.o.; (B): interaction [p=0.257], Bonferroni posttests, #p<0.05 MPTP+vehicle versus MPTP+AEOL 11203 i.p.

The metalloporphyrins attenuated MPTP-induced dopaminergic neuronal loss

Figure 7. The metalloporphyrins attenuated MPTP-induced dopaminergic neuronal loss

The number of dopamine neurons in the substantia nigra pars compacta of C57BL/6 mice 7 days after injection of MPTP (15mg/kg×3, s.c., 24h intervals) alone or in the presence of AEOL 11114 (A) and AEOL 11203(B) 15 mg/kg i.p or p.o. (1 h prior to MPTP and daily thereafter×3). Bars represent mean + S.E.M, n=3–6 mice per group. Statistical analysis: Two-way, ANOVA, (A) interaction [$*p<0.05$], Bonferroni posttests, $\#p<0.01$ MPTP+vehicle versus MPTP+AEOL 11114 i.p. and p.o.; (B): interaction [$*p<0.05$], Bonferroni posttests, $\#p<0.01$ MPTP+vehicle versus MPTP+AEOL 11203 i.p. and p.o.

Figure 8: The metalloporphyrins attenuated MPTP-induced oxidative/nitrative makers

Figure 8. The metalloporphyrins attenuated MPTP-induced oxidative/nitrative makers

GSH, GSSG, GSH/GSSG and 3-NT in the ventral midbrain of the C57BL/6 mice 24 hours after injection of MPTP (15mg/kg $\times$ 3, s.c., daily) alone or in the presence of AEOL 11114 (A) and AEOL 11203 (B) with i.p. or p.o. route (15mg/kg $\times$ 3, daily). Bars represent mean + S.E.M, n=3–7 mice per group. Statistical analysis: Two-way, ANOVA, (A) interaction: GSH [p=0.061]; GSSG [*p<0.05]; GSH/GSSG [p=0.059]; 3-NT [*p<0.01], Bonferroni posttests, GSH: #p<0.05 MPTP+vehicle versus MPTP+AEOL 11114 i.p., GSSG: #p<0.001 MPTP+vehicle versus MPTP+AEOL 11114 i.p. and p.o.; GSH/GSSG: #p<0.01 MPTP+vehicle versus MPTP+AEOL 11114 i.p., 3-NT: #p<0.001 MPTP+vehicle versus MPTP+AEOL 11114 i.p. and p.o.; (B) interaction: GSH [p=0.053]; GSSG [*p<0.01]; GSH/GSSG [p=0.062]; 3-NT [*p<0.01], Bonferroni posttests, GSH: #p<0.001 MPTP+vehicle versus MPTP+AEOL 11203 i.p., GSSG: #p<0.001 MPTP+vehicle versus MPTP+AEOL 11203 i.p. and p.o.; GSH/GSSG: #p<0.01 MPTP+vehicle versus MPTP+AEOL 11203 i.p., 3-NT: #p<0.001 MPTP+vehicle versus MPTP+AEOL 11203 i.p. and p.o.

Table 1Comparison of Plasma Pharmacokinetic Profiles^{*}

Compound	AEOL11114		AEOL11203	
	i.p	p.o.	i.p	p.o.
Half-life (h)	27 ± 2.9	13 ± 2.0	15 ± 0.6	5.6 ± 1.9
AUC (μmol h/L)	1300 ± 64	430 ± 75	440 ± 15	130 ± 14
Cmax (μM)	28 ± 0.9	7.5 ± 1.0	21 ± 0.5	8.0 ± 0.8
Tmax (h)	6.9 ± 1.2	20 ± 1.2	NA	6 ± 0
Bioavailability (%)	NA	33 ± 6	NA	30 ± 3

^{*} Pharmacokinetic data was obtained using a one compartment model with first order input and first order output (PK analyst, model #3, MicroMath, Salt Lake City, UT).

NA, not applicable

Table 2

Comparison of Brain Pharmacokinetic Profiles *

Compound	AEOL11114		AEOL11203	
	i.p	p.o.	i.p	p.o.
Half-life (h)	2.5 ± 0.7	3.7 ± 1.2	11 ± 4.3	6.5 ± 2.4
AUC (μmol h/L)	20.9 ± 9	9.46 ± 1.21	6.84 ± 0.15	3.14 ± 0.84
Cmax (nM)	490 ± 20	230 ± 11	140 ± 5	50 ± 6.3
Tmax (h)	8.5 ± 1.2	9.9 ± 2.0	11 ± 2.1	15 ± 2.1
Bioavailability (%)	NA	45 ± 6	NA	46 ± 1

* Pharmacokinetic data was obtained using a one compartment model with first order input and first order output (PK analyst, model #3, MicroMath, Salt Lake City, UT).

NA, not applicable

Antioxidant Properties of Metalloporphyrins

Table 3

	AEOL 11114	AEOL 11203	AEOL 11207	AEOL 11209	AEOL 11215	AEOL 11216
SOD Activity (U/mg) *	354	138	124	183	130	98
H ₂ O ₂ IC ₅₀ (nM) **	17	3000	104	17	206	93
TBARS IC ₅₀ (nM) ***	200	700	200	200	6700	4000

* Unit of SOD activity is defined as the amount of metalloporphyrin that inhibits one-half the formation of adrenochrome by superoxide at pH 10.2.

** The concentration of the metalloporphyrin that scavenged 50% of H₂O₂ production.

*** The concentration of metalloporphyrin required to inhibit 50% thiobarbituric acid reactive species (TBARS).