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The antitumor thioredoxin-1 inhibitor PX-12 (1-methylpropyl 2-imidazolyl disulfide) decreases thioredoxin-1 and VEGF levels in cancer patient plasma

Amanda F. Baker¹, Tomislav Dragovich^{1,5}, Wendy R. Tate¹, Ramesh Ramanathan², Denise Roe³, Chiu-Hsieh Hsu¹, D. Lynn Kirkpatrick⁴, and Garth Powis¹

¹ Arizona Cancer Center, University of Arizona, Tucson, AZ 85724 USA

² University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213 USA

³ Arizona Cancer Center, College of Medicine, and College of Public Health, University of Arizona, Tucson, AZ 85724 USA

⁴ ProIX Pharmaceuticals, Tucson, AZ 85705 USA

Abstract

Thioredoxin-1 (Trx-1) is a small redox protein that is over-expressed in many human tumors, where it is associated with aggressive tumor growth and decreased patient survival. Trx-1 is secreted by tumor cells and is present at increased levels in the plasma of cancer patients. PX-12 is an irreversible inhibitor of Trx-1 currently in clinical development as an antitumor agent. We have used SELDI-TOF mass spectrometry to measure plasma Trx-1 from patients treated with PX-12 during a Phase I study. Mean plasma Trx-1 levels at pretreatment were significantly elevated in the cancer patients at 182.0 ng/ml compared to 27.1 ng/ml in plasma from healthy volunteers. PX-12 treatment significantly lowered plasma Trx-1 in cancer patients, with the greatest decrease seen in patients having the highest Trx-1 pretreatment levels. High plasma vascular endothelial growth factor (VEGF) levels have been correlated to decreased patient survival. PX-12 treatment also lowered plasma VEGF levels in cancer patients with high pretreatment levels. SELDI-TOF mass spectrometry identified 7 additional plasma proteins whose levels decreased following PX-12 administration, one of which was identified as a truncated form of transthyretin. The results of this study suggest that the lowering of elevated levels of plasma Trx-1 in cancer patients may provide a surrogate for the inhibition of tumor Trx-1 by PX-12. Furthermore, PX-12 decreases plasma VEGF levels which may contribute to the antitumor activity of PX-12.

Keywords

PX-12; thioredoxin-1; VEGF

INTRODUCTION

Thioredoxin-1 (Trx-1) is a low molecular weight redox protein found in both prokaryotic and eukaryotic cells. The cysteine (Cys) residues at the conserved –Cys³²-Gly-Pro-Cys³⁵-Lys active site of human Trx-1 undergo reversible oxidation-reduction catalyzed by the NADPH-dependent flavoprotein thioredoxin reductases [1,2]. Increased cellular Trx-1 has multiple effects including an increase in cell proliferation and inhibited apoptosis (reviewed in [3]).

⁵Co-first author

Mailing address: Garth Powis, D.Phil. Arizona Cancer Center 1515 N. Campbell Ave. Room 3977 Tucson, AZ 85724 (520) 626-6934/4848/2446 (office/fax/lab)

Trx-1 acts as an antioxidant through its ability to reduce thioredoxin peroxidases (peroxiredoxins) that scavenge H₂O₂ and organic hydroperoxides [4]. Trx-1 exerts redox control over a number of transcription factors by increasing their binding to DNA, thus, regulating gene transcription. Transcription factors regulated by Trx-1 include NF-κB [5], the glucocorticoid receptor [6], p53 [7], hypoxia inducible factor-1 (HIF-1) [8,9] and indirectly activator protein-1 (AP-1) via a nuclear redox factor Ref-1/HAPE [10]. Trx-1 also binds to redox sensitive enzymes to regulate their activity including apoptosis signal-regulating kinase-1 (ASK-1) [11], protein kinases C δ, ω, ζ [12], and the dual specificity tyrosine-threonine/phosphatidylinositol-3-phosphatase tumor suppressor protein, PTEN (phosphatase and tensin homolog deleted on chromosome ten) that negatively regulates the phosphatidylinositol-3-kinase/Akt-survival signaling pathway [13].

Trx-1 is over-expressed in many human cancer including lung, colon, cervical, gastric, and pancreatic cancer (reviewed in [3]). Clinically, increased tumor Trx-1 levels have been linked to aggressive tumor growth, inhibition of apoptosis, and decreased patient survival [14]. Trx-1 over-expression has been shown to increase vascular endothelial growth factor (VEGF) production and promote tumor angiogenesis [15]. Trx-1 is secreted by cancer cells through a leaderless secretory pathway [16] and extracellular Trx-1 may act as a growth factor and co-cytokine [17]. Trx-1 levels have been reported to be increased in the plasma of patients with pancreatic and hepatocellular cancers [18,19].

The role Trx-1 plays in promoting cell survival, proliferation and tumor angiogenesis make it an attractive molecular target for therapeutic intervention in cancer [3]. PX-12 (1-methylpropyl 2-imidazolyl disulfide) is a Trx-1 inhibitor that binds irreversibly rendering Trx-1 redox inactive [20]. PX-12 inhibits the expression of VEGF by cells in culture and in human tumor xenografts by preventing Trx-1's stimulation of the HIF-1 transcription factor [21]. PX-12 is the first Trx-1 inhibitor to undergo clinical testing in cancer patients and has recently completed a Phase I clinical trial [22]. Because Trx-1 is secreted by tumors, plasma Trx-1 offers what is a relatively unique opportunity of assessing the effects of a cancer drug on its presumed molecular target in patient plasma without the need for tumor biopsy. We have measured plasma Trx-1 and VEGF in patients receiving PX-12 in the Phase I clinical trial and found that PX-12 treatment decreases both plasma and Trx-1 and VEGF, with the greatest effects being seen in patients with the highest pretreatment Trx-1 and VEGF levels.

METHODS

Materials

Chemicals were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise specified. Recombinant purified human and mouse Trx-1 and mouse monoclonal anti-human Trx-1 antibody (5A3G5) were prepared as previously described [14]. ProteinChips® for surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry were obtained from Ciphergen Biosystems (Fremont, CA).

Mouse Studies

Non-tumor-bearing male C57BL/6 mice were administered PX-12, 25mg/kg intravenously, dissolved in 10% ethanol, 0.9% NaCl intravenously. The mice were euthanized at 0, 0.5, 2, 6, and 24 hr and blood collected into EDTA-coated tubes. Plasma was immediately separated by centrifuging samples at 300 × g for 15 min and stored frozen at -80°C until assay.

Patient Samples

Peripheral blood samples were collected from healthy normal volunteers and from 31 of 38 total patients with advanced solid malignancies receiving PX-12 as part of a Phase I clinical

trial. Informed patient consent was obtained according to Institutional Review Board procedures. The patients we studied were a typical Phase I trial group of advanced malignancies with (number of patients in parenthesis) colorectal (16), squamous cell (1), appendiceal (1), pancreatic (1), renal (1), sarcoma (4), hepatocellular (1), gastroesophageal (1), non-small cell lung cancer (3), and cholangiocarcinoma (2). Patients were administered PX-12 as either a 1 hr or 3 hr infusion, daily for 5 days, every three weeks (one cycle). Blood was collected in heparin-coated tubes, 4 hr post infusion and 3 weeks post infusion before the second course of treatment. Plasma was separated by centrifugation at $200 \times g$ at room temperature for 5 min followed by recentrifugation for 5 min at $300 \times g$ at room temperature to sediment particulate matter. The plasma was stored frozen at -80°C until assay.

Trx-1 measurement by SELDI-TOF mass spectrometry

Plasma Trx-1 was measured by SELDI-TOF mass spectrometry on a Protein Biology System II (PBS II, Ciphergen Biosystems, Fremont, CA). For assay development, a panel of Protein Chips® (weak cationic exchanger, strong anionic exchanger, ion hydrophobic, normal phase) were incubated with normal human plasma and tested for their ability to bind Trx-1 using a low stringency binding buffer according to manufacturer's instructions. The weak cation exchange (CM10) chip was found to be optimal for detecting Trx-1 in plasma. CM10 chips were activated with 2, 5 min incubations with 5 μl 0.1M sodium acetate, pH 4.5, and then 10 μl of a 1:4 dilution of plasma in 0.1M sodium acetate, pH 4.5, added to the chip and incubated in a humidity chamber for 1 hr at room temperature. The chip surface was washed twice with 5 μl 0.1M sodium acetate, pH 4.5, twice with 5 μl double distilled H_2O and allowed to air dry at room temperature before two additions of 1 μl 50% 3,5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile with 0.5% trifluoroacetic acid, allowing air drying between applications. Chips were stored at room temperature and analyzed by SELDI-TOF mass spectrometry within 7 days. Each plasma sample was analyzed in triplicate.

Before each use the SELDI-TOF mass spectrometer was calibrated using eight purified proteins of mass 8.50 kDa to 150.00 kDa, namely bovine ubiquitin (8.56 kDa), bovine cytochrome C (12.23 kDa), bovine superoxide dismutase (15.59 kDa), bovine beta-lactoglobulin A (18.36 kDa), horseradish peroxidase (43.24 kDa), bovine serum albumin (66.41 kDa), chicken conalbumin (77.49 kDa), and bovine IgG (147.30 kDa). The reference proteins were mixed and combined with 3,5-dimethoxy-4-hydroxycinnamic acid on a normal phase chip and the mass spectrometer calibrated according to manufacturers' instructions. Spectra were normalized to the average total ion current of the spectra in the range from 10.00 to 60.00 kDa. The peaks in this mass range were not affected by ion suppression or saturation of the detector. Proteins with a mass of 50.00 to 75.00 kDa were not accurately measured due to the abundance of albumin (66.43 kDa) in plasma. The Biomarker Wizard software program (Ciphergen Biosystems, Fremont, CA) was run with the following parameters: auto-detect peaks; peaks must be present in the percentage equal to one timepoint (30% for human, 20% for mice); signal-to-noise ratio equals 5 in the first pass, 2 in the second pass; peaks clustered within 0.3% of mass.

Identification of Trx-1 in plasma

Immunodepletion studies were performed using mouse monoclonal anti-human Trx-1 antibody cross-linked to cyanogen bromide-activated-Sepharose 4B beads. Normal human plasma was incubated with gentle rotation with 0, 10, 50, 100 and 400 μl of the beads overnight at 4°C and collected by centrifugation at $300 \times g$ for 10 min. Trx-1 in 2 μl supernatant was assayed by SELDI-TOF mass spectroscopy.

Quantitation of Trx-1 in plasma

A standard curve for Trx-1 was generated by the addition of recombinant human purified Trx-1 to normal plasma. The limit of detection of the assay for Trx-1 was 10ng/ml with saturable binding occurring at 500ng/ml. The Trx-1 concentration in a plasma sample was determined by comparison of the relative signal intensity of the test sample with that for recombinant Trx-1 added to plasma as a standard. All of the clinical samples were run on the same chip lot number, spotted and analyzed as a group. When 4 samples with a wide range of Trx-1 peak intensity values were assayed in duplicate on different assay days the trends between samples were consistent, but the peak intensity values on different days varied emphasizing the necessity of running a standard curve with each set of samples. The average coefficient of variation between triplicate samples on the same day, using the same lot number of chip was $\pm 35\%$, which is similar to coefficient of variance values reported by others [23-25].

VEGF ELISA

Plasma VEGF A was measured using a human VEGF ELISA kit (R&D Systems, Minneapolis, MN) according the manufacturers' instructions. A standard curve was run for each plate in duplicate, and each sample was measured in triplicate. Sufficient plasma was available for only 19 of 31. The detection limit of the assay for VEGF was 9 pg/ml plasma.

Identification of transthyretin

To identify the unknown 13.86 kDa plasma peak 0.5ml normal human plasma was diluted 1:1 with 20mM Tris-HCl, pH 9 in 0.1M NaCl and passed through an Anion exchange spin column (Ciphergen Biosystems, Fremont, CA) equilibrated in the same buffer. After washing, the column was eluted with buffers of increasing salt concentration and pH. Each buffer eluant was assayed by SELDI TOF MS to identify the fraction that contained 13.86 kDa protein. The 13.86 kDa protein was further isolated by using 2D SDS polyacrylamide gels (ZOOM® system, Invitrogen, Carlsbad, CA). The first dimension was resolved using a pH 3-10 gel strip and the second dimension using a 4-20% Tris-glycine polyacrylamide gel according to manufacturer's instructions. The gel was silver stained and the spot located at 13.86 kDa cut out, destained and gel pieces were dehydrated with acetonitrile and reswelled with 50 μ L digestion buffer containing 50mM NH₄HCO₃, 10% acetonitrile, and 10 ng/ μ L sequencing grade trypsin (Roche, Mannheim, Germany). Digestions were carried out for overnight at 37°C. Tryptic peptides were extracted using 5% acetic acid. LC-MS/MS analyses of the in-gel digested protein band/spot were carried out using a quadrupole ion trap LCQ DECA XP PLUS mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a Michrom PARADIGM MS4 HPLC and a nano-electrospray ionization source (Auburn, CA). The sequences of individual peptides were identified using the Turbo SEQUEST™ algorithm to search and correlate the MS/MS spectra with amino acid sequences in the non-redundant protein database [26]. Tandem MS spectra of peptides were analyzed with Sequest, a program that allows the correlation of experimental tandem MS data with theoretical spectra generated from known protein sequences.

Statistical analysis

The comparisons of mouse plasma Trx-1 values, and normal to cancer patient baseline Trx-1 values were performed using a two-sided t-test. To test the hypothesis that PX-12 treatment decreases Trx-1 and VEGF levels, a p-value was generated using a one-sided paired t-test. For human plasma difference measurements post-PX-12 administration peaks of interest minus post-treatment, were analyzed by a linear regression model was used with a square root transformation of the dependent variable.

RESULTS

Measurement of plasma Trx-1

Preliminary studies using mouse plasma showed that the CM10 weak cation exchanger chip bound the most plasma proteins in the molecular mass range of 10.00-200.00 kDa and allowed the reproducible detection of Trx-1. Plasma from non-tumor bearing C57BL/6 mice was found to have a peak at 11.68 kDa corresponding to the predicted mass of mouse Trx-1 based on the amino acid sequence. This peak overlaid the peak when recombinant mouse Trx-1 added to mouse plasma (Figure 1A). Normal human plasma obtained from healthy volunteers exhibited a peak at 11.73 kDa that corresponding to the predicted molecular mass of human Trx-1 based on the amino acid sequence, and was at the same position as recombinant human Trx-1 added to the normal plasma (Figure 1B). Human plasma was also immunodepleted using increasing amounts of anti-human Trx-1 monoclonal antibody and the size of the peak decreased as the amount of antibody used increased (Figure 1C) confirming that the 11.73 kDa peak in human plasma was Trx-1.

Effect of PX-12 on Trx-1 in mouse plasma

Trx-1 in mouse plasma was measured in non-tumor-bearing mice before and after the administration of PX-12, 25 mg/kg, intravenously. A significant decrease ($\pm$ S.E) of $61.5 \pm 6.3\%$ ($p < 0.01$ compared to control value) in plasma Trx-1 was observed 2 hr after PX-12 treatment and remained inhibited by 76.3 ± 8.9 ($p < 0.01$) at 24 hr (Figure 2). PX-12 was found to alter 27 other peaks detectable between 10.00 and 200.00 kDa in the mouse plasma. Out of these, 5 peaks were significantly decreased and 11 were significantly increased 2 hr after of PX-12 treatment (data not shown).

Trx-1 in normal and patient plasma

Blood samples from 7 normal, healthy human volunteers and 31 cancer patients enrolled in a Phase I clinical trial of PX-12 were collected, the plasma separated and assayed in triplicate for Trx-1 using SELDI-TOF. The mean Trx-1 level in plasma from healthy volunteers was ($\pm$ SE) 27.6 ± 10.8 ng/ml and in the cancer patients before treatment 182.0 ± 21.8 ($p < 0.003$) (Figure 3). Of the 13 patients with Trx-1 levels above the mean, 6 had colorectal cancer, 2 NSC lung cancer, 1 lung adenoma, 1 hepatocellular carcinoma, 1 cholangiocarcinoma, 1 sarcoma, and 1 pancreatic cancer.

Effect of PX-12 on Trx-1 levels in humans

Trx-1 levels were measured in 31 patients treated with PX-12. As a group there was no significant difference between pretreatment plasma Trx-1, and plasma Trx-1 4 hr or 24 hr after PX-12 administration. There was, however, a significant correlation between pretreatment Trx-1 levels and the decrease in plasma Trx-1 at 4 hr following PX-12 treatment, with larger decreases being seen in patients with the higher pretreatment Trx-1 levels ($R^2 = 0.225$, $p = 0.007$) (Figure 4A). There was still a significant correlation between pretreatment Trx-1 levels and the decrease in plasma Trx-1 up to 3 weeks after PX-12 treatment, that is, immediately before the next course of treatment ($R^2 = 0.223$, $p = 0.007$). Additional analyses explored the relationships between the difference in Trx-1 after PX-12 treatment and infusion time, dose, and drug duration as 1 or 2 treatment cycles (16 patients) versus 3 or more treatment cycles (15 patients), but showed no statistically significant changes.

Effect of PX-12 on VEGF in patient plasma

VEGF levels were measured in plasma collected from 19 patients treated with PX-12, with an average pre-treatment mean of 177.4 ± 35.6 pg/ml (S.E.). Pretreatment plasma VEGF levels in 7 patients were within or below the published mean values for normal plasma VEGF levels

of 48 to 76 pg/ml [27,28]. Of interest, 9 of the 12 patients with the highest levels of plasma VEGF had colorectal cancer. Other tumor types with high plasma VEGF included pancreatic, NSC lung cancer, and renal cancer. There was a significant correlation between pretreatment VEGF levels and the decrease in plasma VEGF at 4 hr following PX-12 treatment with larger decreases in patients with the higher pretreatment VEGF levels ($R^2 = 0.465$, $p = 0.001$) (Figure 4B). There were insufficient patient samples available at the other time points for VEGF analysis. There was no significant correlation between patients with high Trx-1 and high VEGF, or between decreases in plasma Trx-1 and VEGF following PX-12 treatment.

Effect of PX-12 on plasma proteomic profile

The pattern of plasma protein changes resulting from PX-12 treatment of cancer patients was studied. There were 57 unique peaks, but only 8 of these peaks were detectable in all the samples tested (excluding albumin peaks). These peaks were located at 11.73 kDa (Trx-1), 13.86 kDa, 17.35 kDa, 22.20 kDa, 28.00 kDa, 44.46 kDa, 88.75 kDa, and 110.53 kDa. Four peaks, 13.86 kDa, 22.20 kDa, 44.46 kDa, and 110.53 kDa, showed a significant decrease after 4 hr of PX-12 treatment, independent of pretreatment peak intensity. A representative spectrum from a patient showing pre versus post-PX-12 treatment plasma is given in Figure 5. The 13.86 kDa peak was identified using LC MS/MS as a variant of transthyretin. A previous study using SELDI-TOF has identified a 13.86 kDa peak as an S-cysteinylated transthyretin variant [29].

DISCUSSION

SELDI-TOF mass spectrometry can be used to measure changes in protein expression in complex matrices [30]. Changes in human serum proteins have been identified by SELDI-TOF mass spectrometry in a number of different human cancers including ovarian, breast, prostate, head and neck, and liver cancers [31-35]. The technology has also been used to monitor the modulation of human serum proteins in response to treatment and to identify proteins that might be used as discriminators between responders and non-responder in a chemoprevention trial using celecoxib [36]. Plasma as well as serum can be used for protein expression profiling [34]. We employed SELDI-TOF mass spectrometry to measure changes in plasma Trx-1 in mice and in the plasma of Phase I clinical trial patients receiving the Trx-1 inhibitor PX-12. Trx-1 is secreted from cells by a non-classical secretory mechanism [16]. In a study of Trx-1 levels in hepatocellular carcinoma, Trx-1 levels were higher in patients with more advanced disease, and serum Trx-1 levels decreased after surgical removal of the tumor [19]. Therefore, circulating Trx-1 may offer a surrogate biomarker for tumor Trx-1 levels. We were able to demonstrate by co-mass spectrometry and by immunodepletion with anti-human Trx-1 monoclonal antibody that a plasma peak at 11.73 kDa was human Trx-1. The mean level of Trx-1 in plasma of healthy volunteers measured in our study by SELDI-TOF of 27 ng/ml is similar to previous reports of Trx-1 in normal plasma 24 ng/ml measured by ELISA [16]. The mean plasma Trx-1 level for cancer patients in the Phase I study was 182.0 ng/ml, which is higher than that reported for patients with hepatocellular carcinoma of 143 ng/ml [19], and for pancreatic cancer of 55 ng/ml [18].

The Trx-1 inhibitor PX-12 binds irreversibly to human Trx-1 rendering it biologically inactive [20]. Due to the fact that the molecular weight change produced by this modification is below the resolution of the SELDI-TOF mass spectrometer. We found, however, that PX-12 lowered levels of circulating mouse Trx-1 in non-tumor bearing mice suggesting that modified Trx-1 is rapidly removed from the plasma.

Patients receiving PX-12 as part of a Phase I trial showed a correlation between pretreatment Trx-1 level and a PX-12 mediated decrease in plasma Trx-1, with the higher pretreatment Trx-1 levels showing the greatest decrease. The high pretreatment plasma Trx-1 is presumably derived from the patient's tumor. In this study we were not able to distinguish between a PX-12

mediated decrease in plasma Trx-1 due to decreased secretion of Trx-1 from the tumor or increased elimination of modified Trx-1 from the plasma. PX-12 is known to inhibit the redox function of tumor Trx-1 [37], and this may block the secretion of Trx-1.

We explored the use of SELDI-TOF mass spectrometry to study levels of other plasma proteins after PX-12 treatment. There were 8 peaks including Trx-1 whose expression was decreased in patient plasma following PX-12 administration. One of these peaks at 13.86 kDa was identified as a variant of transthyretin, a major carrier for serum thyroxine and triiodothyronine. In addition, transthyretin facilitates the transport of retinol via its interaction with retinol binding protein [38]. Interestingly, serum transthyretin has recently been identified by proteomic analysis as a potential biomarker for ovarian cancer [39].

We found that PX-12 decreased plasma levels of VEGF. VEGF levels have been reported to be a prognostic factor for patient survival in a variety of different tumors [28,40-43]. One mechanism leading to increased tumor VEGF formation is a hypoxia induced increase the HIF-1 transcription factor leading to increased VEGF gene expression [44]. Colorectal, pancreatic, NSC lung cancer, and renal cancers have all been reported to be associated with high HIF-1 expression [44-48]. PX-12 lowers HIF-1 activity in experimental tumor xenografts in mice and decreases tumor VEGF [21]. We have recently shown that Trx-1 increases, and PX-12 inhibits the activity of the Sp-1 transcription factor that can regulate VEGF expression under normoxic conditions [49]. Thus, the decreases in plasma VEGF caused by PX-12 may be due to inhibition of Sp-1 mediated VEGF expression in normoxic cells and HIF-1 mediated VEGF expression by hypoxic cancer cells.

In summary, an exploratory study in mice and in humans shows that both Trx-1 and VEGF plasma levels decreased by treatment with the Trx-1 inhibitor PX-12. Declines in these plasma proteins are most likely to be observed in patients with elevated pretreatment levels of these proteins. The results of this study suggest that lowering of elevated levels of plasma Trx-1 levels in cancer patients may provide a surrogate for the inhibition of tumor Trx-1 by PX-12. The utility of these proteins as biomarkers for Trx-1 modulation may be most beneficial in a tumor type where both VEGF and Trx-1 expression is known to be high, such as colon or pancreatic cancer. Furthermore the PX-12 decreases plasma VEGF levels which may contribute to its antitumor activity

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Abbreviations

PX-12, (1-methylpropyl 2-imidazolyl disulfide); VEGF, (vascular endothelial growth factor); Trx-1, (thioredoxin-1); SELDI-TOF-MS, (surface enhanced laser desorption/ionization time-of-flight mass spectroscopy).

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Figure 1.

Trx-1 in mouse and human plasma. **A.** Recombinant mouse Trx-1 added to mouse plasma (gray line) increased the endogenous peak (black line) at 11.68 kDa corresponding to the predicted molecular weight of mouse Trx-1. **B.** Recombinant purified human Trx-1 added to normal human plasma (gray line) increased the endogenous human Trx-1 peak (black line) at 11.73 kDa. **C.** Overlay of the spectra showing depletion of the 11.73 kDa peak after immunodepletion. Arrows reflect the volume of anti-Trx-1 bound beads used to remove Trx-1 protein from plasma.

Figure 2.
PX-12 decreases Trx-1 in mouse plasma. Each point represents the mean $\pm$ S.E. of four non-tumor bearing mice injected with 25mg/kg PX-12.

Figure 3.
Plasma Trx-1 levels in healthy volunteers and cancer patients. Samples from 7 normal subjects and 31 cancer patients from a Phase I study of PX-12. Plasma was collected before treatment with PX-12. Pretreatment compared to normal controls ($p < 0.001$). The graph shows: mean, 25 and 75 percentiles (gray box), 10 and 90 percentiles (whiskers).

Figure 4.

Decrease in patient plasma Trx-1 and VEGF after PX-12 administration. The change in plasma Trx-1 or VEGF 4 hr after PX-12 administration compared to the pretreatment level in the same patient, plotted against the pretreatment level. The relationship between preand post-PX-12 treatment Trx-1 levels was assessed using a linear regression model with a square root transformation of the dependent variable. **A.** Plasma Trx-1. The line is the correlation ($R^2 = 0.225$), $n = 31$, $p = 0.007$. **B.** Plasma VEGF. The line is the correlation ($R^2 = 0.465$), $n = 19$, $p = 0.001$.

Figure 5. Representative SELDI-TOF spectra from plasma of one patient pre-PX-12 administration (solid line) compared to plasma 4 hrs post-PX-12 treatment (dashed line).