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

Melissa A. Fath¹, Iman M. Ahmad², Carmen J. Smith¹, Jacquelyn Spence¹, and Douglas R. Spitz¹

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.
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). Auronafin 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 auronafin 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 auronafin 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 auronafin 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 + auronafin, supporting the hypothesis that thiol-mediated oxidative stress significantly contributes to outcomes via these drug combinations. The observation that doses of auronafin 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 chemosenstitization. Finally, the results showing that the combination of auronafin + 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 (MediTech 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 auronafin (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 irradiation. In select experiments, cells were treated with 100 μmol/L BSO for 24 hours and/or 0.5 to 5 μmol/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 × 10⁴ A549 or H292 cells were plated in 100-mm dishes, allowed to grow for 24 hours and then treated with 50 to 1,000 μmol/L BSO, 20 mmol/L D2G, 5 μmol/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 μmol/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°C 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 (≈20 μg) 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 chemiluminescent detection (SuperSignal 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 μmol/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 × 10⁶ A549 cells or 3 × 10⁶ H292 cells into the right flank, using 25-gauge needles. When tumor volumes measured approximately 100 mm³, 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 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 × 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 µL 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 resuspended 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 µmol/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 µL of 35 µg/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 chemotherapy agent, carboplatin, in A549 human lung cancer cells. Cells were treated with 20 mmol/L 2DG, 5 µmol/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 µmol/L. Treating with the combination of 2DG and 2 µmol/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 µmol/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 lung 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 thioredoxin homeostasis leading to oxidative stress. Treatment of A549 cells and H292 cells with the combination of 20 mmol/L 2DG and 5 µmol/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 thioredoxin 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 µmol/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.

NCSLCs 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
cells were treated with 20 mmol/L 2DG and 2 Gy IR and assayed for clonogenic survival. Similarly, A549 (B) and H292 (C) (Ptx), and/or 5 µg growing A549 cells were treated with 20 mmol/L 2DG, 1 nmol/L paclitaxel (Ptx), and then either 5 µmol/L carboplatin 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 total GSH levels were determined (Fig. 3D). 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). 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

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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 mmol/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 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). Error bars 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.
presence and absence of BSO and/or 0.5 mmol/L 2DG carboplatin, A549 lung cancer cells were treated with 20 
metabolism could further enhance the toxicity of 2DG cytotoxicity in 2DG auranofin 
Inhibition of hydroperoxide metabolism with 
necessitate simultaneous inhibition of both pathways to 
neccessitate 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 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.

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.

The combination of auranofin and BSO was safely and effectively administered to nude mice
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
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 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 μmol/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. 6B; Supplementary Table S2 and Fig. S1). When protein carbonyl content of the tumors compared with control (Fig. 6A; Supplementary Table S2 and Fig. S1). When protein carbonyl content of the tumors compared with control, carboplatin inhibits A549 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 significantly lower tumor growth rate when compared with control (Fig. 6B; Supplementary Table S2 and Fig. S1). When protein carbonyl content of the tumors compared with control, carboplatin inhibits A549 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 significantly lower tumor growth rate when compared with control (Fig. 6B; Supplementary Table S2 and Fig. S1). When protein carbonyl content of the tumors compared with control, carboplatin inhibits A549 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 significantly lower tumor growth rate when compared with control (Fig. 6B; Supplementary Table S2 and Fig. S1). When protein carbonyl content of the tumors compared with control, carboplatin inhibits A549 tumor growth when compared with control mice (Fig. 6A; Supplementary Table S2 and Fig. S1).
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 μmol/L carboplatin for 24 hours with or without treatment with 100 μmol/L BSO for 24 hours and 0.5 μmol/L auranofin for the last 3 hours followed by clonogenic survival analysis with the data being normalized to control. A, errors represent ±SEM of at least 3 separate experiments. One-way ANOVA with Tukey’s post hoc analysis resulted in *, P < 0.001 versus 2DG + carboplatin. Exponentially growing A549 lung cancer cells were treated with 20 mmol/L 2DG, 5 μmol/L carboplatin, 100 μmol/L BSO, and/or 15 mmol/L NAC for 24 hours with 5 μmol/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 ±SEM of at least 3 treatments dishes. One-way ANOVA with Tukey’s post hoc analysis resulted in *, 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.
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 fact, glucose deprivation has been shown to result in increases in steady-state levels of H$_2$O$_2$ 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 chemoo- 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 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 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.
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 cystene-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 1 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.

Acknowledgments

The authors thank Drs. Sudersan Bhatia and Walter Watson for helpful discussions. The authors also thank Amanda Kalen and Katie Leick for technical assistance.

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Grant Support

This study was supported by NIH grants R01CA133114, R21CA139182, UL1RR024979, T32CA078586, and P30CA086862.

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Received March 25, 2011; revised July 8, 2011; accepted July 30, 2011; published OnlineFirst August 15, 2011.
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