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Determination of plasma levels of the active thiol form of the direct-acting PrC-210 ROS-scavenger using a fluorescence-based assay

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ABSTRACT

PrC-210 is a direct-acting ROS-scavenger. It's active when administered orally, IV, or topically; it has none of the nausea/emesis nor hypotension side effects that have precluded human amifostine use. PrC-210 confers 100% survival to mice and rats that received an otherwise 100% lethal radiation dose and 36% reduction of ischemia-reperfusion-induced mouse myocardial infarct damage, and thus is a viable candidate to prevent human ROS-induced ischemia-reperfusion and ionizing radiation toxicities. We report the first assay for the pharmacologically active PrC-210 thiol in blood. PrC-210 has no double-bonds nor light absorption, so derivatizing the thiol with a UV-absorbing fluorochrome enables quantification. This assay: i) is done on the benchtop; it's read with a fluorescence plate reader, ii) provides linear product formation through 60 min, iii) quantifies μM to low mM rodent blood levels of PrC-210 that confer complete radioprotection, iv) accurately reflects PrC-210 thiol formation of mixed disulfides with other thiols in blood, and v) shows excellent between-day assay outcome with very low standard deviation and coefficient of variation. A fluorescence assay quantifying formation of a PrC-210 thiol-bimane adduct enables measurement of blood PrC-210 thiol. A blood assay will help in the development of PrC-210 for use in the human clinical setting.

1. Introduction

In many clinical settings, including ischemia-reperfusion organ injury [1] and exposure to ionizing radiation [2], the primary toxic event in affected cells is induced by a bolus of reactive oxygen species (ROS), such as hydroxyl radical (·OH). A low toxicity, efficient ROS-scavenger that could be easily and safely administered would have broad application in suppressing or preventing these ROS toxicities.

PrC-210 is a new, direct-acting, aminothiol ROS-scavenger [3–5] that has neither the nausea/emesis nor hypotension side effects [6] that have precluded use of previous aminiothiols like amifostine in humans [7]. In animal models, PrC-210 is orally, IV and topically bioactive; a single systemic dose conferred, 30 min later, 100% survival to mice and rats that received an otherwise 100% lethal radiation dose [3,4]. PrC-210 administration to mice during 45 min coronary artery ligation-induced myocardial infarcts resulted in a 36% reduction in ischemia-reperfusion-induced cardiac muscle death 24 h later. In *ex vivo* human tissue studies, PrC-210 significantly suppressed diagnostic x-ray-induced DNA double-strand breaks (γ-H2AX foci), and also provided 100% suppression of ROS-induced naked DNA damage when added to naked DNA 30 s before the ·OH insult [5]. For these reasons of efficacy and low toxicity in preclinical animal models, PrC-210 is a potential candidate for development as a human pre-exposure and co-exposure ROS-scavenger.

In both preclinical animal development as well as any human clinical trial path, measuring levels of the active PrC-210 thiol drug form in blood, plasma, tissue homogenates and urine is a requirement. Initial studies using [^{14}C]-labeled PrC-210 in both pigs and rats [6] indicated straightforward blood pharmacokinetics and elimination of the small molecule (MW: 148) in urine and feces. Because the PrC-210 molecule has i) no double-bonds, and hence no UV absorbance, and ii) has a free thiol group, bimane derivitization of the thiol to produce a covalent adduct that has both UV absorbance and fluorescence is a logical strategy for measuring levels of the active PrC-210 thiol in plasma and other biological specimens.

In this report we identify reaction conditions in which a stable PrC-210 thiol-bimane adduct is formed, and this reaction enables an efficient and highly sensitive assay for the PrC-210 thiol in a 96 well format to enable measurement of the PrC-210 thiol in biological specimens.

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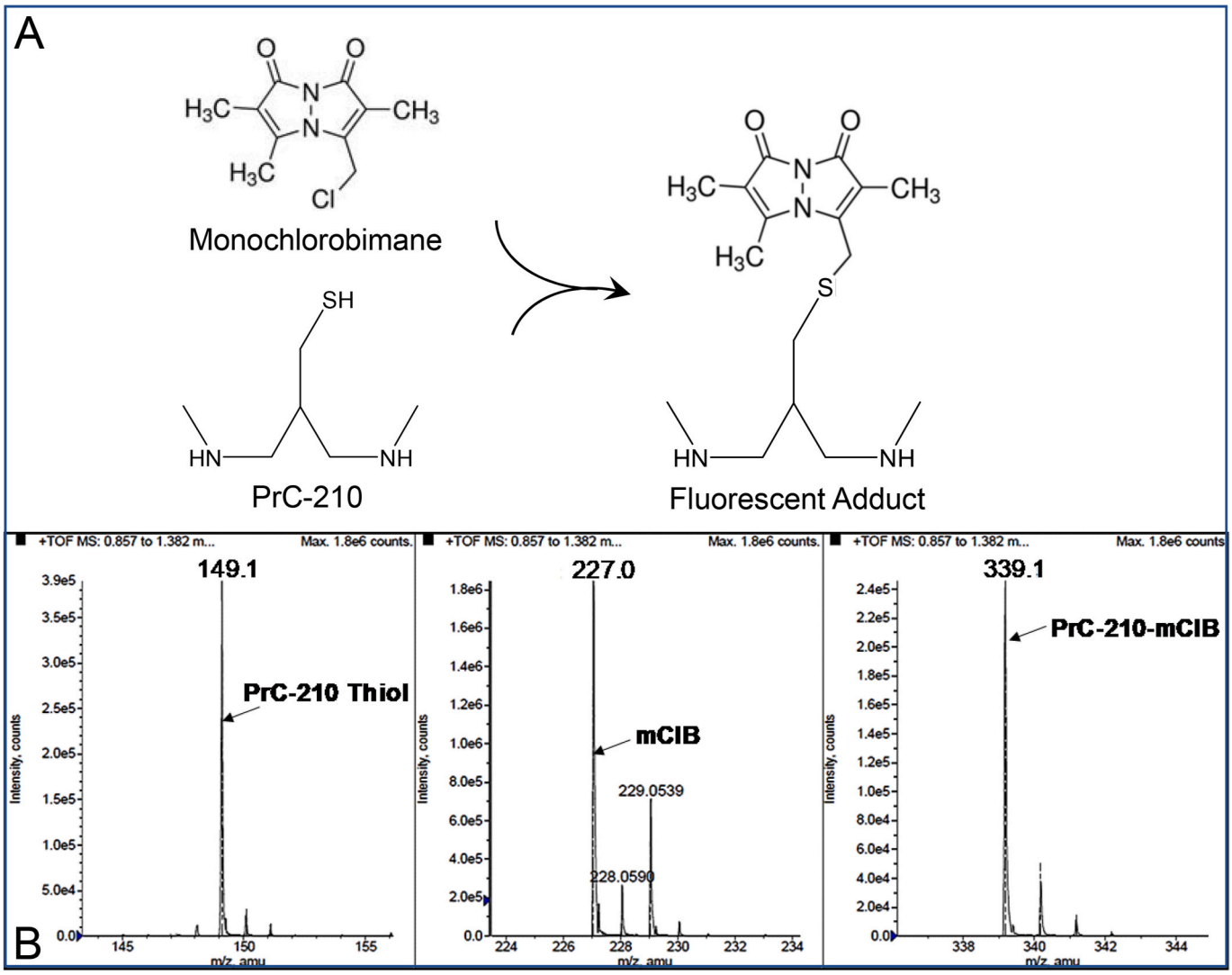


Fig. 1. Chemical structures and characterization of the PrC-210-monochlorobimane covalent conjugate using mass spectrometry. (A) Chemical structures of the pharmacologically-active PrC-210 thiol and the monochlorobimane fluorochrome. (B) Mass spectrograms (left to right) of the PrC-210 thiol form, monochlorobimane, and PrC-210-monochlorobimane conjugate.

2. Materials and methods

2.1. Materials

Synthesis of the PrC-210 aminothiol (3-(methylamino)-2-(methylaminomethyl)propane-1-thiol) is described separately [8,9]. Crystals are stored under a nitrogen atmosphere at −20 °C, and even with routine thawing, use, nitrogen flush of the vial, and re-storage at −20 °C, crystalline PrC-210 is stable for >4 yr by mass spectrometry analysis. Purity of the PrC-210 thiol and disulfide forms was greater than 99% as assayed by mass spectrometry and nmr. Mass spectrometry was performed at the Mass Spectrometry facility in the University of Wisconsin Biotechnology Center. Monochlorobimane (#69899), cysteine (#C5360), glutathione (#G4251) and all other chemical reagents were obtained from Sigma Aldrich (St. Louis, MO). Sprague-Dawley (SD) rats (female, 35–40 g b.w.) that were used as blood donors were from Envigo (Madison, WI). ICR mice (female 20–25 g b.w.) used for plasma PrC-210 concentration and radiation protection experiments were from Envigo (Madison, WI). Rats and mice were maintained on 12 h light/dark cycle and provided *ad lib* water and food. All animal procedures were conducted according to a protocol (#M05744) approved by the University of Wisconsin Institutional Animal Care and Use Committee.

2.2. PrC-210 thiol fluorescence assay

To measure PrC-210 thiol in aliquots of a liquid, such as plasma, a PrC-210 standard curve was first constructed. Aliquots of a 6.66 mM PrC-210 stock were diluted with water to create standards ranging from 0 μM to 666 μM. For the fluorescence assay of known volume aliquots of either plasma or standards, in a 150 μl total volume incubation, i) 90–120 μl of potassium phosphate buffer (0.25 M, pH 7.0) was delivered to wells of black 96 well plates, ii) 15 μl of a 5 mM monochlorobimane stock solution (in methanol:water, 90:10) was added to each of the 96 wells to create a 500 μM final concentration of monochlorobimane, iii) 15 μl of PrC-210 standards or 10–40 μl aliquots of plasma were added to wells via multipipettor, and iv) foil-covered plates were then vigorously rotated at room temperature for 60 min. The pH of the complete reaction mixture, whether it contained PrC-210 standards or plasma, was 7.0–7.1. Fluorescence in the 96 wells (390 excitation, 478 emission) was then determined using a CLARIOStar plate reader.

2.3. Irradiation and PrC-210 treatment

Mice were irradiated in a J.L. Shepherd ^{137}Cs irradiator. For mouse radiation survival assays, unanesthetized mice were placed in small

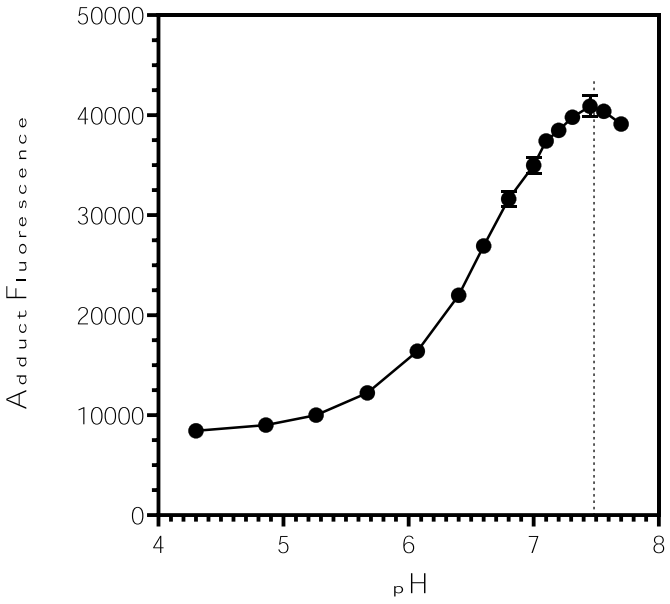


Fig. 2. Conjugation reaction pH optimum. Aliquots of 0.25 M potassium phosphate buffer, at the specified pH's, were combined with 333 μM PrC-210 (final concentration) in water and 500 μM monochlorobimane (final concentration) in methanol:water (90:10) and incubated in the dark at room temperature for 1 h. Fluorescence of the PrC-210-monochlorobimane adduct was monitored on a plate reader with excitation at 390 nm and emission at 470 nm (see Methods).

plastic containers centered in the irradiator and received 9 Gy of whole-body radiation at a dose rate of 2.1 Gy/min. Radiation dose was calibrated using thermoluminescent dosimeters. Mice received either intraperitoneal water or intraperitoneal PrC-210 dissolved in water (pH ~6.0) 30 min prior to irradiation. Mice were observed for 30 days post-irradiation. There were 10–12 mice in each PrC-210 treatment group.

2.4. Statistical analysis

Graphpad Prizm software, was used for plotting data and for analysis of statistical differences between treatment groups.

3. Results

3.1. A stable, fluorescent PrC-210 thiol-bimane adduct

In an initial experiment, we found that mixing monochlorobimane, dissolved in an alcohol:water solution (methanol:water, 90:10) with an equimolar solution of PrC-210 in water at room temperature resulted in the formation of a stable product of the appropriate mass and charge (Fig. 1) to indicate formation of the PrC-210-bimane adduct. Formation of this adduct was expected in the context of published reports of other thiol-bimane adducts [10–14]. PrC-210, when dissolved in aqueous buffer shows the single *m/z* mass at 149 indicating the pure PrC-210 thiol form. At pH 7.2, PrC-210 thiol conversion to disulfide form (*m/z* peaks at 295,148) occurs with a half-life of 3.5 h; in water at pH ≤ 6, the PrC-210 thiol is stable for more than a week. The *m/z* peak at 339 indicates formation of the PrC-210-monochlorobimane conjugate, presumably through a thiol bridge, as previously described for other bimane-thiol adducts [10,11].

3.2. pH-Dependence of the conjugation reaction

To maintain a stable pH within the reaction mixture, to enable both stable reaction rate and stable fluorescence excitation and emission, whether it contained PrC-210 standards in water or various plasma pH-modifiers, 0.25 M potassium phosphate was used. Titration of the KH_2PO_4 stock solution created a family of buffer solutions from pH 4.3 to 7.7 (Fig. 2). Addition of 333 μM PrC-210 and 500 μM monochlorobimane to these buffer aliquots showed that peak adduct

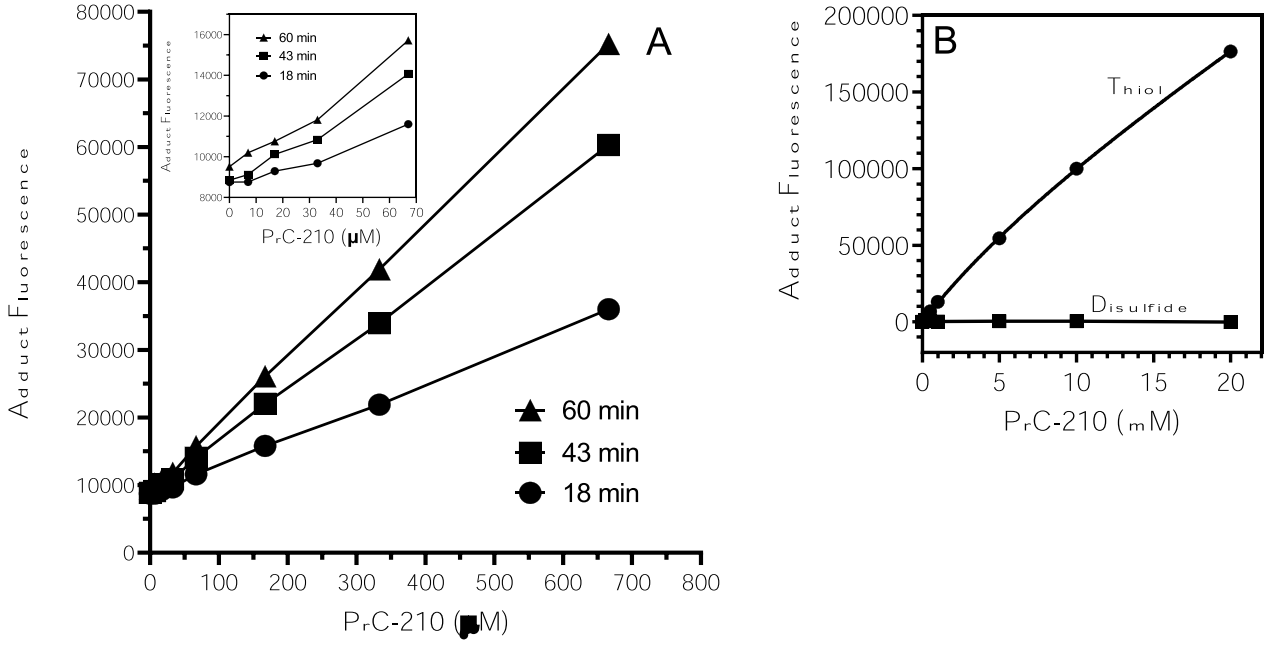


Fig. 3. PrC-210 adduct formation as a function of both incubation time and PrC-210 concentration. (A) A standard curve containing 7–666 μM PrC-210 thiol and 500 μM monochlorobimane in pH 7.0 0.25 M phosphate buffer was shaken at room temperature for the indicated times and adduct fluorescence was monitored. (B) Parallel reaction incubations, with PrC-210 added at the specified concentrations to buffer, either in its thiol form or in its disulfide form, were mixed and fluorescence was determined after 1 h. For illustration purposes, standard deviation bars are shown for three data points on the 60 min curve in panel A.

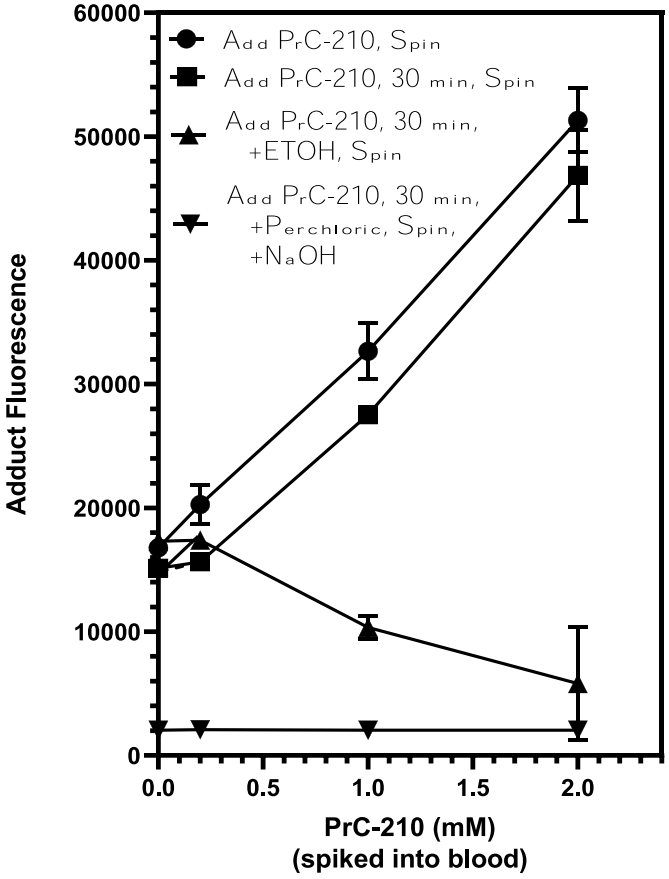


Fig. 4. Determining an optimized processing strategy for PrC-210-spiked into rat blood. 100 μl aliquots of rat blood were spiked with the indicated concentrations of PrC-210. Samples were then processed as indicated in the figure. In two sets, either 55% ethanol (final concentration) or 0.25 M perchloric acid (final concentration) were added to precipitate blood constituents. For all samples, centrifugation at 14,000 rpm in a microfuge for 5 min resulted in clear supernates. A predetermined amount of 5 N NaOH was added to perchloric acid supernates to yield a final pH of 7.0. Aliquots of all final supernates were then added to standard incubations containing pH 7.0 potassium phosphate buffer and monochlorobimane, and adduct fluorescence after 1 h was determined.

formation was achieved in the pH 7.1–7.6 range (Fig. 2).

3.3. A 96 well fluorescence assay

A 96 well assay format was designed to enable routine analysis of small (microliter) aliquots of liquids, such as plasma, serum, tissue culture media, etc. Both the monochlorobimane reactant and the PrC-210 thiol are sufficiently stable to enable routine reagent preparation and dilutions of standards to the 96 well format. Unknown samples, in which PrC-210 thiol may be less stable because of basic pH or other reasons, can be immediately thawed and microliter aliquots added directly to 96 wells pre-charged with monochlorobimane, so that the thiol-bimane reaction can immediately proceed upon admixture in the well. With standardized mixing of PrC-210 thiol standards (0–666 μM final concentration) and monochlorobimane, both linear PrC-210 concentration-dependent, and linear time-dependent, formation of the fluorescent thiol-bimane product were observed (Fig. 3A, and inset). Addition of the pure PrC-210 disulfide, instead of the PrC-210 thiol, to the other reactants produced no detectable fluorescent product (Fig. 3B).

3.4. PrC-210 in blood workup

Three approaches were compared to determine the best way to quickly process rat blood that had been spiked with PrC-210. These included: i) spike blood with PrC-210, immediately microfuge the blood (13,000 rpm, 45 s) and immediately transfer and freeze the supernate plasma using dry ice, i(a)) same as i), except allow the blood to sit at room temperature for 30 min before microfuge, ii) spike blood with PrC-210, wait 30 min at room temperature, add 4 °C ethanol (to achieve 55% ethanol final), microfuge and freeze the supernate, iii) spike blood with PrC-210, wait 30 min at room temperature, add 0.5 M perchloric acid to achieve 0.25 M final concentration, microfuge and neutralize the supernate with added NaOH, and freeze the supernate. The supernates were then thawed and assayed in pH 7.0 reactions, and Fig. 4 shows the adduct formation associated with each procedure. Simple collection of blood into EDTA- or heparin-tubes, spiking with PrC-210, and then rapid microfuge spin and freezing the supernate gives the most consistent outcome. The downward displacement of the curve with a 30 min wait before microfuging is consistent with the previously determined PrC-210 half-life of 3.5 h at pH 7.2[5]. Precipitation steps with either ethanol or perchloric acid were not productive.

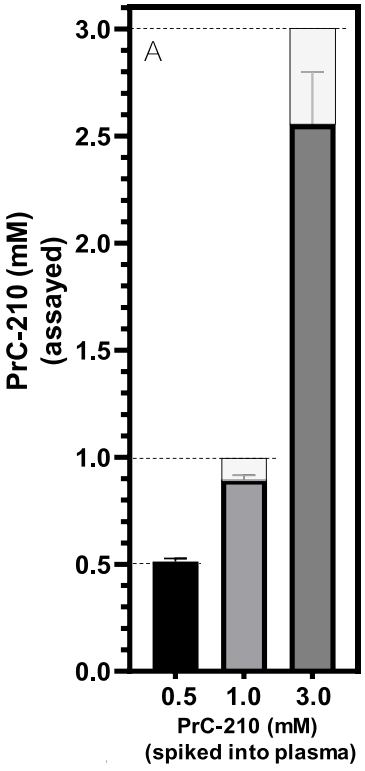
3.5. PrC-210 fate in plasma

To validate this adduct-formation assay, PrC-210 was spiked into rat plasma to achieve three known plasma concentrations, 0.5, 1.0 and 3.0 mM, the tubes sat at room temperature for 15 min, and aliquots of the spiked plasma were then frozen, thawed, and assayed in the 96 well assay described here. At 0.5, 1.0 and 3.0 mM spiking, the assayed values were 0%, 10.5% or 14.5% lower than the specified spike concentration (Fig. 5B).

To explore at least two explanations for “the loss” of PrC-210 thiol spiked into plasma, equimolar concentrations of PrC-210 thiol, cysteine-thiol or glutathione-thiol were incubated together in water, and aliquots of both the pre- and post-reaction mixtures were analyzed by mass spectrometry (Fig. 5C). Ion masses consistent with formation of both PrC-210-cysteine and PrC-210-glutathione mixed disulfides were observed.

3.6. PrC-210 fate in whole blood

When 1.0 mM PrC-210 was spiked into rat plasma the assay standard curve (PrC-210 dissolved in water) extrapolated a concentration of 0.89 mM in the plasma sample (Figs. 5A and 6, center bar). When 1.0 mM PrC-210 was spiked into rat whole blood, the blood was immediately



PrC-210 reaction with Cysteine-thiol and Glutathione-thiol C

Molecules in Reaction	Pre-Reaction Mass spectrometry peaks m/z	Post-Reaction Mass spectrometry peaks m/z
Reaction A*		
PrC-210 thiol	149.1	149.1
Cysteine thiol	122	122
		268.1 (PrC-210-Cysteine disulfide, 1 charge)
		134.5 (PrC-210-Cysteine disulfide, 2 charges)
Reaction B*		
PrC-210 thiol	149.1	149.1
Glutathione (GSH) thiol	308.1	308.1
		456.4 (PrC-210-GSH disulfide)
		615.5 (GSH disulfide)

*Reactions were in water, pH 5.5, 24 hr at room temperature in the dark.

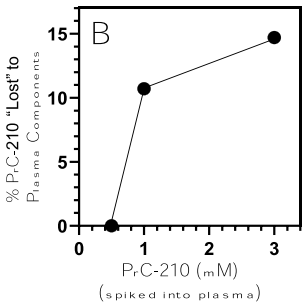


Fig. 5. Fate of PrC-210 thiol when spiked into rat plasma. (A) 95 μl aliquots of rat plasma were spiked with 5 μl aliquots of water containing PrC-210 thiol to achieve the final indicated concentrations. Samples sat at room temperature for 3 min to simulate blood recovery and micro-centrifugation from a rodent or human, and were then frozen on dry ice. Aliquots of the thawed plasma (30 μl) were then assayed versus a PrC-210 standard curve (0–666 μM PrC-210 in buffer), and after a 1 h incubation at room temperature, adduct fluorescence was determined, and the PrC-210 thiol concentrations in plasma, extrapolated from the standard curve were plotted. Shaded boxes highlight the PrC-210 "shortfall" that was observed in the 1.0 mM and 3.0 mM samples. (B) The observed concentration shortfalls in the assayed plasma samples, plotted against the spiked plasma PrC-210 concentration, are shown. (C) In separate, water incubations, PrC-210 thiol was incubated with either cysteine-thiol or glutathione-thiol for 24 h, and reaction samples were then analyzed by mass spectrometry.

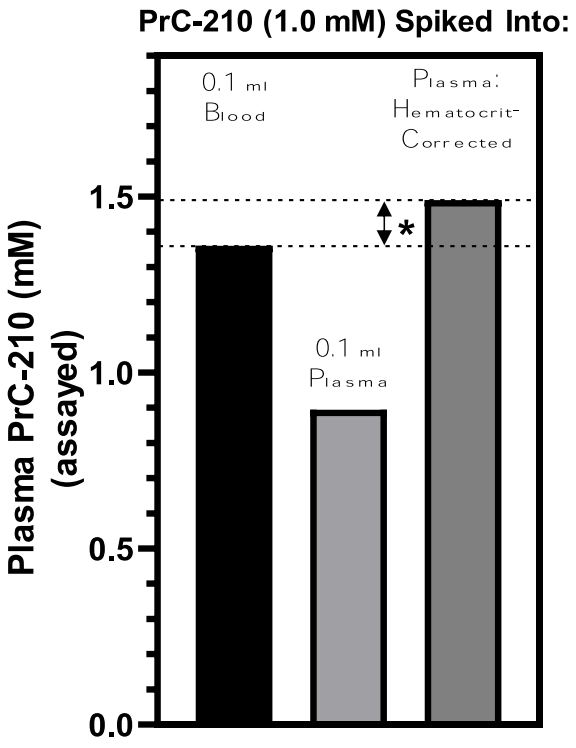


Fig. 6. Fate of PrC-210 thiol when spiked into rat blood or plasma. 95 μl aliquots of rat whole blood or rat plasma were spiked with 5 μl aliquots of water containing PrC-210 to achieve a final concentration of "1 mM" After 3 min at room temperature, blood was micro-centrifuged for 30 s, and both the blood plasma supernate from the spin, and the separate plasma sample were frozen on dry ice, then thawed, and then assayed for PrC-210 content versus a PrC-210 standard curve prepared in pH 7.0 phosphate buffer.

Table 1
Validation parameters of PrC-210 assay in rat plasma.

Spiked Sample Conc (mM)	Measured concentration (mM)			
	Within Day (n = 5)		Between Days (n = 5)	
0.50	Mean	0.502	Mean	0.498
	SD	0.015	SD	0.016

microfuged, and the plasma supernate was then assayed, the assay determined a concentration of 1.36 mM in the plasma (Fig. 6, left bar). If the PrC-210 could not access a portion of the whole blood volume in the time before microfuging and pelleting the blood cells, then its concentration in the available liquid volume would be incrementally higher. If the 0.89 mM PrC-210 concentration found in 0.1 ml of plasma is divided by 0.6, to account for a standard 40% hematocrit in rat blood, the calculated PrC-210 concentration is then 1.49 mM (Fig. 6, right bar). The 0.12 mM difference in PrC-210 ("*" in Fig. 6) between measured blood plasma and hematocrit-corrected blood plasma could result from passage of the PrC-210 molecule into blood cells in the few minutes before microfuging.

3.7. PrC-210 assay validation

The spiked addition of 0.5 mM PrC-210 to rat plasma (Fig. 5A) yielded samples that measured 0.502 mM ± 0.015 SD (n = 5) by assay when the samples were freeze/thawed on the day of the spiking, and 0.498±0.016 SD when the samples were thawed on the day after the spiking. This is a standard deviation for the assay of 3.0% (Table 1).

3.8. PrC-210-conferred radioprotection

Fig. 7A shows that a single intraperitoneal dose of PrC-210 (252 μg/gm b.w.) administered between 7.5 and 60 min before whole-body radiation conferred highly significant radioprotection (*P* values of <0.0001 to 0.001) versus the sham-treated 0 min controls. In Fig. 7B experiment, we treated mice with the same intraperitoneal dose of PrC-210 as used in Fig. 7A radioprotection experiment, and then collected blood, prepared plasma, and measured the PrC-210 concentrations to provide both a picture of the pharmacokinetics of PrC-210 absorption from the peritoneal cavity into blood and its clearance; these blood concentrations and the associated pharmacokinetic parameters that determined them conferred 100% radioprotection. The peak PrC-210 plasma level of 2.3 mM was achieved rapidly, just 3 min following IP injection.

4. Discussion

We report here the first assay for the pharmacologically active form, PrC-210 thiol, in blood and plasma. The assay is also well-suited for assays of the molecule in tissue culture media and other biological fluids to establish half-life and effective drug concentrations. The small PrC-210 molecule, with no double-bonds, has no light absorption, so derivatization of the free thiol with a UV-absorbing fluorochrome provides many options to quantify PrC-210 without the substantial cost, or the accessibility issues during an extended pandemic, of accessing technically-staffed, liquid chromatography-mass spectrometry instruments. The assay described here: i) is conveniently done on the benchtop and read with a standard fluorescence plate reader, ii) provides linear product formation over 1 h, iii) detects and quantifies the μM to low mM blood levels of PrC-210 thiol found in rodents in which PrC-210 confers complete radioprotection, iv) accurately reflects PrC-210 thiol formation of mixed disulfides with other thiols present in blood, and v) shows excellent between-day storage and assay outcome with very low standard deviation and coefficient of variation.

Derivitization of a thiol group with monochlorobimane to form a

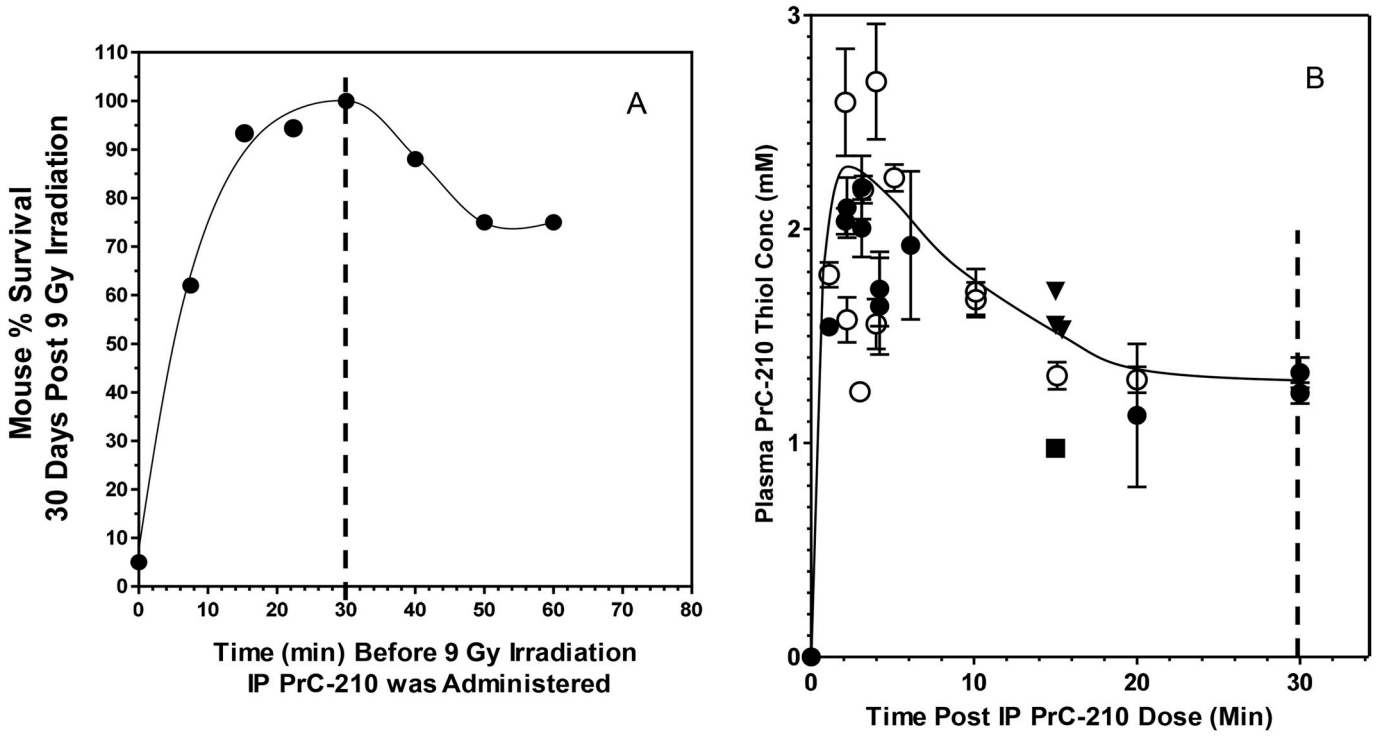


Fig. 7. Mouse plasma PrC-210 thiol levels and their radioprotective impact upon mouse survival following a single intraperitoneal dose of PrC-210. (A) 30-Day survival in groups of mice that were given a single intraperitoneal injection of PrC-210 (252 μg/gm b.w.) at the indicated times prior to a single-dose of whole-body radiation (9 Gy). (B) Individual levels of PrC-210 thiol in plasma samples of individual mice (at indicated times post intraperitoneal injection) that received a single intraperitoneal injection of PrC-210 at "0 Min." Mice received a single dose of 252 μg/gm bw, which equals the 0.5 X Maximum Tolerated PrC-210 Dose [4]. Different symbols represent different experiments.

fluorescent, UV-absorbing adduct of a thiol molecule has been widely used before to quantify plasma and cell levels of the thiols of interest [10–14]. The pH optimum of 7.4 for formation of the PrC-210-monochlorobimane adduct is similar to that of the previous examples as well. There was no detectable reaction between monochlorobimane and the PrC-210 disulfide, so the derivatization reaction provides a simple means to measure levels solely of the pharmacologically-active thiol form of the drug molecule. As described with previous derivatization assays of thiols [14,16], i) reaction of the thiol of interest with physiologic thiols in blood, principally cysteine and glutathione, and ii) reaction of the derivatizing agent with these same thiols to create possible fluorescent background, are always-present aspects of assays such as the one presented here for PrC-210 thiol.

PrC-210 is the prototype of a new family of direct-acting, small molecule aminothiol ROS scavengers [3,4], which can be administered orally or IV, and it has none of the nausea/emesis nor hypotension side effects [6] in animal models that have prevented significant use of earlier aminothiols like amifostine in humans. Because PrC-210 is orally bioactive, where it confers: i) 100% survival to mice and rats that received an otherwise 100% lethal radiation dose, and ii) 36% reduction in ischemia-reperfusion-induced mouse cardiac tissue damage [15], it is a viable candidate for use in humans to suppress or prevent ROS-induced toxicities from both ischemia-reperfusion [1] and ionizing radiation [2]. Having an efficient assay to enable blood and tissue levels of the active PrC-210 thiol form will support and hasten clinical development of this new drug molecule.

5. Conclusion

A simple fluorescence-based assay that quantitates formation of a PrC-210 thiol-bimane adduct has enabled straightforward measurement of the active PrC-210 thiol form in blood plasma. A functional blood assay will help in the clinical and commercial development of PrC-210 for use in a variety of human clinical settings.

Author contributions

Both authors contributed equally to this manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

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