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Intracellular disulfide reduction by phosphine-borane complexes: Mechanism of action for neuroprotection

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ABSTRACT

Phosphine-borane complexes are novel cell-permeable drugs that protect neurons from axonal injury *in vitro* and *in vivo*. These drugs activate the extracellular signal-regulated kinases 1/2 (ERK1/2) cell survival pathway and are therefore neuroprotective, but do not scavenge superoxide. In order to understand the interaction between superoxide signaling of neuronal death and the action of phosphine-borane complexes, their biochemical activity in cell-free and *in vitro* assays was studied by electron paramagnetic resonance (EPR) spectrometry and using an intracellular dithiol reporter that becomes fluorescent when its disulfide bond is cleaved. These studies demonstrated that bis(3-propionic acid methyl ester) phenylphosphine-borane complex (PB1) and (3-propionic acid methyl ester) diphenylphosphine-borane complex (PB2) are potent intracellular disulfide reducing agents which are cell permeable. EPR and pharmacological studies demonstrated reducing activity but not scavenging of superoxide. Given that phosphine-borane complexes reduce cell injury from mitochondrial superoxide generation but do not scavenge superoxide, this implies a mechanism where an intracellular superoxide burst induces downstream formation of protein disulfides. The redox-dependent cleavage of the disulfides is therefore a novel mechanism of neuroprotection.

1. Introduction

Reactive oxygen species (ROS) cause cell death via two disparate mechanisms, direct damage to macromolecules and activation of intracellular pathways that transduce a cell death signal (Simon et al., 2000). In previous studies we demonstrated that superoxide anion is a signaling molecule for death of a specific central nervous system (CNS) neuron, the retinal ganglion cell (RGC), after injury to its axon contained within the optic nerve (Kanamori et al., 2010a, 2010b; Lieven et al., 2006; Scott et al., 2010). Despite near-complete reduction of intracellular levels of superoxide by scavengers such as pegylated superoxide dismutase (PEG-SOD) (Kanamori et al., 2010a) and metallocorroles (Catrinescu et al., 2012; Kanamori et al., 2010b), there is much less neuroprotection of neurons in animal models of optic neuropathy. This disparity between the degree of observed signaling by superoxide and the incomplete neuroprotection when superoxide is scavenged has three possible explanations.

First, the superoxide burst could be a result of apoptosis and not its cause, e.g. if cytochrome c release during apoptosis caused reduction of proximal intermediates in the mitochondrial electron transport chain. The reduced intermediates would react with molecular oxygen to produce superoxide (Cai and Jones, 1998). Against this possibility are data from *in vitro* (Lieven et al., 2012) and longitudinal *in vivo* studies (Kanamori et al., 2010a) demonstrating that the superoxide burst in fact precedes cytochrome c release and

Abbreviations: CNS, central nervous system; DABCO, 1,4-diazobicyclo[2.2.2]octane; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EPR, electron paramagnetic resonance; ERK1/2, extracellular signal-regulated kinases 1/2; P1, bis(3-propionic acid methyl ester) phenylphosphine; P2, (3-propionic acid methyl ester)diphenylphosphine; PAMPA-BBB, parallel artificial membrane permeation assay for the prediction of blood-brain barrier penetration; PAMPA, parallel artificial membrane permeation assay for the prediction of blood-brain barrier penetration; PB1, bis(3-propionic acid methyl ester) phenylphosphine-borane complex; PB2, (3-propionic acid methyl ester) diphenylphosphine-borane complex; PEG-SOD, poly(ethylene glycol)-conjugated superoxide dismutase; RGC, retinal ganglion cell; ROS, reactive oxygen species; TCEP, tris(2-carboxyethyl)phosphine.

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phosphatidylserine externalization, respectively. Second, there are oxidation-independent stress pathways such as the endoplasmic reticulum stress signaling pathways, that operate independently of superoxide signaling. Third, the intracellular superoxide burst could be rapidly followed by activation of the downstream effects of superoxide, e.g. oxidation of one or more critical signaling macromolecules. If scavenging superoxide occurs after the downstream pre-apoptotic pathways are activated, then levels of neuroprotection are likely to be incomplete.

One such downstream target for superoxide induced by axotomy is oxidation of cysteine thiols, with consequent formation of disulfide bonds that modify protein structure and function (Carugo et al., 2003; Park and Raines, 2001). Previous studies demonstrated that the disulfide reducing agent dithiothreitol (DTT) can increase *in vitro* survival of CNS neurons in mixed retinal culture (Geiger et al., 2002). Likewise, studies with tris(2-carboxyethyl)phosphine (TCEP), a disulfide-reducing phosphine, demonstrated that reversing sulfhydryl oxidation prevents neuronal death after axotomy *in vivo* (Geiger et al., 2002) and after optic nerve crush in rats (Swanson et al., 2005). Such results are consistent with disulfide formation being a downstream pathway for cell death induced by axonal injury.

Based on the observed neuroprotection with DTT and TCEP, we synthesized membrane permeable derivatives of TCEP, phosphine-borane complexes bis(3-propionic acid methyl ester) phenylphosphine-borane complex (PB1) and (3-propionic acid methyl ester) diphenylphosphine-borane complex (PB2) (Fig. 1). These compounds have a positive, dose-dependent effect on neuronal viability after axonal injury at concentrations much lower than that of non-derivatized TCEP (Schlieve et al., 2006). PB1 and PB2 are neuroprotective *in vivo* in two rat models of CNS axonal injury, optic nerve transection and experimental glaucoma, with a biological mechanism of action that involves activation of the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway (Almasieh et al., 2011).

Although phosphine itself is toxic, alkyl phosphines are far less toxic. The LD_{50} value of TCEP in rats dosed orally is 3500 mg/kg, and an LD_{50} greater than 1024 mg/kg when administered by i.p injection (Hampton Research, 2016). Previous toxicity testing of PB1 and PB2 *in vitro* and PB1 *in vivo* demonstrated no toxicity to RGCs and retinal endothelial cells up to 1 mM with PB1 and 100 μM with PB2, which are 10^5 and 10^8 times the optimal reducing concentration for neuroprotection (Schlieve et al., 2006).

The phosphine-borane complexes PB1 and PB2 are neuroprotective *in vitro* and *in vivo*, and their structural similarity to TCEP is consistent with an ability to reduce disulfide bonds. Yet their biochemical mechanism of action could result from either scavenging of the upstream superoxide burst that signals neuronal death or reduction of intracellular disulfides. Inhibition of either pathway would be associated with increased neuronal survival. In order to distinguish these two possibilities, electron paramagnetic resonance, intracellular disulfide reducing probes, and superoxide assays were used to assess the biochemical effects of these neuroprotective molecules. Their pharmacological characteristics were tested in order to determine whether they would be able to penetrate the blood-brain barrier and cell membranes, consistent with *in vivo* neuroprotective activity. Their reducing activity was compared to DTT and TCEP at various pH to determine their activity at a biologically relevant pH. Phosphine-borane complexes were potent intracellular reducing agents, with pharmacological and pharmaceutical properties that would predict activity as CNS neuroprotectants.

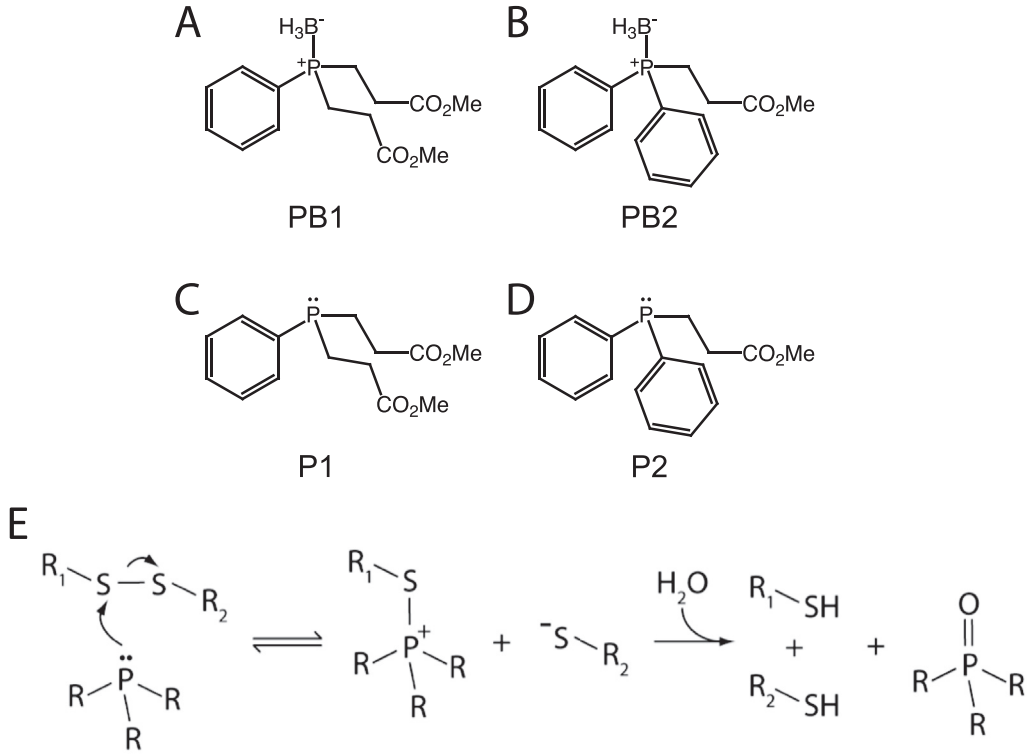

Fig. 1. Biochemistry of phosphine-borane complexes used in the studies. (A) bis(3-propionic acid methyl ester) phenylphosphine-borane complex (PB1); **(B)** (3-propionic acid methyl ester) diphenylphosphine-borane complex (PB2); **(C)** bis(3-propionic acid methyl ester) phenylphosphine (P1); **(D)** (3-propionic acid methyl ester) diphenylphosphine (P2). **(E)** Mechanism of reduction of disulfide by phosphine in water, modified from Burns et al. (1991). Panels A and B are redrawn from Schlieve et al. (2006).

2. Material and methods

2.1. Chemicals

Porcine polar brain lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Dithiothreitol (DTT), 1,4-diazobicyclo[2.2.2]octane (DABCO), and dodecane were obtained from Acros Organics (Geel, Belgium). Poly(ethylene glycol)-conjugated superoxide dismutase (PEG-SOD), xanthine, xanthine oxidase, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), tris(2-carboxyethyl)phosphine (TCEP), and dimethylformamide were from Sigma (St Louis, MO). Hydroethidine was from Invitrogen (Carlsbad, CA). The fluorescent dithiol probe DSSA-1 was a kind gift of Dr. Daniel Sem of Marquette University (Milwaukee, WI). 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was from Radical Vision (Marseilles, France). Unless otherwise stated, all other reagents were from Fisher Scientific (Pittsburgh, PA).

2.2. Synthesis of phosphines

PB1 and PB2 were synthesized according to our published methods (Schlieve et al., 2006) at the Keck-University of Wisconsin Comprehensive Cancer Center Small Molecule Screening Facility (Madison, WI). Unprotected phosphines bis(3-propionic acid methyl ester) phenylphosphine (P1) and (3-propionic acid methyl ester)diphenylphosphine P2, identical to PB1 and PB2 in all but the presence of the protective borane group, were synthesized as follows: P1 was synthesized as the stable intermediate of PB1 synthesis (Schlieve et al., 2006). P2 was synthesized by a method analogous to the published method for PB2 synthesis. Diphenylphosphine (0.190 g) was dissolved in methanol (8 ml) in a flame-dried round bottom flask under argon at room temperature. Potassium hydroxide was added to this mixture, followed by the drop-wise addition of methyl acrylate (0.108 mL). The reaction mixture was allowed to stir at room temperature for 6 h, after which the methanol was removed en vacuo. The white residue was taken up in dichloromethane (10 mL) and washed with 0.5 N HCl (1×5 mL) and brine (1×5 mL). The aqueous layers were washed with dichloromethane (10 mL), and the combined organic layers were dried over MgSO4(s), filtered, and concentrated in vacuo. The residue was purified by flash chromatography (silica gel, 30% v/v ethyl acetate in hexanes). P2 was isolated as a pale yellow oil (0.219 g, 0.76 mmol, 76% yield).

2.3. Measurement of In vitro reducing activity

Solid PB1 and PB2 were dissolved in nitrogen-purged 10 mM DABCO in dimethylformamide to a final concentration of 10 mM and borane-deprotected by refluxing at 60 °C for 2 h. Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) at 30 mM in methanol was reacted with various concentrations of DTT, TCEP, PB1, or PB2 in buffer at pH 5, 7, and 9 in a 96-well plate for 5 min at room temperature. Reduction of DTNB was detected by spectrophotometry at 405 nm using a ThermoMax plate reader (Molecular Devices, Sunnyvale, CA).

2.4. Measurement of intracellular reducing activity

RGC-5 cells (gift of Neeraj Agarwal, Ph.D.) are a cell line derived from retina neurons that despite their name are most similar to the 661W cone photoreceptor cell line, a type of retinal neuron (Al-Ubaidi, 2014; Krishnamoorthy et al., 2013; Thompson et al., 2015). They were incubated in 20 μM DSSA-1 for 1 h. Cells were thoroughly rinsed 5 times with Hanks balanced salt solution to remove extracellular probe, and PB1, PB2 or TCEP added. Serial fluorescent micrographs images were longitudinally captured over 60 min with an Axiovert 135 microscope (Carl Zeiss, Dublin, CA) equipped with a Nikon D5000 DSLR camera (Nikon, Melville, NY), and analyzed off-line using NIH ImageJ. Images were processed to yield mean fluorescence of individual cells. Images were processed to identify individual cells with the ImageJ Auto Threshold plug-in, choosing the thresholding algorithm selected on a picture-by-picture basis between Li (22 images), Moments (32 images), Huang (1 image), and Triangle (1 image) in order to achieve the greatest combination of cell detection and separation of individual cells. The intensities of the identified cells were then measured, and non-normalized intensities averaged across three separate experiments to assess the extent of probe reduction.

2.5. Superoxide detection by oxidation of hydroethidine

For fluorescence assays, superoxide was generated by reaction of xanthine (1 mM) with xanthine oxidase (0.05 U/mL) and detected by fluorescence of its reaction product with hydroethidine (1 mM), 2-hydroxyethidium (excitation 396 nm, emission 590 nm), on a Wallac 1420 VICTOR 2 T Multilabel Counter (PerkinElmer, Inc., Wellesley, MA). Reactions were carried out at room temperature in the presence or absence of PEG-SOD, PB1, or PB2.

2.6. Superoxide detection by electron paramagnetic resonance

Superoxide was generated by reaction of hypoxanthine (200 μM) with xanthine oxidase (0.0025 U/mL) in the presence of the spin trap DEPMPO. A reference spectrum for the hydroxyl adduct was generated through the Fenton reaction of FeSO4 (100 μM), diethylenetriaminepentacetic acid (100 μM), and hydrogen peroxide (1 mM) in the presence of DEPMPO (100 mM). The formation of superoxide-DEPMPO and hydroxyl-DEPMPO adducts were monitored using electron paramagnetic resonance (EPR) spectrometry. EPR spectra were recorded at room temperature using a Bruker EMX spectrometer operating at 9.85 GHz and equipped with a Bruker ER 4119HS-WI high sensitivity resonator. Typical spectrometer parameters were: scan range 100 G, time constant 1.28 ms, sweep time 42 s, modulation amplitude 1.0 G, modulation frequency 100 kHz, microwave power 20 mW. Micropipettes (50 μL) were used as sample tubes. Spectra were averaged over 5 scans. Observations were repeated 2–3 times.

2.7. Aqueous solubility

One mg of pure compound was dissolved in 10 mL ultrapure water and mixed by inversion for 72 h. Samples were taken at 48 and 72 h, and solubility was determined by detection of PB1 and PB2 in solution using high performance liquid chromatography (ThermoFinnigan SpectraSYSTEM P4000 quaternary pump; AS3000 autosampler, UV2000 detector; and Shimadzu CR501 Chromatopac integrator. Phenomenex Luna C_{18}, 5 μ, 250 × 4.6 mm column) with the following program: 5% acetonitrile/95% ddH_2O 0–5 min, 50% acetonitrile/50% ddH_2O 15–45 min, 5% acetonitrile/95% ddH_2O 50 min-hold.

2.8. Octanol/water partitioning

The method of Sohn and Park (2003) was used with minor modification to determine the partitioning coefficients of PB1 and PB2. Equal volumes of octanol and milliQ deionized water were mixed by inversion for 30 min and allowed to equilibrate overnight at room temperature. PB1 or PB2 (1 mg) were dissolved in 5 mL of equilibrated octanol and mixed by inversion with an equal amount of equilibrated water overnight. Samples were allowed to

equilibrate for 24 h, and then concentrations in octanol were determined by absorbance at 265 nm using a NanoDrop-1000 spectrophotometer. Partitioning coefficients were determined by comparison to absorbance of unmixed octanol controls.

2.9. Blood-brain barrier permeability

A parallel artificial membrane permeation assay for the prediction of blood-brain barrier penetration (PAMPA-BBB) was used to determine log P_e for predicted blood-brain barrier permeability of PB1 and PB2. Several groups have shown accurate prediction of blood-brain barrier permeability using this assay (Di et al., 2003). Artificial membranes were created on a 96-well filter plate using porcine polar brain lipids dissolved in dodecane (20 mg/mL). PB1 or PB2 (200 μM) dissolved in 200 μL milliQ deionized water was placed in the donor well above the membrane, and 300 μL pure water was placed in the acceptor well below the membrane. Concentration of compound in acceptor wells after 18 h at room temperature was assessed by absorbance at 265 nm using a NanoDrop-1000 spectrophotometer. Comparison to absorbance of equilibrium mixtures of 200 μL PB1 or PB2 solutions with 300 μL water were then used to determine log P_e. Three independent experiments were performed, each with 3–6 replicates.

2.10. Accelerated stability

The method of Sohn and Park (2003) was used with minor modification to determine the accelerated stability of PB1 and PB2. Pure PB1 or PB2 (4 mg) were incubated in sand baths at 40 °C and 60 °C for 37 and 125 days. 0.5 mg from each sample was dissolved in acetonitrile and concentration of undegraded compound determined by high performance liquid chromatography as above.

2.11. Statistical analyses

Comparison between two groups was by two-sample Student's t-test. Comparison of a group with a population mean was by one-sample Student's t-test. Comparison of more than 2 groups was by ANOVA followed by *post hoc* testing with Tukey-Kramer HSD. All data are reported as mean ± SEM.

3. Results

3.1. Reduction of disulfide bonds in cell-free assays

Phosphine-borane complexes were hypothesized to reduce disulfide bonds, similar to the activity seen with the parent molecule TCEP. Such an activity could explain the *in vivo* efficacy of PB1 and PB2 in optic nerve transection and experimental glaucoma (Almasieh et al., 2011). To study this, the protective borane group was chemically removed with DABCO (Brisset et al., 1993) and the *in vitro* reactivity of deprotected PB1 and deprotected PB2 measured spectrophotometrically with DTNB, a colorless disulfide that becomes yellow when reduced). There was a linear relation between concentration and reducing power for each compound (Fig. 2, representative of 3 independent experiments). Deprotected PB2 had the highest reducing capability at pH 7, possibly explaining its activity at very low (picomolar to nanomolar concentrations) in cultured neurons (Schlieve et al., 2006). Deprotected PB1 was a less potent reducing agent than PB2, but was minimally affected by changes in pH values. The non-deprotected (borane-complexed) forms PB1 and PB2 did not reduce disulfides in cell-free assays.

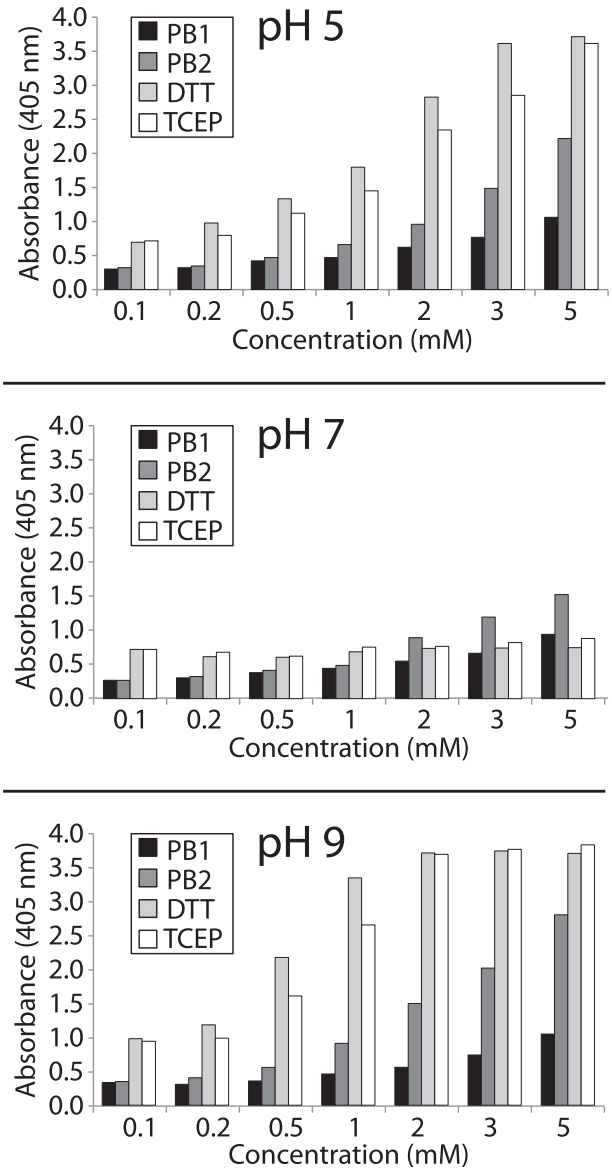

Fig. 2. Phosphine-borane complexes are disulfide reducing agents at physiological pH. PB1, PB2, DTT, and TCEP (0.1, 0.2, 0.5, 1, 2, 3, 5 mM) were reacted with 7.5 mM DTNB at pH 5, 7, and 9. While all four compounds reduced disulfides more effectively at pH 5 and pH 9, PB2 reduced disulfides more effectively than DTT or TCEP at the biologically relevant pH of 7. Results are representative of three independent experiments.

3.2. Intracellular reduction of disulfide bonds in vitro

Given that PB1 and PB2 were chemically able to reduce disulfides when deprotected, the ability of these phosphines to enter cells and reduce disulfides when the borane was present was studied. To establish whether PB1 and PB2 were able to reduce disulfide bonds of proteins within cells, the intracellular redox sensitive probe DSSA-1 (Pullela et al., 2006) was used. DSSA-1 becomes fluorescent (excitation 485–495 nm, emission 516–525 nm) when reduced (Fig. 3A). Cells were loaded with DSSA-1 and incubated with to PB1, PB2, or TCEP. Addition of 10 μM PB1 for 1 h resulted in an increase in mean cell fluorescence intensity compared to DSSA alone (26.7 ± 0.3 vs. 22.3 ± 0.2; $p < 0.001$; Fig. 3B–C, based on mean of 3 independent experiments). A 1 h incubation with PB2 (10 μM) induced a greater increase in intracellular reduction of DSSA-1 (36.6 ± 0.2 vs. 22.3 ± 0.2; $p < 0.001$).

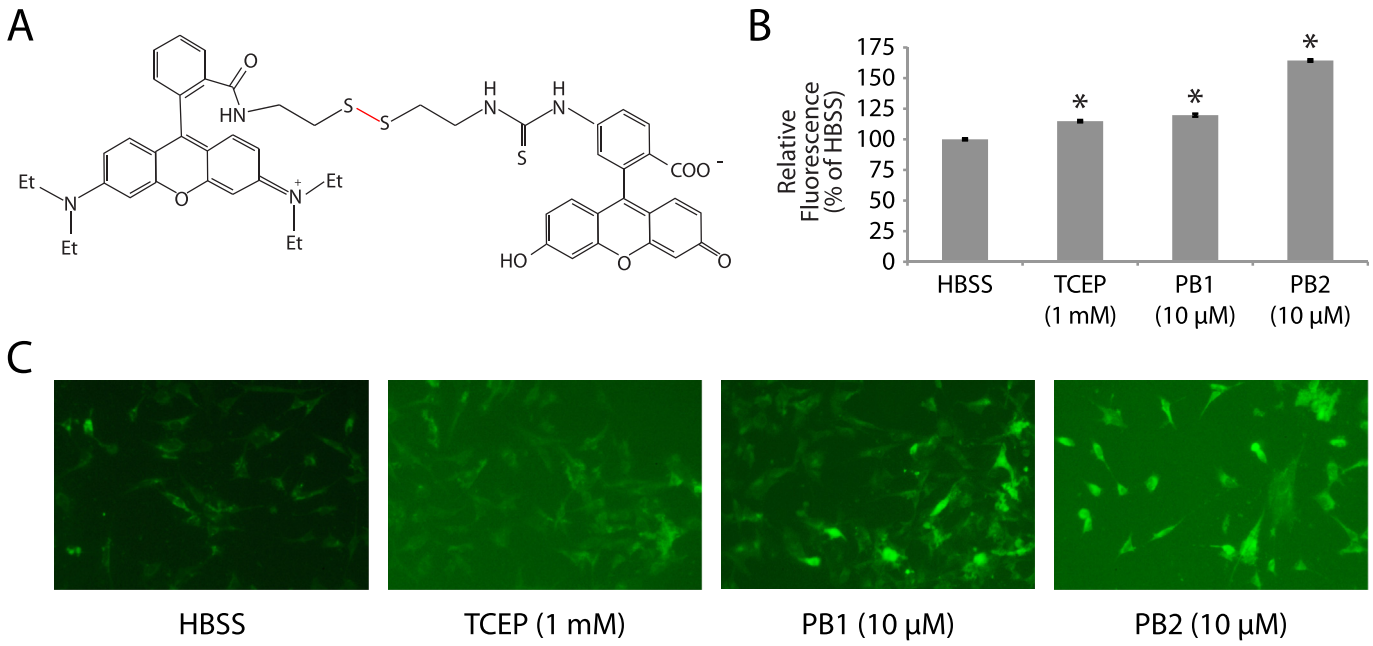

Fig. 3. Phosphine-borane complexes are intracellular disulfide reducing agents *in vitro*. **(A)** DSSA-1 is a redox-sensitive probe, which fluoresces upon reduction of its disulfide linkage. **(B)** Cells preloaded with DSSA-1 showed a significant increase in fluorescence upon treatment with PB1 and PB2 after 1 h. **(C)** Representative micrographs of DSSA-1 fluorescence after treatment with PB1, PB2, TCEP, and media alone. Results are the means of three independent experiments ± SEM. Differences in background reflect reduction of DSSA-1 in the medium, and not differences in camera exposure.

Treatment with 1 mM TCEP for 1 h resulted in an increase in intracellular brightness (25.6 ± 0.2 vs. 22.3 ± 0.2; $p < 0.001$) that was less pronounced than what was observed with PB1 or PB2, despite a 100-fold increase in concentration of reductant.

3.3. Superoxide scavenging

Reactivity with endogenous superoxide anion is a theoretical alternative mechanism of action for the neuroprotective effect of phosphine-borane complexes. To test this hypothesis, superoxide was generated using xanthine/xanthine oxidase and detected by fluorescence of 2-hydroxyethidium. PB1 and PB2 did not appreciably scavenge superoxide when compared to PEG-SOD control (Fig. 4A, representative of 3 independent experiments), confirming previous experiments (Seidler et al., 2010).

Since 2-hydroxyethidium fluorescence is not completely specific for the reaction product of HEt and superoxide (Zhao et al., 2005), electron paramagnetic resonance (EPR) was used as an alternate method for detecting superoxide. P1 and P2, corresponding to PB1 and PB2 but without the protective borane groups, were synthesized and their effect on superoxide measured by EPR. The spin trap DEPMPO was used to detect superoxide generated by xanthine/xanthine oxidase. This produces a signal characteristic of the superoxide adduct (DEPMPO/OOH) (Fig. 4B), and is readily distinguishable from the hydroxyl adduct generated by the Fenton reaction (Fig. 4C). The presence of SOD (30 U/mL) eliminated the signal from superoxide (Fig. 4D). Addition of P1 or P2 did not diminish the spectra of superoxide (Fig. 4E and F), and instead, the observed spectra were identical to that of DEPMPO/OH, the hydroxyl adduct of DEPMPO. The same result was seen with the addition of catalase, indicating that the hydroxyl-DEPMPO adduct was not generated by the Fenton reaction from H_2O_2. Results are representative of 2 independent experiments. Together, these results indicate that these phosphines are reducing agents and not superoxide dismutase mimetics, with activity analogous to the glutathione-dependent reduction of a hydroperoxide to an alcohol (Karoui et al., 1996).

3.4. Pharmacological properties

Despite demonstration of disulfide-reducing activity by these phosphine-borane complexes in cell-free and *in vitro* models, there was little evidence that their pharmacological properties were consistent with their observed neuroprotective activity *in vivo* in models of CNS axonal injury, optic nerve transection and experimental glaucoma (Almasieh et al., 2011). To address this disparity, their pharmacological properties relevant to CNS activity and use as clinical neuroprotectants were studied.

The ability to penetrate the blood-brain barrier is critical to pharmacological activity in the CNS. Using an artificial membrane system of porcine brain lipids (PAMPA-BBB), log P_e was as −4.93 ± 0.04 for PB1 and −5.00 ± 0.06 for PB2. Both values are significantly ($p = 0.008$ and 0.025, respectively) more permeable than the threshold (−5.34) designated as CNS-penetrant (Di et al., 2003). Water solubility critically influences the route by which a drug can be administered as well as its absorption and distribution in the body. Detection of compound dissolved in water over 72 h was by high performance liquid chromatography. PB1 was soluble in water up to 237.8 µM, and PB2 was soluble in water up to 3.7 µM, demonstrating limited water solubility.

Hydrophobicity is another important parameter for structure-activity relations and biological activity of a compound. Using octanol/water partitioning, log $P_{oct/wat}$ was 0.6 (somewhat hydrophobic) for PB1 and 1.1 (highly hydrophobic) for PB2, signifying high potential biological activity. Long-term stability is correlated with accelerated stability at elevated temperatures. Heated samples were maintained over a period of 125 days and then analyzed by high performance liquid chromatography. Peak areas for PB1 at 40 °C and 60 °C were 80% and 72% of control respectively at 37 days. PB2 peak areas were 100% of control for both conditions at 37 days. At 125 days, the peak area for PB1 at 40 °C was 58% and PB2 was 86%; at 60 °C peak areas for PB1 and PB2 were 12% and 14% respectively (Fig. 5, representing 1 experiment). Extrapolation using the Arrhenius equation yielded a calculated half-life for solid PB1 of 2 years and for solid PB2 of 18 years at 20 °C. Finally, both

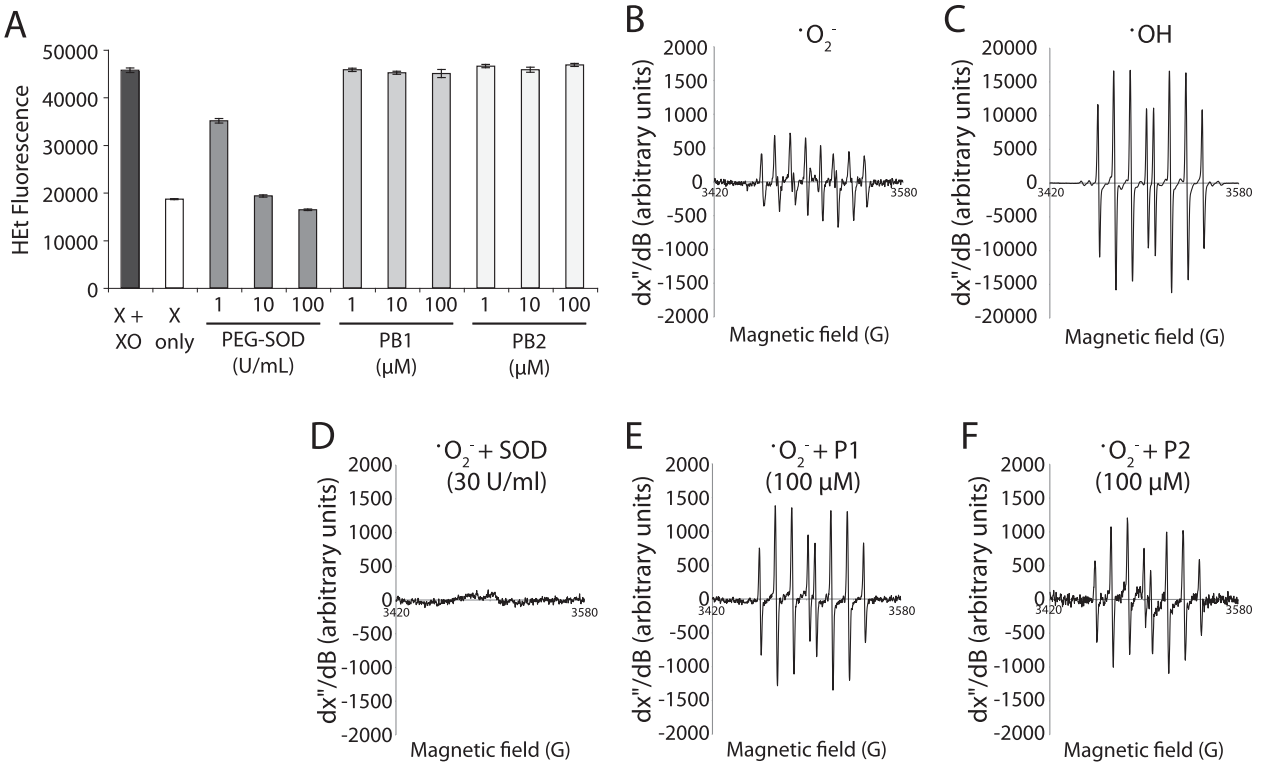

Fig. 4. PB1 and PB2 do not scavenge superoxide. (A) PB1 and PB2 at 1, 10, and 100 μM, and PEG-SOD at 1, 10, and 100 U/mL were allowed to react with superoxide radical generated by the reaction of 0.05 U/mL xanthine oxidase (XO) with 1 mM xanthine (X). Superoxide was measured as fluorescence of its reaction product with hydroethidine, 2-hydroxyethidium, at 465 nm and results are reported as the mean ± SEM. Asterisk indicates $p < 0.05$ compared to X/XO. **(B)** EPR spectroscopy confirmed that superoxide is generated by xanthine oxidase, and it can be distinguished from hydroxyl radical **(C)** with the spin trap DEPMPO. **(D)** Carrying out the reaction in the presence of 30 U/mL SOD eliminated the spectra. The presence of P1 **(E)** and P2 **(F)**, borane-deficient versions of PB1 and PB2 respectively, convert the spectra to that of the hydroxyl adduct of DEPMPO, consistent with a reduction mechanism. Hydroethidine results are representative of 3 independent experiments. EPR spectroscopy results are representative of two independent experiments.

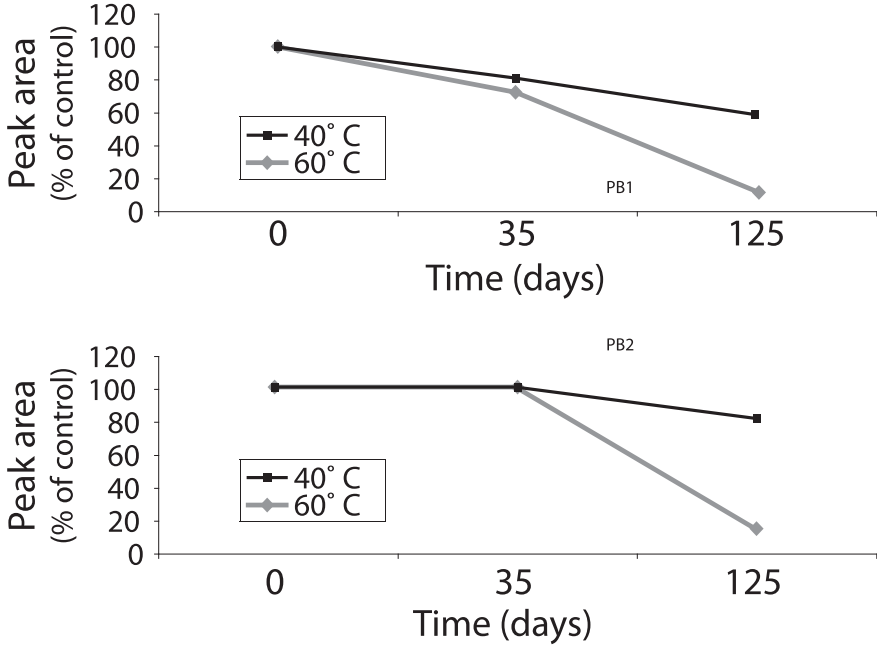

Fig. 5. PB1 and PB2 are stable under accelerated stability conditions. Peak areas for PB1 at 40 °C and 60 °C were 80% and 72% of control respectively at 37 days. PB2 peak areas were 100% of control for both conditions at 37 days. At 125 days, the peak area for PB1 at 40 °C was 58% and PB2 was 86%; at 60 °C peak areas for PB1 and PB2 were 12% and 14% respectively. Results are from 1 experiment.

PB1 and PB2 met the requirements of Lipinksi's Rule of 5 for druglikeness (Ghose et al., 1999; Lipinski et al., 2001): having fewer than 5 hydrogen bond donors, fewer than 10 hydrogen bond acceptors, a log P_e between −0.4 and 5.6, and a molecular weight between 160 and 480.

4. Discussion

4.1. Mechanism of action

These results provide a mechanism by which phosphine-borane complexes are able to protect CNS cells from injury. Use of a fluorescent dithiol reporter demonstrate that this class of drugs is able to enter cells and reduce intracellular disulfides. CNS-specific PAMPA-BBB assays showed that they penetrate brain lipids, consistent with their observed neuroprotective potency *in vivo* (Almasieh et al., 2011). Finally, although phosphine-borane complexes inhibit a pathway for neuronal death that is superoxide-dependent (Kanamori et al., 2010a; Lieven et al., 2006; Scott et al., 2010), EPR and fluorescent reporter experiments indicate that they are not superoxide scavengers but rather disulfide reducing agents.

Previous studies demonstrated that axonal injury is associated with a superoxide burst within the RGC soma (Kanamori et al., 2010a; Lieven et al., 2006). Consistent with those findings, knockdown of the endogenous mitochondrial superoxide scavenger superoxide dismutase-2 increases neuronal cell death (Scott et al., 2010). In the present study PB1 and PB2 had no superoxide scavenging activity, as manifested by absence of decrease in superoxide produced in a xanthine/xanthine oxidase system and detected by 2-hydroxyethidium fluorescence or electron paramagnetic resonance. The partial rescue of retinal ganglion cell death with PB1 and PB2 seen in *in vitro* (Schlieve et al., 2006) and *in vivo* (Almasieh et al., 2011) is therefore likely the result of restoration of redox homeostasis by disulfide reduction and not superoxide scavenging. Given that phosphine-borane complexes inhibit neuronal death induced by elevations of superoxide in mitochondria external to the matrix (Seidler et al., 2010), yet do not scavenge superoxide, this implies that their disulfide-reducing activity is downstream of superoxide generation.

Phosphines reduce protein disulfides through nucleophilic attack by the reactive lone-pair electrons of the phosphorous. Following hydrolysis this results first in a thiophosphonium salt and then a free thiol and oxidized phosphine (Cline et al., 2004). PB1 and PB2 were equally or more effective at reducing the disulfide bond of DTNB at pH 7 than equimolar concentrations of commonly used reducing agents DTT and TCEP. PB1 and PB2 were also able to reduce intracellular disulfides on a shorter time scale than TCEP in tissue culture. P1 and P2, similar to PB1 and PB2 but without borane protection, had strong reducing activity by EPR spectroscopy, converting the superoxide adduct of the spin trap to a hydroxyl adduct.

The increased effectiveness of PB1 and PB2 compared to the parent compound TCEP results from specific structural modifications. Borane protection of the reactive phosphine protects the compounds from extracellular oxidation. Intracellular phosphine reactivity is restored when the borane protecting group is removed by tertiary amines within tissues. Replacement of TCEP's organic acid groups by methyl esters results in intracellular sequestration upon polarization of the molecule due to cleavage by esterases. In addition, replacement of organic acid groups by phenyl rings results in increased cell membrane permeability and lower steric hindrance of the lone-pair phosphorous electrons. Overall, these modifications result in their concentration within cells where they can interact with accessible protein thiols. This allows effective reduction of critical disulfides formed as a result of oxidation by superoxide or other reactive oxygen species.

4.2. Upstream vs. downstream therapies

A critical issue with respect to cytoprotective therapies is the timing between the onset of the injury, the commitment point for dying (i.e. "point of no return"), and the point at which cell death becomes manifest. If a drug only becomes bioavailable after the commitment point, then it will not be cytoprotective, even if administered long before cell death is manifest. This is analogous to the fuse of a bomb, which will continue to burn even if the match that ignited it is extinguished. In the case of cytoprotective therapies, those that act on proximal or upstream pathways in cell death need to be given early to be effective. Those that act on distal or downstream pathways in cell death can be given later. The relation between injury and the timing of cytoprotection is critical to clinical trial design (Levin, 2004).

The neuroprotective action of phosphine-borane complexes is associated with activation of ERK1/2 (Almasieh et al., 2011), a downstream mechanism that also underlies the neuroprotection mediated by brain-derived neurotrophic factor and the TrkB receptor (Cheng et al., 2002). Activation of this pathway with PB1 protects both the somal and axonal compartment after axonal injury in two different animal models (Almasieh et al., 2011). The intermediate steps between the reduction of a disulfide(s) and the activation of the ERK1/2 pathway are not known, and presumably reflect interruption of a cascade of redox signaling in one or more critical death pathways, initiated by superoxide-dependent disulfide oxidation of key protein(s). Although c-Jun N-terminal kinase (JNK) signaling is critical to RGC death after axonal injury (Fernandes et al., 2012), the lack of effect of PB1 on phospho-JNK1/2/3 levels indicates that its prosurvival effect bypasses JNK (Almasieh et al., 2011).

The paradigm of upstream and downstream therapies can be applied to the case of axonal injury in the CNS (Fig. 6). Our previous work demonstrated that optic nerve transection activated an intracellular pathway which caused elevated levels of superoxide within the soma of the RGC (Kanamori et al., 2010a; Lieven et al., 2006). This superoxide-dependent pathway is similar to that seen in growth factor-deprived sympathetic neurons (Greenlund et al., 1995) and renal proximal tubular cells (Lieberthal et al., 1998). It likely represents an upstream pathway for cell death because it precedes the appearance of cell death markers such as phosphatidylserine externalization (Kanamori et al., 2010a) and cytochrome c release (Lieven et al., 2012). However, cell-permeant superoxide scavengers such as pegylated superoxide dismutase (Kanamori et al., 2010a) or iron(III) 2,17-bis-sulfonato-5,10,15-tris(pentafluorophenyl)corrole (Fe(tpfc)(SO(3)H)(2)) (Fe-corrole) (Catrinescu et al., 2012) only incompletely protect RGCs against axonal injury.

The present study provides a mechanism by which phosphine-borane complex drugs would be more efficacious than superoxide scavenging, based on the fact that they chemically reduce protein disulfides which form under oxidative conditions (Fig. 6). This redox chemistry is downstream of superoxide and other reactive oxygen species, and therefore its reversal would be effective even if late in the course of neuronal pathophysiology. Furthermore, if the reactivity of reactive oxygen species with sulfhydryl groups on critical signaling molecules is rapid, then this would help explain why clinical trials based on scavenging superoxide and other oxidative molecules fail to demonstrate efficacy (Louwerse et al., 1995; Shuaib et al., 2007; Parkinson Study Group, 1993). It is unknown whether therapies such as phosphine-borane complexes are sufficiently downstream to be efficacious in randomized clinical

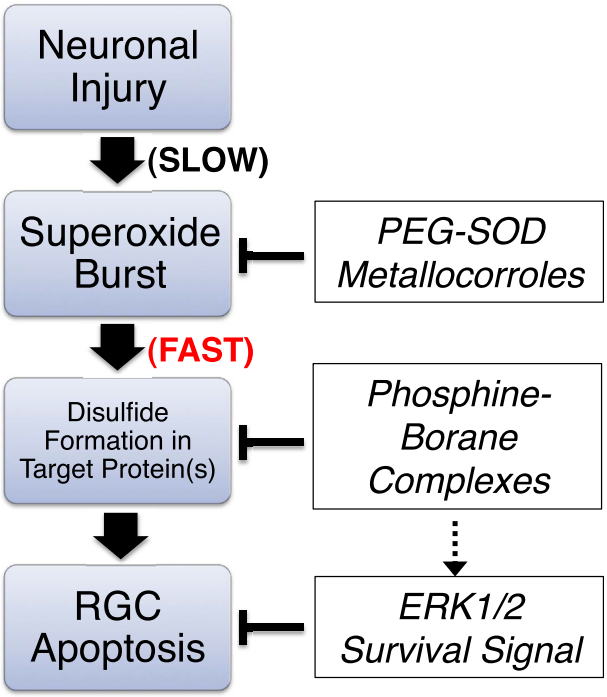

Fig. 6. Schematic for theoretical pathway linking neuronal injury with cell death via superoxide and formation of disulfide bonds. The effect of phosphine-borane complexes on the signaling of retinal ganglion cell death after axonal injury is distal to an oxidative burst, with axonal injury inducing a burst of superoxide within the retinal ganglion cell (RGC) soma (Kanamori et al., 2010a; Lieven et al., 2006). The time course for superoxide induction is slow, and even a small amount of residual superoxide after scavenging will induce downstream oxidative disulfide formation and subsequent neuronal death. Inhibiting downstream signaling events with phosphine-borane complexes (Almasieh et al., 2011) reduces cell death by reducing critical disulfides and indirectly inducing activation of prosurvival ERK1/2 pathways.

trials, although their ability to chemically reduce protein disulfides and protect neurons in animal models of traumatic optic neuropathy and glaucoma is encouraging (Almasieh et al., 2011). It is also possible that combined upstream and downstream therapies (e.g. a superoxide scavenger and a phosphine-borane complex reducing agent) would be more effective than either alone.

4.3. Neuroprotection vs. axoprotection

Therapies for primary axonal diseases are necessarily different from diseases where the initial site of injury is the cell body because protection of the cell body may not be sufficient to rescue the injured axon. This issue of somaprotection vs. axoprotection has been reviewed in the past (Levin, 2001; Schwartz et al., 1999), and is relevant to the discovery of effective neuroprotective therapies. For example, it is possible to completely block RGC death by knocking out *bax*, resulting in preservation of the cell body in experimental glaucoma yet not preventing axonal degeneration (Howell et al., 2007; Libby et al., 2005). Conversely, slowing down axonal degeneration in the Wallerian degeneration slow (Wld^S) rat does not prevent loss of the cell body in experimental glaucoma (Beirowski et al., 2008).

In the case of phosphine-borane complexes, PB1 delay both cell body and axonal loss in optic nerve transection and experimental glaucoma (Almasieh et al., 2011). This indicates that the disulfide-reduction mechanism of action either inhibits the early pathology at the site of injury (the axon) and/or interrupts a subsequent signaling process for axonal and cell body degeneration. Support for the latter alternative is the mechanism by which the Wld^S mutation prevents axonal degeneration. These animals have a fusion of nicotinamide mononucleotide adenylyl transferase 1 (NMNAT-1) to ubiquitination factor e4b (UBE4B) by an 18 amino acid linkage. The mechanism of Wld^S axoprotection involves increased axonal NMNAT-1 after axotomy (Babetto et al., 2010). NMNAT-1 is critical to synthesis of NAD^+, a key electron-accepting redox agent, and it is possible that one of the redox reactions by which elevated local NAD^+ protects axons interacts with phosphine-borane complex redox chemistry.

In summary, phosphine-borane complexes interfere with injury-mediated signals by reducing intracellular disulfide(s) and not by superoxide scavenging. Pharmacological characterization of these molecules is consistent with their being candidates for use in the clinic based on their mechanism of action in optic nerve disease, lipophilic character, blood-brain barrier permeability, and accelerated stability. These drugs may have efficacy and utility for treating diseases where there is upstream superoxide signaling of cell death.

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References

Al-Ubaidi, M.R., 2014. RGC-5: are they really 661W? the saga continues. Exp. Eye Res. 119, 115.
Almasieh, M., Lieven, C.J., Levin, L.A., Di Polo, A., 2011. A cell-permeable phosphine-borane complex delays retinal ganglion cell death after axonal injury through activation of the pro-survival extracellular signal-regulated kinases 1/2 pathway. J. Neurochem. 118, 1075–1086.
Babetto, E., Beirowski, B., Janeckova, L., Brown, R., Gilley, J., Thomson, D., Ribchester, R.R., Coleman, M.P., 2010. Targeting NMNAT1 to axons and synapses transforms its neuroprotective potency in vivo. J. Neurosci. 30, 13291–13304.
Beirowski, B., Babetto, E., Coleman, M.P., Martin, K.R., 2008. The WldS gene delays axonal but not somatic degeneration in a rat glaucoma model. Eur. J. Neurosci. 28, 1166–1179.
Brisset, H., Gourdel, Y., Pellon, P., Le Corre, M., 1993. Phosphine-borane complexes; direct use in asymmetric catalysis. Tetrahedron Lett. 34, 4523–4526.
Burns, J.A., Butler, J.C., Moran, J., Whitesides, G.M., 1991. Selective reduction of disulfides by tris(2-carboxyethyl)phosphine. J. Org. Chem. 56, 2648–2650.
Cai, J., Jones, D.P., 1998. Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss. J. Biol. Chem. 273, 11401–11404.
Carugo, O., Cemazar, M., Zahariev, S., Hudaky, I., Gaspari, Z., Perczel, A., Pongor, S., 2003. Vicinal disulfide turns. Protein Eng. 16, 637–639.
Catrinescu, M.M., Chan, W., Mahammed, A., Gross, Z., Levin, L.A., 2012. Superoxide signaling and cell death in retinal ganglion cell axotomy: effects of metallocorroles. Exp. Eye Res. 97, 31–35.
Cheng, L., Sapieha, P., Kittlerova, P., Hauswirth, W.W., Di Polo, A., 2002. TrkB gene transfer protects retinal ganglion cells from axotomy-induced death in vivo. J. Neurosci. 22, 3977–3986.
Cline, D.J., Redding, S.E., Brohawn, S.G., Psathas, J.N., Schneider, J.P., Thorpe, C., 2004. New water-soluble phosphines as reductants of peptide and protein disulfide bonds: reactivity and membrane permeability. Biochemistry 43, 15195–15203.
Di, L., Kerns, E.H., Fan, K., McConnell, O.J., Carter, G.T., 2003. High throughput artificial membrane permeability assay for blood-brain barrier. Eur. J. Med. Chem. 38, 223–232.
Fernandes, K.A., Harder, J.M., Fornarola, L.B., Freeman, R.S., Clark, A.F., Pang, I.H., John, S.W., Libby, R.T., 2012. JNK2 and JNK3 are major regulators of axonal injury-induced retinal ganglion cell death. Neurobiol. Dis. 46, 393–401.
Geiger, L.K., Kortuem, K.R., Alexejun, C., Levin, L.A., 2002. Reduced redox state

allows prolonged survival of axotomized neonatal retinal ganglion cells. Neuroscience 109, 635–642.
Ghose, A.K., Viswanadhan, V.N., Wendoloski, J.J., 1999. A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. J. Comb. Chem. 1, 55–68.
Greenlund, L.J., Deckwerth, T.L., Johnson, E.M., 1995. Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. Neuron 14, 303–315.
Hampton Research, 2016. Material Safety Data Sheet for Tris(2-carboxyethyl) Phosphine Hydrochloride. Accessed April 26, 2016. http://hamptonresearch.com/documents/product/hr007314_2-651_sds.pdf.
Howell, G.R., Libby, R.T., Jakobs, T.C., Smith, R.S., Phalan, F.C., Barter, J.W., Barbay, J.M., Marchant, J.K., Mahesh, N., Porciatti, V., Whitmore, A.V., Masland, R.H., John, S.W., 2007. Axons of retinal ganglion cells are insulted in the optic nerve early in DBA/2J glaucoma. J. Cell Biol. 179, 1523–1537.
Kanamori, A., Catrinescu, M.M., Kanamori, N., Mears, K.A., Beaubien, R., Levin, L.A., 2010a. Superoxide is an associated signal for apoptosis in axonal injury. Brain 133, 2612–2625.
Kanamori, A., Catrinescu, M.M., Mahammed, A., Gross, Z., Levin, L.A., 2010b. Neuroprotection against superoxide anion radical by metallocorroles in cellular and murine models of optic neuropathy. J. Neurochem. 114, 488–498.
Karoui, H., Hogg, N., Frejaville, C., Tordo, P., Kalyanaraman, B., 1996. Characterization of sulfur-centered radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite. ESR-spin trapping and oxygen uptake studies. J. Biol. Chem. 271, 6000–6009.
Krishnamoorthy, R.R., Clark, A.F., Daudt, D., Vishwanatha, J.K., Yorio, T., 2013. A forensic path to RGC-5 cell line identification: lessons learned. Invest Ophthalmol. Vis. Sci. 54, 5712–5719.
Levin, L.A., 2001. Relevance of the site of injury of glaucoma to neuroprotective strategies. Surv. Ophthalmol. 45, S243–S249.
Levin, L.A., 2004. Extrapolation of animal models of optic nerve injury to clinical trial design. J. Glaucoma 13, 1–5.
Libby, R.T., Li, Y., Savinova, O.V., Barter, J., Smith, R.S., Nickells, R.W., John, S.W., 2005. Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. PLoS Genet. 1, 17–26.
Lieberthal, W., Triaca, V., Koh, J.S., Pagano, P.J., Levine, J.S., 1998. Role of superoxide in apoptosis induced by growth factor withdrawal. Am. J. Physiol. 275, F691–F702.
Lieven, C.J., Schlieve, C.R., Hoegger, M.J., Levin, L.A., 2006. Retinal ganglion cell axotomy induces an increase in intracellular superoxide anion. Invest Ophthalmol. Vis. Sci. 47, 1477–1485.
Lieven, C.J., Thurber, K.A., Levin, E.J., Levin, L.A., 2012. Ordering of neuronal apoptosis signaling: a superoxide burst precedes mitochondrial cytochrome c release in a growth factor deprivation model. Apoptosis 17, 591–599.
Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 46, 3–26.
Louwerse, E.S., Weverling, G.J., Bossuyt, P.M., Meyjes, F.E., de Jong, J.M., 1995. Randomized, double-blind, controlled trial of acetylcysteine in amyotrophic lateral sclerosis. Arch. Neurol. 52, 559–564.
Park, C., Raines, R.T., 2001. Adjacent cysteine residues as a redox switch. Protein Eng. 14, 939–942.
Parkinson Study Group, 1993. Effects of tocopherol and deprenyl on the progression of disability in early Parkinson's disease. N. Engl. J. Med. 328, 176–183.
Pullela, P.K., Chiku, T., Carvan 3rd, M.J., Sem, D.S., 2006. Fluorescence-based detection of thiols in vitro and in vivo using dithiol probes. Anal. Biochem. 352, 265–273.
Schlieve, C.R., Tam, A., Nilsson, B.L., Lieven, C.J., Raines, R.T., Levin, L.A., 2006. Synthesis and characterization of a novel class of reducing agents that are highly neuroprotective for retinal ganglion cells. Exp. Eye Res. 83, 1252–1259.
Schwartz, M., Yoles, E., Levin, L.A., 1999. 'Axogenic' and 'somagenic' neurodegenerative diseases: definitions and therapeutic implications. Mol. Med. Today 5, 470–473.
Scott, C.J., Seidler, E.A., Levin, L.A., 2010. Cell-autonomous generation of mitochondrial superoxide is a signal for cell death in differentiated neuronal precursor cells. Brain Res. 1306, 142–148.
Seidler, E.A., Lieven, C.J., Thompson, A.F., Levin, L.A., 2010. Effectiveness of novel borane-phosphine complexes in inhibiting cell death depends on the source of superoxide production induced by blockade of mitochondrial electron transport. ACS Chem. Neurosci. 1, 95–103.
Shuaib, A., Lees, K.R., Lyden, P., Grotta, J., Davalos, A., Davis, S.M., Diener, H.C., Ashwood, T., Wasiewski, W.W., Emeribe, U., 2007. NXY-059 for the treatment of acute ischemic stroke. N. Eng. J. Med. 357, 562–571.
Simon, H.U., Haj-Yehia, A., Levi-Schaffer, F., 2000. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5, 415–418.
Sohn, Y.T., Park, B.Y., 2003. Characterization of the physicochemical properties of KR-31378. Arch. Pharm. Res. 26, 526–531.
Swanson, K.I., Schlieve, C.R., Lieven, C.J., Levin, L.A., 2005. Neuroprotective effect of sulfhydryl reduction in a rat optic nerve crush model. Invest Ophthalmol. Vis. Sci. 46, 3737–3741.
Thompson, A.F., Crowe, M.E., Lieven, C.J., Levin, L.A., 2015. Induction of neuronal morphology in the 661W cone photoreceptor cell line with staurosporine. PLoS One 10, e0145270.
Zhao, H., Joseph, J., Fales, H.M., Sokoloski, E.A., Levine, R.L., Vasquez-Vivar, J., Kalyanaraman, B., 2005. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. Proc. Natl. Acad. Sci. U. S. A. 102, 5727–5732.