Significant Suppression of CT Radiation-Induced DNA Damage in Normal Human Cells by the PrC-210 Radioprotector

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While computed tomography (CT) is now commonly used and considered to be clinically valuable, significant DNA double-strand breaks (γ -H2AX foci) in white blood cells from adult and pediatric CT patients have been frequently reported. In this study to determine whether γ -H2AX foci and X-ray-induced naked DNA damage are suppressed by administration of the PrC-210 radioprotector, human blood samples were irradiated in a CT scanner at 50–150 mGy with or without PrC-210, and γ -H2AX foci were scored. X-ray-induced naked DNA damage was also studied, and the DNA protective efficacy of PrC-210 was compared against 12 other common “antioxidants.” PrC-210 reduced CT radiation-induced γ -H2AX foci in white blood cells to near background ($P < 0.0001$) at radiation doses of 50–150 mGy. PrC-210 was most effective among the 13 “antioxidants” in reducing naked DNA X-ray damage, and its addition at 30 s before an $\cdot\text{OH}$ pulse reduced to background the $\cdot\text{OH}$ insult that otherwise induced $>95\%$ DNA damage. A systemic PrC-210 dose known to confer 100% survival in irradiated mice had no discernible effect on micro-CT image signal-to-noise ratio and CT image integrity. PrC-210 suppressed DNA damage to background or near background in each of these assay systems, thus supporting its development as a radioprotector for humans in multiple radiation exposure settings. © 2018 by Radiation Research Society

INTRODUCTION

Human lifetime exposure to ionizing radiation has risen steadily in recent decades, primarily with the increased use

of diagnostic radiation, in particular, computed tomography (CT). More than 81-million CT scans were done in the U.S. in 2014 (1, 2), and 3.1-billion radiologic exams were performed worldwide in 2007 (3). The increased use of diagnostic radiation has contributed significantly to radiation exposure in humans and has led to studies concerning the cancer risk associated with radiologic examinations (4, 5), particularly in children (6–8). Estimates of potential cancer cases from diagnostic X rays have ranged from 0.6% to 2.0% of annual cases in developed countries (9). Diagnostic radiation risks are particularly pertinent to patients who are genetically predisposed to cancer; for this reason, useful radiologic procedures are often excluded from the medical care provided to these individuals (10). The development and administration of a low-toxicity, oral or intravenous (i.v.) radioprotector for particular at-risk human populations, in a variety of medical and industrial settings, remains a valid goal.

Irradiation of tissue generates reactive oxygen species (ROS) that cause the large majority of radiation-induced DNA damage (11). DNA double-strand breaks (DSBs) are regarded as the most serious consequence of radiation exposure (12), resulting in chromosomal translocations and carcinogenesis (13, 14). Addition of phytonutrient (15) or synthetic antioxidants (16, 17) to cells to induce production of endogenous ROS scavenging products like glutathione, glutathione S-transferase and glutathione peroxidase over hours to days, has shown some radioprotective efficacy in preclinical settings.

PrC-210 is the prototype of a new family of direct-acting, small molecule aminothioli radioprotectors (18, 19), which can be administered orally and has no measurable nausea/emesis nor hypotension side effects (20, 21) in animal models that accurately recreate amifostine toxicities. To evaluate the ability of PrC-210 to suppress X-ray-induced DNA damage in normal human cells, we measured γ -H2AX foci formation in white blood cells from CT-irradiated normal human blood samples. These foci are well recognized as a sensitive biomarker of radiation-induced DNA DSBs (22, 23). The efficacy, potency and time dependency of PrC-210-conferred radioprotection of naked

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DNA was also directly compared to 12 other commonly studied "antioxidants." Micro-CT imaging of animals done before and after PrC-210 dosing was also done to assess how a 100% radioprotective systemic dose of PrC-210 affects CT image quality.

MATERIALS AND METHODS

This study was performed after University of Wisconsin Institutional Review Board approval. Informed consent was obtained from each volunteer. Blood was obtained from five healthy volunteers, mean age of 31 years.

PrC-210

PrC-210 (MW: 148; see Fig. 2 structure) has two (+) charged amines in the flexible, alkyl backbone that cause the backbone to hover over the (−) charged DNA helix; a thiol group is projected away from the DNA helix to scavenge X-ray-induced ROS (18, 19). PrC-210 synthesis as an HCl salt is described elsewhere (21). To determine what representative human blood PrC-210 concentration to test here, we estimated that a single PrC-210 intraperitoneal (i.p.) injection at the mouse 0.5× maximum tolerated dose (0.5 MTD) of PrC-210 HCl (0.5 MTD = 252 µg/g bodyweight), if fully distributed in the total blood volume of a 21-g mouse, achieves a blood concentration of ~20 mM (i.e., $0.252 \text{ mg} \times 21 \text{ g} = 5.29 \text{ mg}$; $1.22 \text{ ml blood} = 4.4 \text{ mg/ml} = 19.7 \text{ mM}$). For human blood irradiations in this study, 20 mM PrC-210 HCl in blood was tested.

In Vitro Blood Irradiation Experiments

Blood samples (~8 ml blood/person for each experiment) were drawn using EDTA-vacutainers, aliquoted into sealed, plastic 2.0-ml blood vials (Corning® Inc., Corning, NY) with or without added PrC-210, and then incubated on a rotator for 60 min under standard 37°C conditions immediately prior to irradiation. For X-ray irradiation, the Corning vials containing 2.0 ml blood were positioned radially in an acrylic holder that was centered in the field of a GE Discovery CT750HD CT scanner (GE Healthcare, Pittsburgh, PA) (see Fig. 1A). At least three vials within the radial configuration contained thermoluminescent dosimeters to provide corroborated X-ray dose calibration. X-ray irradiation was performed using the axial scan mode with a 40-mm beam width, 120 kV and a 2 s exposure time. The mA was varied to obtain the desired dose to the vials. Workup of the blood samples to immunostain γ -H2AX foci began 5 min postirradiation and was done with samples at 4°C until the cells were fixed in 4°C methanol. For each volunteer, levels of γ -H2AX foci in nonirradiated blood cells were measured in parallel to irradiated samples. PrC-210 was added to blood samples 60 min before 50, 100 or 150 mGy irradiation.

γ -H2AX Immunofluorescence Microscopy

Staining of γ -H2AX foci in white blood cells is based on phosphorylation of the histone variant H2AX after formation of DNA DSBs (24).² Irradiated (or nonirradiated control) blood samples (2.0 ml) were layered onto 5 ml of lymphocyte separation medium 1.077 (Lonza, Morristown, NJ) and centrifuged at 1,200g for 13 min at a temperature of 4°C. The separated and washed white blood cells were plated by cytopspin onto glass slides and then fixed with 4°C methanol and then acetone, dried, rehydrated and stained overnight using a specific γ -H2AX antibody [dilution 1:1,500, anti-H2A.X-Phosphorylated (Ser 139); BioLegend® Inc., San Diego, CA] in 1% fetal bovine serum in phosphate buffered saline (PBS; Gibco®, Grand

Island, NY). After washing and a 60-min incubation with an Alexa Fluor® 488-conjugated goat anti-mouse secondary antibody (dilution 1:400; Thermo Fisher Scientific™ Inc., Madison, WI) the white blood cells were mounted with Vectashield® mounting medium containing 4,6-diamidino-2-phenylindole (Vector® Laboratories, Burlingame, CA). Fluorescence microscopy was performed with a Nikon Eclipse Ti microscope (Melville, NY) using a 40× objective. For each microscopy session, to ensure consistent fluorescence sensitivity between sessions, a slide containing a 10-µl aliquot of fluorescent beads (Molecular Probes®, Eugene, OR) was first photographed separately using either green or blue fluorescence parameters, to establish the same exposure times between microscopy sessions. Blue or green fluorescence images of cells were saved as TIFF files, and ImageJ software (NIH, Bethesda, MD) was used to superimpose a circle of standard diameter onto the images (Fig. 1B). The number of blue nuclei or green γ -H2AX foci within the circle were then counted and divided to produce a frequency of γ -H2AX foci per nucleus. Each microscope slide was counted at least two times by two independent observers. Mean values with standard deviations were calculated for each blood sample. The frequency of γ -H2AX foci observed in nonirradiated white blood cells is indicated in Fig. 1C; this background is not subtracted from irradiated samples.

PrC-210 Thiol Fluorescence Assay

At set time points after addition of PrC-210 thiol (>99% thiol form by mass spectrometry) to human blood, human plasma or PBS, concentrations of PrC-210 thiol were determined using a new 96-well assay³ based on the fluorescence of a monochlorobimane-PrC-210 thiol conjugate. Briefly, PrC-210 biological samples were taken from 37°C incubations and immediately frozen in liquid nitrogen. After the wells of a black 96-well plate received 90 µl of PBS and 10 µl of monochlorobimane (5 mM in 90:10 methanol:water), 2.5 µl of PrC-210 thiol standards (0–50 nmols/2.5 µl) or 0–30-µl aliquots of immediately thawed PrC-210 biological samples were added directly to 96 wells. The plate was covered with foil and rotated vigorously at room temperature for 10 min. The fluorescence of each well (excitation = 390 nm; emission = 478) was measured using a CLARIOstar® plate reader (BMG LABTECH, Ortenburg, Germany). PrC-210 thiol concentrations in samples were interpolated from a standard curve using GraphPad Prism software (LaJolla, CA).

Plasmid DNA Damage Assays

pUC19 plasmid DNA was purified from overnight cultures of *E. coli* using a QIAprep Spin Miniprep Kit (QIAGEN®, Valencia, CA). For radioprotection assays, pUC19 DNA (913 ng) was added to 40 µl incubations in PCR tubes containing 30 mM sodium acetate buffer (pH 7.0). To assess radioprotective efficacy, 10 µl of each tested molecule, at the same 12 increasing concentrations (0–100 mM final), were added to triplicate, plasmid-containing tubes (assay TV = 50 µl). As shown in Fig. 3, these concentrations represent a 12–384-fold molar excess over the moles of dG calculated to be present in the 913 ng of pUC19 plasmid in the incubation. If the test molecule was not sufficiently water soluble (β -carotene, 2-methylthio-propane) Triton™ X-100 was added to facilitate dispersion. After a 15-min incubation, the 36 experimental PCR tubes and controls were positioned in an X-RAD 320 X-ray irradiator and were irradiated at 90 Gy over 30 min. Aliquots of each irradiated tube were then mixed with gel loading dye and electrophoresed in 1% agarose gel at 60 volts for 90 min. Gels were stained with ethidium bromide, quantitatively imaged, and ImageJ software was used to quantify supercoiled and nicked band intensity (both pre- and postirradiation) in each of the 36 sample lanes. Plots of "% X-Ray-Damaged Plasmid DNA" versus protective drug

² Brand *et al.* manuscript submitted for publication.

³ Dreischmeier E, Fahl WE. Manuscript submitted for publication.

FIG. 1. PrC-210 suppression of γ -H2AX foci in CT X-ray-irradiated human white blood cells. Panel A: Human blood samples (2.0 ml) in plastic vials, with or without 20 mM PrC-210 added 60 min before 50, 100 or 150 mGy irradiation in the GE Discovery CT750HD CT scanner. Panel B: All of the DAPI-stained blue fluorescent nuclei or immunostained green γ -H2AX foci within the standardized circle superimposed on the 40 $\times$ fluorescent ImageJ images were counted to establish foci/nucleus frequencies in all cell images. Panel C: Green γ -H2AX foci/blue nuclei frequencies were established for each subject at no irradiation ("foci background") and 50, 100 or 150 mGy irradiation, with or without added 20 mM PrC-210. *P* values between groups are indicated. Panel D: Gamma-H2AX foci abundance in both 100 mGy and 100 mGy with PrC-210 samples are shown.

concentrations enabled direct comparison of the radioprotective efficacy and potency of each tested molecule.

To establish the lead time needed for PrC-210 to radioprotect naked plasmid DNA, a nearly instantaneous pulse of the $\cdot$ OH DNA insult was needed, rather than producing a steady stream of $\cdot$ OH over the course of a 30-min X-ray irradiation. For these experiments, pUC19 DNA (880 ng) was incubated with PrC-210 (20 mM) added at various times (–60 min to +1 min) prior to addition of 5 mM H_2O_2 and immediate irradiation of the tubes with UV light to catalyze nearly instantaneous conversion of H_2O_2 to $\cdot$ OH (25). Immediately after the 60-s $\cdot$ OH pulse that is achieved during the 60-s UV irradiation, triplicate samples of the irradiated plasmid DNA (260 ng) were electrophoresed on a 1% agarose gel in Tris-acetate buffer for 90 min at 60 volts. Gels were stained with ethidium bromide, digitally imaged, and supercoiled versus

nicked/ $\cdot$ OH damaged DNA band intensities were quantified using ImageJ software.

CT Image Analysis of PrC-210-Treated Mice

Two 23-g C57/BL6 naïve mice were induced with 4% anesthetic isoflurane gas and maintained with 2% isoflurane during whole-body micro-computed tomography (μ CT) imaging using a Siemens Inveon (Siemens Medical Solutions, Knoxville, TN) at the University of Wisconsin-Madison Small Animal Imaging Facility. One mouse then received an i.p. PrC-210 dose (252 $\mu\text{g/g}$ bodyweight) and the other mouse an oral gavage PrC-210 dose (900 $\mu\text{g/g}$ bodyweight); both doses were previously shown to confer 100% protection against a 100% lethal dose of whole-body irradiation. Micro-CT was subsequently performed 30 min after i.p. injection and 60 min after oral gavage delivery of PrC-210. Both pre and post PrC-210 delivery μ CT

FIG. 2. Time-dependent decay of PrC-210 thiol to its oxidized metabolite after its addition to 37°C PBS (panel A), human plasma (panel B) or human blood (panel C). Samples at each incubation time point were immediately frozen in liquid nitrogen, and then thawed and assayed using a monochlorobimane-PrC-210 thiol conjugate-based fluorescence assay. Best fit curves of the data points were assigned using GraphPad Prism software; half-lives of PrC-210 thiol disappearance rates are indicated.

images were analyzed by Dr. Brand, and statistical analyses of signal-to-noise ratios in like sites in both images were compared.

CT Radiologist Survey

In 2014, the University of Wisconsin (<http://www.warf.org/>) assembled a mailing list of 400 practicing radiologists in the U.S. A survey containing 12 questions was emailed to each of the contacts on the list. Of those contacted, 7.25% responded by completing and returning the survey. In the survey, PrC-210 was referred to as “P04014.” In addition to a summary of the results provided in Fig. 6, the entire survey response is assembled and summarized in the Supplementary Information (<http://dx.doi.org/10.1667/RR14928.1.S1>).

Statistical Analysis

Student’s *t* test was used to compare the foci frequencies between PrC-210-pretreated cells and non-PrC-210-pretreated cells, as well as for DNA damage comparisons between plasmid DNA treatment groups. *P* < 0.05 was considered statistically significant. Statistical analysis was performed using Prism version 7 (2015; GraphPad Software, San Diego, CA).

RESULTS

Two second irradiations (at different mA settings) of human blood samples without added PrC-210 in a GE Discovery CT750HD CT scanner (Fig. 1A) resulted in the X-ray dose-dependent induction of γ -H2AX foci in blood samples from all five donors. (Fig. 1B and C). Scoring all of the DAPI-

stained blue cell nuclei and immunostained green γ -H2AX foci within a circle that was superimposed on the 40 $\times$ microscope images gave sufficient numbers of foci and nuclei to produce green foci/blue nucleus frequencies for all blood samples, both with and without the addition of 20 mM PrC-210. The background foci frequency (in nonirradiated cells), which varied little among five subjects, is shown but not subtracted from the irradiated samples (Fig. 1C). Addition of PrC-210 to whole blood samples 60 min before CT irradiation reduced the incidence of γ -H2AX foci to levels not statistically different from the nonirradiated background.

The monophasic half-life (Fig. 2) for disappearance of the active thiol form of PrC-210 in PBS (3.7 h) was longer than the monophasic half-life seen in human plasma (1.5 h) or the biphasic half-lives (16 min and 1.5 h) seen in human blood.

The gel-based assay of X-ray-induced plasmid DNA breaks (Fig. 3) allowed direct comparison of the ROS-scavenging potency of most of the molecules that have commonly been tested for “radioprotection.” PrC-210 and WR-1065 were equally potent, cysteamine less so, and the other molecules virtually without effect in protecting the naked DNA against the ROS generated during the X-ray insult. At >10 mM (a blood concentration that is probably achieved in clinical settings), N-acetylcysteine increased X-ray-induced DNA damage.

The gel-based assay of $\cdot$ OH-induced plasmid DNA breaks (Fig. 4), in which a 60-s pulse of $\cdot$ OH was generated (25), showed that addition of PrC-210 at all time points before the $\cdot$ OH insult, including as little as 30 s before, conferred complete protection against the $\cdot$ OH pulse that otherwise induced >95% damage to the naked plasmid DNA. PrC-210 addition 1 min after the ROS insult had little or no protective effect.

To address potential concerns that the systemic and cellular presence of PrC-210, at concentrations that confer radioprotection, could reduce CT image quality, mice were: 1. micro-CT imaged (Fig. 5); 2. then administered either i.p. or oral gavage doses of PrC-210 previously shown to confer 100% suppression of a 100% lethal radiation dose (18, 20); and 3. then re-imaged. Analysis of signal-to-noise ratios (Fig. 5E) at several sites within the CT images indicated that no statistical differences were observed between before and after PrC-210 images.

The results, compiled from the 29 CT radiologists who responded to a study survey, are summarized in Fig. 6, and the entire survey results are available in the Supplementary Information (<http://dx.doi.org/10.1667/RR14928.1.S1>). The results indicate that: 1. pediatric patients would be the preferred PrC-210 radioprotector recipients; and 2. even with introduction of the “low-dose” CT scanning concept, there is still a strong interest in the protective capacity conferred by PrC-210.

DISCUSSION

Our results indicate that addition of PrC-210 to human whole blood reduces to near background the level of CT X-

FIG. 3. Small molecule protection of naked plasmid DNA from X-ray-induced damage. At 15 min after test molecules were added to pUC19 plasmid incubations, tubes were irradiated at 90 Gy over 30 min. Panel A: Aliquots of incubations were electrophoresed and quantitative imaging of ethidium bromide-stained gels was performed. Intensities of lower (supercoiled) and upper (nicked) bands were quantified using ImageJ software. Panel B: Band intensities were plotted using GraphPad Prism software. Panel C: Structures of some of the small molecules are shown.

ray-induced γ -H2AX foci in white blood cells, a sensitive cell marker of DNA damage, at radiation doses (50–150 mGy) that are routinely encountered in clinical practice. PrC-210 was the most potent of the 13 “antioxidants” screened in an assay that scored the ability of molecules to prevent X-ray-induced damage to naked DNA. In a related assay, addition of PrC-210 at 30 s before a 1-min pulse of $\cdot\text{OH}$ provided complete protection against the $\cdot\text{OH}$ insult that induced >95% DNA damage in unprotected controls. With respect to CT imaging, systemic PrC-210 at a known 100% radioprotective dose in a mouse, had no effect on the CT image signal-to-noise ratio or image integrity.

The biphasic disappearance of the active PrC-210 thiol in 37°C human blood can best be explained by the following. For the 16-min $T_{1/2}$, there is rapid diffusion of the PrC-210 thiol (MW:148) into blood cells that are pelleted before the plasma is assayed. For the 87-min $T_{1/2}$, either there is oxidation of the PrC-210 thiol to the disulfide or it reacts with other thiols in plasma to form mixed disulfides (19). Rapid cell uptake of the PrC-210 thiol is consistent with our earlier observations of: 1. A 74% suppression of X-ray-induced γ -H2AX foci when added 15 min before blood irradiation;² and 2. a 100% suppression of radiation-induced

FIG. 4. Agarose gel separation of supercoiled and nicked/[•]OH-damaged forms of pUC19 plasmid DNA after exposure of plasmid DNA to a 60-s pulse of an [•]OH generator [H₂O₂ + UV light (25)]. Supercoiled DNA was incubated with water (lanes A, B) or 20 mM PrC-210 (lanes C–H) for the indicated times and then exposed for 1 min to the [•]OH generator. Aliquots of each reaction were electrophoresed, stained with EtBr and digitally imaged. Three replicate reactions and gels were done, and band intensities were quantified using ImageJ software. *P* value for comparison of supercoiled band intensities in lanes A vs. G is indicated.

death in mice 15 min after receiving an i.p. dose of PrC-210 (19).

Based on the screening of common “antioxidants” (Fig. 3) we conclude that PrC-210 and WR-1065 are equally potent and effective in suppressing X-ray-induced damage to naked DNA. However, PrC-210 has shown zero incidence of either the retching/vomiting or hypotension toxicities that are induced by WR-1065 or amifostine in ferret and rat models (20) or in 60–90% of patients treated clinically with amifostine (26). Another conclusion is that *N*-acetylcysteine has no ROS-scavenging capability, and actually enhanced X-ray-induced DNA damage at >10 mM, a concentration that may be achieved in a patient’s blood in clinical settings. Rather, *N*-acetylcysteine protection, when conferred, is better explained by its ability to enhance synthesis of intracellular glutathione (27) and induce the time-dependent (hours/days) expression of protective phase 2 drug metabolizing gene products (28).

Several observations, from both preclinical animal models and the *ex vivo* human experiments in this report, support the future use of PrC-210 in humans. Among these

FIG. 5. Micro-CT image integrity between animals given (panel A) no PrC-210 or (panel B) an oral gavage systemic PrC-210 dose (900 µg/g bodyweight) previously shown to confer 100% protection against an otherwise 100% lethal dose of X rays (20). Micro-CT imaging was done using a Siemens Inveon on mice anesthetized with isoflurane gas. Analysis of signal-to-noise ratio (panel E) in µCT images both before (panel C) and after (panel D) systemic PrC-210 was done. *P* values were determined using GraphPad Prism version 7.

Sample of radiologist survey comments

1. "I would imagine that pediatric pts would either be req'd to have the drug, or documentation instituted as to why it wasn't used."
2. "if medication were cheap enough it would have to be recommended for every patient for every study."
3. "if safe to use, it should be made as routine protocol before any CT scan and many fluoro studies."

FIG. 6. Summary results from a 2014 survey of 29 CT radiologists practicing in the U.S. Answers from three (Panels A–C) of the 12 survey questions are shown here. Three of the nine verbatim statements from the survey respondents are also shown. The survey was conducted by the University of Wisconsin. PrC-210 was given the name of "P04014" for the survey. The entire survey content is available in the Supplementary Information (<http://dx.doi.org/10.1667/RR14928.1.S1>).

observations are that PrC-210 significantly suppresses: 1. diagnostic X-ray-induced DNA DSBs (γ -H2AX foci) (Fig. 1C); 2. ROS-mediated X-ray and $\cdot$ OH naked DNA damage (Figs. 3 and 4); and 3. mouse and rat deaths induced by whole-body irradiation to background (18–20). Additional observations are that PrC-210: 4. shows neither nausea/emesis nor hypotension toxicities in animal models; 5. is deliverable by oral (20) i.p./i.v. (19) and dermatotopical (18) routes; 6. shows extended plasma half-life of the active thiol form consistent with the conferred animal radioprotective effect; and 7. shows no negative effect on CT images at systemic PrC-210 dose levels that were previously shown to confer 100% radioprotection in mice [Fig. 5 and ref. (18)].

So, with PrC-210 as an effective, orally-active ROS scavenger that apparently does not induce nausea nor hypotension, should it be developed as a radioprotector for use in diagnostic radiology patients, including CT scan patients? This question presents interesting challenges and leads to subsequent questions. While we do not have the answers, among these questions there are three in particular worthy of further discussion:

1. Is it humanly possible to design a clinical trial path to determine the efficacy of PrC-210 in suppressing diagnostic X-ray-induced cancers? The enormous sizes of the studies done to assess the cancer risks of CT radiation (29) indicate that it is not practical to perform even larger studies to determine cancer suppression; what company would pay for it? In a related challenge, even if PrC-210 CT risk reduction cannot be directly pursued, 90% of the surveyed radiologists (Fig. 6) still want such a protective molecule, preferentially for their pediatric patients.
2. Will the toxicities found to be associated with PrC-210 in clinical trials, extrapolated to millions of diagnostic radiation users, outweigh the burden of the projected annual cancers from diagnostic radiation, i.e., what will be the medical cost/benefit of PrC-210? With regards to the PrC-210 toxicities one could anticipate in humans, both the causes of death and the maximum tolerated doses of PrC-210 and amifostine in mice are very similar (19), and amifostine has been an FDA-approved drug for decades. In addition, there was no detectable PrC-210 nor PrC-211-induced mutagenesis when they were tested in either frameshift or point mutation *Salmonella typhimurium* strains (18). This observation is consistent with an aminothioli molecule that "hovers" around DNA based on charge, but does not chemically react with DNA. Furthermore, we are currently studying the ability of PrC-210 to suppress X-ray-induced lymphomas using the $p53^{-/-}$ mouse model (30) where tumors occur in 100% of the mice 5–14 weeks after 4 Gy irradiation of the six-day-old neonate mice. After 225 days of observation, nonirradiated six-day-old mouse pups (both $p53^{-/-}$ and $p53^{+/+}$ that received the 0.5 $\times$ maximum tolerated dose (0.252 mg PrC-210/g body-

weight) show no differences in health from nonirradiated control pups that received no PrC-210; in addition, tumor occurrence is reduced and tumor latency is increased, both significantly, in p53^{-/-} mice that received PrC-210 at 26 min before 4 Gy irradiation on day 6 of life.

3. Should PrC-210 follow an entirely different clinical development path, with a clinical indication that has a clear short-term clinical end point, to establish human efficacy, such as suppression of radiotherapy-induced hearing loss (31)? And, once PrC-210 becomes an approved drug for this or another indication, then perhaps over a decade, should we explore PrC-210 as a diagnostic radiation radioprotector.

In this study, the PrC-210 suppression of DNA damage that was observed in each of the assay systems in which it was tested support its development as a radioprotector for humans in multiple radiation exposure settings.

SUPPLEMENTARY INFORMATION

Supplementary materials: Notes from research study, questions and responses and physician verbatim statements.

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