Enhancement of carboplatin-mediated lung cancer cell killing by simultaneous disruption of glutathione and thioredoxin metabolism

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Pages: 28
Figures: 6
Supplemental Tables: 2
Supplemental Figures: 1
Words: 4995

Keywords: 2-Deoxyglucose, carboplatin, glutathione, lung cancer, thioredoxin reductase, auranofin, buthionine sulfoximine, oxidative stress

Running Title: Chemo-sensitization of lung cancer using GSH and TR inhibitors

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Abstract

Purpose: Cancer cells (relative to normal cells) demonstrate increased steady-state levels of hydroperoxides that are compensated for by increased glucose and hydroperoxide metabolism. The current study determined if inhibitors of glucose and hydroperoxide metabolism could induce chemo-radio-sensitization by enhancing oxidative stress in lung cancer cells.

Experimental Design: A549 and NCI-H292 human lung carcinoma cells were treated with 2-Deoxy-D-glucose (2DG) combined with carboplatin (carbo) + ionizing radiation (IR). Lung cancer cells were further sensitized with inhibitors of glutathione- and thioredoxin-dependent metabolism [buthionine sulfoximine (BSO) and auranofin (Au), respectively] in vitro and in vivo.

Results: When 2DG was combined with carbo+IR, clonogenic cell killing was enhanced in A549 and NCI-H292 cells and this combination was more effective than paclitaxel+carbo+IR. The thiol antioxidant (N-acetylcysteine, NAC) was capable of protecting cancer cells from 2DG+carbo-induced cell killing. Simultaneous treatment of cancer cells with BSO and Au, at doses that were not toxic as single agents also enhanced lung cancer cell killing and sensitivity to 2DG+carbo. This treatment combination also increased both glutathione and thioredoxin oxidation which were inhibited by NAC. Mice treated with Au+BSO showed no alterations in circulating leukocytes or red blood cells. Xenograft lung tumor growth in mice was more effectively inhibited by treatment with Au+BSO+carbo when compared to animals treated with carbo or Au+BSO alone.

Conclusions: These results show in vitro and in vivo that simultaneous inhibition of glutathione and thioredoxin metabolism can effectively inhibit lung cancer cell growth and induce chemo-sensitization by a mechanism that involves thiol mediated oxidative stress.
Statement of Translational Relevance

Glutathione and thioredoxin dependent metabolism have both been associated with the acquisition of cancer cell resistance to therapy for the last twenty five years. Clinical strategies for inhibiting glutathione or thioredoxin dependent metabolism individually for the purpose of sensitizing cancer cells to therapy have been attempted with limited success and this strategy has not yet lead to FDA approved cancer therapies. The current report clearly demonstrates that simultaneous inhibition of both glutathione- and thioredoxin-dependent metabolism is necessary to consistently sensitize human lung cancer cells to oxidative stress and cell killing mediated by 2-deoxyglucose combined with carboplatin in vitro and in vivo. These findings could have significant implications for the development of combined modality cancer therapies targeting oxidative metabolism for the purpose of sensitizing human lung cancer cells to chemotherapy.
Introduction

Lung cancer results in approximately 180,000 deaths per year with a majority of these stemming from non-small cell lung cancer (NSCLC) (1). NSCLC has a poor prognosis with a 5 year survival rate of 17% (2). Currently the common therapies for NSCLC include platinum-based combined modality chemotherapy with concurrent ionizing radiation (IR) (3). Platinum based therapies not only have high incidences of serious side effects but also are characterized by both intrinsic and adaptive cancer cell resistance which limits clinical utility (4). It is clear that improvements to the current therapies are needed to decrease normal tissue toxicity, prevent development of resistance, and provide for better outcomes.

Cancer cells have been shown to have increased rates of glucose uptake when compared to non-malignant cells. In addition to its role in energy production, significant evidence is accumulating that glucose metabolism plays a role in the metabolism of reactive oxygen species (ROS) via the formation of pyruvate that scavenges hydroperoxides directly through deacetylation reactions (5, 6) as well as via the regeneration of NADPH in the pentose phosphate pathway (7-9). NADPH is an important cofactor for glutathione reductase and thioredoxin reductase (TR) which are essential for maintaining glutathione (GSH) and thioredoxin (Trx) in their reduced state (7-9). GSH and Trx are two of the main cellular thiol redox circuits that are responsible for decomposition of hydroperoxides and other ROS, maintaining cell redox potential while preventing and repairing oxidative damage (10). Many cancer cells, including lung cancer, have been shown to up-regulate GSH and Trx (11, 12) metabolism and this up-regulation has been associated with resistance to IR as well as platinum based therapies (13-15). Other studies have demonstrated that cisplatin-
resistant cells have higher levels of TR expression as well as activity and inhibition of TR activity or GSH synthesis increased the cellular sensitivity to cisplatin (16, 17).

2-deoxy-D-glucose (2-DG) is a glucose analog that cannot undergo glycolysis nor advance beyond the reaction with glucose-6-phosphate dehydrogenase in the pentose phosphate pathway (18-20). Previous investigations have shown that treatment of cancer cells with 2DG mimics glucose deprivation and results in depletion of NADPH, disruptions in GSH metabolism, as well as increases in steady-state levels of hydroperoxides (18-20). Treatment with 2DG also sensitizes head and neck, osteosarcoma, lung, and pancreas cancer xenografts to agents that are known to induce metabolic oxidative stress including IR and chemotherapy (19, 21, 22).

Glutamate cysteine ligase is the rate limiting step in GSH synthesis which is reversibly inhibited by buthionine-sulfoximine (BSO). Treatment of multiple cancer cell types, including lung cancer, with clinically relevant doses of BSO results in the depletion of GSH, enhancement of cytotoxicity, and decreased resistance to chemotherapy (15, 23, 24). Auronafin (Au) is a gold phosphine that has been used to treat rheumatoid arthritis patients for over 20 years. Although the mechanism of action of Au is not fully understood, Au was recently shown to inhibit both cystolic and mitochondrial TR at therapeutically relevant doses (25-27). Treatment of ovarian cancer cells, including cisplatin resistant ovarian cancer cells, with Au has been suggested to increase steady-state levels of H$_2$O$_2$ and decrease cancer cell viability (28).

In the current report the hypothesis that lung cancer cells could be sensitized to oxidative stress and chemotherapy was tested using inhibitors of glucose and hydroperoxide metabolism including
2-DG, BSO, and Au in combination with carboplatin (carbo). All these agents have been used safely in humans and therefore potentially represent clinically relevant interventions for sensitizing cancer cells and decreasing resistance to standard therapies. The results demonstrate that 2DG decreases clonogenic cell survival in two non-small cell lung cancer (NSCLC) cell lines (A549 and H292) as well as enhancing the toxicity of carbo+IR. The thiol antioxidant N-acetylcysteine (NAC) is capable of protecting lung cancer cell lines from the enhanced cytotoxicity seen with 2DG+carbo as well as BSO+Au supporting the hypothesis that thiol-mediated oxidative stress significantly contributes to outcomes using these drug combinations. The observation that doses of Au and BSO that do not cause toxicity as single agents, cause significant toxicity and oxidative stress when combined, supports the notion that both GSH- and Trx-dependent metabolism must be simultaneously inhibited to achieve maximal chemo-sensitization. Finally the results showing that the combination of Au+BSO can be given safely to mice to achieve chemo-sensitization in A549 xenograft tumors exposed to carbo, supports the hypothesis that combined manipulation of GSH+Trx metabolism represents a promising approach for enhancing chemotherapy responses in the treatment of lung cancer.

**Methods**

*Cells and Culture Conditions:* A549 and H292 human lung carcinoma cells were obtained from American Type Culture Collection (ATCC) and maintained in DMEM or RPMI 1640 media (Mediatech Inc., Manassas, VA) respectively with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA). Cultures were maintained in 5% CO₂ and humidified in a 37°C incubator. Carboplatin 10 mg/ml (Hospira, Lake Forest, IL) and paclitaxel 6 mg/ml (Bedford Labs, Bedford, OH) were further diluted in normal saline immediately prior to use to achieve the desired
concentrations. Stock solutions of auranofin (Axxora, SanDiego, CA) were dissolved in absolute ethanol then further diluted in normal saline before use to achieve the desired concentration. Stock solutions of 2DG grade III and L-butathionine-sulfoximine (Sigma Chemical Company, St. Louis, MO) were made in PBS and kept at 4°C prior to use. A 1 M solution of N-acetyl-L-cysteine (>99% TLC, Sigma Chemical Co.) was made in water and adjusted to pH 7.1 with 1 M bicarbonate, sterile filtered, and used immediately.

Clonogenic cell survival assay: For clonogenic experiments, 120,000 A549 or H292 cells were plated in 60 mm dishes and allowed to grow in their respective stock culture media for 24 h. Cells were then treated 24 h with or without 20 mM 2DG, 2-5 μM carbo and 1 nM paclitaxel. Ionizing radiation was delivered using a J.L. Shepherd cesium irradiator (J.L. Shepherd, San Fernando, CA) with a dose rate of 0.805 Gy/min. Cells were irradiated with 2 Gy at room temperature following drug treatment. Cells were plated for clonogenic survival immediately after radiation. In select experiments cells were treated with 100 μM BSO for 24 hours and/or 0.5 – 5 μM Au for the last 3 hours. Floating cells in medium were collected and combined with the attached cells from the same dish that were trypsinized with 1 ml trypsin-EDTA (CellGro, Herndon, VA). Samples were centrifuged and cells were counted using a Beckman Coulter Counter. Cells were plated at low density (150-150,000 cells per dish), and clones were allowed to grow for 10-14 days in maintenance media with 0.1% gentamycin added. Cells were then fixed with 70% ethanol and stained with coomassie blue for analysis of clonogenic survival. Individual assay colony counts were normalized to that of control with at least 3 cloning dishes per condition, repeated in at least 3 separate experiments.
Glutathione Assay: 440,000 A549 or H292 cells were plated in 100 mm dishes, allowed to grow for 24 hours then treated with 50-1000 μM BSO, 20 mM 2DG, 5 μM carbo for 24 hours and 15 mM NAC for 22 hours. Au was added for the last three hours of treatment at 0.5 – 15 μM. Immediately following treatment the cultures were washed in cold PBS and then cells were scraped into 300 μL of 5% 5-sulfosalicylic acid (Sigma) in water and stored at -20°C for a maximum of 72 hours. Total glutathione (GSH) content was determined as described previously (29). Glutathione disulfide (GSSG) was determined by adding 35 µl of a 1:1 mixture of 2-vinylpyridine and ethanol to 175 µl of sample and incubating for 2 h prior to assaying as described previously (30). The rates of the reaction were compared to similarly prepared GSH and GSSG standard curves. Glutathione determinations were normalized to the protein content of the insoluble pellet from the SSA extracts dissolved in 2.5% SDS in 0.1 N bicarbonate using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Thioredoxin Western. Separation of the oxidized and reduced forms of thioredoxin was based on the method by Holmgren and Fagerstedt (31). Cells were plated and treated identically to the glutathione assay then harvested by scraping directly into G-Lysis buffer containing 50 mM iodoacetic acid sodium salt (Sigma I9148) with a final pH of 8.3 (32). After incubating at 37° for 30 minutes excess iodoacetic acid was removed using desalting columns (GE Healthcare MicroSpin G-25 columns). Protein concentration was then determined using Bio-Rad protein assay dye reagent and equal amounts of total protein (approximately 20 μg) in a non-reducing, non-denaturing sample buffer was loaded into wells on a 15% Ready-Gel (Bio-Rad). Gels were electroblotted to nitrocellulose membranes and probed for Trx1 using anti-Trx1 primary antibody (American Diagnostica, Greenwich, CT) and horseradish peroxidase-conjugated anti-goat.
immunoglobulin G secondary antibody followed by chemoiluminescent detection (SuperSignal West Pico, Pierce) with x-ray film and/or a Typhoon FLA 7000 fluorescent detection system. Band integrated densities were determined using image J software.

**Thioredoxin Reductase Assay.** TR activity was determined spectrophotometrically using the method of Holmgren and Bjornstedt (31). (Sigma-Aldrich CS0170) Enzymatic activity was determined by subtracting the time dependent increase in absorbance at 412 nm in the presence of the TR activity inhibitor, aurothioglucose from total activity. One unit of activity was defined as 1 \( \mu \text{M} \) TNB formed/(min·mg protein). Protein concentrations were determined by the Lowry assay (33).

**Tumor xenograft growth in vivo.** Female 6–8 week old athymic-nu/nu nude mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were housed in a pathogen-free barrier room in the Animal Care Facility at the University of Iowa and handled using aseptic procedures. All procedures were approved by the IACUC committee of the University of Iowa and conformed to the guidelines established by NIH. Mice were allowed at least 1 week to acclimate prior to beginning experimentation, and food and water were made freely available. Tumor cells were inoculated into nude mice by subcutaneous injection of 0.1 mL aliquots of saline containing 5 \( \times \) 10^6 A549 cells or 3 \( \times \) 10^6 H292 cells into the right flank using 25-gauge needles. When tumor volumes measured approximately 100 mm^3 treatments with drugs were started. The mice were divided into 6 groups, 5-7 mice/group and given the drugs intraperitoneally (i.p.) every other day (weekends off) for 6 doses. BSO was diluted in sterile normal saline to a concentration of 50 mg/ml pH adjusted with bicarbonate and then administered at 450 mg/kg. Au was dissolved in...
0.25 ml of absolute ethanol then 0.2 ml Cremaphor EL brought to a final concentration of 0.2 mg/ml in normal saline and administered i.p. at 1.6 mg/kg. Carboplatin 10 mg/ml was further diluted in saline and administered i.p. at 15 mg/kg. Mice in the control group were administered i.p. saline/Cremaphor mixture every other day. In some experiments blood was obtained via cardiac puncture, diluted 1:5 in Sysmex buffer and CBC obtained using Sysmex XT2000i Automated Hematologic Analyzer (Mundelein, IL). Mice were sacrificed 2 hours after the last dose of drug and the tumors and organs were frozen in liquid nitrogen for later evaluation for TR activity and carbonyl assay or homogenized in 5% SSA for GSH quantification. Mice in the tumor growth study were monitored and tumors measured daily using Vernier calipers (Vol = (Length x Width^2 )/2) and euthanized when tumor length exceeded 1.5 cm in any dimension.

**Protein Carbonyl Assay.** Approximately 100 mg of mouse tumor or muscle was washed and homogenized in 500 μl PBS containing EDTA pH 6.7 then processed for carbonyl content by reaction with dinitrophenylhydrazine using carbonyl assay kit instructions from Cayman Chemical Company (Ann Arbor, MI) with the following exception. After the final wash protein pellets were re-suspended in 400 μL 6 M guanidine HCL and transferred to a quartz cuvette and the absorbance read at 370 nM. The sample was then further diluted 1:4 in guanidine and the absorbance read at 280 nM and the protein concentration determined against an albumin standard curve.

**Propidium Iodide Staining for DNA content.** A549 cells were plated and treated as in Figure 1. After 24 hours of treatment cells were washed and scraped in ice cold PBS then fixed in 70% ethanol over-night at 4°C. Cell pellet was washed in PBS then incubated in 100 μM 1 mg/ml Rnase A, that had been boiled and cooled for 20 min. at 37°C prior to use. 500 μL of 35 μg/ml
propidium iodide was then added and cells were incubated an additional 30 min. at 37°C. Cells were then strained through a 50 micron mesh filter and analyzed using a FACScan flowcytometer (Becton Dickinson Immunocytometry System). Data from a minimum of 10,000 cells were acquired and were processed using Cellquest Pro software (Immunocytometry Systems) and analyzed ModFit software (Verity Software House).

Results

2DG enhances carboplatin-induced clonogenic cell killing. 2DG is known to enhance cisplatin-induced cell killing in human head and neck cancer cells (21). To extend this observation to lung cancer cells, 2DG was combined with another commonly used platinum containing chemotherapeutic agent, carbo in A549 human lung cancer cells. Cells were treated with 20 mM 2DG, 5 μM carbo, 1 nM paclitaxel (ptx) or 2 Gy IR and subjected to clonogenic cell survival assay (Fig. 1A). The dose of 2DG was chosen to achieve a physiologically relevant ratio of 2DG to glucose in DMEM media, relative to what is achievable in human blood (0.8 2DG:glucose) (34). The combination of ptx, carbo and IR was chosen for comparison because this combination is considered part of the standard of care in the treatment of NSCLC at some institutions. The doses of drugs were adjusted until approximately 80% survival was achieved as single agents in order to reveal interactions between various agents. The combination of carbo+2DG resulted in better than additive cell killing (82% killing; compared to 15% cell killing with either agent alone) which was significantly greater than carbo+ptx+IR (67%, p< 0.01 Fig. 1A). These results demonstrate that inhibition of glucose metabolism is an effective method of enhancing carbo-induced cytotoxicity in lung cancer cells.
In order to demonstrate interactions with radiation, the dose of carbo was reduced to 2 μM. Treating with the combination of 2DG and 2 μM carbo resulted in clonogenic cell killing of 35% in A549 cells (Fig. 1B) as well as another NSCLC human mucoepidermoid cell line, NCI-H292 (Fig. 1C). When these NSCLC cell lines were exposed to 2 Gy IR A549 and H292 cells demonstrated 44% and 54% cell killing, respectively (Fig. 1B, C). Interestingly, exposure to 2DG or 2 μM carbo for 24 h prior to 2 Gy IR did not result in a significant enhancement of cytotoxicity in either cell line. However when cells were exposed to the combination of 2DG+carbo for 24 h followed by 2 Gy IR there was a significant decrease in clonogenic cell survival when compared to either IR alone or carbo+IR (22% A549 p<.001; 30% H292 p<0.01 Fig. 1B,C). Radio-sensitization after 24 hour exposure to 2DG+carbo was seen in the absence of any decrease in the percent of cells in the radio-resistant S-Phase of the cell cycle (see Supplemental Table 1), which suggests that cell cycle redistribution at the time of radiation is an unlikely contributor to the observed effects. These results demonstrate that the combination of 2DG+carbo can radio-sensitize both A549 and H292 human NSCLC cells in vitro.

Carboplatin combined with 2DG enhances cytotoxicity of lung cancer cells via a thiol-dependent mechanism. Disruptions in thiol metabolism indicative of oxidative stress have been observed in multiple human cancer cell lines during inhibition of glucose metabolism (21, 35, 36). GSH is a tripeptide containing a reactive cysteine which acts as an intracellular reducing agent and the accumulation of glutathione disulfide (GSSG) is considered a parameter indicative of disruptions in thiol redox homeostasis leading to oxidative stress. Treatment of A549 cells and H292 cells with the combination of 20 mM 2DG and 5 μM carbo for 24 hours resulted in 40% (p<0.001) and 30% (p<0.05) decreases in total GSH content respectively (data not shown). To
probe for the possible involvement of disruptions in thiol redox homeostasis as a causal mechanism responsible for 2DG+carbo-induced cytotoxicity, 15 mM of the thiol antioxidant, NAC, was added to cultures 2 hours after the addition of 20 mM 2DG and 5 μM carbo and allowed to incubate for 24 hours (Fig. 2A,B). In both A549 cells (Fig. 2A) and H292 cells (Fig. 2B) NAC treatment was capable of significantly inhibiting the cytotoxicity of 2DG+carbo (Fig. 2A, B). The fact that NAC was capable of inhibiting toxicity even when added 2 h following 2DG+carbo (when the drugs have presumably already entered the cells), strongly supports the hypothesis that clonogenic cell killing mediated by 2DG+carbo in lung cancer cells is mediated by disruptions in thiol metabolism.

NCSLCs are susceptible to simultaneous inhibition of thioredoxin and glutathione metabolism. The intracellular redox environment is largely maintained in a highly reduced state though the actions of GSH and Trx-dependent metabolism. To investigate the roles of these inter-dependent thiol metabolic pathways; BSO, an inhibitor of GSH synthesis, and Au, a gold phosphine thioredoxin reductase (TR) inhibitor, were tested in the A549 and H292 lung cancer cell models (25, 27). TR activity was measured as a function of Au dose in homogenates from exponentially growing H292 and A549 cells treated with the vehicle control or Au at 0.5, 1, 5 or 15 μM for 3 hours. Interestingly, H292 cells had approximately 50% of the baseline TR activity, as compared to A549 cells (Fig. 3A). Treatment of A549 and H292 cells with 0.5 μM Au for 2 h resulted in 60-70% inhibition of TR activity (Fig. 3A) and 15 μM Au for 3 hours was able to completely inhibit TR activity in both cell lines (Fig. 3A). Similarly, exponentially growing cells were treated with BSO at 50, 100, or 1000 μM for 24 hours and then harvested in 5% sulfosalicylic acid for total GSH content (Fig. 3B). As was seen with TR activity, A549 cells had greater levels of total
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GSH (167 nmol GSH/mg protein) when compared to H292 (98 nmol GSH/mg protein). Treatment with BSO at 50 μM for 24 hours was capable of depleting total GSH content by >80% but even 1000 μM BSO for 24 h was not capable of depleting GSH to undetectable levels in either lung cancer cell line (Fig. 3B).

To determine the effects of inhibition of GSH- and Trx-dependent metabolism on clonogenic survival, A549 and H292 cells were treated with 100 μM BSO for 24 hours, and then either 5 μM Au (A549, Fig. 3C) or 0.5 μM Au (H292, Fig 3D) was added to cell cultures for 3 hours in the continued presence of BSO. These doses of Au were chosen to cause similar levels of TR activity inhibition in both cell lines (Fig. 3A). Treatment with Au or BSO alone resulted in no decrease in clonogenic survival in A549 cells and only a 20% decrease in clonogenic survival in H292 cells. In stark contrast, when Au was combined with BSO greater than 99.9% clonogenic cell killing was noted in both lung cancer cell lines (Fig. 3C, D). To determine if disruptions in thiol metabolism were causally related to the toxicity seen with Au+BSO, cells were treated as described above and 15 mM NAC was added for the entire incubation period (Fig. 3C,D). NAC was capable of nearly completely rescuing both H292 and A549 cells from clonogenic cell killing mediated by treatment with Au+BSO treatment (Fig. 3C,D p< 0.001). The fact that neither Au nor BSO alone caused significant toxicity while simultaneous treatment with Au+BSO was extremely toxic, suggests that redundancies between GSH and Trx metabolism necessitate simultaneous inhibition of both pathways in order to get the maximal anti-cancer effect.

Inhibition of hydroperoxide metabolism with Au+BSO enhances oxidative stress and cytotoxicity in 2DG+carbo treated A549 cells. To determine if inhibition of Trx and GSH metabolism could further enhance the toxicity of 2DG+carbo, A549 lung cancer cells were treated
with 20 mM 2DG + 2 μM carbo for 24 hours in the presence and absence of BSO and/or 0.5 μM Au for the last 3 h of incubation (Fig. 4A). BSO or Au treatments individually did not result in a significant decrease in clonogenic cell survival in A549 cells and the addition of either BSO or Au at these doses of 2DG+carbo did not result in increased cytotoxicity (Fig. 4A). When A549 cells were treated with the combination of Au+BSO+2DG+carbo there was a significant enhancement of cell killing, when compared to 2DG+carbo (p < 0.01, Fig. 4A). These results suggest that inhibition of both GSH and Trx metabolism does enhance the toxicity of 2DG+carbo in lung cancer cells.

To further probe the involvement of oxidative stress in the enhancement of 2DG+carbo-induced cell killing in the presence of BSO and Au, A549 cells were again treated with the four drugs as in Figure 4A and analyzed for total GSH and GSSG. The combination of 2DG+carbo resulted in a 45% decrease in total GSH and a slight but insignificant increase in %GSSG in A549 cells. Treatment with Au alone did not result in a significant change in the either total GSH or %GSSG while treatment with BSO effectively depleted 90% of total GSH in A549 cells and resulted in a 15% increase in %GSSG (Fig. 4BC). Treatment of A549 cells with the combination of Au + BSO also resulted in >90% depletion total GSH with 60% of the remaining GSH being in the form of GSSG (Fig. 4BC). Consistent with the cell killing seen in Figure 4A, treatment of A549 cells with all four drugs resulted in a >90% depletion in total GSH and essentially all the remaining GSH was converted to GSSG (Fig. 4BC). Treatment with NAC alone did not have a significant affect on either total GSH or GSSG in A549 cells (Fig. 4BC). However, NAC suppressed the increase %GSSG (Fig. 4C) in A549 cell treated with the combination of all four drugs.

To further investigate the effects the drugs had on the oxidation of Trx, A549 cells were treated in an identical manner as in Figure 4A and were then analyzed for oxidized/reduced
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thioredoxin by a redox western blot (Fig. 4DE). The combination of all four drugs resulted in a significant increase in oxidized Trx/reduced Trx and this effect was completely suppressed by treatment with NAC (Fig. 4DE). Taken together, Figures 3 and 4 support the hypothesis that the combination of 2DG+carbo induces disruptions in GSH- and Trx-dependent thiol metabolism consistent with oxidative stress, which is reversed by NAC, and enhanced by Au+BSO clearly leading to the conclusion that the cytotoxicity of these drug combinations in human lung cancer cells is mediated by thiol dependent oxidative stress.

The combination of Au and BSO was safely and effectively administered to nude mice.

Because the combination of Au and BSO was effective in decreasing lung cancer cell survival *in vitro* and both agents have been used safely as single agents in humans, these studies were expanded to xenograft models of H292 and A549 cells grown in nude mice. H292 and A549 cells were injected subcutaneously into the flanks of female athymic nude mice and when the tumors reached 4 mm in diameter, animals were treated with 450 mg/kg BSO and 1.6 mg/kg Au i.p. every other day for two weeks. Two hours after the final injection animals were euthanized and tumors, blood, and organs harvested for GSH and TR activity (Fig. 5A, B). Neither total GSH nor TR activity was significantly altered in the brain tissue, however the combination of Au+BSO resulted in a significant decrease in both TR activity and total GSH in thigh muscle, and H292 tumors. Treatment with Au+BSO also resulted in a decrease in TR activity and total GSH in A549 tumors, but the decrease in total GSH decrease did not reach statistical significance. Mice were observed daily during treatment and adverse effects as measured by weight changes or behavioral activity level were not observed (data not shown). Blood analysis done on mice immediately following 2 weeks of treatment with Au+BSO also demonstrated no decreases in any circulating white or red
blood cells (Fig. 5C,D). However, Au + BSO treatment did result in a 30% increase in circulating neutrophils (Fig. 5D). This finding is in agreement with other investigators who showed that less than 1 μM Au increased neutrophil viability \textit{in vitro} (37). These results clearly indicated that Au+BSO treatment was well-tolerated by nude mice bearing H292 and A549 human tumor xenografts and the drugs effectively decreased total GSH and TR activity in tumors.

\textbf{Au+BSO+carbo inhibits A549 tumor growth \textit{in vivo}.} Mice bearing A549 xenografts were divided into six groups and treated with vehicle control, carbo 15 mg/kg or the combinations of 1.6 mg/kg Au + 450 mg/kg BSO or the combination of Au+BSO+carbo, 5 days a week for 2 weeks. None of the 24 mice demonstrated toxicity as measured by weight loss and the mice appeared healthy for the duration of the experiment (data not shown). Treatment with carbo or Au+BSO alone did not result in a significant difference in the rate of tumor growth when compared to control mice (Figure 6A, Supplemental Table 2 and Supplemental Figure 1). Most importantly, the combination of Au+BSO+carbo resulted in a highly significant decrease in tumor growth rate when compared to control (Figure 6A, Supplemental Table 2 and Supplemental Figure 1). When protein carbonyl content of the tumors and thigh muscle of treated animals was used was as a marker of protein oxidation, muscle and tumors from mice treated with Au+BSO+carbo demonstrated a significant increase in protein carbonyls compared to control or Au+BSO treated mice (Fig. 6B). These data support the conclusion that the combination of inhibitors of hydroperoxide metabolism (i.e. Au+BSO) with carboplatin can enhance tumor growth inhibition and parameters of oxidative stress \textit{in vivo} without causing overt signs of morbidity and mortality.

\textbf{Discussion}
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Platinum containing chemotherapeutics including cisplatin, oxaliplatin, and carboplatin are used with some success clinically to treat many types of cancer including lung cancer; however treatment with these agents is limited by a narrow therapeutic window and both acquired and intrinsic resistance. Although a number of factors contribute to resistance to these agents, there is a great deal of evidence implicating the GSH and Trx pathways in resistance to these agents (13, 14, 38) and protection from oxidative stress. In the current study the simultaneous inhibition of glucose and hydroperoxide metabolism mediated by Trx- and GSH-dependent pathways was shown to enhance the anticancer effects of carboplatin through thiol-mediated oxidative stress.

Lung cancer cells have been shown to have increased utilization of glucose by the pentose phosphate pathway (39). Glucose is metabolized through the pentose phosphate pathway resulting in the regeneration of two molecules of NADPH from two molecules of NADP+ and ribose-5-phosphate for the synthesis of nucleotides. NADPH acts as the ultimate cofactor providing reducing equivalents for the all the GSH-dependent peroxidase pathways as well as all the Trx-dependent peroxidase pathways. GSH- and Trx-dependent pathways are two of the major pathways utilized by many cancer cells to regulate the toxic effects of therapy agents that induce oxidative stress. In fact glucose deprivation has been shown to result in increases in steady-state levels of H₂O₂ in cancer cells, rendering them more susceptible to cell death, presumably by inhibiting the endogenous metabolism of hydroperoxides (18, 40).

2DG, is a safe and well tolerated (41) glucose analog, that can only undergo the first enzymatic step in the pentose phosphate pathway (glucose-6-phosphate dehydrogenase) to regenerate one molecule of NADPH from NADP+ but is not capable of further metabolism in the second step in the pentose cycle that recycles a second molecule of NADP+ to NADPH. Theoretically this action of 2DG would compromise the ability of cancer cells to metabolize
hydroperoxides through GSH- and Trx-dependent pathways resulting in lower steady-state levels of NADPH as well as mimicking the effects of glucose deprivation (18, 19, 42). It has been previously demonstrated that exposure to 2DG causes cytotoxicity, chemo- and radio-sensitization in head and neck, colon and breast cancer cells via a mechanism involving perturbations in thiol metabolism in cell culture (20, 21, 36, 42). In this study we demonstrate that 2DG is capable of enhancing carbo- and radiation-induced toxicity in two human NSCLC lines (A549 and NCI-H292). This finding is also consistent with a previous finding that 2DG could sensitize lung cancer cells to radiation-induced clonogenic cell killing (43). We went on to show that the addition of the thiol antioxidant, NAC, to the culture media is capable of significantly protecting both cell lines from the toxicity of 2DG + carbo supporting the hypothesis that 2DG + carbo cytotoxicity is mediated by thiol-dependent oxidative stress in human lung cancer cells (Figure 2).

GSH is a tripeptide consisting of glutamate-cysteine-glycine with the thiol group of the cysteine molecule capable of donating reducing equivalents as a co-factor for all glutathione peroxidase enzymes that regulate hydroperoxide toxicity, as well as participating in the redox regulation of signaling molecules, such as apoptosis signaling kinase-1 (ASK-1), by glutaredoxin (36, 44). Glutaredoxin shares several thiol redox functions, including the redox regulation of ASK-1, with the related thiol containing peptide, Trx (44). Trx and GSH metabolism also both rely on NADPH as a co-factor for the redox regulation of cysteine-disulfied exchange reactions necessary for redox signaling (44). It has also previously been demonstrated that both Trx1 and Trx2 knockout embryonic fibroblasts are highly susceptible to cell killing mediated by inhibition of GSH synthesis with BSO (45, 46) clearly demonstrating the redundancy of Trx- and GSH-dependent protective functions.
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The data in the current report also support the hypothesis that redundancy between GSH- and Trx-dependent metabolisms is a major consideration when developing biochemical rationales for sensitizing lung cancer cells to oxidative stress-induced cell killing mediated by combined radio-chemo-therapies. When GSH was depleted >80% using BSO or TR activity was inhibited >90% using Au, only minimal clonogenic cell killing or chemo-sensitization using carbo was noted in A549 or H292 human lung cancer cells. In contrast, Au+BSO is highly effective at inducing thiol mediated oxidative stress as well as inducing cell killing and chemo-sensitization in lung cancer cells both *in vitro* and *in vivo*.

There is much evidence that Trx and GSH pathways are up regulated in non-small cell lung cancers and up regulation correlates positively with increased aggressiveness of tumors (11). Multiple studies have attempted manipulating either GSH or Trx metabolism to enhance responses to chemotherapy. Phase I clinical trials using BSO alone or in combination with melphalan concluded that BSO infusions are relatively non-toxic and resulted in depletion of tumor GSH as well as showing the potential for some therapeutic benefit (24, 47, 48). As early as 1981 it was demonstrated that Au prolonged the lifespan of mice inoculated with lymphocytic leukemia (49). A more recent study has demonstrated that Au was effective at decreasing viability of cisplatin resistant ovarian cancer cells *in vitro* (28), however several other studies have failed to demonstrate Au posses anticancer effects *in vivo* (50). In the current report we demonstrate, for the first time, that Au+BSO can be given safely and effectively in combination with carbo *in vivo* to improve efficacy of radio-chemo-therapy in preclinical models of lung cancer *in vitro* and *in vivo*.

Overall, the current results support the biochemical rationale of simultaneous inhibition of both Trx and GSH as a promising approach for inducing thiol-mediated oxidative stress for the purpose of chemo-sensitizing human lung cancer cells that is worthy of further investigation.
Acknowledgements: The authors would like to thank Dr. Sudersan Bhatia and Dr. Walter Watson for helpful discussions. We would also like to thank Amanda Kalen and Katie Leick for technical assistance. The authors would like to acknowledge the support of NIH grants R01CA133114, R21CA139182, UL1RR024979, T32CA078586, and P30CA086862.
Figure Legends

Figure 1. Clongenic cell killing mediated by carbo and carbo+IR was significantly enhanced by 2DG. (A) Exponentially growing A549 cells were treated with 20 mM 2DG, 1 nM ptx and/or 5 μM carbo for 23 h followed by exposure to 2 Gy IR and assayed for clonogenic survival. Similarly, A549 (B) and H292 (C) cells were treated with 20 mM 2DG and 2 μM carbo for 24 hrs. After 23 hrs of drug exposure, cells were exposed to 2 Gy IR and assayed for clonogenic survival. All colony counts were normalized to the sham treated control group (designated Control in each panel). Errors represent ± 1 SEM of at least 3 separate experiments. One way ANOVA with Tukey’s post hoc analysis resulted in *p<0.01 versus control; # p<0.01 versus carbo+ptx+IR; **p<0.001 versus similar treatment without IR.

Figure 2. The thiol antioxidant, NAC, is capable of protecting A549 (A) and H292 (B) cells from clongenic cell killing caused by 2DG+carbo. A549 and H292 cells were treated with 20 mM 2DG and 5 μM carbo for 24 h. NAC (15 mM pH adjusted) was added 2 hrs after the beginning of 2DG+carbo treatment. Cells were assayed for clonogenic survival. Colony counts are normalized to respective controls. Errors represent ± 1 SEM, at least 3 separate experiments. One way ANOVA with Tukey’s post hoc analysis resulted in *p<0.05 versus control; **p<0.05 versus respective treatment without NAC.

Figure 3. Au combined with BSO is effective at inhibiting TR and depleting total GSH, respectively, as well as inducing cytotoxicity in A549 and H292 cells. Cells were treated with 0 – 15 μM Au for three hours and then scrape harvested and assayed for TR activity as described in methods. Error bars represent ± 1 SEM of at least four separate treatment dishes assayed in
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two separate experiments (A). Cells were treated with 0 - 1000 μM BSO for 24 hours and then scrape harvested and assayed for total GSH content as described in methods. Error bars represent ± 1 SEM of at least four separate treatment dishes assayed in two separate experiments (B). Cells were treated with 100 μM BSO for 24 hours and with 5 μM (A549, C) or 0.5 μM (H292, D) Au for the last 3 hours of drug exposure. NAC (15 mM, pH adjusted) was added 2 hrs after BSO addition. Following treatment, cells were assayed for clonogenic survival and normalized to the respective sham treated controls. Errors represent ± 1 SEM of at least 3 separate experiments. One way ANOVA with Tukey’s post hoc analysis resulted in *p<0.0001 versus control; **p<0.0001 versus respective treatment without NAC.

**Figure 4. The combination of Au and BSO decreases the clonogenic survival of 2DG+carbo treated A549 cells as well as increasing thiol oxidation that is inhibited by NAC.**

Exponentially growing cells were treated with 20 mM 2DG and 2 μM carbo for 24 hours with or without treatment with 100 μM BSO for 24 hours and 0.5 μM Au for the last 3 hours followed by clonogenic survival analysis with the data being normalized to control (A). Errors represent ± 1 SEM of at least 3 separate experiments. One way ANOVA with Tukey’s post hoc analysis resulted in # p<0.05 versus 2DG+carbo. Exponentially growing A549 lung cancer cells were treated with 20 mM 2DG, 5 μM carbo, 100 μM BSO and/or 15 mM NAC for 24 hours with 5 μM Au for the last 2 hours. Cells were scraped in 5% SSA and assayed for total glutathione [GSH+GSSG] (B) and glutathione disulfide (GSSG) (C) content as described in methods. Percent glutathione disulfide was calculated by dividing nmol GSSG in GSH equivalents by nmol total glutathione. Errors represent ± 1 SEM of at least three treatments dishes. One way ANOVA with Tukey’s post analysis resulted in * = p<0.001, **p=0.05 versus control. For Trx analysis (D, E) cells were scraped into G-lysis buffer
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and derivatized in iodoacetic acetate and assayed via Trx redox Western analysis as described in methods. Treatment of A549 cells with all four drugs resulted in increases in oxidized Trx that were reversed by treatment with NAC (D). Quantification of four separate experiments where four separate redox Westerns were analyzed is shown in panel E. Error bars represent the average of (integrated density of oxidized bands)/(integrated density of the reduced band), normalized to the control. One way ANOVA with Tukey’s post hoc analysis resulted in ** = p < 0.01 versus treatment without NAC, # p<0.05 versus all other treatment groups. Student’s t test resulted in *p<0.05 verses control.

**Figure 5. BSO+Au is effective at decreasing total GSH and TR activity without causing myelosuppression in nude mice bearing human lung cancer xenografts.** Five mice from each treatment group were implanted with either A549 or H292 lung cancer cells in the hind leg and when tumors reach approximately three mm in diameter mice were treated with 1.6 mg/kg Au and 450 mg/kg BSO injected i.p. every other day for two weeks. Brain, thigh muscle and xenograft tumors were harvest two hours after the final injection and total GSH (B) and TR (A) activity analysis was performed on homogenized tissue as described in methods. Errors represent ± 1 SEM. Student’s t test resulted in *p<0.05. Blood was drawn via a cardiac puncture from 10 anesthetized mice and diluted with Sysmex buffer and analyzed via Sysmex XT2000i Automated Hematology Analyzer (C,D) per manufacturer’s instructions. Errors represent ± 1 SEM. Student’s t test resulted in *p<0.05.

**Figure 6. The combination of carbo+Au+BSO inhibits A549 tumor growth as well as resulting in an increase in protein carbonyls.** Mice (6-7 per group) were injected with 5 million
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A549 tumor cells in the right flank. When tumors reached approximately 3-4 mm in diameter (Day 1) mice were given carbo (15 mg/kg), BSO (450 mg/kg) and/or Au (1.6 mg/kg) simultaneously i.p. every other day for two weeks. Control mice received a Cremaphor EL ® mixture (Au vehicle) 0.1 ml every other day. Linear and quadratic effects of time were included in the mixed effects regression modeling. Pairwise treatment group comparisons from the analysis are summarized on the panel showing the average tumor volume from each group (A). Statistically significant differences were found between Control and Au+BSO+carbo (p=0.0051). Marginally but nonsignificant differences were observed between Control and Au+BSO (p=0.0562) as well as carbo alone (p=0.0712). At the end of treatment tumors and thigh muscles from the opposite leg of three or four mice (muscle and tumor respectively) were harvested and subjected to protein carbonyl assay as described in methods and analyzed spectrophotometrically (B). Errors represent ± 1 SEM. Student’s t-test resulted in *p<0.05.

**Supplementary Table 1.** 2DG, carbo, and the combination of 2DG+carbo does not decrease the percent of cells in S phase in A549 cells. Exponentially growing A549 cells were treated with 20 mM 2DG and/or 5 μM carbo for 24 h followed by propidium iodide staining for DNA content for cell cycle analysis. Errors represent ± 1 SEM of at least 3 separate experiments. One way ANOVA with Tukey’s post hoc analysis resulted in *p<0.05 versus control.

**Supplemental Table 2.** Pairwise comparisons of tumor growth rates between treatment groups shown in Supplemental Figure 1. Linear and quadratic effects of time were included in the mixed effects regression modeling. Pairwise treatment group comparisons with p values from this analysis are summarized.
Supplemental Figure 1: Plots of tumor growth curves for each individual mouse used to generate the analysis shown in Figure 6 and Supplemental Table 2. Linear and quadratic effects of time were included in the mixed effects regression modeling.
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Figure 1.

A. 

B. 

C. 

Normalized Surviving Fractions

Control 20 mM 2DG, 5 μM Carbo 1 nM Pt x 2 Gy IR Carbo + Pt + IR 2GD + Carbo

Normalized Surviving Fraction

Control 2DG - Carbo 2 Gy IR 2GD - IR Carbo - IR 2GD - Carbo IR

Normalized Surviving Fraction

Control 2DG - Carbo 2 Gy IR 2GD + IR Carbo - IR 2GD - Carbo IR
Figure 2.

A. Normalized Surviving Fraction

B. Normalized Surviving Fraction
Figure 3.

A. B.

C. D.

Normalized Surviving Fraction

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<td>Au 5 μM</td>
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<tr>
<td>Au + BSO + NAC</td>
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Normalized Surviving Fraction

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<tr>
<td>NAC 15 mM</td>
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<tr>
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<td>Au + BSO + NAC</td>
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A. Graph showing TR activity expressed as units/mg of protein.
B. Graph showing nM GSH/mg of protein.
C. Graph showing normalized surviving fraction for different conditions.
D. Graph showing total GSH levels for different conditions.

Legend:
- H449
- H292
- Control
- NAC 15 mM
- Au 5 μM
- BSO 100 μM
- Au + BSO
- Au + BSO + NAC

Significance levels:
- * p < 0.05
- ** p < 0.01
- NA: not applicable

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Figure 4.

A. Normalized Surviving Fraction

B. Normalized moles GSH per mg protein

C. % GSSG

D. 

E. Normalized Density TrxS2/Trx(SH)2

Pr(S-S)Trx

(S-S)2Trx

(S-S)Trx

Trx

Control 2DG + Carbo Au 5 μM BSO 100 μM Au + BSO all 4 drugs NAC all 4 + NAC Control + H2O2 Mol wt

Control 2DG + Carbo Au 5 μM BSO 100 μM Au + BSO all 4 drugs NAC all 4 + NAC

GSH

NAC

2DG + Carbo

H2O2

Mol wt
Figure 5.
Figure 6.

A. 

![Graph showing tumor volume over days with different treatments.](image)

B. 

![Bar graph showing muscle and tumor total protein levels.](image)
Clinical Cancer Research

Enhancement of carboplatin-mediated lung cancer cell killing by simultaneous disruption of glutathione and thioredoxin metabolism

Melissa Fath, Iman M. Ahmad, Carmen J. Smith, et al.

Clin Cancer Res  Published OnlineFirst August 15, 2011.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-0736

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