

Inhibition of Glutamate Cysteine Ligase Activity Sensitizes Human Breast Cancer Cells to the Toxicity of 2-Deoxy-D-Glucose

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Abstract

It has been hypothesized that cancer cells increase glucose metabolism to protect against metabolic fluxes of hydroperoxides via glutathione-dependent peroxidases. 2-Deoxy-D-glucose, inhibits glucose metabolism and has been shown to cause cytotoxicity in cancer cells that is partially mediated by disruptions in thiol metabolism. In the current study, human breast cancer cells were continuously treated (24 hours) with 2-deoxy-D-glucose, and total glutathione content as well as the expression of the first enzyme in the glutathione synthetic pathway [glutamate cysteine ligase (GCL)] were found to be induced 2.0-fold. Inhibiting GCL activity during 2-deoxy-D-glucose exposure using L-buthionine-[S,R]-sulfoximine (BSO) significantly enhanced the cytotoxic effects of 2-deoxy-D-glucose and caused increases in endpoints indicative of oxidative stress, including % oxidized glutathione and steady-state levels of pro-oxidants as assayed using an oxidation-sensitive fluorescent probe. These results show that treatment of human breast cancer cells with 2-deoxy-D-glucose causes metabolic oxidative stress that is accompanied by increases in steady-state levels of GCL mRNA, GCL activity, and glutathione content. Furthermore, inhibition of 2-deoxy-D-glucose-mediated induction of GCL activity with BSO increases endpoints indicative of oxidative stress and sensitizes cancer cells to 2-deoxy-D-glucose-induced cytotoxicity. These results support the hypothesis that drug combinations capable of inhibiting both glucose and hydroperoxide metabolism may provide an effective biochemical strategy for sensitizing human cancer cells to metabolic oxidative stress. (Cancer Res 2006; 66(3): 1605-10)

Introduction

Glucose metabolism has been suggested to be an integral component of the metabolic detoxification pathways, which protect tumor cells from H_2O_2 (1–4). The increased detoxification of H_2O_2 is believed to be facilitated by glucose through increases in the capacity to regenerate NADPH from $NADP^+$ via the pentose phosphate pathway as well as increases in pyruvate production through glycolysis (1–7). NADPH produced from the pentose phosphate pathway serves as a source of electrons for H_2O_2 detoxification through the glutathione peroxidase/glutathione reductase and thioredoxin peroxidase/thioredoxin reductase sys-

tems (1–8). Pyruvate can interact directly to scavenge hydroperoxides through deacetylation reactions (7). It has been suggested that tumor cells produce more H_2O_2 than their normal cell counterparts because of defects in mitochondrial oxidative energy metabolism (1–4). Therefore, it can be hypothesized that increased steady-state levels of metabolically derived hydroperoxides inside tumor cells would cause an increased demand for glucose metabolism directed towards peroxide detoxification.

Although it is possible to completely deprive cells of glucose in culture, this is not practical *in vivo*. It is possible to treat tumor-bearing animals with 2-deoxy-D-glucose, a clinically relevant drug that competes with glucose for entry into glucose metabolic pathways (9). This competition would be expected to cause inhibition of glucose metabolism, thereby creating a drug-induced state of glucose deprivation. Although there are reports that 2-deoxy-D-glucose can proceed through the first reaction in the pentose phosphate pathway (glucose 6-phosphate dehydrogenase), leading to the regeneration of one NADPH (10), 2-deoxy-D-glucose is incapable of further metabolism in the pentose phosphate pathway and cannot be metabolized to pyruvate (3). 2-Deoxy-D-glucose administered in animals has been shown to be an effective way to inhibit glucose metabolism without causing toxicity until very high levels are achieved (>2 g/kg body weight; ref. 9). 2-Deoxy-D-glucose administered in humans has also been shown to be well tolerated up to a level of 200 mg/kg (11, 12), which results in blood levels of 5 to 6 mmol/L at 1 hour after treatment (12). Therefore, using 2-deoxy-D-glucose as a partial inhibitor of glucose metabolism may provide an effective addition to combined modality therapies designed to limit hydroperoxide metabolism and increase metabolic oxidative stress in cancer cells.

Reduced glutathione (GSH) acts as a free radical scavenger, a reducing cofactor, and is essential for all glutathione peroxidase enzymes that act to regulate H_2O_2 toxicity (13, 14). Total GSH [GSH + glutathione disulfide (GSSG)] represents the most abundant small molecular weight thiol pool in cells and as such steady-state levels of % $GSSG_{in\ GSH\ equivalents}$ can be used as an endpoint indicative of oxidative stress (1–4, 13). Increases in steady-state levels of GSH and the rate-limiting enzyme in GSH synthesis [glutamate cysteine ligase (GCL)] are thought to represent a protective cellular response to glucose deprivation-induced metabolic oxidative stress (1–4), but these observations have not been rigorously extended to studies of 2-deoxy-D-glucose-induced cytotoxicity.

L-Buthionine-[S,R]-sulfoximine (BSO) is a drug being tested in clinical trials for cancer therapy that reversibly inhibits GCL activity, thereby inhibiting GSH synthesis resulting in GSH depletion and chemosensitization (13–17). It has been shown that levels up to 1 mmol/L BSO can be measured in the blood at 2 hours after i.v. injection (16). Because GCL is the rate-limiting enzyme in GSH synthesis (13–17), we hypothesized that GCL activity would be up-regulated by human breast cancer cells

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treated with 2-deoxy-D-glucose in an attempt to compensate for increased 2-deoxy-D-glucose-induced metabolic oxidative stress. Furthermore, if GCL activity protected against 2-deoxy-D-glucose-induced metabolic oxidative stress, it would be predicted that combining BSO treatment with 2-deoxy-D-glucose should increase 2-deoxy-D-glucose-induced cytotoxicity as well as endpoints indicative of metabolic oxidative stress in MDA-MB231 human breast carcinoma cells.

The results of the current studies showed that treatment of human breast cancer cells with 2-deoxy-D-glucose causes metabolic oxidative stress that is accompanied by increases in steady-state levels of GCL mRNA, GCL activity, and glutathione content. Furthermore, inhibition of 2-deoxy-D-glucose-mediated induction of GCL activity with BSO increased endpoints indicative of oxidative stress and sensitized cancer cells to 2-deoxy-D-glucose-induced cytotoxicity. These results support the hypothesis that combining drugs that inhibit glucose and hydroperoxide metabolism may provide an effective biochemical strategy for sensitizing human cancer cells to metabolic oxidative stress.

Materials and Methods

Cells and culture conditions. MDA-MB231 human breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cultures were maintained in 5% CO₂ and humidified in a 37°C incubator.

2-Deoxy-D-glucose and BSO treatments. For experiments, 100,000 to 300,000 MDA-MB231 cells were plated in 60-mm dishes, and 600,000 to 900,000 cells were plated in 100-mm dishes and grown for 2 days. At time 0, medium was removed, and fresh medium was added. All control dishes were sham treated with a medium change; 20 mmol/L 2-deoxy-D-glucose (Sigma, St. Louis, MO) was dissolved in medium, sterile filtered, and added to treatment dishes; 1 mmol/L BSO (Sigma) was dissolved in medium, sterile filtered, and added to appropriate treatment dishes. Cells were placed in a 37°C incubator and harvested at the time points indicated.

Cell pellet collection. Following treatment, medium was collected and centrifuged to harvest floating cells, and attached cells were scrape harvested in cold PBS and centrifuged at 4°C, the supernatant was discarded, the cell pellets were transferred to 1.5 mL tubes and frozen at -20°C until biochemical analysis was performed.

Glutathione assay. Cell pellets were thawed and homogenized in 50 mmol/L PO₄ buffer (pH 7.8) containing 1.34 mmol/L diethylenetriamine-pentaacetic acid (DETAPAC buffer). Total glutathione content was determined by the method of Anderson (18). GSH and GSSG were distinguished by addition of 2 µL of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 µL of sample followed by incubation for 2 hours and assayed as described previously (19). All glutathione determinations were normalized to the protein content of whole homogenates using the method of Lowry et al. (20).

Clonogenic cell survival experiments. Floating cells in medium from the experimental dishes were collected and combined with the attached cells from the same dish that were trypsinized with 1× trypsin-EDTA (CellGro, Herndon, VA) and inactivated with RPMI 1640 containing 10% fetal bovine serum (Hyclone). All cells from each dish were then centrifuged, resuspended in fresh medium, and counted using a Coulter counter. Cells were then plated at low density (500-5,000 per plate), and clones were allowed to grow for 14 days in complete medium, in the presence of 0.1% gentamicin. Cells were then fixed with 70% ethanol and stained with Coomassie blue for analysis of clonogenic cell survival as previously described (21).

GCL activity assay. GCL activity was determined using whole-cell homogenates in DETAPAC buffer as described previously (22). Each assay reaction contained 250 µL reaction buffer (0.1 mol/L Tris, 20 mmol/L MgCl₂, 0.75 mmol/L L-glutamic acid, 6 mmol/L ATP, 50 mmol/L KCl, 6 mmol/L DTT, 0.75 mmol/L cysteine) and 50 µL of cell homogenate. Cell pellets were thawed and homogenized in DETAPAC buffer, and protein was determined

by the method of Lowry et al. (20). Homogenate containing 300 to 400 µg of protein was added to each reaction tube with reaction buffer (final volume, 300 µL) and allowed to incubate for 0 to 20 minutes at 37°C. At each time point during the assay (0-20 minutes), 20 µL of the assay reaction was put into 230 µL Nanopure water (Barnstead-Thermolyne Corp., Dubuque, IA), and 750 µL of 0.5 mmol/L ThioGlo 3 (dissolved in acetonitrile; Covalent Technologies, Inc., Walnut Creek, CA) was added immediately to derivatize the thiols in the sample. After 10 minutes, the samples were acidified with a 1:6 dilution of 12 N HCl, to obtain a pH of 2.5. Samples were filtered through 0.45-µm nylon syringe filters and separated on a Shimadzu high-pressure liquid chromatography (HPLC) system using a mobile phase of 65% acetonitrile, 35% H₂O with 0.05% glacial acetic acid and 0.05% 85% O-phosphoric acid with a flow rate to 0.5 ml/min and a fluorescent detector with excitation/emission wavelengths of 365 and 445 nm, respectively. Data was obtained using Shimadzu Class VP software and analyzed using linear regression of data points representing the area under the peak corresponding to increasing concentrations of γ-glutamylcysteine from a standard curve that was linear from 200 to 10,000 fmol/20 µL injection. Units of GCL activity were expressed in fmol GCL produced/µg protein/min.

Pro-oxidant measurements. Steady-state levels of pro-oxidants were determined as previously described (4) using the oxidation-sensitive [5-(and-6)-carboxy-2, 7-dichlorodihydrofluorescein diacetate (DCFH), 10 µg/mL] or oxidation insensitive [5-(and-6)-carboxy-2, 7-dichlorofluorescein diacetate (DCF), 10 µg/mL] fluorescent dyes (dissolved in DMSO) obtained from Molecular Probes (Eugene, OR). The oxidized form of the dye (DCF) acts as a control for changes in uptake, ester cleavage, and efflux. Any changes in fluorescence seen between the groups with the oxidation sensitive dye are presumed to represent changes in steady-state levels of pro-oxidants if no change is seen under similar conditions with the oxidation insensitive probe. After cells received drug treatment, they were washed once with PBS and labeled with the fluorescent dyes for 15 minutes at 37°C in PBS. At the end of the incubation time, the plates were placed on ice to stop the labeling reaction. Cells then were harvested on ice using phenol red-free trypsin-EDTA. Trypsin was inactivated with phenol red-free EMEM containing 10% FBS. Cell suspensions were placed in tubes and centrifuged at 1,000 rpm for 5 minutes. After centrifugation, cells were resuspended in PBS and placed on ice and analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry System, Inc., Mountain View, CA; excitation, 488 nm; emission, 530 nm band-pass filter). Analysis was done using CellQuest software. The mean fluorescence intensity of 10,000 cells was analyzed in each sample and corrected for autofluorescence of unlabeled cells.

Real-time PCR measurements of catalytic and modifier subunits of GCL. MDA-MB231 cells (600,000) were plated in a 100-mm dish and allowed to grow for 2 days; 20 mmol/L 2-deoxy-D-glucose was added to appropriate dishes and collected at the time points indicated through scrape harvesting in PBS. Total RNA was isolated using the Qiagen RNeasy Mini kit (Valencia, CA) according to the manufacturer's suggested protocol. RNA was quantified using a BioPhotometer (Eppendorf, Hamburg, Germany), and 1 µg of RNA from each sample was reverse transcribed using the RT kit (BD Biosciences, San Jose, CA) for 2 hours at 37°C. The cDNA was subjected to real-time PCR analysis with specific primers to the catalytic subunit of GCL (GCLC; GCLCF, 5'-TGCTGTCTCCAGGTGACATTC-3'; GCLCR, 5'-CCCAGCGACAATCAATGTCT-3'), the modifier subunit of GCL (GCLM; GCLMF, 5'-TTGGAGTTGCACAGCTGGATTC-3'; GCLMR, 5'-TG-GTTTAC-CTGTGCCACTG-3'), and 18S rRNA (18SF, 5'-CCTTGGATGTGGTAGCC-GTTT-3'; 18SR, 5'-AACTTTCGATGGTAGTCGCCG-3') in conjunction with the 2× SYBR real-time master mix (Applied Biosystems, Foster City, CA). SYBR green dye is incorporated into double-stranded DNA and allows real-time monitoring of PCR product by fluorescence. The reactions were carried out by running an initial step of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A threshold of amplification in the linear range was then selected and the cycle threshold (C_t) was determined for each sample. Amplification of GCLC and GCLM were then normalized to the 18S control to account for input differences between samples. The fold difference between samples was then determined using the ΔΔC_t method. The average and SD of each sample were then calculated.

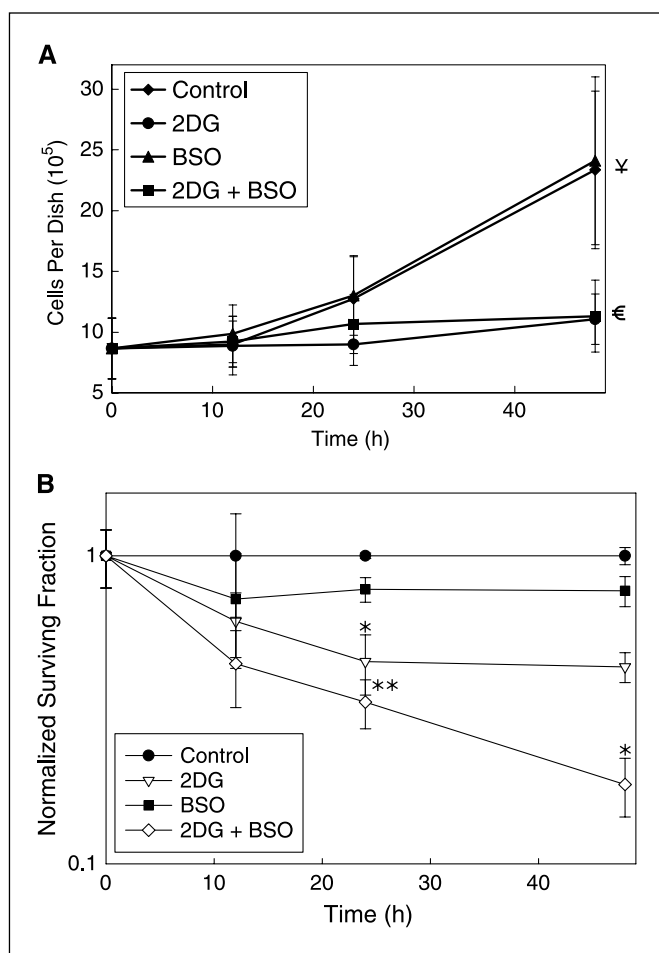


Figure 1. A, MDA-MB231 cell growth is decreased following treatment with 2-deoxy-D-glucose (2DG) and BSO. The cells treated with 20 mmol/L 2-deoxy-D-glucose and the combination of 20 mmol/L 2-deoxy-D-glucose + 1 mmol/L BSO showed a significant growth delay. Cells treated with 1 mmol/L BSO show no differences in growth compared with the control cells. There are significant differences in the growth rates of the cells treated with 2-deoxy-D-glucose and 2-deoxy-D-glucose + BSO and the BSO and control cells. ¥, $P < 0.05$ versus 2-deoxy-D-glucose; €, $P < 0.05$ versus BSO. Points, average; bars, $1 \pm$ SD. Represent three independent experiments, where at least three cell counts were averaged from each treatment dish at each time point. B, decreases in clonogenic survival caused by exposure to 2-deoxy-D-glucose and BSO in human breast carcinoma cells. Cells treated with 2-deoxy-D-glucose showed a decrease in clonogenic survival over time that was significantly different from control cells. Cells treated with the combination of 2-deoxy-D-glucose and BSO showed an even greater decrease in clonogenic survival over time, which was also significantly different from control cells. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control. Points, mean; bars, ± 1 SE. Represent among three and six cloning dishes taken from one treated dish per group in three independent experiments.

Statistical analysis. Statistical analysis was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).¹ To determine differences among three or more means, one-way ANOVA with Bonferroni posttest was done. Two-way ANOVA with Bonferroni posttest was done to determine differences over various time points and treatment groups. Error bars represent ± 1 SD unless otherwise specified.

Results

In humans treated with 200 mg/kg 2-deoxy-D-glucose, circulating levels of 5 to 6 mmol/L 2-deoxy-D-glucose are achievable in

the context of blood levels of 8 mmol/L glucose (12). The medium (RPMI) used to maintain MDA-MB231 human breast carcinoma cells contains 11 mmol/L glucose. Therefore, for the current study using MDA-MB231 cells, 20 mmol/L 2-deoxy-D-glucose was chosen to ensure that glucose metabolism was maximally inhibited. In addition, human blood levels of 1 mmol/L BSO are clinically achievable 2 hours after infusion; thus, this concentration was chosen for cell experiments (16). MDA-MB231 cells treated with 20 mmol/L 2-deoxy-D-glucose had a delay in cell growth over the 48-hour period (Fig. 1A). Cells treated with 1 mmol/L BSO showed no difference in the growth patterns and were comparable in growth with the control cells (Fig. 1A). Using the combination of 20 mmol/L 2-deoxy-D-glucose and 1 mmol/L BSO also caused inhibition of growth that was similar to 2-deoxy-D-glucose alone (Fig. 1A). These differences in growth inhibition were significant at the 48-hour time point when comparing 2-deoxy-D-glucose versus BSO and 2-deoxy-D-glucose + BSO versus BSO.

Figure 1B shows the results of clonogenic survival assays following treatment with 20 mmol/L 2-deoxy-D-glucose for 0 to 48 hours. 2-Deoxy-D-glucose caused a significant decrease in cell survival compared with cells treated with BSO and control cells. Cells treated with BSO showed a slight but not significant decrease in cell survival over the time course of the experiment compared with control cells (Fig. 1B). The cells treated with the combination of 2-deoxy-D-glucose and BSO showed significant cell killing over the time course of the experiment and a significantly greater proportion of the cells were clonogenically inactivated by the combined treatment at both 24 and 48 hours, relative to either agent alone (Fig. 1B). Given that more of the cells remained attached to the culture plates at 24 hours, relative to 48 hours, the 24-hour time point was chosen for subsequent experiments to elucidate biochemical mechanisms causing toxicity and GCL induction.

Figure 2 shows the combined data from 10 experiments where 20 mmol/L 2-deoxy-D-glucose for 24 hours caused 25% cell killing as detected with clonogenic cell survival assays, relative to untreated control cells. Treatment for 24 hours with the inhibitor of GCL activity (BSO) caused a slight but not significant decrease in survival (12% cell killing) compared with control. BSO + 2-deoxy-D-glucose

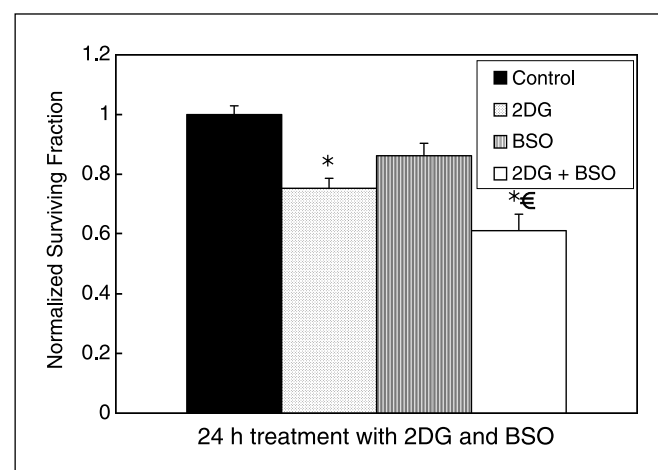


Figure 2. Treatment of MDA-MB231 with 20 mmol/L 2-deoxy-D-glucose (2DG) and 1 mmol/L BSO caused clonogenic cell killing at 24 hours. Cells treated with 2-deoxy-D-glucose show a decrease in survival of 25%, whereas cells treated with 2-deoxy-D-glucose and BSO showed decreased survival to 39%, relative to control. BSO-treated cells showed a slight but not significant decrease in survival compared with control ($n = 10-11$). *, $P < 0.05$ versus control; €, $P < 0.05$ versus BSO. Columns, mean; bars, ± 1 SE.

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¹ <http://www.graphpad.com>.

Table 1. Measurements of total GSH and GSSG and calculations of % GSSG in MDA-MB231 cells treated for 24 hours with 2-deoxy-D-glucose and BSO

	Control	2-Deoxy-D-glucose	BSO	2-Deoxy-D-glucose + BSO
Total GSH	20.40 ± 2.54	34.74 ± 2.63*	1.41 ± 0.40* [†]	0.99 ± 0.26* [†]
GSSG	0.84 ± 0.27	1.86 ± 0.32*	0.14 ± 0.06 [†]	0.14 ± 0.05 [†]
% GSSG	4.00 ± 0.87	5.94 ± 1.15	17.79 ± 6.30	21.22 ± 5.73* [†]

**P* < 0.05 versus control.†*P* < 0.05 versus 2-deoxy-D-glucose; errors represent ± 1 SD.

for 24 hours caused a significant increase in clonogenic death (to 39% cell killing; Fig. 2) compared with BSO alone. These results combined with the results in Fig. 1 show that inhibition of GCL activity with BSO significantly enhances the cytotoxicity seen with continuous exposure to 2-deoxy-D-glucose in human breast carcinoma cells.

To determine if oxidative stress could be contributing to the toxic effects of 2-deoxy-D-glucose and BSO, the GSH/GSSG redox couple was evaluated in cell homogenates, and the % total GSH that was oxidized was used to calculate % GSSG. Because GSH represents the major small molecular weight thiol redox buffer in cells, increases in % GSSG are believed to signify a shift towards a more highly oxidizing intracellular environment indicative of oxidative stress. In these experiments, MDA-MB231 cells were treated for 24 hours with 20 mmol/L 2-deoxy-D-glucose and/or 1 mmol/L BSO. Treatment with 2-deoxy-D-glucose caused a significant 1.8-fold increase in total GSH, relative to untreated control, whereas BSO caused significant GSH depletion (>90%) as well as inhibiting the 2-deoxy-D-glucose-induced increases in total GSH (Table 1). These results suggest that, similar to glucose deprivation (1–4), 2-deoxy-D-glucose treatment induces the synthesis and accumulation of total GSH, and this can be inhibited using BSO.

Because the recycling assay used to detect total GSH recognizes both the reduced form (GSH) and the oxidized form (GSSG) of glutathione in the cell homogenates, 2-vinylpyridine was used to conjugate GSH before running the assay to allow for the measurement of GSSG. Cells treated with 2-deoxy-D-glucose showed a 2-fold increase in GSSG compared with the untreated control cells (Table 1), indicating that 2-deoxy-D-glucose treatment was inducing a condition of oxidative stress. BSO treated cells and the combination of 2-deoxy-D-glucose- and BSO-treated cells showed significant decreases in GSSG content (Table 1). However, cells treated with BSO as well as BSO + 2-deoxy-D-glucose showed a 3- to 4-fold increase in % GSSG, indicating an increase in oxidative stress, relative to control (Table 1). Interestingly, at this time point, 2-deoxy-D-glucose alone did not cause an increase in % GSSG, relative to control. These results (Table 1) are consistent with the hypothesis that 2-deoxy-D-glucose was inducing oxidative stress that was resulting in the increased synthesis of GSH in an attempt to maintain the redox buffering capacity of the intracellular reducing environment. However, when 2-deoxy-D-glucose and BSO were combined, the results indicate that inhibition of the 2-deoxy-D-glucose-induced synthesis of GSH resulted in increased metabolic oxidative stress as measured by % GSSG.

To obtain further evidence supporting this hypothesis, steady-state levels of intracellular pro-oxidants were assayed by measuring the oxidation of an oxidation sensitive fluorescent probe (DCFH). In these experiments, MDA-MB231 cells were treated with 20

mmol/L 2-deoxy-D-glucose and/or 1 mmol/L BSO for 24 hours. Cells were labeled with DCFH for flow cytometric analysis as described in Materials and Methods. Exposure of cells to 2-deoxy-D-glucose or BSO for 24 hours caused a 1.5- to 2-fold increase in the probe oxidation during the labeling period (Fig. 3A). Furthermore,

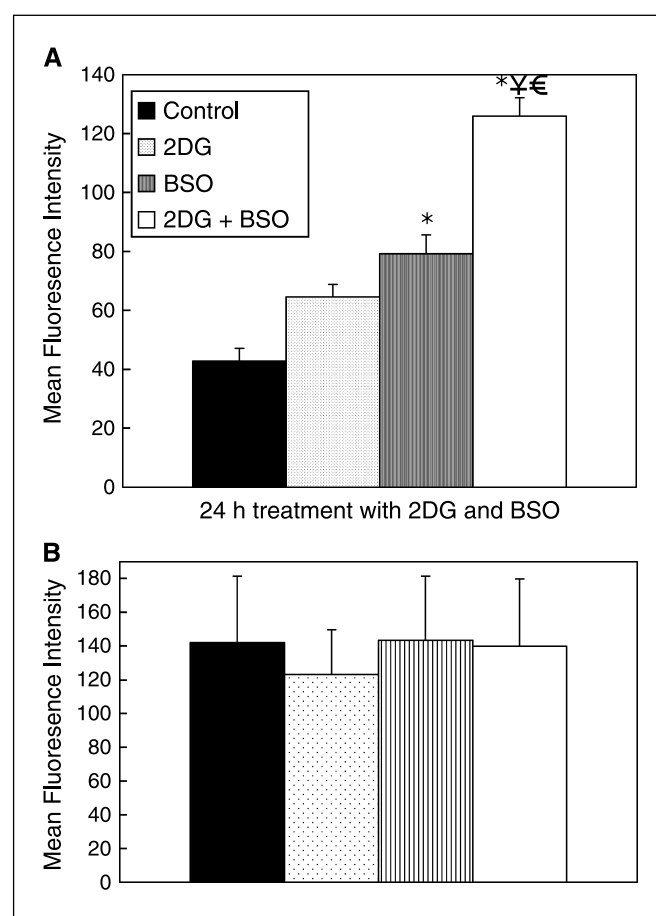


Figure 3. A, MDA-MB231 cells treated with 20 mmol/L 2-deoxy-D-glucose (2DG) and 1 mmol/L BSO show an increase in steady-state levels of pro-oxidants as measured via DCFH oxidation. Cells treated with 2-deoxy-D-glucose showed a slight increase in oxidation of the probe, whereas treatment with BSO showed a significant 2-fold increase in probe oxidation. The combination of 2-deoxy-D-glucose + BSO caused a significant 3-fold increase in probe oxidation (*n* = 6). *, *P* < 0.05 versus control; †, *P* < 0.05 versus 2-deoxy-D-glucose; ‡, *P* < 0.05 versus BSO. Bars, ± 1 SD. B, breast carcinoma cells treated over 24 hours with 2-deoxy-D-glucose and BSO showed no change in fluorescence when labeled with the oxidation insensitive probe. Samples showed no statistical differences between treatment groups (*n* = 3–4). Bars, ± 1 SD.

a significant 3-fold increase in probe oxidation was noted in cells treated with the combination of 2-deoxy-D-glucose and BSO, relative to control cells. To confirm that we were truly measuring changes in DCFH oxidation and not changes in DCFH uptake, efflux, or ester cleavage, the experiments shown in Fig. 3A were repeated using the oxidation-insensitive analogue of DCFH (DCF). Figure 3B shows the results of the same experiments done with DCF, suggesting that no significant differences between control cells or cells treated with any of the drugs were observed. Because the oxidation-insensitive probe showed no significant difference, the observed differences in treatment groups seen with the oxidation sensitive probe (Fig. 3A) can be directly ascribed to changes in the oxidation of the probe in the cells. These results further support the hypothesis that treatment of these human cancer cells with 2-deoxy-D-glucose or BSO causes a measurable increase in steady-state levels of pro-oxidants in the cells and the combination of 2-deoxy-D-glucose + BSO acts to significantly enhance this effect. These results combined with the thiol analysis in Table 1 also confirm that 2-deoxy-D-glucose induces a condition of metabolic oxidative stress in human breast cancer cells that is enhanced by inhibiting GCL activity and GSH synthesis.

To directly measure the ability of 2-deoxy-D-glucose to induce GCL activity, MDA-MB231 cells were treated with 20 mmol/L 2-deoxy-D-glucose for 24 hours when the cells were scraped harvested and collected for GCL activity analysis using a previously described HPLC assay (22) to determine the fmol of γ -glutamylcysteine (GGC) produced/ μ g sample protein/min. Figure 4 shows that control cells in these experiments had GCL activity of 150 fmol GGC/ μ g protein/min, and treatment with 2-deoxy-D-glucose for 24 hours caused a significant 1.7-fold increase in GCL activity, relative to control. Treatment with BSO inhibited the 2-deoxy-D-glucose-induced increase in GCL activity (data not shown). Interestingly, BSO did not inhibit 2-deoxy-D-glucose-induced increases in GCL activity in the *in vitro* assay to the same extent as it depleted GSH levels in the cells (50% versus >90%). This is most likely due to the reversible nature of the BSO-induced inhibition of GCL (15), suggesting that when the cell pellet was homogenized and diluted in the reaction buffer the BSO no longer completely bound the active site of the enzyme.

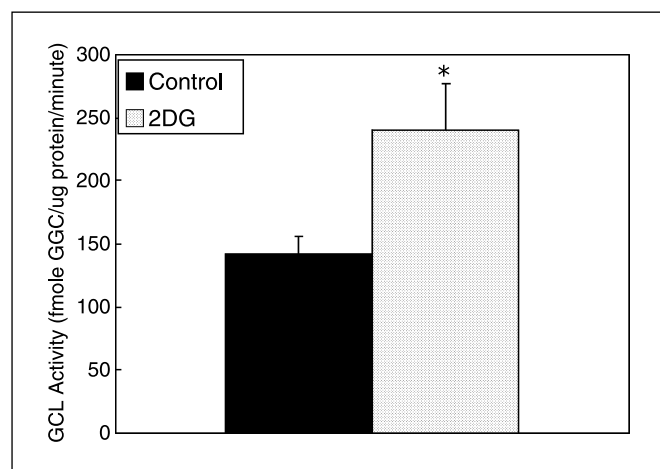


Figure 4. Increased GCL activity in MDA-MB231 cells was detected by HPLC analysis following 2-deoxy-D-glucose (2DG) treatment. Cells treated for 24 hours with 20 mmol/L 2-deoxy-D-glucose showed a significant increase in the GCL activity (250 fmol GGC/ μ g protein/min) over control cells (150 fmol GGC/ μ g protein/min; $n = 5$)*, $P < 0.05$ versus control. Bars, ± 1 SD.

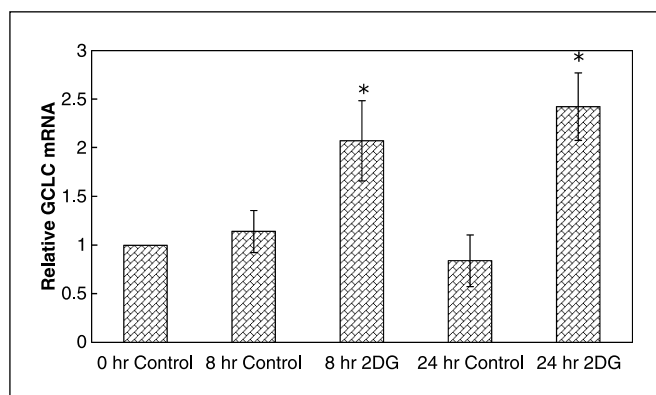


Figure 5. GCLC mRNA expression was increased in breast carcinoma cells treated with 2-deoxy-D-glucose (2DG) as determined by real-time PCR. Cells treated with 20 mmol/L 2-deoxy-D-glucose showed a 2-fold increase in mRNA over control cells at 8 hours after 2-deoxy-D-glucose treatment and a 2.5-fold increase in mRNA levels at 24 hours after 2-deoxy-D-glucose treatment ($n = 3$).*, $P < 0.05$ versus control. Bars, ± 1 SD.

This 2-deoxy-D-glucose-induced increase in GCL activity was accompanied by a similar increase in steady-state levels of RNA coding for the GCLC enzyme as evidenced by the quantitative real-time reverse transcription-PCR data shown in Fig. 5. A significant increase in GCLC mRNA was first noted after 8 hours of treatment with 2-deoxy-D-glucose and persisted at 24 hours of 2-deoxy-D-glucose exposure. BSO treatment had no effect on GCLC mRNA expression in any treatment group (data not shown). Interestingly, a similar analysis of the RNA levels of the modifier subunit of the GCL enzyme (GCLM) showed no significant increases at the 8- or 24-hour time points of 2-deoxy-D-glucose exposure (data not shown). Overall, these results show that treatment of human breast cancer cells with 2-deoxy-D-glucose induced the increased expression of GCLC as well as GCL activity. These results provide further support for the hypothesis that GCL is a protective enzyme up-regulated in response to metabolic oxidative stress induced by exposure of human cancer cells to 2-deoxy-D-glucose.

Discussion

Cancer cells metabolize more glucose than their normal cell counterparts, and this metabolic characteristic is exploited in positron emission tomography imaging to localize cancerous tissue using F^{18} -2-deoxy-D-glucose. In previous animal studies, treatment with 2-deoxy-D-glucose has been shown to be initially effective at causing some tumor growth inhibition (23), but tumors seem to become refractory to 2-deoxy-D-glucose exposure. In clinical trials, patients tolerated treatment with 2-deoxy-D-glucose (12), but because of an incomplete understanding of the mechanism of the antitumor action, no effective therapies have yet been developed using 2-deoxy-D-glucose. Determining effective biochemical manipulations that could inhibit resistance of cancer cells to 2-deoxy-D-glucose-induced toxicity would therefore represent a significant advancement for designing combined modality cancer therapy protocols using 2-deoxy-D-glucose.

Glucose deprivation has been shown to increase cancer cell killing via increases in oxidative stress (4). In the current report, 2-deoxy-D-glucose caused significant cell killing over 24 and 48 hours (Figs. 1 and 2) that was significantly enhanced by the GCL inhibitor BSO that resulted in the depletion of total GSH (Table 1). In addition, increases in endpoints indicative of oxidative stress

were also noted in 2-deoxy-D-glucose-treated human breast cancer cells as evidenced by increases in total GSH, GSSG, and increases in % GSSG (Table 1). These observations were further supported by studies using an oxidation sensitive dye (Fig. 3A) that detects steady-state increases in pro-oxidants. When total GSH (Table 1) was depleted with BSO during 2-deoxy-D-glucose treatment, % GSSG (Table 1) and measurements of steady-state pro-oxidant levels using the oxidation sensitive dye (Fig. 3A) showed significant increases in oxidative stress that correlated with increased cell killing (Figs. 1 and 2). Finally, 2-deoxy-D-glucose caused a significant increase in the GCL activity (Fig. 4) that was accompanied by increased steady-state levels of mRNA coding for GCLC (Fig. 5).

Taken together, these data are consistent with the hypothesis that MDA-MB231 human breast cancer cells induce an adaptive response to treatment with 2-deoxy-D-glucose that involves the up-regulation of GSH synthesis via increased GCL activity, resulting in enhanced 2-deoxy-D-glucose resistance. These data also show that combining 2-deoxy-D-glucose with the GSH synthesis inhibitor (BSO) significantly enhanced cell killing by 2-deoxy-D-glucose, suggesting that inhibiting hydroperoxide detoxification via the GSH-dependent peroxidases inhibited the induction of this protective pathway.

Therefore, using these principles, inhibitors of hydroperoxide metabolism could represent a significant target for enhancing the effectiveness of therapies based on 2-deoxy-D-glucose-induced toxicity. Because it has been suggested that 2-deoxy-D-glucose could cause radiosensitization (3, 12, 24) and chemosensitization (25), these results also suggest that inhibition of glucose metabolism combined with inhibition of GSH-dependent hydroperoxide metabolism could represent an effective biochemical strategy for improving outcome in combined modality protocols involving conventional cancer therapy agents that induce oxidative stress.

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