

Enhancement of Carboplatin-Mediated Lung Cancer Cell Killing by Simultaneous Disruption of Glutathione and Thioredoxin Metabolism

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Abstract

Purpose: Cancer cells (relative to normal cells) show increased steady-state levels of hydroperoxides that are compensated by increased glucose and hydroperoxide metabolism. The current study determined whether inhibitors of glucose and hydroperoxide metabolism could induce chemoradiosensitization 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 + ionizing radiation (IR). Lung cancer cells were further sensitized with inhibitors of glutathione (GSH)- and thioredoxin (Trx)-dependent metabolism [buthionine sulfoximine (BSO) and auranofin, respectively] *in vitro* and *in vivo*.

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

Conclusions: These results show *in vitro* and *in vivo* that simultaneous inhibition of GSH and Trx metabolism can effectively inhibit lung cancer cell growth and induce chemosensitization by a mechanism that involves thiol-mediated oxidative stress. *Clin Cancer Res*; 17(19); 6206–17. ©2011 AACR.

Introduction

Lung cancer results in approximately 180,000 deaths per year, with a majority of these stemming from non-small-cell lung cancer (NSCLC; ref. 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; ref. 3). Platinum-based therapies not only

have high incidences of serious side effects but are also characterized by intrinsic and adaptive cancer cell resistance that 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 with nonmalignant 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) both via the formation of pyruvate that scavenges hydroperoxides directly through deacetylation reactions (5, 6) and 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 2 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

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Translational Relevance

Glutathione (GSH)- and thioredoxin (Trx)-dependent metabolism have both been associated with the acquisition of cancer cell resistance to therapy for the last 25 years. Clinical strategies for inhibiting GSH- or Trx-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 shows that simultaneous inhibition of both GSH- and Trx-dependent metabolism pathways 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.

cancer cells, including lung cancer, have been shown to upregulate GSH and Trx (11, 12) metabolism, and this upregulation has been associated with resistance to IR as well as platinum-based therapies (13–15). Other studies have shown 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 (2DG) is a glucose analogue that cannot undergo glycolysis or 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, and 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). Auranofin is a gold phosphine that has been used to treat patients with rheumatoid arthritis for more than 20 years. Although the mechanism of action of auranofin is not fully understood, it was recently shown to inhibit both cystolic and mitochondrial TR activities at therapeutically relevant doses (25–27). Treatment of ovarian cancer cells, including cisplatin-resistant ovarian cancer cells, with auranofin has been suggested to increase steady-state levels of H₂O₂ 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 with inhibitors of glucose and hydroperoxide metabolism including 2-DG, BSO, and auranofin in combination with carboplatin. 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 show that 2DG decreases clonogenic cell survival in 2 NSCLC cell lines (A549 and H292) and enhances the toxicity of carboplatin + IR. The thiol antioxidant *N*-acetylcysteine (NAC) is capable of protecting lung cancer cell lines from the enhanced cytotoxicity seen with 2DG + carboplatin as well as BSO + auranofin, supporting the hypothesis that thiol-mediated oxidative stress significantly contributes to outcomes via these drug combinations. The observation that doses of auranofin 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 pathways must be simultaneously inhibited to achieve maximal chemosensitization. Finally, the results showing that the combination of auranofin + BSO can be given safely to mice to achieve chemosensitization in A549 xenograft tumors exposed to carboplatin 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 Dulbecco's Modified Eagle's Medium (DMEM) or RPMI 1640 media (Mediatech Inc.), respectively, with 10% FBS (Atlanta Biologicals). Cultures were maintained in 5% CO₂ and humidified in a 37°C incubator. Carboplatin 10 mg/mL (Hospira) and paclitaxel 6 mg/mL (Bedford Labs) were further diluted in normal saline immediately prior to use to achieve the desired concentrations. Stock solutions of auranofin (Axxora) were dissolved in absolute ethanol and then further diluted in normal saline before use to achieve the desired concentration. Stock solutions of 2DG grade III and l-buthionine sulfoximine (Sigma Chemical Company) were made in PBS and kept at 4°C prior to use. A 1 mol/L solution of *N*-acetyl-l-cysteine (>99% TLC; Sigma Chemical Co.) was made in water and adjusted to pH 7.1 with 1 mol/L 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 hours. Cells were then treated 24 hours with or without 20 mmol/L 2DG, 2 to 5 μmol/L carboplatin, and 1 nmol/L paclitaxel. Ionizing radiation was delivered with a J.L. Shepherd cesium

irradiator (J.L. Shepherd) at 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 $\mu\text{mol/L}$ BSO for 24 hours and/or 0.5 to 5 $\mu\text{mol/L}$ auranofin 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). Samples were centrifuged, and cells were counted with a Beckman Coulter counter. Cells were plated at low density (150–150,000 cells per dish), and clones were allowed to grow for 10 to 14 days in maintenance media containing 0.1% gentamycin. 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.

GSH assay

A total of 44×10^4 A549 or H292 cells were plated in 100-mm dishes, allowed to grow for 24 hours and then treated with 50 to 1,000 $\mu\text{mol/L}$ BSO, 20 mmol/L 2DG, 5 $\mu\text{mol/L}$ carboplatin for 24 hours, and 15 mmol/L NAC for 22 hours. Auranofin was added for the last 3 hours of treatment at 0.5 to 15 $\mu\text{mol/L}$. 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 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 hours before assaying as described previously (30). The rates of the reaction were compared with similarly prepared GSH and GSSG standard curves. GSH determinations were normalized to the protein content of the insoluble pellet from the 5-sulfosalicylic acid dissolved in 2.5% SDS in 0.1N bicarbonate using the BCA Protein Assay Kit (Thermo Scientific).

Trx Western

Separation of the oxidized and reduced forms of Trx was based on the method by Holmgren and Fagerstedt (31). Cells were plated and treated identically to the GSH assay and then harvested by scraping directly into G-lysis buffer containing 50 mmol/L 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 with desalting columns (GE Healthcare MicroSpin G-25 columns). Protein concentration was then determined with Bio-Rad protein assay dye reagent, and equal amounts of total protein ($\sim 20 \mu\text{g}$) in a nonreducing, nondenaturing 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) and horseradish peroxidase-conjugated anti-goat immunoglobulin G secondary anti-

body followed by chemoiluminescent detection (Super-Signal West Pico; Pierce) with X-ray film and/or a Typhoon FLA 7000 fluorescent detection system. Band-integrated densities were determined with ImageJ software.

TR assay

TR activity was determined spectrophotometrically using the method of Holmgren and Fagerstedt (ref. 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 $\mu\text{mol/L}$ 5'-thionitrobenzoic acid formed/(min mg protein). Protein concentrations were determined by the Lowry assay (33).

Tumor xenograft growth *in vivo*

Female 6- to 8-week-old athymic *nu/nu* nude mice were purchased from Harlan Laboratories. Mice were housed in a pathogen-free barrier room in the Animal Care Facility at the University of Iowa and handled by aseptic procedures. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa and conformed to the guidelines established by NIH. Mice were allowed at least 1 week to acclimate before 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 5×10^6 A549 cells or 3×10^6 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, with 5 to 7 mice per 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. Auranofin was dissolved in 0.25 mL absolute ethanol and then 0.2 mL Cremaphor EL and brought to a final concentration of 0.2 mg/mL in normal saline and administered intraperitoneally at 1.6 mg/kg. Carboplatin 10 mg/mL was further diluted in saline and administered intraperitoneally at 15 mg/kg. Mice in the control group were administered intraperitoneally saline/Cremaphor mixture every other day. In some experiments, blood was obtained via cardiac puncture, diluted 1:5 in Sysmex buffer, and complete blood cell count was obtained with Sysmex XT2000i Automated Hematology Analyzer. 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 were measured daily with Vernier calipers [Volume = (Length \times Width²)/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, and then processed for carbonyl content by reaction with dinitrophenylhydrazine, using carbonyl assay kit instructions from Cayman Chemical Company, with the following exception. After the final wash, protein pellets were resuspended in 400 μ L 6 mol/L guanidine HCl and transferred to a quartz cuvette and the absorbance was read at 370 nm. The sample was then further diluted 1:4 in guanidine, the absorbance was read at 280 nm, and the protein concentration was 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 and then fixed in 70% ethanol overnight at 4°C. Cell pellet was washed in PBS and then incubated in 100 μ mol/L 1 mg/mL RNase A that had been boiled and cooled for 20 minutes at 37°C prior to use. A solution of 500 μ L of 35 μ g/mL propidium iodide was then added and cells were incubated for an additional 30 minutes at 37°C. Cells were then strained through a 50-micrometer mesh filter and analyzed with a FACScan flow cytometer (Becton Dickinson Immunocytometry System). Data from a minimum of 10,000 cells were acquired and were processed and analyzed with Cellquest Pro software (Immunocytometry Systems) and ModFit software (Verity Software House), respectively.

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, carboplatin, in A549 human lung cancer cells. Cells were treated with 20 mmol/L 2DG, 5 μ mol/L carboplatin, 1 nmol/L paclitaxel, 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 (2DG/glucose = 0.8; ref. 34). The combination of paclitaxel, carboplatin, 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 rate was achieved as single agents to reveal interactions between various agents. The combination of carboplatin + 2DG resulted in better than additive cell killing (82% killing; compared with 15% cell killing with either agent alone), which was significantly greater than the combination of carboplatin + paclitaxel + IR (67%, $P < 0.01$ Fig. 1A). These results show that inhibition of glucose metabolism is an effective method of enhancing carboplatin-induced cytotoxicity in lung cancer cells.

To show interactions with radiation, the dose of carboplatin was reduced to 2 μ mol/L. Treating with the combi-

nation of 2DG and 2 μ mol/L carboplatin 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 showed 44% and 54% cell killing, respectively (Fig. 1B and C). Interestingly, exposure to 2DG or 2 μ mol/L carboplatin for 24 hours 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 + carboplatin for 24 hours followed by 2 Gy IR, there was a significant decrease in clonogenic cell survival when compared with either IR alone or carboplatin + IR (22% A549 $P < 0.001$; 30% H292 $P < 0.01$ Fig. 1B and C). Radiosensitization after 24-hour exposure to 2DG + carboplatin was seen in the absence of any decrease in the percentage of cells in the radioresistant S-phase of the cell cycle (see Supplementary Table S1), which suggests that cell-cycle redistribution at the time of radiation is an unlikely contributor to the observed effects. These results show that the combination of 2DG + carboplatin can radiosensitize 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 that acts as an intracellular reducing agent, and the accumulation of 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 mmol/L 2DG and 5 μ mol/L carboplatin 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 + carboplatin -induced cytotoxicity, 15 mmol/L of the thiol antioxidant NAC was added to cultures 2 hours after the addition of 20 mmol/L 2DG and 5 μ mol/L carboplatin and allowed to incubate for 24 hours (Fig. 2A and B). In both A549 cells (Fig. 2A) and H292 cells (Fig. 2B), NAC treatment was capable of significantly inhibiting the cytotoxicity of 2DG + carboplatin (Fig. 2A and B). The fact that NAC was capable of inhibiting toxicity even when added 2 hours following 2DG + carboplatin (when the drugs have presumably already entered the cells) strongly supports the hypothesis that clonogenic cell killing mediated by 2DG + carboplatin in lung cancer cells is mediated by disruptions in thiol metabolism.

NSCLCs are susceptible to simultaneous inhibition of Trx and GSH metabolism

The intracellular redox environment is largely maintained in a highly reduced state by the actions of GSH and Trx-dependent metabolism. To investigate the roles of

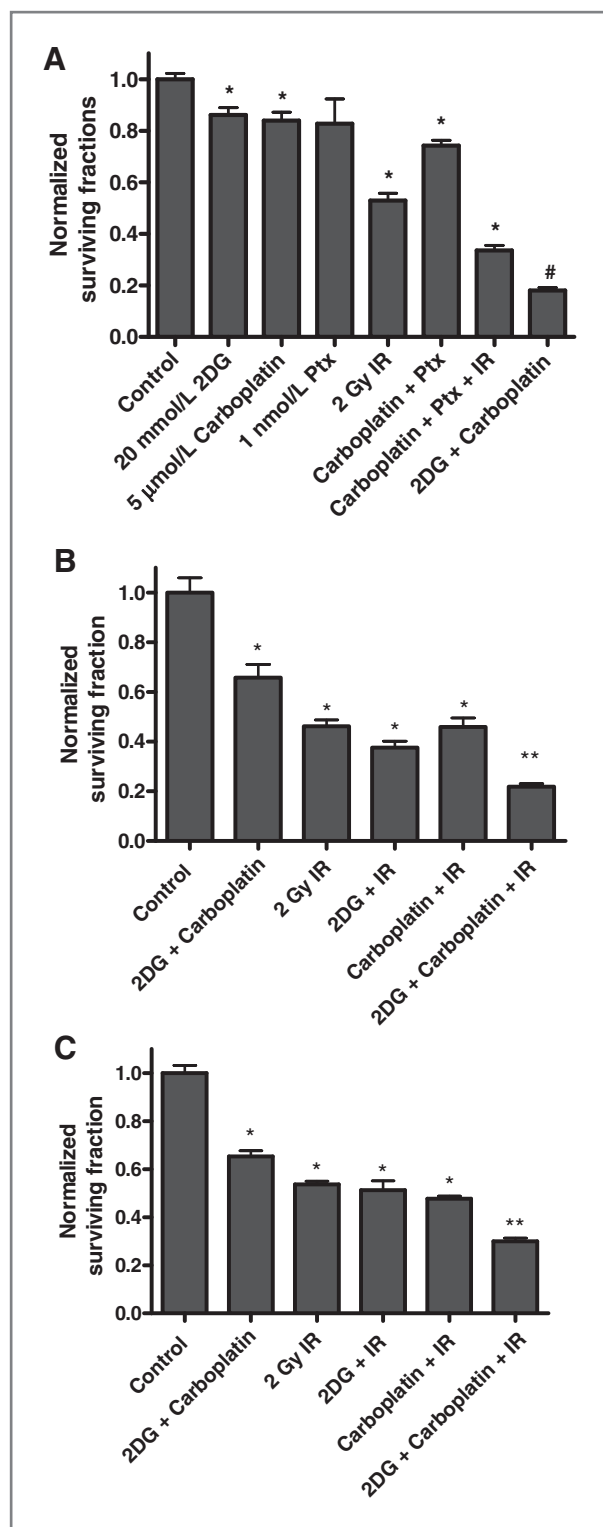


Figure 1. Clonogenic cell killing mediated by carboplatin (Carbo) and carboplatin + IR was significantly enhanced by 2DG. A, exponentially growing A549 cells were treated with 20 mmol/L 2DG, 1 nmol/L paclitaxel (Ptx), and/or 5 μmol/L carboplatin for 23 hours followed by exposure to 2 Gy IR and assayed for clonogenic survival. Similarly, A549 (B) and H292 (C) cells were treated with 20 mmol/L 2DG and 2 μmol/L carboplatin for

these interdependent thiol metabolic pathways; BSO, an inhibitor of GSH synthesis, and auranofin, a gold phosphine TR inhibitor, were tested in the A549 and H292 lung cancer cell models (25, 27). TR activity was measured as a function of auranofin dose in homogenates from exponentially growing H292 and A549 cells treated with the vehicle control or auranofin at 0.5, 1, 5, or 15 μmol/L for 3 hours. Interestingly, H292 cells had approximately 50% of the baseline TR activity as compared with A549 cells (Fig. 3A). Treatment of A549 and H292 cells with 0.5 μmol/L auranofin for 3 hours resulted in 60% to 70% inhibition of TR activity (Fig. 3A), and 15 μmol/L auranofin for 3 hours could completely inhibit TR activity in both cell lines (Fig. 3A). Similarly, exponentially growing cells were treated with BSO at 50, 100, or 1,000 μmol/L 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 GSH (167 nmol GSH/mg protein) than H292 cells (98 nmol GSH/mg protein). Treatment with 50 μmol/L BSO for 24 hours could deplete total GSH content by more than 80%, but even 1,000 μmol/L BSO for 24 hours 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 μmol/L BSO for 24 hours and then either 5 μmol/L auranofin (A549; Fig. 3C) or 0.5 μmol/L auranofin (H292; Fig. 3D) was added to cell cultures for 3 hours in the continued presence of BSO. These doses of auranofin were chosen to cause similar levels of TR activity inhibition in both cell lines (Fig. 3A). Treatment with auranofin 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 auranofin was combined with BSO, greater than 99.9% clonogenic cell killing was noted in both lung cancer cell lines (Fig. 3C and D). To determine whether disruptions in thiol metabolism were causally related to the toxicity seen with auranofin + BSO, cells were treated as described earlier and 15 mmol/L NAC was added for the entire incubation period (Fig. 3C and D). NAC was capable of nearly completely rescuing both H292 and A549 cells from clonogenic cell killing mediated by treatment with auranofin + BSO (Fig. 3C and D $P < 0.001$). The fact that neither auranofin nor BSO alone caused significant toxicity whereas simultaneous treatment with auranofin + BSO was extremely toxic suggests that redundancies between GSH and Trx metabolism

24 hours. After 23 hours 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 carboplatin + paclitaxel + IR; **, $P < 0.001$ versus similar treatment without IR.

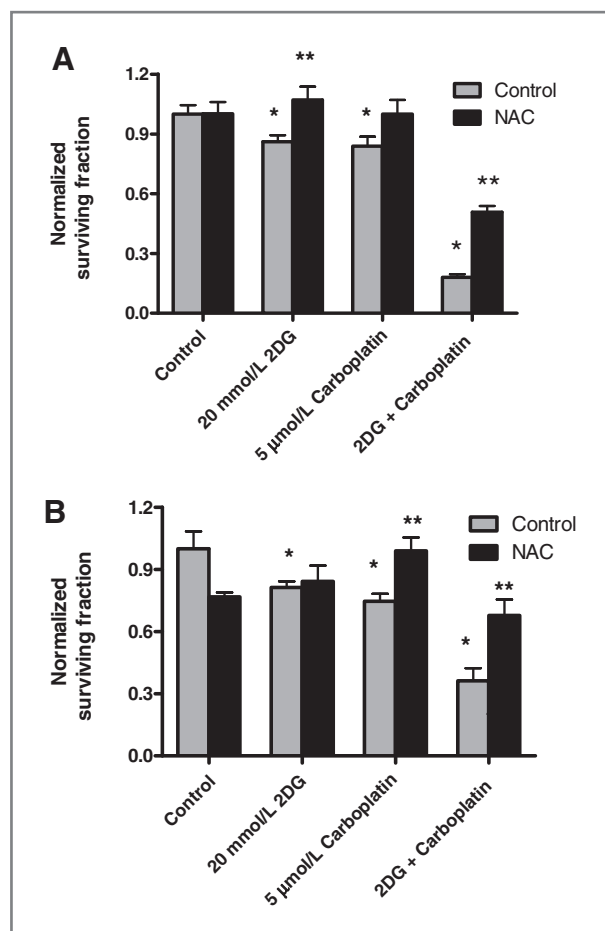


Figure 2. The thiol antioxidant, NAC, is capable of protecting A549 (A) and H292 (B) cells from clonogenic cell killing caused by 2DG + carboplatin (Carbo). A549 and H292 cells were treated with 20 mmol/L 2DG and 5 μmol/L carboplatin for 24 hours. NAC (15 mmol/L, pH adjusted) was added 2 hours after the beginning of 2DG + carboplatin treatment. Cells were assayed for clonogenic survival. Colony counts are normalized to respective controls. 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; **, $P < 0.05$ versus respective treatment without NAC.

necessitate simultaneous inhibition of both pathways to get the maximal anticancer effect.

Inhibition of hydroperoxide metabolism with auranofin + BSO enhances oxidative stress and cytotoxicity in 2DG + carboplatin-treated A549 cells

To determine whether inhibition of Trx and GSH metabolism could further enhance the toxicity of 2DG + carboplatin, A549 lung cancer cells were treated with 20 mmol/L 2DG + 2 μmol/L carboplatin for 24 hours in the presence and absence of BSO and/or 0.5 μmol/L auranofin for the last 3 hours of incubation (Fig. 4A). BSO or auranofin treatments individually did not result in a significant decrease in clonogenic cell survival in A549 cells and the addition of either BSO or auranofin at these doses of 2DG + carboplatin did not result in increased cytotoxicity (Fig. 4A). When A549 cells were treated with the combination of auranofin + BSO + 2DG + carboplatin, there was a significant enhancement of cell killing when compared with 2DG + carboplatin ($P < 0.01$, Fig. 4A). These results suggest that inhibition of both GSH and Trx metabolism pathways does enhance the toxicity of 2DG + carboplatin in lung cancer cells.

To further probe the involvement of oxidative stress in the enhancement of 2DG + carboplatin-induced cell killing in the presence of BSO and auranofin, A549 cells were again treated with the 4 drugs as in Figure 4A and analyzed for total GSH and GSSG. The combination of 2DG + carboplatin resulted in a 45% decrease in total GSH and a slight but insignificant increase in the percentage of GSSG in A549 cells. Treatment with auranofin alone did not result in a significant change in either total GSH or the percentage GSSG, whereas treatment with BSO effectively depleted 90% of total GSH in A549 cells and resulted in a 15% increase in the percentage of GSSG (Fig. 4B and C). Treatment of A549 cells with the combination of auranofin + BSO also resulted in more than 90% depletion of total GSH, with 60% of the remaining GSH being in the form of GSSG (Fig. 4B and C). Consistent with the cell killing seen in Figure 4A, treatment of A549 cells with all 4 drugs resulted in a more than 90% depletion in total GSH and essentially all the remaining GSH was converted to GSSG (Fig. 4B and C). Treatment with NAC alone did not have a significant affect on either total GSH or GSSG in A549 cells (Fig. 4B and C). However, NAC suppressed increase in the percentage of GSSG (Fig. 4C) in A549 cell treated with the combination of all 4 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 Trx by a redox Western blotting (Fig. 4D and E). The combination of all 4 drugs resulted in a significant increase in oxidized Trx/reduced Trx, and this effect was completely suppressed by treatment with NAC (Fig. 4D and E). Taken together, Figures Fig. 3 and Fig. 4 support the hypothesis that the combination of 2DG + carboplatin induces disruptions in GSH- and Trx-dependent thiol metabolism consistent with oxidative stress, which is reversed by NAC and enhanced by auranofin + 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 auranofin and BSO was safely and effectively administered to nude mice

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Because the combination of auranofin 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 auranofin i.p. every other day for

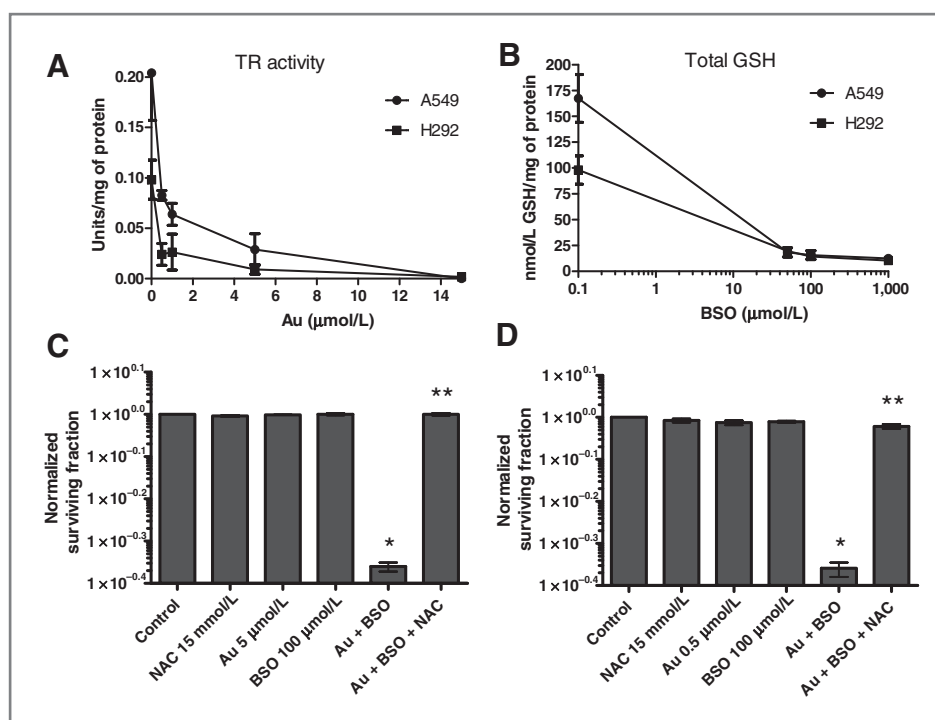


Figure 3. Auranofin (Au) combined with BSO is effective at inhibiting TR and depleting total GSH, respectively, as well as inducing cytotoxicity in A549 and H292 cells. Cells were treated with 0 to 15 $\mu\text{mol/L}$ auranofin for 3 hours and then scrape harvested and assayed for TR activity as described in Methods. Error bars represent ± 1 SEM of at least 4 separate treatment dishes assayed in 2 separate experiments. A, cells were treated with 0 to 1,000 $\mu\text{mol/L}$ 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 4 separate treatment dishes assayed in 2 separate experiments. B, cells were treated with 100 $\mu\text{mol/L}$ BSO for 24 hours and with 5 $\mu\text{mol/L}$ (A549; C) or 0.5 $\mu\text{mol/L}$ (H292; D) auranofin for the last 3 hours of drug exposure. NAC (15 mmol/L, pH adjusted) was added 2 hours 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.

2 weeks. Two hours after the final injection, animals were euthanized and tumors, blood, and organs were harvested for GSH and TR activity (Fig. 5A and B). Neither total GSH nor TR activity was significantly altered in the brain tissue; however, the combination of auranofin + BSO resulted in a significant decrease in both TR activity and total GSH in thigh muscle and H292 tumors. Treatment with auranofin + BSO also resulted in a decrease in TR activity and total GSH in A549 tumors, but the decrease in total GSH 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 auranofin + BSO also showed no decreases in any circulating white or red blood cells (Fig. 5C and D). However, auranofin + 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 $\mu\text{mol/L}$ auranofin increased neutrophil viability *in vitro* (37). These results clearly indicated that auranofin + 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.

Auranofin + BSO + carboplatin inhibits A549 tumor growth *in vivo*

Mice bearing A549 xenografts were divided into 6 groups and treated with vehicle control, carboplatin 15 mg/kg, or the combinations of 1.6 mg/kg auranofin + 450 mg/kg BSO or of auranofin + BSO + carboplatin, 5 days a week for 2 weeks. None of the 24 mice showed toxicity as measured by weight loss, and the mice seemed healthy for the duration of the experiment (data not shown). Treatment with carboplatin or auranofin + BSO alone did not result in a significant difference in the rate of tumor growth when compared with control mice (Fig. 6A; Supplementary Table S2 and Fig. S1). Most important, the combination of auranofin + BSO + carboplatin resulted in a highly significant decrease in tumor growth rate when compared with control (Fig. 6A; Supplementary Table S2 and Fig. S1). When protein carbonyl content of the tumors and thigh muscle of treated animals was used as a marker of protein oxidation, muscle and tumors from mice treated with auranofin + BSO + carboplatin showed a significant increase in protein carbonyls compared with control or auranofin + BSO-treated mice (Fig. 6B). These data support the conclusion that the combination of inhibitors of hydroperoxide metabolism (i.e., auranofin +

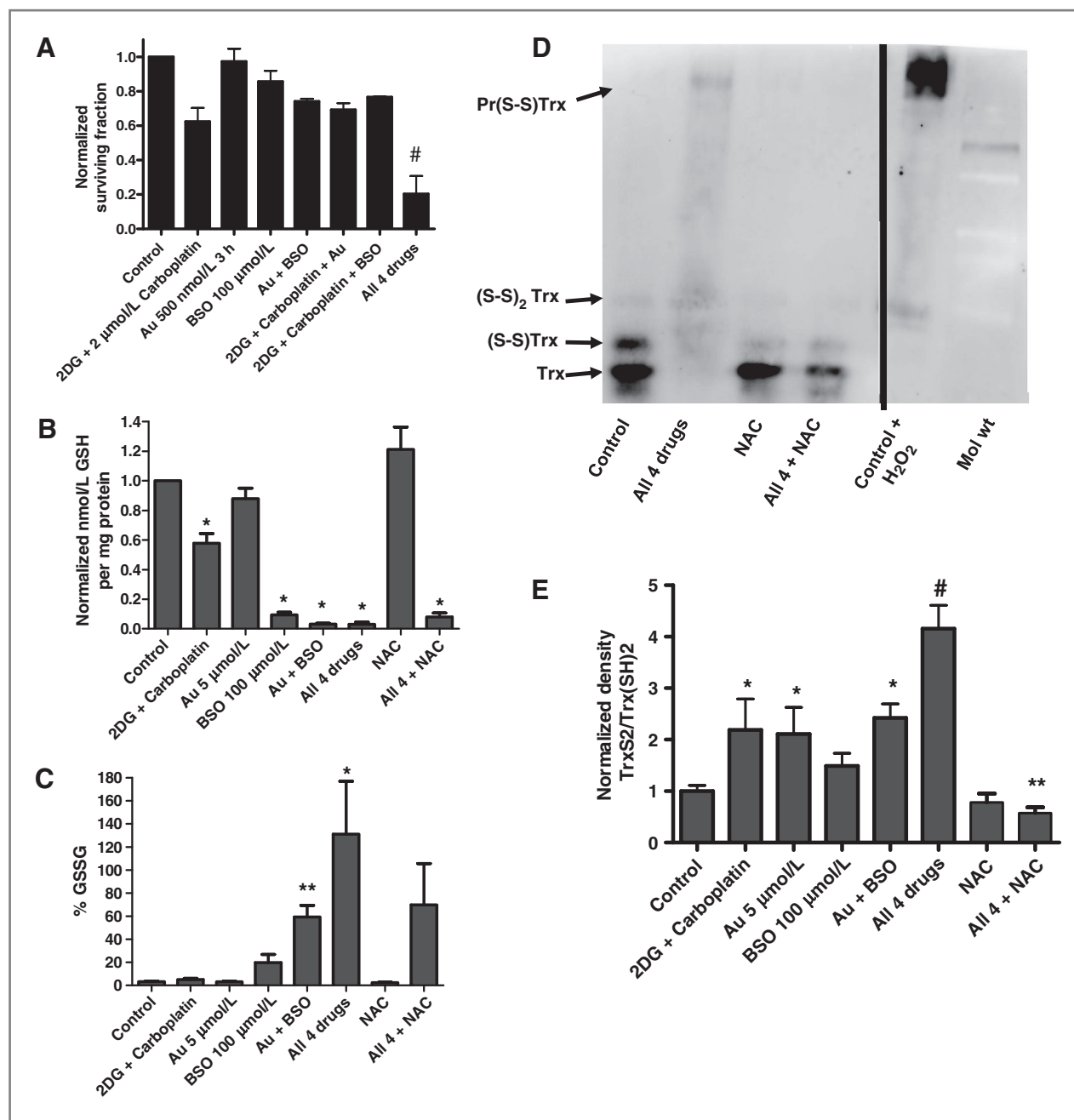


Figure 4. The combination of auranofin (Au) and BSO decreases the clonogenic survival of 2DG + carboplatin treated A549 cells and increasing thiol oxidation that is inhibited by NAC. Exponentially growing cells were treated with 20 mmol/L 2DG and 2 $\mu\text{mol/L}$ carboplatin for 24 hours with or without treatment with 100 $\mu\text{mol/L}$ BSO for 24 hours and 0.5 $\mu\text{mol/L}$ auranofin 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 + carboplatin. Exponentially growing A549 lung cancer cells were treated with 20 mmol/L 2DG, 5 $\mu\text{mol/L}$ carboplatin, 100 $\mu\text{mol/L}$ BSO, and/or 15 mmol/L NAC for 24 hours with 5 $\mu\text{mol/L}$ auranofin for the last 2 hours. Cells were scraped in 5% SSA and assayed for total glutathione [GSH + GSSG; (B)] and GSSG; (C) content as described in Methods. Percent GSSG was calculated by dividing nmol GSSG_{in GSH equivalents} by nmol total GSH. Errors represent ± 1 SEM of at least 3 treatments dishes. One-way ANOVA with Tukey's post hoc analysis resulted in *, $P < 0.001$, **, $P = 0.05$ versus control. For Trx analysis (D and E) cells were scraped into G-lysis buffer and derivatized in iodoacetic acetate and assayed via Trx redox Western analysis as described in Methods. Treatment of A549 cells with all 4 drugs resulted in increases in oxidized Trx that were reversed by treatment with NAC (D). Quantification of 4 separate experiments where 4 separate redox Western blots were analyzed is shown in 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 *, $P < 0.05$ versus control.

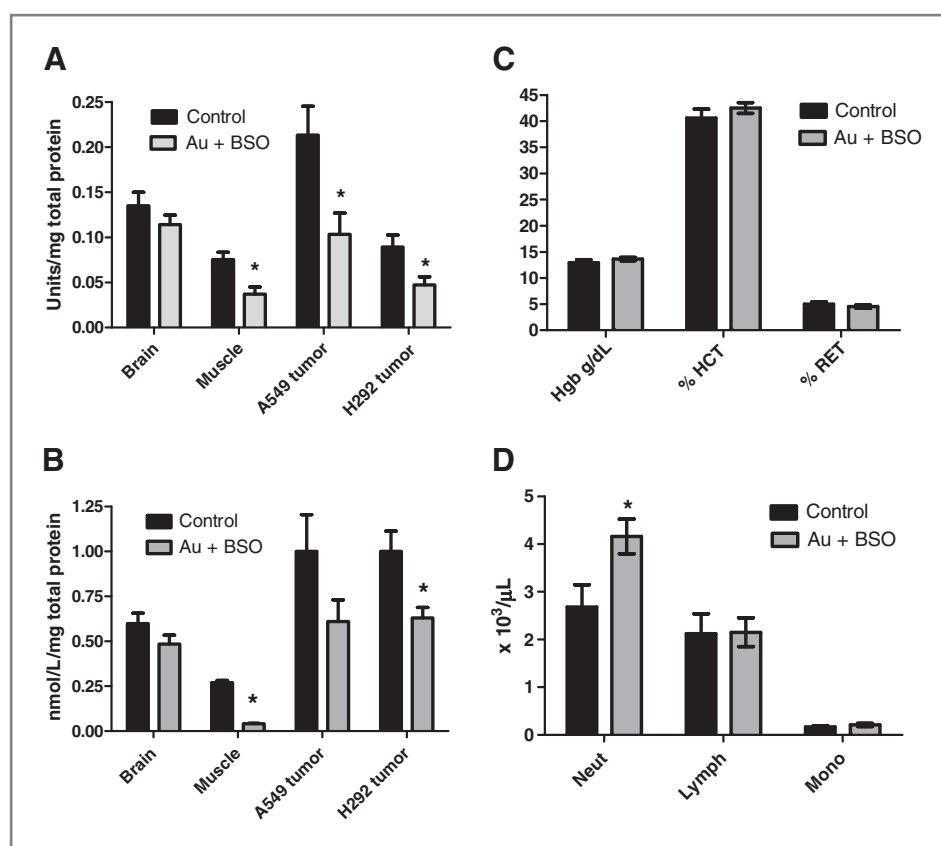


Figure 5. BSO+ auranofin (Au) is effective at decreasing total GSH and TR activity without causing myelosuppression in nude mice bearing human lung cancer xenografts. Five mice from each treatment group were implanted with either A549 or H292 lung cancer cells in the hind leg and when tumors reach approximately 3 mm in diameter, mice were treated with 1.6 mg/kg auranofin and 450 mg/kg BSO injected intraperitoneally every other day for 2 weeks. Brain, thigh muscle, and xenograft tumors were harvested 2 hours after the final injection and total GSH (B) and TR (A) activity analysis was conducted 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, and D) per the manufacturer's instructions. Errors represent ± 1 SEM. Student's *t* test resulted in *, $P < 0.05$. Hgb, hemoglobin; HCT, hematocrit; RET, reticulocyte; Neut, neutrophil; Mono, monocyte.

BSO) with carboplatin can enhance tumor growth inhibition and parameters of oxidative stress *in vivo* without causing overt signs of morbidity and mortality.

Discussion

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 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 2 molecules of NADPH from 2 molecules of NADP⁺ and ribose-5-phosphate for the synthesis of nucleotides. NADPH acts as the ultimate cofactor providing reducing equivalents for all the GSH-dependent peroxidase pathways and all the

Trx-dependent peroxidase pathways. GSH- and Trx-dependent pathways are 2 of the major pathways used by many cancer cells to regulate the toxic effects of therapeutic 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 analogue that can undergo only the first enzymatic step in the pentose phosphate pathway (glucose-6-phosphate dehydrogenase) to regenerate 1 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 shown that exposure to 2DG causes cytotoxicity and chemo- and radiosensitization 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 show that 2DG is capable of enhancing carboplatin- and radiation-induced toxicity in 2 human NSCLC lines (A549 and NCI-H292). This finding is also consistent with a previous finding that 2DG could

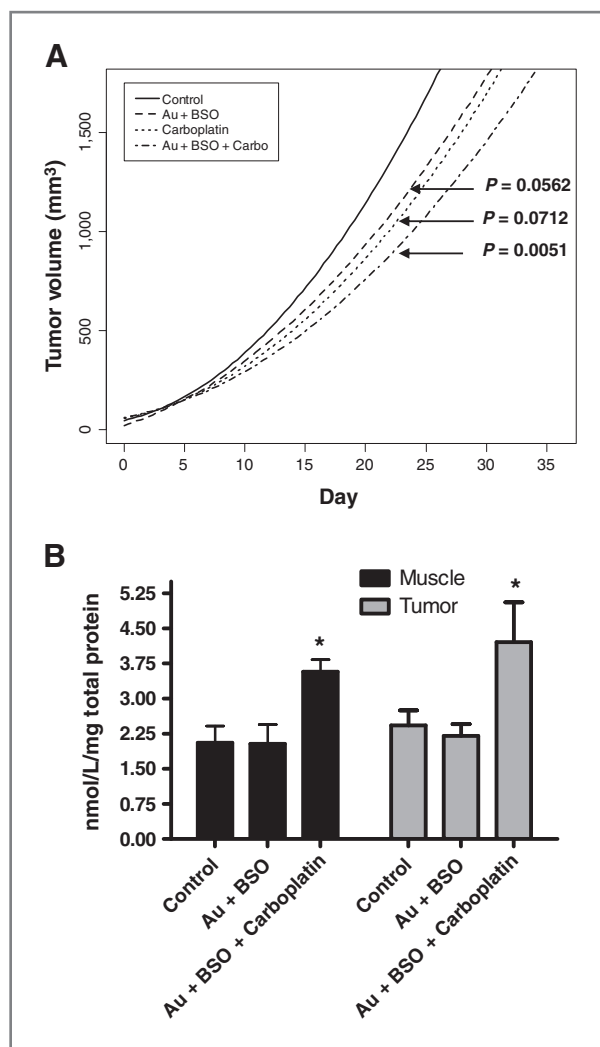


Figure 6. The combination of carboplatin (Carbo) + auranofin (Au) + BSO inhibits A549 tumor growth and results in an increase in protein carbonyls. Mice (6–7 per group) were injected with 5 million A549 tumor cells in the right flank. When tumors reached approximately 3 to 4 mm in diameter (day 1), mice were given carboplatin (15 mg/kg), BSO (450 mg/kg), and/or auranofin (1.6 mg/kg) simultaneously intraperitoneally every other day for 2 weeks. Control mice received 0.1 mL Cremaphor EL (auranofin vehicle) 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 auranofin + BSO + carboplatin ($P = 0.0051$). Marginal but nonsignificant differences were observed between control and auranofin + BSO ($P = 0.0562$) as well as carboplatin alone ($P = 0.0712$). At the end of treatment, tumors and thigh muscles from the opposite legs of 3 or 4 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 *, $P < 0.05$.

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 + carboplatin, supporting the

hypothesis that 2DG + carboplatin cytotoxicity is mediated by thiol-dependent oxidative stress in human lung cancer cells (Fig. 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 cofactor for all GSH 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 rely on NADPH as a cofactor for the redox regulation of cysteine-disulfide exchange reactions necessary for redox signaling (44). It has also previously been shown 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 showing the redundancy of Trx- and GSH-dependent protective functions.

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 radiochemotherapies. When GSH was depleted more than 80% by BSO or TR activity was inhibited more than 90% by auranofin, only minimal clonogenic cell killing or chemosensitization by carboplatin was noted in A549 or H292 human lung cancer cells. In contrast, auranofin + BSO is highly effective at inducing both thiol-mediated oxidative stress and cell killing and chemosensitization in lung cancer cells both *in vitro* and *in vivo*.

There is much evidence that Trx and GSH pathways are upregulated in NSCLC and upregulation 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 nontoxic and result in depletion of tumor GSH as well as show the potential for some therapeutic benefit (24, 47, 48). As early as 1981, it was shown that auranofin prolonged the life span of mice inoculated with lymphocytic leukemia (49). A more recent study has shown that auranofin was effective at decreasing viability of cisplatin-resistant ovarian cancer cells *in vitro* (28); however, several other studies have failed to show that auranofin possesses anticancer effects *in vivo* (50). In the current report, we show, for the first time, that auranofin + BSO can be given safely and effectively in combination with carboplatin *in vivo* to improve efficacy of radiochemotherapy 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 chemosensitizing human lung cancer cells that is worthy of further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. *CA Cancer J Clin* 2005;55:10–30.
- Raz DJ, Zell JA, Ou SH, Gandara DR, Anton-Culver H, Jablons DM. Natural history of stage I non-small cell lung cancer: implication for early detection. *Chest* 2007;132:193–9.
- Jett JR, Schild SE, Keith RL, Kesler KA. Treatment of non-small cell lung cancer, stage IIIB: ACCP evidence-based clinical practice guidelines (2nd edition). *Chest* 2007;132 Suppl:266S–76S.
- Rabik CA, Dolan ME. Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat Rev* 2007;33:9–23.
- Roudier E, Bachelet C, Perrin A. Pyruvate reduces DNA damage during hypoxia and after reoxygenation in hepatocellular carcinoma cells. *FEBS J* 2007;274:5188–98.
- Wang X, Perez E, Liu R, Yan LJ, Mallet RT, Yang SH. Pyruvate protects mitochondria from oxidative stress in human neuroblastoma SK-N-SH cells. *Brain Res* 2007;1132:1–9.
- Lord-Fontaine S, Averill-Bates DA. Heat shock inactivates cellular antioxidant defenses against hydrogen peroxide: protection by glucose. *Free Radic Biol Med* 2002;32:752–65.
- Aw TY, Rhoads CA. Glucose regulation of hydroperoxide metabolism in rat intestinal cells. Stimulation of reduced nicotinamide adenine dinucleotide phosphate supply. *J Clin Invest* 1994;94:2426–34.
- Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 2001;30:1191–212.
- Holmgren A, Johansson C, Berndt C, Lonn ME, Hudemann C, Lillig CH. Thiol redox control via thioredoxin and glutaredoxin systems. *Biochem Soc Trans* 2005;33:1375–7.
- Ceccarelli J, Delfino L, Zappia E, Castellani P, Borghi M, Ferrini S, et al. The redox state of the lung cancer microenvironment depends on the levels of thioredoxin expressed by tumor cells and affects tumor progression and response to prooxidants. *Int J Cancer* 2008;123:1770–8.
- Perquin M, Oster T, Maul A, Froment N, Untereiner M, Bagrel D. The glutathione-related detoxification system is increased in human breast cancer in correlation with clinical and histopathological features. *J Cancer Res Clin Oncol* 2001;127:368–74.
- Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc Natl Acad Sci U S A* 1992;89:3070–4.
- Arner ES, Nakamura H, Sasada T, Yodoi J, Holmgren A, Spyrou G. Analysis of the inhibition of mammalian thioredoxin, thioredoxin reductase, and glutaredoxin by *cis*-diamminedichloroplatinum(II) and its major metabolite, the glutathione-platinum complex. *Free Radic Biol Med* 2001;31:1170–8.
- Meijer C, Mulder NH, Timmer-Bosscha H, Sluiter WJ, Meersma GJ, de Vries EG. Relationship of cellular glutathione to the cytotoxicity and resistance of seven platinum compounds. *Cancer Res* 1992;52:6885–9.
- Sasada T, Nakamura H, Ueda S, Sato N, Kitaoka Y, Gon Y, et al. Possible involvement of thioredoxin reductase as well as thioredoxin in cellular sensitivity to *cis*-diamminedichloroplatinum(II). *Free Radic Biol Med* 1999;27:504–14.
- Spitz DR, Phillips JW, Adams DT, Sherman CM, Deen DF, Li GC. Cellular resistance to oxidative stress is accompanied by resistance to cisplatin: the significance of increased catalase activity and total glutathione in hydrogen peroxide-resistant fibroblasts. *J Cell Physiol* 1993;156:72–9.
- Aykin-Burns N, Ahmad IM, Zhu Y, Oberley LW, Spitz DR. Increased levels of superoxide and H₂O₂ mediate the differential susceptibility of cancer cells versus normal cells to glucose deprivation. *Biochem J* 2009;418:29–37.
- Coleman MC, Asbury CR, Daniels D, Du J, Aykin-Burns N, Smith BJ, et al. 2-deoxy-d-glucose causes cytotoxicity, oxidative stress, and radiosensitization in pancreatic cancer. *Free Radic Biol Med* 2008;44:322–31.
- Fath MA, Diers AR, Aykin-Burns N, Simons AL, Hua L, Spitz DR. Mitochondrial electron transport chain blockers enhance 2-deoxy-d-glucose induced oxidative stress and cell killing in human colon carcinoma cells. *Cancer Biol Ther* 2009;8:1228–36.
- Simons AL, Ahmad IM, Mattson DM, Dornfeld KJ, Spitz DR. 2-Deoxy-d-glucose combined with cisplatin enhances cytotoxicity via metabolic oxidative stress in human head and neck cancer cells. *Cancer Res* 2007;67:3364–70.
- Maschek G, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF, et al. 2-deoxy-d-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers *in vivo*. *Cancer Res* 2004;64:31–4.
- Bailey HH. L-S,R-buthionine sulfoximine: historical development and clinical issues. *Chem Biol Interact* 1998;111–112:239–54.
- Anderson CP, Seeger RC, Satake N, Monforte-Munoz HL, Keshe-lava N, Bailey HH, et al. Buthionine sulfoximine and myeloablative concentrations of melphalan overcome resistance in a melphalan-resistant neuroblastoma cell line. *J Pediatr Hematol Oncol* 2001;23:500–5.
- Rigobello MP, Scutari G, Folda A, Bindoli A. Mitochondrial thioredoxin reductase inhibition by gold(I) compounds and concurrent stimulation of permeability transition and release of cytochrome c. *Biochem Pharmacol* 2004;67:689–96.
- Rigobello MP, Folda A, Baldoiu MC, Scutari G, Bindoli A. Effect of auranofin on the mitochondrial generation of hydrogen peroxide. Role of thioredoxin reductase. *Free Radic Res* 2005;39:687–95.
- Omata Y, Folan M, Shaw M, Messer RL, Lockwood PE, Hobbs D, et al. Sublethal concentrations of diverse gold compounds inhibit mammalian cytosolic thioredoxin reductase (TrxR1). *Toxicol In Vitro* 2006;20:882–90.
- Marzano C, Gandin V, Folda A, Scutari G, Bindoli A, Rigobello MP. Inhibition of thioredoxin reductase by auranofin induces apoptosis in cisplatin-resistant human ovarian cancer cells. *Free Radic Biol Med* 2007;42:872–81.
- Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:502–22.
- Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 1980;106:207–12.
- Holmgren A, Fagerstedt M. The *in vivo* distribution of oxidized and reduced thioredoxin in *Escherichia coli*. *J Biol Chem* 1982;257:6926–30.
- Watson WH, Pohl J, Montfort WR, Stuchlik O, Reed MS, Powis G, et al. Redox potential of human thioredoxin 1 and identification of a second dithiol/disulfide motif. *J Biol Chem* 2003;278:33408–15.

33. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
34. Mohanti BK, Rath GK, Anantha N, Kannan V, Das BS, Chandramouli BA, et al. Improving cancer radiotherapy with 2-deoxy-d-glucose: phase I/II clinical trials on human cerebral gliomas. *Int J Radiat Oncol Biol Phys* 1996;35:103–11.
35. Mattson DM, Ahmad IM, Dayal D, Parsons AD, Aykin-Burns N, Li L, et al. Cisplatin combined with zidovudine enhances cytotoxicity and oxidative stress in human head and neck cancer cells via a thiol-dependent mechanism. *Free Radic Biol Med* 2009;46:232–7.
36. Hadzic T, Aykin-Burns N, Zhu Y, Coleman MC, Leick K, Jacobson GM, et al. Paclitaxel combined with inhibitors of glucose and hydroperoxide metabolism enhances breast cancer cell killing via H₂O₂-mediated oxidative stress. *Free Radic Biol Med* 2010;48:1024–33.
37. Liu J, Akahoshi T, Namai R, Matsui T, Kondo H. Effect of auranofin, an antirheumatic drug, on neutrophil apoptosis. *Inflamm Res* 2000;49:445–51.
38. Rudin CM, Yang Z, Schumaker LM, VanderWeele DJ, Newkirk K, Egorin MJ, et al. Inhibition of glutathione synthesis reverses Bcl-2-mediated cisplatin resistance. *Cancer Res* 2003;63:312–8.
39. Boros LG, Torday JS, Lim S, Bassilian S, Cascante M, Lee WN. Transforming growth factor beta2 promotes glucose carbon incorporation into nucleic acid ribose through the nonoxidative pentose cycle in lung epithelial carcinoma cells. *Cancer Res* 2000;60:1183–5.
40. Ahmad IM, Aykin-Burns N, Sim JE, Walsh SA, Higashikubo R, Buettner GR, et al. Mitochondrial O₂⁻ and H₂O₂ mediate glucose deprivation-induced stress in human cancer cells. *J Biol Chem* 2005;280:4254–63.
41. Vijayaraghavan R, Kumar D, Dube SN, Singh R, Pandey KS, Bag BC, et al. Acute toxicity and cardio-respiratory effects of 2-deoxy-d-glucose: a promising radio sensitiser. *Biomed Environ Sci* 2006;19:96–103.
42. Lin X, Zhang F, Bradbury CM, Kaushal A, Li L, Spitz DR, et al. 2-Deoxy-d-glucose-induced cytotoxicity and radiosensitization in tumor cells is mediated via disruptions in thiol metabolism. *Cancer Res* 2003;63:3413–7.
43. Sinthupibulyakit C, Grimes KR, Domann FE, Xu Y, Fang F, Ittarat W, et al. p53 is an important factor for the radiosensitization effect of 2-deoxy-d-glucose. *Int J Oncol* 2009;35:609–15.
44. Song JJ, Rhee JG, Suntharalingam M, Walsh SA, Spitz DR, Lee YJ. Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H₂O₂. *J Biol Chem* 2002;277:46566–75.
45. Conrad M, Jakupoglu C, Moreno SG, Lippl S, Banjac A, Schneider M, et al. Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function. *Mol Cell Biol* 2004;24:9414–23.
46. Owusu-Ansah E, Yavari A, Mandal S, Banerjee U. Distinct mitochondrial retrograde signals control the G₁-S cell cycle checkpoint. *Nat Genet* 2008;40:356–61.
47. Bailey HH, Ripple G, Tutsch KD, Arzoomanian RZ, Alberti D, Feierabend C, et al. Phase I study of continuous-infusion I-S,R-buthionine sulfoximine with intravenous melphalan. *J Natl Cancer Inst* 1997;89:1789–96.
48. O'Dwyer PJ, Hamilton TC, LaCreta FP, Gallo JM, Kilpatrick D, Halbherr T, et al. Phase I trial of buthionine sulfoximine in combination with melphalan in patients with cancer. *J Clin Oncol* 1996;14:249–56.
49. Simon TM, Kunishima DH, Vibert GJ, Lorber A. Screening trial with the coordinated gold compound auranofin using mouse lymphocyte leukemia P388. *Cancer Res* 1981;41:94–7.
50. Mirabelli CK, Johnson RK, Sung CM, Faucette L, Muirhead K, Crooke ST. Evaluation of the *in vivo* antitumor activity and *in vitro* cytotoxic properties of auranofin, a coordinated gold compound, in murine tumor models. *Cancer Res* 1985;45:32–9.