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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

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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–poly-ethylene 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