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Research Paper

Borane-protected phosphines are redox-active radioprotective agents for endothelial cells

Megan E. Crowe [a], Christopher J. Lieven [a,b], Alex F. Thompson [a], Nader Sheibani [a], Leonard A. Levin [a,b,c,*]

[a] Department of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States
[b] Cytodefense, Madison, WI, United States
[c] Department of Ophthalmology, McGill University, Montreal, QC, Canada

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ABSTRACT

Exposure to radiation can damage endothelial cells in the irradiated area *via* the production of reactive oxygen species. We synthesized phosphine–borane complexes that reduce disulfide bonds and had previously been shown to interfere with redox-mediated signaling of cell death. We hypothesized that this class of drugs could interfere with the downstream effects of oxidative stress after irradiation and rescue endothelial cells from radiation damage. Cultured bovine aortic endothelial cells were plated for clonogenic assay prior to exposure to varying doses of irradiation from a ^{137}Cs irradiator and treated with various concentrations of bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1) at different time points. The clone-forming ability of the irradiated cells was assessed seven days after irradiation. We compared the radioprotective effects of PB1 with the aminothiol radioprotectant WR1065 and known superoxide scavengers. PB1 significantly protected bovine aortic endothelial cells from radiation damage, particularly when treated both before and after radiation. The radioprotection with 1 μM PB1 corresponded to a dose-reduction factor of 1.24. Radioprotection by PB1 was comparable to the aminothiol WR1065, but was significantly less toxic and required much lower concentrations of drug (1 μM *vs.* 4 mM, respectively). Superoxide scavengers were not radioprotective in this paradigm, indicating the mechanisms for both loss of clonogenicity and PB1 radioprotection are independent of superoxide signaling. These data demonstrate that PB1 is an effective redox-active radioprotectant for endothelial cells *in vitro*, and is radioprotective at a concentration approximately 4 orders of magnitude lower than the aminothiol WR1065 with less toxicity.

1. Introduction

Tissue damage following ionizing radiation injuries occurs *via* multiple mechanisms, including damage to endothelial cells [1–3]. Irradiated intestine [4], kidney [5], lung [6], brain [7,8], and optic nerve [9] exhibit decreased numbers or abnormal morphology of endothelial cells. Among the changes noted in endothelial cells following irradiation are reductions in extracellular matrix proteins, changes in adhesion and binding, and loss of integrity at tight cell junctions [10]. This damage can lead to capillary rupture or loss of small vessels. Other late effects on vessels include abnormal endothelial proliferation and fibrosis [1]. In neuronal tissues, this damage results in a disruption of the blood–brain barrier [11]. Pulmonary tissue undergoes a similar disruption of the blood–alveolus barrier subsequent to endothelial cell damage [12]. The effects of radiation-dependent endothelial cell injury cause tissue-specific pathophysiology. For example, radiation to optic nerve endothelial cells causes radiation optic neuropathies, while that to retinal endothelial cells causes radiation retinopathy. These diseases can lead to blindness, and are frequently seen in patients receiving radiation treatment for ocular tumors.

Ionizing radiation acts on multiple targets within the cell. Ionizing radiation leads to damage of DNA bases and strand breaks, as well as the generation of radical nucleic acids and reactive oxygen species (ROS), including hydroxyl and superoxide ions

Abbreviations: PB1, bis(3-propionic acid methyl ester)phenylphosphine borane complex; PB2, (3-propionic acid methyl ester)diphenylphosphine borane complex; TCEP, tris(2-carboxyethyl)phosphine; PEG-SOD, superoxide dismutase–polyethylene glycol from bovine erythrocytes; MnTMPyP, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin; ROS, reactive oxygen species; BAEC, bovine aortic endothelial cells

* Correspondence to: Department of Ophthalmology and Visual Sciences, University of Wisconsin Medical School, 1300 University Avenue, Room 694, Madison, WI 53706, United States.

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generated by the radiolysis of water [13]. The wide variety of injuries makes pharmacological radioprotection an elusive goal. One common approach is to try to minimize the effects of ROS, which can comprise as much as 70% of the damage from irradiation, by the addition of supplemental antioxidants in order to minimize indirect damage to macromolecules.

Over the past two decades it has become clear that ROS are not only chemically reactive, but act as signal transduction agents, transducing intracellular signals *via* several mechanisms. Protein targets for ROS transduction most commonly have a redox-sensitive moiety, often a cysteine sulfhydryl, at the active site. Proteins can also be covalently modified by ROS, e.g. S-nitrosylation with NO+, S-nitration with peroxynitrite, or glutathiolation with glutathione. Redox modulation of vicinal cysteine sulfhydryls is an efficient means of modulating protein function, because the oxidative cross-linking results in a disulfide bond that can dramatically change the conformation of the active site [14,15]. Some targets for ROS-mediated cysteine oxidation are involved in the induction of apoptosis, e.g. creatine kinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [16].

Approaches for counteracting ROS fit into three categories: prevention of ROS formation, scavenging of ROS, and reversal of oxidative damage. ROS-mediated cell death can be prevented by decreasing their formation from molecular oxygen [17,18], an example of preventing the oxidation before it can occur. The limitation of this approach is that the inhibitors of formation must be present before the generation of ROS. Cells maintain a wide range of natural systems to reduce levels of free radical species *in vivo*, including catalase (H_2O_2), superoxide dismutase (SOD)-1, -2, -3 (superoxide), and glutathione peroxidases (R-OOH). Both upregulation [19] and addition of exogenous scavengers [20] have been shown to protect cells from radiation-induced oxidative damage. Again, the effectiveness of this approach is dependent on the presence of the scavengers at the time of ROS generation. The third approach, the reversal of oxidative damage, is perhaps the most promising of the three. As described above, ROS may induce cysteine modification to elicit changes in protein conformation and function [14,15]. Chemical reduction of the cross-linked sulfhydryl residues can counteract and reverse the oxidative damage before cell death signals are transduced. Of the three approaches, methods like chemical reduction of oxidized moieties have the greatest window of opportunity for radiomitigation because they would be effective when given before, during, or soon after oxidative injury.

Many compounds that protect against the damages of radiation are antioxidants [21]. Thiol-containing molecules were first found to be effective radioprotectants *in vivo* in 1949, when cysteine was used to protect mice from X-ray irradiation [22]. Further studies demonstrated similar protective effects with other compounds containing sulfhydryl groups, including glutathione and β-mercaptoethylamine. These studies were performed in mice undergoing irradiation and exposed to elevated oxygen levels. Mice exposed to elevated oxygen levels at or within 2 h after irradiation showed increased toxicity, establishing a link between the antioxidant characteristics of thiols and radioprotection [23]. Currently, the only FDA-approved radioprotectant antioxidant is the phosphothioate amifostine [24]. Upon reaching the cell surface, amifostine is converted by alkaline phosphatase to its active, free thiol form, or WR1065.

The mechanism by which thiols are radioprotective is still not completely understood. Hypothesized pathways include direct scavenging of free radicals [25], reduction of disulfide bonds [26], and activation of transcription factors driving cytoprotective gene expression [19]. It is possible that a combination of these actions is responsible for the observed protection from radiation. However, these drugs are radioprotective, not radiomitigative, and have significant systemic toxicity.

We have developed a class of new chemical entities that are able to interfere with downstream effects of ROS. Novel sulfhydryl reducing agents bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1) and (3-propionic acid methyl ester) diphenylphosphine borane complex (PB2) are able to protect retinal ganglion cells against apoptosis following axonal injury, a superoxide-dependent process [27], without direct scavenging of superoxide. These phosphines are alternatives to thiol drugs, and are structurally similar to the reducing agent tris(2-carboxyethyl) phosphine (TCEP), which is neuroprotective for retinal ganglion cells [17,28] and photoreceptors [29]. PB1 and PB2 were designed to have low reactivity in the extracellular compartment, high rates of transmembrane diffusion, and a side-group that can be cleaved by intracellular enzymes, resulting in an intracellular accumulation of PB1 or PB2. PB1 and PB2 are neuroprotective *in vitro* in axotomized primary retinal ganglion cells [27] and a neuronal cell line where the mitochondrial electron transport chain components are inhibited [30]. They are also neuroprotective *in vivo* in rat optic nerve crush and ocular hypertension models [31].

Given that axotomy-induced superoxide-dependent retinal ganglion cell apoptosis is decreased by treatment with the reducing agent PB1 and that irradiation-induced endothelial cell death is decreased through treatment with antioxidants and thiol-based compounds, we hypothesized that treatment with PB1 would reduce clonogenic death in endothelial cells exposed to ionizing radiation. PB1 was radioprotective of endothelial cells to a similar degree as WR1065 but at much lower effective concentrations and without the associated toxicity that limits the use of WR1065. Unlike WR1065, PB1 was also protective against direct oxidative stress with t-butyl hydroperoxide and showed some evidence of radiomitigative effects.

2. Materials and methods

2.1. Chemicals

The aminothiol WR1065 and superoxide dismutase–polyethylene glycol from bovine erythrocytes (PEG-SOD) were from Sigma-Aldrich (St. Louis, MO). Manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) was from Adipogen (San Diego, CA). Hydroethidine (HEt) was from Anaspec Solutions (Waddinxveen Zuid-Holland, Netherlands). Phenylphosphine was from Strem Chemicals Inc. All other chemicals and solvents were from Sigma-Aldrich (Milwaukee, WI).

2.2. Synthesis of bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1)

To a flame-dried round bottom flask fitted with magnetic stirbar was added phenylphosphine (5.00 ml, 45.0 mmol) to degassed acetonitrile (5.00 ml) under argon atmosphere. The solution was cooled to 0 °C and methyl acrylate (8.10 ml, 90 mmol) was added slowly drop-wise. After complete addition of methyl acrylate, the solution was warmed to room temperature, and stirred overnight. After 19 h, the solvent was removed *in vacuo* and dry tetrahydrofuran was added (15 ml). The solution was placed under argon, cooled to 0 °C and borane-dimethylsulfide (4.5 ml, 45.0 mmol) was added slowly drop-wise. The reaction was warmed to room temperature to stir for 45 min. The solvent was removed *in vacuo* and the crude product was purified by flash chromatography (silica gel, gradient elution 10–20% EtOAc in hexanes). Phosphine–borane complex 1 (PB1) was isolated as a clear oil (7.83 g, 26.4 mmol, 58% yield over two steps).

Reactions were monitored by thin-layer chromatography and visualized by ultraviolet light or staining with I_2. ^1H and ^{13}C NMR

spectra were obtained on Varian VI-400 and VI-500 spectrometers using $CDCl_3$ with TMS or residual solvent as standard unless otherwise noted. Low-resolution mass spectra were obtained using an Agilent 1100 series LS/MSD using atmospheric pressure chemical ionization (APCI).

PB1: R_f=0.30 [33% EtOAc in hexanes]; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.67 (d, J=122.7 Hz, 3H), 2.14–2.39 (m, 6H), 2.53–2.69 (m, 2H), 3.63 (d, J=0.7 Hz, 6H), 7.43–7.60 (m, 3H), 7.67–7.78 (m, 2H); 13C NMR (126 MHz, $CDCl_3$) δ 21.06 (d, J=38.3 Hz), 27.67 (d, J=1.6 Hz), 52.13, 126.41 (d, J=52.0 Hz),129.22 (d, J=9.7 Hz), 132.10 (d, J=2.4 Hz), 132.20 (d, J=9.2 Hz), 172.61 (d, J=15.6 Hz). 31P NMR (162 MHz, $CDCl_3$) δ 17.85 (d, J=70.0 Hz); mass spectrum (APCI) m/e 295.1 (65) (M–H)+, 283.2 (100) (M–BH_2)+.

2.3. Cell culture

Bovine aortic endothelial cells (BAEC) were purchased from Cell Applications, Inc. (San Diego, CA) and harvested up to passage 9. Cells were grown at 37 °C in 5% CO_2 on tissue-culture flasks in bovine EC basal medium supplemented with 10% bovine EC growth supplement, both from Cell Applications, Inc.

2.4. Irradiation

Before treatment, BAEC were plated on 6-well plates at 100 cells per well. After 24 h in culture, all plates were removed from the incubator for the duration of irradiation treatment. Treatment conditions received single doses of radiation between 1 and 6 Gy using a ^{137}Cs irradiator (JL Shepherd & Associates; San Fernando, CA) delivering radiation at a rate of 4.3905 Gy per minute. PB1 was administered 24 and 2 h before, at the time of, and/or 24, 48, 72, and 120 h after irradiation. It was also administered 2 h before irradiation and removed immediately after irradiation. WR1065 was administered 2 h before irradiation and removed immediately after as a positive control. In some experiments, WR1065 was allowed to remain in the medium to assess toxicity.

2.5. Clonogenic assays

Cells were grown in 6-well tissue culture plates at a density of approximately 100 cells/well, and treated in sextuplicate. Cell viability was assessed by measuring cells' ability to propagate *via* clonogenic assay, *i.e.* clonogenicity [32]. A week after irradiation, media was removed and the wells rinsed with 1 × PBS. Cells were fixed with 6% glutaraldehyde and stained with 0.5% crystal violet solution. Thirty minutes later, the crystal violet solution was removed and the wells rinsed with water. Blue-staining colonies (1–5 mm in diameter) were manually counted and results tabulated. Plating efficiency of non-irradiated, untreated controls ranged from 20-57% and all clone counts were normalized to that condition within each experiment for comparison across repetitions. Rescue of clonogenicity was calculated by determining the number of colonies in treated cells compared to irradiated controls as a fraction of the number of colonies in non-irradiated cells compared to irradiated controls, *i.e.*

$$\frac{C_{tx} - C_{irr}}{C_{nonirr} - C_{irr}}.$$

2.6. Assessment of intracellular superoxide levels

Levels of intracellular superoxide were assessed using dihydroethidium (HEt), which is converted to fluorescent 2-hydroxyethidium in the presence of superoxide [33]. In order to test whether ionizing radiation results in an increase in intracellular superoxide, cells were plated in black 96-well plates (Corning, Tewksbury, MA) at a density of 2000 cells per well 24 h prior to irradiation. Cells were treated with 3.2 μM HEt in medium 30 min before irradiation, followed by received single doses of radiation between 0 and 6 Gy. Fluorescence was assessed 5 min after irradiation using a 1420 Victor 2 T Multilabel Counter (excitation 485 nm, emission 580 nm). After subtracting background fluorescence seen in medium-only wells, fluorescence readings were compared among cells not subjected to radiation, irradiated cells, and irradiated cells treated with PEG-SOD (0.3–30 U/ml). Conditions were performed in sextuplicate.

2.7. Statistics

Comparisons between groups were by unpaired t-test. The clonogenic rescue percentages were used for comparison in order to minimize between-experiment variability in radiation response in untreated cells. All results are presented as mean ± SEM.

3. Results

3.1. PB1 is radioprotective for bovine aortic endothelial cells

Cultured BAEC were exposed to irradiation ranging from 1 Gy to 6 Gy and their ability to divide was measured by clonogenic assay, with colony counts performed seven days later. There was a near-linear toxicity associated with increasing doses of radiation, with colony-forming potential (as a percentage of non-irradiated cells) ranging from 84.3% ± 2.7% with 1 Gy to 7.1% ± 0.8% with 6 Gy ($p < 0.001$ for all comparisons; Fig. 1).

To assess radioprotection with PB1 (Fig. 2A), cultured BAEC were exposed to irradiation in the presence of varying concentrations of PB1. A single dose of 3 Gy irradiation resulted in a 64.4% ± 2.2% reduction in colony formation compared to non-irradiated cells seven days after irradiation. Treatment with 10 nM, 100 nM, 1 μM, or 10 μM PB1 at the time of irradiation resulted in a significant dose-dependent improvement in colony formulation (Fig. 2B), with corresponding radioprotective rescue of 15.4% ± 4.2%, 11.1% ± 4.0%, 21.5% ± 3.5%, and 20.3% ± 4.9% (p=0.002, 0.02, < 0.001, and < 0.001 respectively). Treatment with PB1 2 h prior to irradiation also conferred significant protection to colony formation (Fig. 2C), with corresponding radioprotective rescue values of 15.7% ± 4.7%, 21.4% ± 4.1%, 22.4% ± 3.3%, and 26.6% ± 4.8% for 10 nM through 10 μM PB1 respectively ($p < 0.01$). The

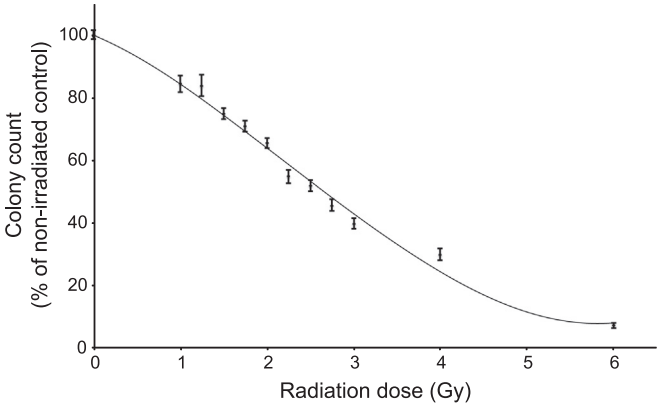

Fig. 1. Radiation exposure leads to a decrease in colony-forming potential of BAEC. Cells were assayed for clonogenic activity following exposure to 0–6 Gy of ionizing radiation and exhibited a near-linear decrease in colony-forming capacity with increasing radiation. Results are from 12 independent experiments, and are presented as mean ± SEM.

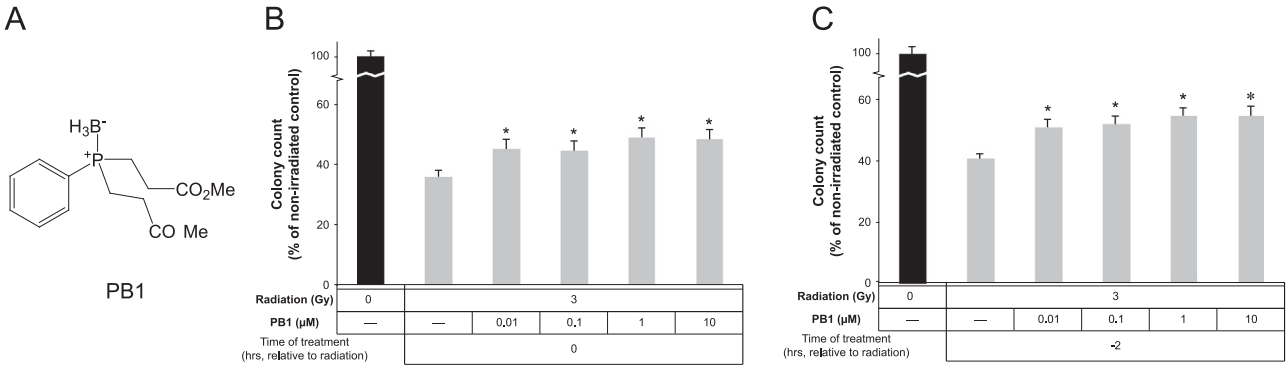

Fig. 2. PB1 is radioprotective for BAEC. (A) Chemical structure of bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1). (B) PB1 (10 nM to 10 μM) was administered to cultured cells at the time of irradiation (3 Gy). Treatment resulted in significant recovery of colony-forming potential of the cells in a dose-dependent fashion. Asterisks represent values significantly ($p < 0.02$) different from untreated, irradiated cells. (C) PB1 (10 nM to 10 μM) was administered to cultured cells 2 h prior to irradiation (3 Gy). Treatment resulted in significant recovery of colony-forming potential of the cells in a dose-dependent fashion. Asterisks represent values significantly ($p < 0.01$) different from untreated, irradiated cells. Results are from 8 (panel B) or 5 (panel C) independent experiments, and are presented as mean ± SEM.

radioprotection with 1 μM PB1 when administered 2 h prior to irradiation corresponded to a dose-reduction factor of 1.24.

3.2. PB1 is significantly less toxic than WR1065

WR1065 is a thiol-based radioprotectant that is the effector molecule for amifostine, the only FDA-approved radioprotectant. The radioprotective activities of PB1 and WR1065 were compared. BAEC were treated with PB1 or WR1065 2 h before 3 Gy irradiation and the cells incubated for a further 7 days in the presence of radioprotectant. There was significant radioprotection with PB1 but no colonies were seen with WR1065 (Fig. 3; middle bars). However, when drugs were removed immediately after irradiation, both PB1 and WR1065 were radioprotective (Fig. 3; rightmost bars). These data indicate that although WR1065 was radioprotective, it was toxic to cells when present for 7 days. PB1, on the other hand, retained its radioprotective qualities and had minimal toxicity when incubated with cells for 7 days.

3.3. Time course of PB1-mediated radioprotection

To assess the effectiveness of PB1 as both a radiomitigator and a radioprotector, PB1 (final concentration 1 μM) was added to BAEC cultures at various time points before and after irradiation. Treatment after irradiation at either +24 h or +24, 48, 72, and 120 h resulted in statistically insignificant rescue of clonogenicity of 4.9% ± 6.1% and 6.7% ± 5.5% (Fig. 4). Treatment before irradiation at either −2 h or −24 h and −2 h resulted in greater radioprotective rescue (15.6% ± 4.9% and 11.9% ± 5.2%; $p = 0.02$). Optimal radioprotection was seen with PB1 when added both before and after irradiation, at −24, −2, +24, +48, +72 and +120 h, with radioprotective rescue of 23.8% ± 7.0% ($p = 0.008$). In summary, treatment with PB1 before and after irradiation was more effective than at either time alone.

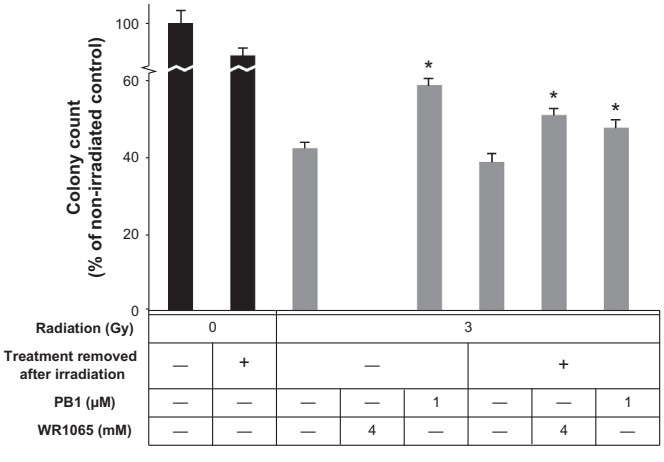

Fig. 3. PB1 is less toxic than WR1065. WR1065, the effector molecule of the radioprotective pro-drug amifostine (4 mM), was compared to PB1 (1 μM). PB1 significantly restored colony-forming potential when not removed following irradiation, while WR1065 resulted in complete toxicity (0 surviving colonies). When treatments were removed immediately following irradiation, WR1065 and PB1 showed comparable levels of protection. Asterisks represent values significantly ($p < 0.02$) different from untreated, irradiated cells. Results are from 2 independent experiments, and are presented as mean ± SEM.

3.4. PB1 radioprotection is independent or downstream of irradiation-induced superoxide

3.4.1. Irradiation of BAEC results in an increase in intracellular superoxide levels

We had previously shown that PB1 interferes with retinal

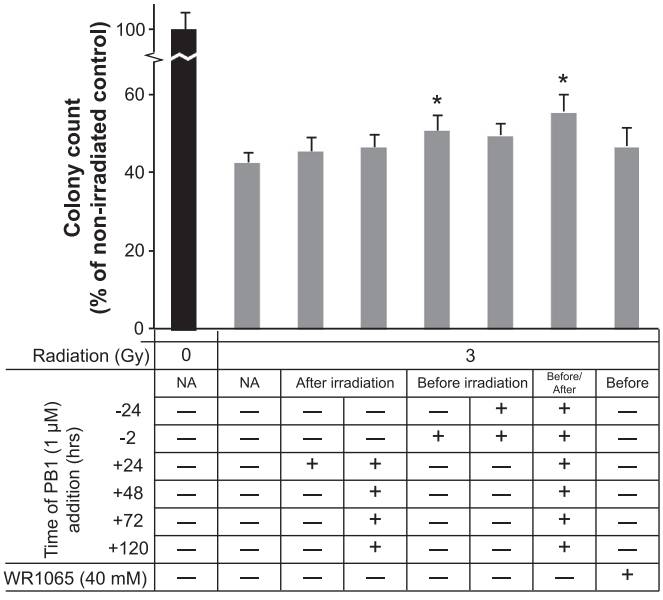

Fig. 4. Time course of PB1-mediated radioprotection. PB1 (1 μM) was added at various times ranging from 24 h before to 120 h after radiation to investigate the potential for PB1 as a radiomitigant. While PB1 did not confer significant protection when added only following irradiation, multiple additions before and after exposure resulted in significant recovery of colony formation. Asterisk represents a value significantly ($p < 0.02$) different from untreated, irradiated cells. Results are from 2 independent experiments, and are presented as mean ± SEM.

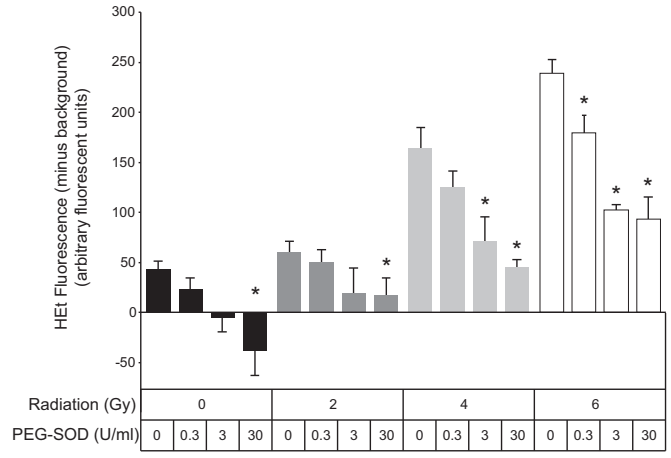

Fig. 5. Radiation induces superoxide in BAEC. BAEC were irradiated (0, 2, 4, or 6 Gy) after loading with a superoxide-sensitive fluorescent marker. Increasing radiation doses led to higher fluorescence levels, which were significantly reduced in the presence of PEG-SOD. Results shown are from a single representative experiment. Asterisks represent values significantly ($p < 0.05$) different from irradiated cells in the absence of PEG-SOD. Results are from 3 independent experiments, and are presented as mean ± SEM.

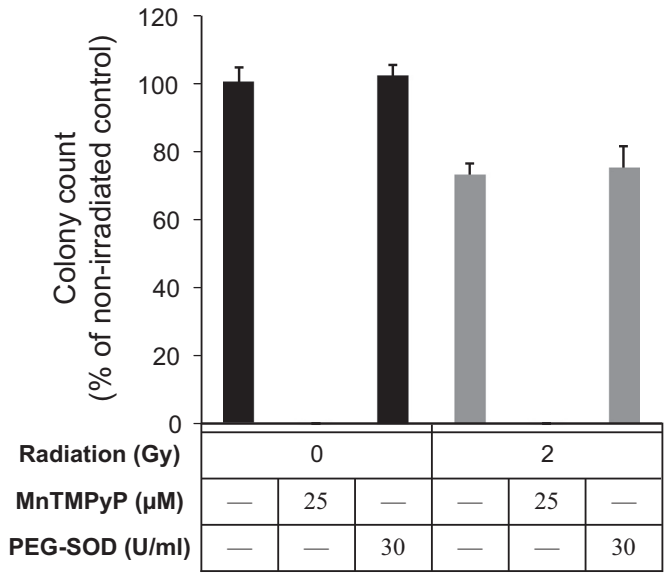

Fig. 6. Superoxide scavenging is not radioprotective for BAEC. BAEC were irradiated (2 Gy) in the presence or absence of the SOD mimetic MnTMPyP (25 μM) or PEG-SOD (30 U/ml). MnTMPyP showed toxicity (0 surviving colonies) and PEG-SOD failed to confer protection for colony-forming potential. Results are from 2 independent experiments, and are presented as mean ± SEM.

ganglion cell death after axonal injury [27,34], which is mediated by superoxide [35–37]. In order to determine whether irradiation-induced cell death was associated with generation of superoxide, hydroethidine (HEt) was used measure intracellular superoxide levels following irradiation. Single doses of irradiation (2 Gy, 4 Gy, or 6 Gy) resulted in a dose-dependent increase in fluorescence readings (Fig. 5), indicating higher levels of superoxide, compared to non-irradiated cells ($p=0.13$, $p < 0.001$, $p < 0.001$, respectively). Treatment with 0.3, 3, and 30 U/ml PEG-SOD, a superoxide scavenger, before 4 Gy or 6 Gy irradiation resulted in significantly lower HEt fluorescence levels than those seen in irradiated cells without PEG-SOD.

3.4.2. *Superoxide scavenging is not radioprotective for bovine aortic endothelial cells*

Given that irradiation of BAEC increased intracellular levels of superoxide and WR1065 induces MnSOD expression [19], experiments were performed to see if modulating superoxide levels improved their survival. BAEC were treated with the known superoxide scavengers PEG-SOD and MnTMPyP, followed by irradiation and clonogenic assay 7 days later. Neither scavenger was significantly radioprotective, determined by clonogenic assay of irradiated cells (Fig. 6). Irradiation with 2 Gy was used to increase the likelihood of detecting a small effect on clonogenic potential. Treatment with PEG-SOD minimally affected the clonogenic potential of irradiated cells, increasing slightly from 73.2% ± 3.3% to 75.3% ± 6.2 (p=NS). Similar results were seen when cells were irradiated with 3 Gy. Treatment with a different superoxide dismutase mimetic, MnTMPyP, resulted in toxicity to cells in all conditions. Therefore, reducing superoxide levels in these cells was not radioprotective.

3.4.3. *PB1 protects from induced oxidative stress*

PB1 is a redox-active disulfide reducing agent. Given that PB1 was radioprotective but does not scavenge superoxide [30], we hypothesized that it would protect against oxidative damage not directly associated with superoxide. We assessed this by testing whether PB1 could rescue endothelial cells from direct oxidative damage. BAEC were treated with tert-butyl hydroperoxide (tBHP) at concentrations from 100 nM to 1 μM for 24 h in the presence or absence of 1 μM PB1. Oxidative damage mediated by 100 nM, 316 nM, and 1 μM tBHP resulted in decreased clonogenic potential

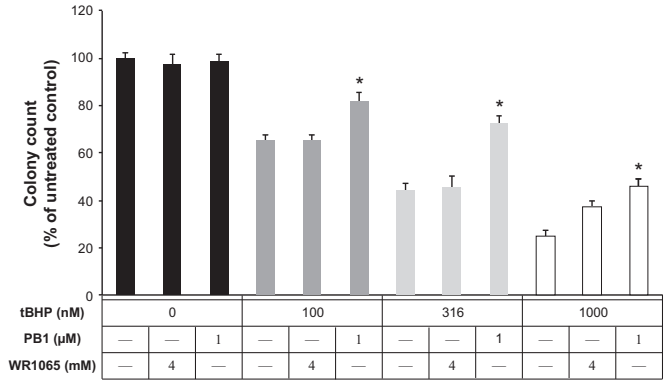

Fig. 7. PB1 protects BAEC from induced oxidative stress. Direct oxidative stress was induced in BAEC through addition of tBHP (0, 100, 316 or 1000 nM). Treatment with PB1 (1 μM) significantly ameliorated the loss in colony-forming potential at all tested levels of oxidative stress. Asterisks represent values significantly ($p < 0.001$) different from tBHP-treated cells in the absence of pharmacological treatment. Results are from 2 independent experiments, and are presented as mean ± SEM.

that was dose-dependent (Fig. 7). Treatment with 1 μM PB1 significantly rescued lost clonogenic potential at all concentrations of tBHP, rescuing 46.7% ± 8.8%, 50.1% ± 7.3%, and 32.9% ± 4.3% of clones respectively ($p < 0.001$). WR1065 (4 mM) showed non-significant protection from tBHP at all doses. These data indicate that radioprotection by PB1 is independent or downstream of an irradiation-induced oxidative signal.

4. Discussion

These data demonstrate that the novel reducing agent bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1) is an effective radioprotectant for endothelial cells *in vitro*. PB1 restored clonogenicity to an equal or greater degree than the clinical radioprotectant WR1065 at a concentration approximately 4 orders of magnitude lower and without any of the observed toxicity [38] that has limited the clinical applications of the pro-drug for WR1065, amifostine. Although radiation induced

generation of superoxide in these cells, dismutation of superoxide anion was not radioprotective. Finally, treatment with PB1 after irradiation had a small positive effect on clonogenicity, suggesting a potential role for this class of drugs as a radiomitigant.

Irradiation-induced endothelial damage is a limiting factor for radiation dosing in radiation therapy. In the treatment of ocular, orbital, or intracranial tumors, for example, exposure to therapeutic irradiation may result in delayed and irreversible loss of vision due to radiation optic neuropathy [39]. Clinically, there is subacute visual loss occurring months to years after irradiation, with radiographic evidence of breakdown of the blood-nerve barrier [40]. The mechanism responsible for these features is not known, but endothelial cell damage is a consistent finding. Similar findings have been reported in a variety of other healthy tissues following irradiation [4–7]. The development of an endothelial radioprotectant would allow for more aggressive treatment of tumors while minimizing off-target effects.

The mechanism by which PB1 is radioprotective is unlikely to be related to superoxide scavenging, given that superoxide scavenging in these cells is not radioprotective and PB1 does not scavenge superoxide [30]. The radioprotective mechanism is more likely related to its phosphine group, which has functionality that parallels those of thiols. The active thiol (WR1065) and disulfide (WR33278) forms of amifostine are radioprotective *via* a variety of mechanisms [41]. WR1065 and WR33278 are positively-charged polyamines similar in structure to spermine. They localize to the nucleus where they associate with DNA as a positively charged counterion to negatively charged phosphates [42,43], modulate the cell cycle and DNA repair through regulation of a host of genes, and act to stabilize DNA while scavenging hydroxyl and DNA radicals. The effectiveness of various thiols as radioprotectants *in vivo* corresponds directly to their charge, suggesting that localization to negatively-charged organelles (e.g. nuclei and mitochondria) plays a role in their radioprotective capacity [44].

In contrast, PB1 undergoes reduction to a negatively-charged diacid, which would not be expected to localize to negatively-charged organelles. In fact, we observed that PB1 has a greater capacity for protection against ROS generated outside the mitochondrial matrix than inside [30], suggesting that charge affects subcellular localization of PB1.

Thiols chelate a variety of metals and in some cases remove metal cofactors from enzymes [45]. This is an unlikely mechanism for PB1. Phosphines vary greatly in their ability to chelate metals at biological pH levels, depending on their electronic and steric properties. The diacid metabolite of PB1 is similar to TCEP, which is a fairly weak metal chelator [46]. Therefore, PB1 would be unlikely to compete with metal cofactors at the low concentrations used in our study.

The most likely mechanism for PB1-mediated radioprotection relates to effects on cellular redox state, which is affected both by thiols and phosphines. Modulation of redox state can result in signal transduction, gene induction, and chemical reduction of oxidized protein disulfides. While a number of redox-sensitive transcription factors are possible targets (p53, NF-kB, AP-1, Nrf2) [47–49], the ability of PB1 to confer a high degree of protection at a micromolar dose suggests either high specificity of PB1 for an effector molecule or mediation by an alternate pathway.

Although PB1 is effective against irradiation in healthy endothelial cells *in vitro*, it is unknown how well it would function in the milieu of tumor cells or *in vivo*. Identification of the targets for reduction is crucial for medicinal chemistry optimization of PB1 for radioprotection, with goals of diminishing off-target effects, and increasing efficacy. It may be possible to achieve subcellular localization and targeting of compounds based on PB1 with appropriate choices of substituted side chains and charged functional groups. We are currently investigating the effect of different side chains as a means of optimizing organelle affinity for phosphine–borane complexes. The ideal compound would be highly radioprotective for normal but not tumor cells.

In addition to radiotherapeutic use, the potential for phosphine–borane complexes to act as radiomitigants creates a window for treating acute radiation toxicity due to unanticipated exposure to high levels of ionizing radiation, e.g. terrorism or accidents. When exposure cannot be predicted in advance, it is only through blocking or reversal of downstream cell-death signaling pathways that protection can be achieved, limiting the usefulness of compounds which function only as radioprotectants. The modest amount of radiomitigative activity seen with PB1 would require optimization in order for it to be suitable for treatment of acute radiation toxicity.

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