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S-Glutathionylated Serine Proteinase Inhibitors as Plasma Biomarkers In Assessing Response to Redox-Modulating Drugs.

Running Title: Serpins as Pharmocodynamic Biomarkers

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Conflict of Interest: The authors declare that they have no conflict of interest.
Abstract

Many cancer drugs impact cancer cell redox regulatory mechanisms and disrupt redox homeostasis. Pharmacodynamic biomarkers that measure therapeutic efficacy or toxicity could improve patient management. Using immunoblot analyses and mass spectrometry, we identified that serpins A1 and A3 were S-glutathionylated in a dose- and time-dependent manner following treatment of mice with drugs that alter reactive oxygen or nitrogen species. MS/MS analyses identified Cys^{256} of serpin A1 and Cys^{263} of serpin A3 as the S-glutathionylated residues. In human plasma from cancer patients there were higher levels of unmodified serpin A1 and A3, but following treatments with redox active drugs, relative S-glutathionylation of these serpins was higher in plasma from normal individuals. Serpin S-glutathionylation was both dose and time dependent. There is potential for S-glutathionylated serpins A1 and A3 to act as pharmacodynamic biomarkers for evaluation of patient response to drugs that target redox pathways.
Introduction

Preclinical and early clinical testing of novel anticancer drugs can be significantly enabled by the inclusion of direct or surrogate biomarkers that correlate with pharmacological effects (1, 2). Incorporation of such biomarkers can enhance data interpretation, provide an explanation for either positive or negative correlations with efficacy or toxicity and aid in early decision candidate selection. In complex diseases such as cancer, recent trends in drug development have moved towards targeted therapies to provide maximal therapeutic response while minimizing side effects. Failure of early stage clinical trials incurs great expense and there is merit in including biomarkers, both to estimate treatment efficacy and to identify patients with phenotypic characteristics that might predict response outcomes or toxicities.

In evaluating drug response, a single biomarker can lend itself to quantitative measurements through dose and time response studies. The metabolism of many drugs leads to production of electrophilic species that can generate reactive oxygen or nitrogen species (ROS; RNS). These can directly influence redox balance in both blood and tissue compartments. Biological “sensing” of redox changes is most actively monitored through select cysteine residues in various proteins. While there is debate as to the precise definition of what constitutes redox “sensing” versus redox “signaling” (3) there is little doubt that cysteines at various oxidation states are integral. S-glutathionylation is a post-translational modification that occurs when a cysteine in a low pK environment forms a disulfide bond with GS\(^{2-}\). This is reversible and the resultant
S-glutathionylation cycle has the potential to selectively regulate the function of numerous enzymes, receptors, structural proteins, transcription factors, and transport proteins. Moreover, post-translational modification may alter a variety of protein-protein interactions (4). The activities of serine protease inhibitors (serpins) are regulated by modifications of key cysteines (5, 6), where for example, S-glutathionylation of serpin A1 results in conformational changes that weaken its affinity for its target protease thereby reducing its effectiveness in preventing proteolytic activity (7).

Vertebrate serpins are classified into six sub-groups and represent ~2% of the total protein in human plasma (8). While most are defined by their inhibitory activities, certain serpins have non-inhibitory roles as hormone transporters (9), molecular chaperones (10) or tumor suppressors (11). Moreover, serpins can influence myeloproliferation and hematopoietic progenitor cell mobilization. Serpins A1 and A3 have redox-sensitive cysteines and are down-regulated in bone marrow during mobilization of hematopoietic progenitor cells into the peripheral bloodstream (12), implicating a regulatory function for S-glutathionylation and redox homeostasis in the marrow compartment. This is consistent with the fact that certain redox active drugs (including the glutathione disulfide mimetic NOV-002 (13) and the GSH peptidomimetic, Telintra (14)) have both preclinical and clinical myeloproliferative effects (15).

In the present study we have identified the S-glutathionylation of plasma serpins A1 and A3 as potential quantifiable response biomarkers for assessing response to two different types of drugs that either directly or indirectly impact
redox status. NOV-002 is a formulation of disodium glutathione disulfide (GSSG) that in combination protocols is in Phase II trials for the treatment of breast cancer. NOV-002 causes a redox regulation of protein thiols that persists in plasma for ~4hrs and in rodents and humans stimulates proliferation of bone marrow progenitor cells (13). PABA/NO is a diazeniumdiolate that acts as a direct nitrogen monoxide (NO) donor and is in development as an anticancer drug. We show that plasma serpin S-glutathionylation correlates with drug treatment in vivo and in vitro. Furthermore, analysis of cancer patient plasma samples suggests disease specific variation in unmodified and S-glutathionylated serpin profiles correlating with drug concentrations.

Materials and Methods

Reagents

Reduced GSH was from Sigma (St. Louis, MO). NOV-002 provided by Novelos Therapeutics (Newton, MA), PABA/NO from Dr. Larry Keefer (16, 17).

Animal and human plasma studies

Mice were treated with an intravenous bolus of 25mg/kg NOV-002 and after 1h blood was collected via orbital bleed in heparin-coated tubes. Plasma was separated by centrifugation and proteins separated on SDS-PAGE and transferred for immunoblot analyses. Human blood samples were obtained with informed consent from 8 cancer-free volunteers and 47 oncology patients with
various types/stages of cancer: 20 acute myeloid leukemia (AML); 10 acute lymphoblastic leukemia (ALL); 1 multiple myeloma (MM); 4 myelodysplastic syndrome (MDS); 3 chronic lymphoblastic leukemia (CLL); 1 chronic myeloid leukemia (CML); 2 lung carcinomas; 2 urothelial carcinomas; 1 leiomyosarcoma; 1 neuroblastoma; 1 spindle cell carcinoma, 1 invasive ductal carcinoma. 8-10cc’s of blood was collected into Vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA). Plasma was separated via centrifugation and treated with NOV-002 or PABA/NO at 37°C.

**Immunoblot analysis**

Equal amounts of purified recombinant serpins A1/A3 or total plasma protein, as determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), were separated on non-reducing SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) and probed with a monoclonal anti-GSSG antibody (Virogen, Watertown, MA) to detect S-glutathionylation, or polyclonal antibodies for serpins A1 and A3 (R&D systems Minneapolis, MN), glutaredoxin1 (Abcam, Cambridge, MA) or GSTP (MBL, Woburn, MA). Equal loading was estimated by polyclonal rabbit albumin antibody (Abcam, Cambridge, MA). Secondary antibodies were from Amersham Biosciences (Piscataway, NJ).

**Identification of S-glutathionylated plasma proteins**
Simultaneously separated plasma S-glutathionylated protein bands were isolated from gels run simultaneously with immunoblots and trypsin digested. Peptide mass was analyzed by matrix-assisted laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrometry at the Proteomics Core Facility. Protein identification was performed using software from the NCBI protein database.

**Immunoprecipitation**

Human plasma samples (2mg) were treated with 50μM of NOV-002 for 60min at 37°C. Biotinylated rabbit polyclonal antibodies to serpins A1 and A3 (Abcam, 5μg) were used to pull-down respective proteins by overnight incubations at 4°C. Antibody complexes were isolated through incubation with NeutrAvidin agarose resin (Thermo Scientific) for 2hrs. Resin-bound complexes were washed 5x with immunoprecipitation buffer (20mM HEPES, 300mM NaCl, 1% Triton, 10% glycerol) and proteins eluted in non-reducing SDS-sample buffer. Immunoprecipitated proteins were separated by non-reducing SDS-PAGE and S-glutathionylation of serpins A1 and A3 evaluated by immunoblot.

**In vitro S-glutathionylation assays**

Purified serpin A1 or A3 (50ng) was incubated at 37°C in 50mmol/L potassium phosphate buffer (pH 7.2), 1mmol/L GSH with either NOV-002 or PABA/NO. Samples were run on non-reducing SDS-PAGE gels for subsequent transfer and immunoblot analysis.
Tandem Mass spectroscopy

Purified serpins A1 and A3 were treated with 100μmol/L PABA/NO or 1mmol/L NOV-002 for 30min at 37°C, digested with Lys-C and analyzed via liquid chromatography (LC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) on a linear ion trap mass spectrometer (LTQ, Thermo Finnigan) coupled to an LC Packings nano LC system. S-glutathionylated serpin A3 peptides required additional trypsin digestion. See Supplemental Data for instrument settings.

Spectroscopic Analysis of Serpin A1 and A3 in vitro

The effect of serpin S-glutathionylation on enzyme secondary structure was examined by circular dichroism (CD) measurements carried out on a 202 AVIV Associates CD spectrometer (Lakewood, NJ) using a semi-micro quartz rectangular 1×10×40mm cuvette. Serpins A1 and A3 (~2mg/ml) were S-glutathionylated by treatment with 40μmol/L PABA/NO and 1mM GSH for 30min in 20mM PB, pH 7.4, at 37°C. Excess PABA/NO and GSH were eliminated using Biospin-6 (Bio-Rad) SEC micro-spin columns.

Purified native and S-glutathionylated serpins A1 and A3 (~95% homogeneous, 40μM in 20mM PB, pH 7.4) were maintained at 22°C using a Pelletier element. Spectra were recorded while scanning in the far-ultraviolet region (190–260 nm), with bandwidth of 1.0nm, step size of 0.5nm, integration time of 30sec. Protein tryptophanyl fluorescence was recorded on a QM-4
spectrofluorometer (PTI, Piscatway, NJ) using 10x10x40-mm quartz cuvette, excitation and 2.5 and 5.0 nm emission slits. To minimize effects of protein tyrosines and phenylalanines an excitation wavelength of 295 nm was used. Background spectra were subtracted from final emission data.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). P-values lower than 0.05 were considered significant. Parametric data were statistically evaluated using T-tests and non-parametric data were evaluated using Mann-Whitney or Wilcoxon matched-pairs signed rank test tests, based on data distribution. Multiple comparisons were analyzed using ANOVAs with Dunnet’s Multiple Comparison tests or Kruskall-Wallis tests with Dunn’s multiple Comparsion test. Corrections were applied based on program recommendation. Relative S-glutathionylation levels induced in cancer were analyzed using Two-Way ANOVAs.

**Results**

*In vivo* treatment with NOV-002, leads to S-glutathionylation of a limited sub-set of plasma proteins in mice (Fig. 1A). Immunoblots from animals detected four prevalent S-glutathionylated proteins within 15 mins of treatment. Excised bands were trypsin digested and peptide mass analyzed by MALDI-TOF. Protein
Complement C3 appears as a common S-glutathionylated protein throughout the analysis, even before drug treatments. Proteins bands B and D were identified as serpin A1, while band C was identified as serpin A3. Murine contrapsin is homologous to human serpin A3. Ex vivo treatment of mouse plasma with various concentrations of NOV-002 showed similar S-glutathionylation induction. Quantification of band densities showed increasing serpin S-glutathionylation with dose exposure (Fig. 1B).

Extending these analyses purified human plasma samples were similarly treated ex vivo with NOV-002. Anti-PSSG immunoblots showed the induction of several distinct S-glutathionylated protein bands with a profile similar to in vitro and in vivo in mouse plasma assays. Basal levels of S-glutathionylated proteins were detected in both mouse and human untreated samples, most likely the result of ex vivo processing. Treatment with 40μM NOV-002 caused a time-dependent increase in the relative amount (as normalized to albumin loading control) of S-glutathionylated proteins that peaked between approximately 40-60min and gradually decreased with additional time (Fig. 1D). This indicates a relatively rapid turnover rate involving the S-glutathionylation and deglutathionylation of proteins following drugs.

Immunoprecipitation of serpins A1 and A3 from human plasma confirmed that both these proteins are S-glutathionylated (Fig. 1E). Due to high sequence homology (~46%) between human serpins A1 and A3, a certain extent of antibody cross-reactivity may be anticipated. In particular, the
immunoprecipitating serpin A3 antibody also pulled down serpin A1, as evidenced in the PSSG blot. Protein bands correspond with the approximate expected MW’s of serpins A1 and A3 (seen on PSSG immunoblots). Detection of multiple protein bands on immunoblots probed with serpin antibodies is not uncommon. Serpins and serpin/protease complexes undergo gradual proteolysis at the serpin active site producing cleavage products perhaps explaining the multiple protein bands detected on serpin immunoblots (18).

Purified recombinant A1 and A3 protein was treated in vivo and in vitro with agents known to induce protein S-glutathionylation (13, 19). In the presence of PABA/NO and GSH, both serpins A1 and A3 were S-glutathionylated in a time- (Figure 2A) and concentration-dependent manner (Figure 2B). Serpin A1 was rapidly S-glutathionylated within 1 min and peaked at approximately 5-10 min. Following this, S-glutathionylated protein levels gradually declined. Alternatively, serpin A3 showed a more gradual increase in S-glutathionylation over a 30 min time course, suggesting unique protein properties despite high sequence homology. Similar drug concentrations were needed to induce minimum (at ~25 μmol/L PABA/NO) and maximum (at ~75 μmol/L PABA/NO) S-glutathionylation levels of both serpins A1 and A3. However, drug-induced S-glutathionylation levels of serpin A3 became relatively saturated between 25 and 50 μmol/L PABA/NO, suggesting that serpin A3 S-glutathionylation may be more time-dependent. We evaluated the effects of S-glutathionylation on serpin secondary structure. Tryptophanyl fluorescence of serpin A3 was lower than that
of serpin A1 indicating a quenching in the former. Tryptophanyl fluorescence of S-glutathionylated serpin A3 had an increased intensity and a shift of emission maximum from 328 to 336nm, interpreted as tryptophanyl exposure to a more polar environment, decrease of quenching and structural change (Fig. 2C). The CD spectrum (far UV, 190–260nm) of S-glutathionylated serpin A1 was similar to that of native protein, consistent with a minimal increase in the α-helical content (206–220nm) of the protein (Fig. 2D). This suggests a minor decrease in tryptophanyl fluorescence caused by S-glutathionylation, indicating a reasonably intact tertiary and quaternary structure. In contrast, the CD spectrum of S-glutathionylated serpin A3 was distinct from that of the native protein, consistent with an increase in the β-strand content of the protein and altered protein folding (Fig. 2D). These fluorescent analyses confirm that S-glutathionylation affects the tertiary structure of serpin A3.

S-glutathionylated recombinant serpins A1 and A3 were analyzed using tandem mass spectrometry in order to identify S-glutathionylated cysteines. Purified recombinant serpins A1 and A3 were incubated at 37°C for 30min the presence of 100μmol/L PABA/NO and 10mmol/L GSH. Analyses of (RLGMFNIQHCK) of serpin A1 (Fig. 3A,B) indicated that an additional ~305 Da at Cys256, representing GSH, in addition to the Cys alone (~103 Da) and water (~18 Da), was present only in the drug treated Serpin A1 (Figure 3B). Tandem mass spectrometry analyses similarly identified Cys263 of (DEELSCTVVELK; Fig. 3C,D) treated Serpin A3 to be modified by the addition of GSH, indicated by an
additional ∼305 Da (Figure 3D). These data provide a platform to develop analytical methods for quantification of S-glutathionylated serpins in biological fluids.

Human plasma samples from 47 cancer patients undergoing chemotherapy treatments and 8 cancer-free patients were analyzed for total and S-glutathionylated serpin A1 and A3 protein. Immunoblot densitometry showed significantly (P<0.05) elevated amounts of each unmodified serpin in cancer patients (Fig. 4A, 4B). All protein bands were normalized to an albumin loading control as well as an internal standard, permitting comparison across membranes. Levels of S-glutathionylated serpins A1 and A3 did not correlate with total serpin levels. In fact, serpin S-glutathionylation profiles were distinct between patients suggesting that individual patients with specific disease and treatment profiles have unique serpin S-glutathionylation. However, when analyzing all cancers in a group, the overall ratio of S-glutathionylated serpin A1 (PSSGa and PSSGc) and A3 (PSSGb) to unmodified serpin A1 and A3, respectively, was significantly decreased (P<0.05) in cancer patients (Fig. 4C). Thus, in reference to the total amount of serpin, the fraction of serpin protein subject to S-glutathionylation was lower in cancer patients. Table 1 summarizes the relative fold change in protein expression of unmodified serpins and S-glutathionylated serpins in total cancer as compared to cancer-free, in addition to AML an ALL as individual groups. While general trends remain consistent between all cancers, discrepancies in significance may suggest disease specific
profiles. Of particular interest, levels of basal S-glutathionylated serpins were significantly lower in ALL patients as compared to AML and cancers as a group.

*Ex vivo* treatment of patient plasma with NOV-002 for 60 min at 100μmol/L proportionally enhanced levels of S-glutathionylated A1 and A3 [Fig. 5A, B and Supplemental Data - Fig. 1 (lighter exposures)]. Panel 5B shows the relative quantities of S-glutathionylated serpin A1 (PSSGa and PSSGc) and A3 (PSSGb) after NOV-002 treatment from samples of 47 cancer and 8 cancer-free patients, determined by densitometry measurements normalized to both albumin loading controls and an internal standard incorporated on all gels. S-glutathionylated serpin A1 (PSSGa and PSSGc) and A3 (PSSGb) levels were significantly increased (P<0.05) in response to NOV-002. Two-way ANOVA analyses show that *ex vivo* treatment of plasma with 100μmol/L NOV-002 resulted in significantly higher relative increases (P<0.05) in serpin A1 S-glutathionylation (PSSGa/A1 and PSSGc/A1) in cancer-free plasma as compared to cancer patients (Fig. 5C). Thus, in response to treatment, serpin S-glutathionylation and disease status are interrelated and demonstrate altered redox homeostasis in cancer patients. NOV-002 treatment also induced S-glutathionylation of albumin, as evidenced by the doublet band was detected only at high drug concentrations. Critically, NOV-002 treatment did not alter serpin stability, as indicated by total serpin immunoblotting. Treatment with 40μmol/L NOV-002 for 0-240 min did not change levels of unmodified serpin A1 or A3 (P>0.05) (Fig. 5D). Additional analyses using treated and untreated plasma samples from cancer and cancer-free
patients support these data (Fig. 5E). Treatment with Velcade, vinblastine, Taxol, or tamoxifen did not induce changes in plasma serpin A1 or A3 S-glutathionylation (Supplemental Data, Fig. 2A, B, C and D).

GSTP and Grx1 are involved in the forward and reverse reactions of S-glutathionylation. Relative protein levels were determined via densitometry and then normalized to an albumin loading control, as well as an internal standard incorporated into all gels. Plasma protein levels of GSTP were significantly decreased (P<0.05) in cancer patients as compared to cancer-free. Conversely, plasma Grx1 levels were significantly elevated (P<0.05) in cancer patients (Fig 6). These data are consistent with the interpretation that the decreased relative ratio of S-glutathionylated to unmodified serpin in cancer patient plasma may be linked with diminished GSTP levels and a relatively enhanced rate of serpin deglutathionylation, as a consequence of elevated Grx1.

Discussion

There is expanding interest in adopting a redox-modulating platform in drug discovery/development in cancer (20). In this study we used two drugs that alter redox homeostasis through distinct mechanisms. NOV-002 is an oxidized glutathione mimetic that modifies extra- and intracellular ratio of GSH:GSSG (13, 21). PABA/NO releases NO to raise levels of RNS and ROS (19). Increased efficacy and limited off-target toxicities could be facilitated by the identification of plausible pharmacodynamic biomarkers (preferably through non-invasive
approaches) that serve as predictors of drug response. Initially using mice, we identified S-glutathionylated serpins A1 and A3 as quantifiable response biomarkers. These preclinical observations were extended to human plasma samples where correlative associations with drug exposure and disease status were identified. Moreover, variations in the expression of unmodified and/or S-glutathionylated serpin profiles were identified and may reflect aberrant redox homeostasis in some cancers.

Because of the variable valence states of sulfur, cysteine-targeted oxidation has evolved as a critical regulator of protein function in a number of signal transduction pathways. Protein S-glutathionylation is cyclical in nature (the forward reaction is catalyzed by GST, the reverse by glutaredoxin (22)), providing the framework for reversible signaling (23). A number of protein clusters with roles in cell survival pathways are characteristically sensitive to post-translational modification, including enzymes with catalytically important cysteines, signaling proteins and transcription factors. Serine proteinase inhibitors are susceptible to modulation by redox conditions (7, 24, 25). MS/MS analyses identified Cys$^{256}$ of serpinA1 and Cys$^{263}$ of serpin A3 as S-glutathionylation targets. Cys$^{256}$ of full-length serpin A1 has previously been demonstrated to be redox sensitive (7, 26). However, the present data provide are the first to report a similar redox-sensitive cysteine in serpin A3. Oxidation of cysteine residues in serpins inhibits their activity (7, 24-26). From a functional basis, members of the serpin family are involved in regulation of myeloproliferation and hematopoetic progenitor cell (HPC) mobilization (12, 27). Down-regulation of serpins A1 and A3 in bone
marrow occurs during progenitor cell mobilization and influences marrow microenvironment and migratory behavior of HPC (12). Our data show that serpin A1 and A3 S-glutathionylation following NOV-002 or PABA/NO treatment alters their structure and such factors may alter the role of serpins involved in myeloproliferative pathways. To interrogate the specificity of these effects for ROS/RNS active drugs, we investigated (supplemental Figure 2) the effects of Velcade (proteasome inhibitor) vinblastine and Taxol (antimicrotubule drugs) or tamoxifen (estrogen-receptor antagonist). While these drugs may produce low levels of ROS indirectly, the data suggest that there is no dose effect response on plasma serpin S-glutathionylation profiles.

Immunoblots of S-glutathionylated serpins revealed proteins of various molecular weights. Different segments of the A1 molecule may possess unique biologic properties that may or may not be linked to protease inhibitor activity. Low molecular weight fragments of serpin A1, generated by complexing with neutrophil elastase or by macrophage metalloelastase attack, possess chemoattractant properties (28). In this context, serpin S-glutathionylation may have a mechanistic connection with the in vivo myeloproliferative activity of NOV-002 (13). The redox-sensitive serpin, bomapin, is directly involved in the responsiveness of myeloid progenitor cells to their microenvironment (29). Post-translational regulation of serpins in the peripheral circulation may indicate a role in the control of proteolytic pathways. Whatever the functional association, since the plasma half-life of S-glutathionylated proteins is ~4hrs (19), our mouse studies provided evidence that these post-translationally modified proteins could
be viable as pharmacodynamic biomarkers in a clinical setting. There is precedent for using post-translationally modified proteins as biomarkers. In initial trials of the CML therapeutic imatinib levels of phosphorylated CRKL correlated directly with BCR-ABL inhibition and proved useful in determining appropriate dosing strategies (30, 31). In addition, success of small-molecule inhibitors of EGFR (gefitinib, erlotinib) and Src kinases (dasatinib) can be linked with phosphorylation of downstream markers like ERK1/2, FAK or paxillin (32, 33).

Our patient profile data showed that baseline protein levels of both serpin A1 and A3 were elevated in the plasma of cancer patients. Elevated serpin A1 expression in tumors has been correlated with poor prognosis and highly invasive, metastatic adenocarcinomas (34-36) and with poor response in multiple myeloma (37). While the functional importance of serpins A1 and A3 in cancer is not yet clearly defined, serpin A1 has inhibitory activity against cytotoxic T lymphocytes and natural killer cells (38), suggesting that the tumor-promoting effect of serpin A1 could be related to a decreased immune response against malignant cells. Serpin A1 overexpression may also be a useful biomarker in insulinomas (39). In leukemias, where the cancer is of bone marrow origin, further defining a role of serpin A1 and A3 S-glutathionylation in myeloproliferation may be valuable with regards to development of tailored treatment strategies. Chronic oxidative stress, characterized by excessive cellular ROS levels, occurs in several hematopoietic malignancies including ALL, MDS, CML, and AML (40-42). While ROS is involved in normal hematopoiesis, imbalances of ROS results in redox dysregulation and may influence
downstream redox mediated signaling events, including S-glutathionylation of serpins. Our data suggest that ALL patients have lower basal levels of glutathionylated serpins A1 and A3, perhaps indicating that certain myeloid leukemias might respond preferentially to redox-targeted therapeutics.

Analyses of cancer patients as a group showed that their overall ratio of S-glutathionylated serpins to unmodified serpins was decreased. Further, ex vivo drug-induced S-glutathionylation was blunted in cancer patient plasma, results consistent with a disrupted redox homeostasis associated with cancer. High levels of oxidative stress markers in patient sera often correlate with similar markers within tumors, suggesting the utility of patient serum in assessing redox state. Further correlative analysis of patient samples estimated levels of enzymes associated with the forward and reverse reactions of the S-glutathionylation cycle. Levels of GSTP (forward reaction) were significantly decreased in cancer patient plasma compared to cancer-free, while Grx1 (reverse reaction) levels were elevated. These results are consistent with the relative ratios of S-glutathionylated to unmodified serpin proteins found in cancer patient plasma samples. Modulation of Grx1 levels, either by over-expression or RNAi knockdown, directly influences S-glutathionylation of inhibitory NF-kappaβ kinase by respectively decreasing or catalyzing its post-translational modification (43). However, reports indicate a dual role for Grx1, as a glutathionylating enzyme under ROS or as a deglutathionylase when the oxidative signal or stress subsides (44). Earlier results have shown that the glutathione conjugate of cisplatin can inhibit the enzyme activity of Grx (45). However, there were no
indications that the parent drug (or related platinum analogues) had inhibitory effects. The drug concentrations employed in the present study are generally too low to cause enzyme inhibition, either for GSTP (13) or of Grx (45).

The present report provides evidence supporting the utility of post-translationally modified serpins as pharmacodynamic biomarkers. Optimization of detection and characterization protocols will provide promising opportunity in a clinical setting where serpin S-glutathionylation may be directly used to infer patient response to redox-modulating therapeutics. The ability to detect S-glutathionylated serpin peptides, as we have demonstrated in our MS/MS studies, may provide a platform to develop increased sensitivity of detection for quantification of S-glutathionylated serpins in biological fluids.

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**TABLE 1.** Relative fold change in protein expression (cancer vs cancer-free) of unmodified serpins and S-glutathionylated serpins. SDS-PAGE and
western blot analyses were used to analyze levels of unmodified and S-glutathionylated serpins A1 and A3. The relative quantity of unmodified serpins and S-glutathionylated serpin A1 (PSSGa and PSSGc), and serpin A3 (PSSGb) were determined by densitometry measurements normalized to both albumin loading controls and an internal standard incorporated on all gels. n=8, cancer-free; n=47, cancer; n= 20, AML; n=10, ALL. Data are presented as fold change vs. cancer-free ± standard error. *p<0.05.

**Figure legends**

**Figure 1.** NOV-002 treatment induces serpin A1 and A3 S-glutathionylation *in vivo* in mouse plasma and *ex vivo* in human plasma. Immunoblot analyses of S-glutathionylated proteins (PSSG) from (A) wild-type mice treated *in vivo* with an i.v. bolus of 25mg/kg NOV-002 at time-points following 1hr; and (B) wild-type mouse plasma treated *ex vivo* with various concentrations of NOV-002. MALDI-TOF mass spectrometry identified S-glutathionylated mouse plasma proteins (Table, C). Human plasma was treated *ex vivo* with 40μmol/l NOV-002 for 0-240min and evaluated by immunoblot for S-glutathionylated proteins (D). Albumin immunoblotting determined equal protein loading. For immunoprecipitation (IP), human plasma was treated with 40μmol/L NOV-002 for 1h and biotinylated serpin A1 and A3 antibodies were used to pull-down total unmodified and modified serpins. S-glutathionylated proteins were evaluated by
immunoblot (E). Blots were stripped and re-probed with anti-serpin A1 or A3 as a loading control. Due to high homology, there is significant cross reactivity of anti-A1 and A3.

Figure 2. S-glutathionylation of serpins A1 and A3 is time and dose dependent and impacts protein structure. Recombinant serpin A1 and A3 were treated with 1mmol/L GSH and (A) 40μmol/L of PABA/NO for 0 to 30min or (B) 0 to 100μmol/L of PABA/NO for 30min. Immunoblot analyses were used to detect S-glutathionylation (PSSG) and total serpin. Spectroscopic analyses of secondary and tertiary (quaternary) structure of native (control; solid curves) and PABA/NO + GSH– treated (Glut; dashed curves) A1 (red) and A3 (green) protein were done in vitro using (C) tryptophanyl fluorescence and (D) circular dichroism.

Figure 3. Serpin A1 is S-glutathionylated at Cys$^{256}$ and serpin A3 is S-glutathionylated at Cys$^{263}$. Recombinant serpin A1 and A3 proteins were treated with 1mmol/L GSH and 100μmol/L PABA/NO for 30min. To identify specific cysteine residues susceptible to S-glutathionylation, control unmodified serpin A1 (A) PABA/NO-treated serpin A1 (B); control unmodified serpin A3 (C), and PABA/NO-treated serpin A3 (D) were digested under non-reducing conditions and analyzed via liquid chromatography (LC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) to identify modification GSH addition. A single S-glutathionylated modification [+305.6] was detected at of Cys$^{256}$ of
serpin A1 (B) and Cys<sup>263</sup> of treated serpin A3 (D). Red arrows indicate modified peaks. The two arrows in panel D represent both forward and reverse analyses.

Figure 4. Unmodified serpin is elevated in certain cancers while the ratio of S-glutathionylated to unmodified serpin is decreased. Human plasma samples from cancer-free patients (n=8; represented in lanes 1,3,5 of Panel A) and cancer patients undergoing chemotherapeutic treatment (n=47; represented in lanes 2,4,6 of Panel A) were analyzed by immunoblotting for unmodified serpin A1 and A3 protein levels (B) as well as relative levels of S-glutathionylated serpin compared to total unmodified serpin (C). Relative quantities of unmodified serpins and S-glutathionylated serpin A1 (PSSGa and PSSGc), and serpin A3 (PSSGb) were determined by densitometry measurements normalized to both albumin loading controls and an internal standard incorporated on all gels. Data are +/- SEM.

Figure 5. Ex vivo treatment with NOV-002 induces serpin A1 and A3 S-glutathionylation and results in greater relative increases in serpin A1 glutathionylation in cancer-free human plasma. Cancer (samples 2-8) and cancer-free (sample 1) human plasma samples were treated with 100μmol/L NOV-002 for 60min (A). S-glutathionylation (PSSG), serpin A1, serpin A3 and albumin levels (loading control) were evaluated by immunoblot. Levels of S-glutathionylated serpin A1 (PSSGa and PSSGc), and serpin A3 (PSSGb) after NOV-002 treatment (both cancer and cancer-free) were determined by
densitometry normalized to both albumin loading controls and an internal standard on all gels \((B)\). Relative S-glutathionylation levels in reference to total unmodified serpin levels induced in response to NOV-002 treatment in cancer patient plasma as compared to those induced in cancer free plasma were analyzed using Two-Way ANOVAs \((C)\). To confirm that S-glutathionylation of serpins does not alter serpin A1 and A3 protein stability, plasma was treated with 40\(\mu\)mol/L NOV-002 for 0-240min \((D)\). The relative quantity of serpins A1 and A3 in treated and untreated samples (both cancer and cancer-free) after 100\(\mu\)mol/L NOV-002 treatment for 1hr was normalized to both albumin loading controls and an internal standard on all gels \((E)\). Data are mean for 8 cancer-free and 47 cancer samples +/- SEM.

**Figure 6. Cancer patient plasma samples have decreased levels of GSTP protein and elevated levels of Grx1.** Human plasma samples from cancer-free patients (represented in lanes 1 and 3) and cancer patients undergoing chemotherapeutic treatment (represented in lanes 2 and 4) were evaluated for GSTP and Grx1 using immunoblots. Relative quantities of GSTP and Grx1 were normalized to both albumin loading controls and an internal standard on all gels. Data are mean for 8 cancer-free and 47 cancer samples +/- SEM.

**TABLE 1. Relative fold change in protein expression (cancer vs cancer-free) of unmodified serpins and S-glutathionylated serpins.** SDS-PAGE and immunoblot analyses analyzed levels of unmodified and S-glutathionylated
serpins A1 and A3. Relative quantities of unmodified serpins and S-glutathionylated serpin A1 (PSSGa and PSSGc), and serpin A3 (PSSGb) were determined by densitometry normalized to both albumin loading controls and internal standards incorporated on all gels. n=8, cancer-free; n=47, cancer; n=20, AML; n=10, ALL. Data are fold change vs. cancer-free ± standard error. *p<0.05.
Figure 4
Figure 5