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2DG enhances the susceptibility of breast cancer cells to doxorubicin

Research Article

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Abstract: 2DG causes cytotoxicity in cancer cells by disrupting thiol metabolism while Doxorubicin (DOX) induces cytotoxicity in tumor cells by generating reactive oxygen species (ROS). Here we examined the combined cytotoxic action of 2DG and DOX in rapidly dividing T47D breast cancer cells *vs.* slowly growing MCF-7 breast cancer cells. T47D cells exposed to the combination of 2DG/DOX significantly decreased cell survival compared to controls, while 2DG/DOX had no effect on MCF-7 cells. 2DG/DOX also disrupted the oxidant status of T47D treated cells, decreased intracellular total glutathione and increased glutathione disulfide (%GSSG) compared to MCF-7 cells. Lipid peroxidation increased in T47D cells treated with 2DG and/or DOX, but not in MCF-7 cells. T47D cells were significantly protected by NAC, indicating that the combined treatment exerts its action by increasing ROS production and disrupting antioxidant stores. When we inhibited glutathione synthesis with BSO, T47D cells became more sensitive to 2DG/DOX-induced cytotoxicity, but NAC significantly reversed this cytotoxic effect. Finally, 2DG/DOX, and BSO significantly increased the %GSSG in T47D cells, an effect which was also reversed by NAC. Our results suggest that exposure of rapidly dividing breast cancer cells to 2DG/DOX enhances cytotoxicity *via* oxidative stress and *via* disruptions to thiol metabolism.

Keywords: *2DG • Doxorubicin • Oxidative stress • Breast cancer • GSH • BSO*

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1. Introduction

One of the main characteristics of cancer cells is their altered metabolism when compared to normal cells [1-3]. The metabolic disruptions appear to involve metabolism of glucose and the loss of regulation between glycolytic metabolism and respiration [1-3]. Several studies have shown that glucose deprivation can induce cytotoxicity in transformed human cell types *via* metabolic oxidative stress [4-7]. In addition, transformed human cell types appear to be more sensitive to glucose deprivation-induced cytotoxicity and metabolic oxidative stress than non-transformed human cell types [5].

2DG is an analog of glucose, and is a competitive inhibitor of glycolysis [8]. 2DG is cytotoxic to mammalian tumor cells *in vitro* and inhibits tumor growth *in vivo* [9-11]. 2DG was shown to inhibit the growth of fibrosarcoma in rats, and these tumors had the greatest uptake of 2DG, which surpassed that of most glucose-dependent organs: brain, liver, and muscle [9]. Studies *in vitro* have also shown that 2DG is cytotoxic and capable of inducing radiosensitization in human cancer cells [11]. Importantly, NAC, a thiol antioxidant, has been shown to suppress the cytotoxicity and radiosensitization as well as partially reverse the disruptions in thiol metabolism induced by 2DG in mouse and human cancer cells [11,12]. The effects of 2DG are likely more pronounced in cancer cells (exhibiting high rates of glycolysis) than in normal cells [10,12]. To date one of the biochemical characteristics most closely associated with cancer

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cells is the increased utilization of glucose. The rate of glucose utilization in tumors is generally correlated with the rate of proliferation and degree of aggressiveness [13,14]. Since the 1920's it has been noted that cancer cells use more glucose than normal cells [2,3].

Doxorubicin, is a member of cytotoxic anthracyclin antibiotics, a group of antibiotics commonly used in cancer treatment [15]. Doxorubicin is obtained from *streptomyces peucetius*, and can also be commercially synthesized [16]. Tumor cells are believed to be killed in part by the redox cycling capability of Doxorubicin [17]. This redox cycling capability generates oxygen radicals that in turn can kill cancer cells [17]. Free radical damage from treatment with Doxorubicin may occur *via* two mechanisms. The first mechanism involves the formation of the semiquinone free radical [16]. In the presence of oxygen this free radical will form superoxide anions. Superoxide dismutation, catalyzed by superoxide dismutase (SOD), yields hydrogen peroxide, which undergoes the Fenton reaction by reduced metals to form the highly toxic OH radical. The second mechanism is a reaction of DOX with iron to form a Fe^{2+}-Doxorubicin free radical complex [16]. This iron complex can reduce oxygen to hydrogen peroxide and other active ROS. The collective damage in the target cell due to such oxygen radical formation will depend on the rate of formation of ROS and on the efficacy of the cellular defense mechanisms. Doxorubicin has been used to treat cancers for over 30 years and it is an effective therapy [15]. However, there are many chronic side effects that limit the amount of Doxorubicin that can be given to a patient, the most detrimental side effect is the cardiomyopathy [15,18,19]. Therefore decreasing the amount of Doxorubicin given to patients by using combination therapy that enhances efficacy would be highly desirable.

The glucose analog, 2DG, is a clinically relevant inhibitor of glucose metabolism believed to mimic the effects of glucose deprivation [20-22]. Therefore, we hypothesized that applying 2DG and DOX and modulating intracellular thiol levels using BSO will inhibit critical aspects of thiol-mediated hydroperoxide metabolism. In turn, the inhibition of thiol-mediated hydroperoxide metabolism will lead to increased steady-state levels of ROS and enhance tumor cell killing *via* metabolic oxidative stress (Figure 1). Here we test this hypothesis using human breast carcinoma cells of different glycolytic capacity: T47D cells (rapidly dividing) and MCF-7 cells (slowly growing).

2. Experimental Procedures

2.1 Cells and culture conditions

T47D and MCF-7 human breast cancer cells were a gift from Dr Said Ismail (Jordan University). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's

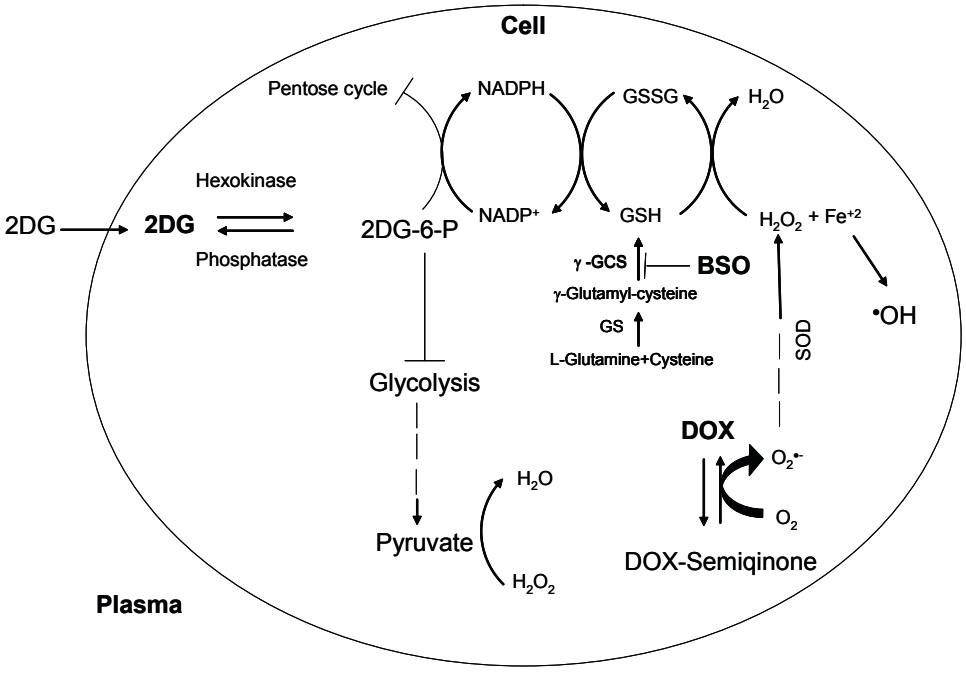

Figure 1. Hypothetical biochemical rationale to explain the interaction between 2DG, DOX and the Glutathione metabolism system and its inhibitors.

medium and Ham's F12 medium (DMEM:F12; Lonza, Belgium) containing 4.5 g/L glucose and supplemented with 10% heat-inactivated (50°C, 30 min) fetal bovine serum, 1% L-glutamine, 1% penicillin – streptomycin and 15 mM HEPES. Stock cultures were maintained in 5% CO_2 and humidified in a 37°C incubator.

2.2 Drug treatment

2DG, NAC, BSO and DOX were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All drugs were used without further purification. Drugs were added to cells at a final concentration of 20 mM 2DG, 1 mM BSO, 0.1 μM DOX and 20 mM NAC. Stock solution of 2DG and BSO were dissolved in phosphate buffer saline (PBS), NAC was dissolved in 1 M sodium bicarbonate (pH 7.0) and DOX was dissolved in normal saline (NaCl); the required volume of each drug was added directly to complete cell culture medium on cells to achieve the desired final concentration. All cells were incubated with a specific treatment in 37°C incubation and harvested according to the time framework that determined in each experiment.

2.3 Measurement of Glutathione

Following treatment with 2DG and/or DOX or vehicle, cells were scrape-harvested in phosphate buffered saline (PBS) at 4°C and centrifuged (1200 rpm for 5 min). The PBS was discarded and the cell pellets were frozen at –80°C. Glutathione disulfide (GSSG) and total glutathione (GSH + GSSG) were determined using a previously described spectrophotometric recycling assay [23,24]. All biochemical determinations were normalized to protein content using the Bradford method.

2.4 MTT assay

The MTT assay was performed as described previously [25]. Briefly, the MTT solution was prepared by dissolving 2 mg MTT/mL PBS and sterilized by filtering through a 0.45 μm syringe filter. 15 μL of MTT solution was added to each well, and the plates were incubated at 37°C for 3 h in a 5% CO_2 incubator. The formation of formazan crystals was checked using inverted microscope. Plates were then taken and 150 μL of each well was discarded carefully using micropipette leaving the crystals only. These crystals were dissolved with 160 μL dissolving solution (1:1 mixture of DMSO and iso-propanol) and incubated at 37°C for 1 h. The absorbance of the dark violet color solution developed in the wells was measured at 570 nm using a Jasco V-530 spectrophotometer. Percent (%) survival was calculated as the proportion between absorbance of treated cells and control cells multiplied by 100.

2.5 Assessment of lipid peroxidation

Lipid peroxidation was determined by measuring thiobarbituric acid-reactive species (TBARS) as described by Ohkawa *et al*., 1979 with modifications [26]. Briefly, samples of control and treated dishes were scraped in cold PBS centrifuged for 5 min to obtain cell pellets that were frozen at –80°C. Pellets were then thawed and homogenized in 250 μL 1% sodium dodecyl sulfate (SDS). Homogenates were then taken and mixed with 1 mL of 20% trichloroacetic acid (TCA; Sigma, USA), 2 mL 0.8% TBA (ACROS, USA) and 250 μL 1% phosphoric acid, and boiled in a water bath at 95°C for 1 hour till a pink color appeared. The reaction was stopped on ice and the color was extracted in n-butanol by centrifugation at 15000 rpm. TBARS levels were measured at 530 nm using a Jasco V-530 spectrophotometer. The values are expressed in nmole/mg protein MDA.

2.6 Statistical analysis

Statistical analysis was done using GraphPad Prism version 4 for Windows (GraphPad Software San Diego, CA). To determine differences between three or more means, we used one-way ANOVA with Bonferroni *post-hoc* tests. Two-way ANOVA was used to determine differences over different time points and treatment groups. Error bars represent mean ± SEM. All statistical analyses are significant at $P<0.05$.

3. Results

3.1 Inhibition of cell growth with 2DG and DOX

The cell growth curves shown in Figure 2 show the growth delay of T47D cells (A) and MCF-7 cells (B) treated with 20 mM 2DG and/or 0.1 μM DOX over the 48-h exposure period. T47D cells treated with 2DG and/or DOX caused a significant growth delay compared with the control cells at 24 and 48 h (Figure 2A), while no effect was seen in MCF7 cells at 24 h as compared to the control (Figure 2B). The combination of 2DG and DOX inhibited cell growth and was significantly different from either treatment alone at 48 h in both cell lines (Figure 2).

3.2 2DG and DOX-induced cytotoxicity are inhibited by NAC

Treatment with 2DG or DOX alone caused ~27% cell killing relative to untreated control T47D cells (Figure 3A), whereas no significant effect was seen in MCF-7 cells (Figure 4A). The combination of 2DG

and DOX caused a significant increase to 36% cell killing, when compared with 2DG and DOX alone in T47D cells (Figure 3A), while no effect was seen in MCF-7 cells (Figure 4A). NAC significantly inhibited the cytotoxicity induced by treatment with 2DG and/ or DOX in T47D cells. To assess the ability of NAC to rescue the cells from 2DG in combination with DOX after the drugs were allowed to interact with the cells, treatment with NAC was given either 1 h before or at the same time of the addition of 2DG and DOX. Consequently, we found that there was no statistically significant difference in the ability of NAC to rescue cells from the toxicity seen when NAC was added at the same time of 2DG and DOX addition, relative to when NAC was added 1 h before 2DG and DOX (data not shown). These results support the hypothesis that the direct reaction of DOX/ 2DG with NAC does not seem to completely account for the protective effects of NAC and that some other mechanism (which could include inhibition of oxidative stress) seems to play a role in the toxicity seen with the combination of 2DG+DOX in T47D cells (Figure 3A).

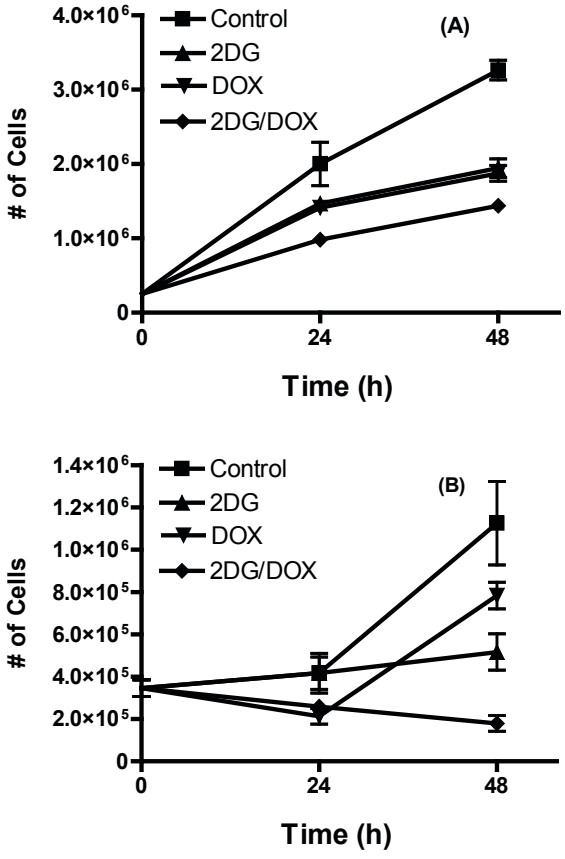

Figure 2. Effect of 2DG and DOX alone and in combination on growth of T47D cells (A) and MCF-7 cells (B). The cells were treated with 20 mM 2DG, 0.1 μM DOX, and their combination for 24 and 48 h. (A) showed a significant growth delay relative to controls ($P<0.05$, $P<0.001$, respectively), while no effect was seen in MCF7 cells at 24 h as relative to controls (B). The combination of 2DG and DOX for 48 h showed a significant growth delay compared with either treatment alone ($P<0.05$) in both (A) and (B). Points, average cell counts from four treatment dishes at each time point; bars, SEM.

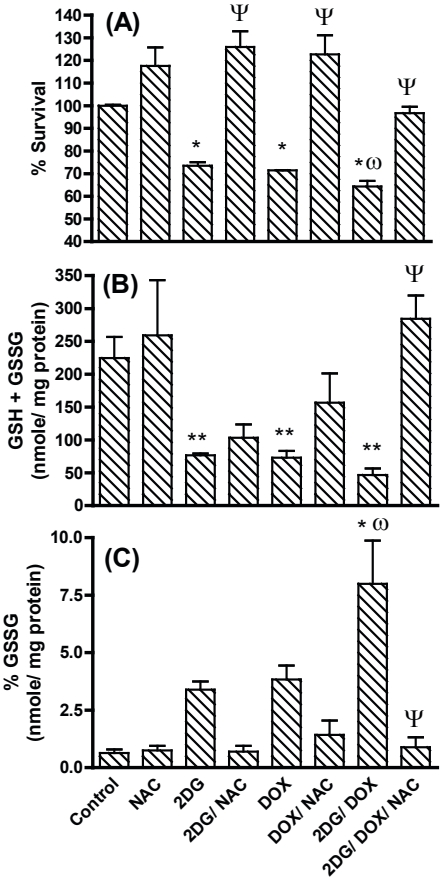

Figure 3. Effect of 2DG, DOX, and NAC on cytotoxicity (A), total glutathione (B), and percentage of oxidized glutathione (%GSSG) levels (C) in T47D cells. In (A), cells were treated with 20 mM 2DG and/or 0.1 μM DOX for 24 h with or without treatment with 20 mM NAC for 1 h before and during 2DG and DOX exposure. Cell survival data were normalized to control. Columns, mean of N=4 experiments; bars, SEM. In (B) and (C), cells were treated as stated above and harvested for glutathione analysis using the spectrophotometric recycling assay. Columns, mean of N=3 experiments; bars, SEM. *$P<0.001$, *versus* control; **$P<0.05$, *versus* control; Ψ, $P<0.01$, *versus* respective treatment without NAC; ω, $P<0.05$, *versus* 2DG and DOX alone.

3.3 2DG and DOX–induced alterations in thiol pools are inhibited by NAC

Glutathione is a major intracellular redox buffer such that the ratio of GSH to GSSG can be used as a reflection of intracellular redox status [27]. Because glucose deprivation has previously been shown to alter GSH/GSSG levels consistent with causing oxidative stress [4-7,9], thiol analysis was done to determine if NAC caused any effects on intracellular GSH/GSSG in cells treated with 2DG and DOX. Exposure of T47D cells to 2DG or/and DOX caused a 25% to 54% decrease in total glutathione (Figure 3B), while no significant difference was seen in MCF-7 cells (Figure 4B). Coincubation with 20 mM NAC inhibited the effects of 2DG and DOX on total glutathione compared with the same treatment groups without NAC (Figure 3B). The combination of 2DG and DOX caused the percentage of GSSG to increase >5-10 fold compared with either treatment or control, respectively in T47D cells (Figure 3C), indicating that this drug combination was causing oxidative stress. However, no changes were seen in the percentage of GSSG in treated MCF-7 cells as compared to control cells (Figure 4C). When the T47D cells were treated with the combination of 2DG and DOX in the presence of NAC, percentage of GSSG significantly decreased near to control levels (Figure 3C). Taken together, the data in Figure 3 and Figure 4 suggest that the cytotoxic effects of 2DG in combination with DOX were mediated by disruptions in thiol metabolism consistent with oxidative stress, which was reversed by the thiol antioxidant NAC in rapidly dividing cells, T47D.

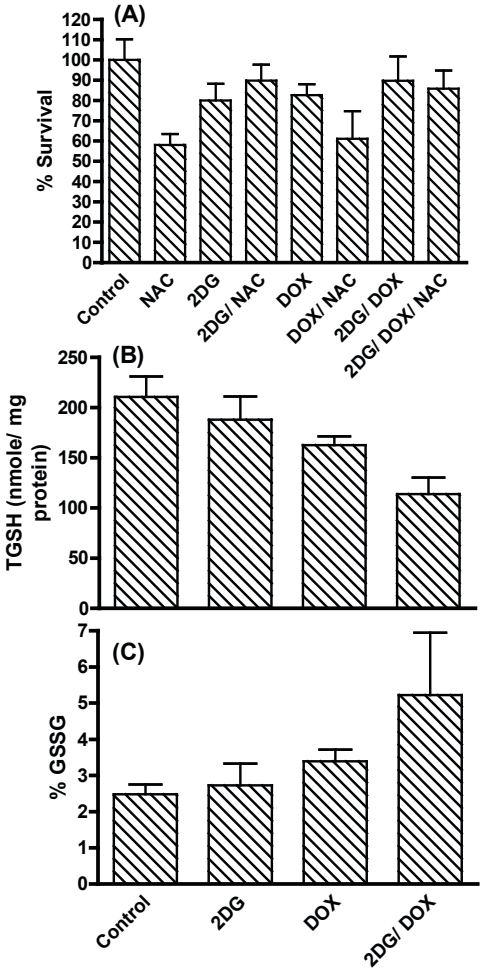

Figure 4. Effect of 2DG and DOX, on cytotoxicity (A), total glutathione (B), and %GSSG levels (C) in MCF-7 cells. In (A), cells were treated with 20 mM 2DG and/or 0.1 μM DOX for 24 h with or without treatment with 20 mM NAC for 1 h before and during 2DG and DOX exposure. Cell survival data were normalized to control. Columns, mean of N=3 experiments; bars, SEM. In (B) and (C), cells were treated as stated above without NAC and harvested for glutathione analysis using the spectrophotometric recycling assay. Columns, mean of N=3 experiments; bars, SEM.

3.4 Effect of 2DG and/or DOX on lipid peroxidation

Oxidative stress that occurs in the cells, as a consequence of an inequity between the prooxidant/antioxidant systems, causes injure to biomolecules such as nucleic acids, proteins, structural carbohydrates, and lipids [28]. Among these targets, the peroxidation of lipids is basically damaging because the formation of lipid peroxidation products leads to spread of free radical reactions. To explore the effect of 2DG and/or DOX on lipid peroxidation, malondialdehyde (MDA) levels (secondary products of lipid peroxidation) was measured using thiobarbituric acid reactive substances (TBARS) method. As shown in Figure 5, MDA levels were significantly elevated in all treated cells, T47D and MCF-7 with 2DG and/or DOX as compared to control, $P<0.001$, $P<0.05$, respectively. The combination of 2DG and DOX caused a significant increase in MDA levels when compared with 2DG or DOX alone in T47D cells, $P<0.05$ (Figure 5A).

3.5 2DG and DOX–induced cytotoxicity is enhanced by BSO

To determine if GSH depletion would enhance the toxicity and oxidative stress induced by treatment with 2DG and DOX, T47D cells were treated with 1 mM BSO for 2 h before and during treatment with 2DG and DOX for 24 h. The results indicate that BSO sensitized cells to the cytotoxicity of the combination of 2DG and DOX (2DG + DOX + BSO, >46% killing, *versus* 2DG + DOX, 36% killing). Furthermore, NAC significantly protected against the cytotoxicity of 2DG/ DOX/ BSO (Figure 6A).

The control of BSO/NAC was not different from BSO alone in our cell model system (data not shown). These data support the hypothesis that inhibition of GSH synthesis with BSO significantly enhanced the cytotoxicity observed with 2DG and DOX in T47D cells.

3.6 2DG and DOX–induced oxidative stress is enhanced by BSO

To determine if oxidative stress contributed to the cytotoxic effect of 2DG, DOX, and BSO, thiol analysis was done on T47D cells treated with the three drugs alone and in combination (Figure 6B and C). BSO caused significant GSH depletion in cells treated with 2DG or 2DG/DOX compared with treatments without BSO (Figure 6B). In addition, cells treated with 2DG/ DOX/ BSO showed significant increases in %GSSG compared with the other treatments (Figure 6C). Furthermore, exposure to NAC significantly decreased the %GSSG induced by treatment with 2DG / DOX/ BSO (Figure 6C). These results indicate that BSO effectively decreased total GSH and increased a parameter indicative of oxidative stress (%GSSG) when co-incubated with the combination of 2DG and DOX. Furthermore, the fact that NAC suppressed the increase in %GSSG in 2DG/DOX/BSO treated cells (Figure 6C), as well as suppressed the cytotoxicity of 2DG/DOX/ BSO (Figure 6A), supports the hypothesis that oxidative stress was causally related to the enhanced toxicity seen with these three drugs.

4. Discussion

Cells that have undergone neoplastic transformation (cancer cells) demonstrate altered metabolism when compared to untransformed (normal) cells

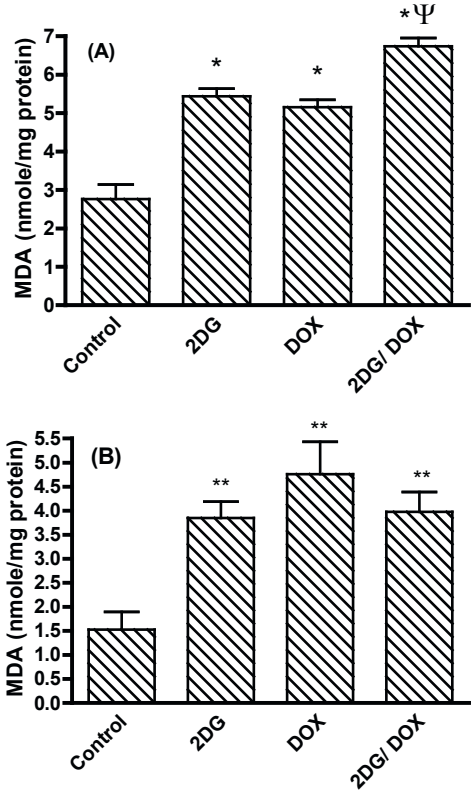

Figure 5. Effect of 2DG and/or DOX on lipid peroxidation level in T47D (A) and MCF-7 cells (B). Cells were treated with 20 mM 2DG and/or 0.1 μM DOX for 24 h then harvested for MDA assay. Columns, mean of N=3 experiments; bars, SEM. *P<0.001, *versus* control; **P<0.05, *versus* control; Ψ, P<0.05, *versus* 2DG and DOX alone.

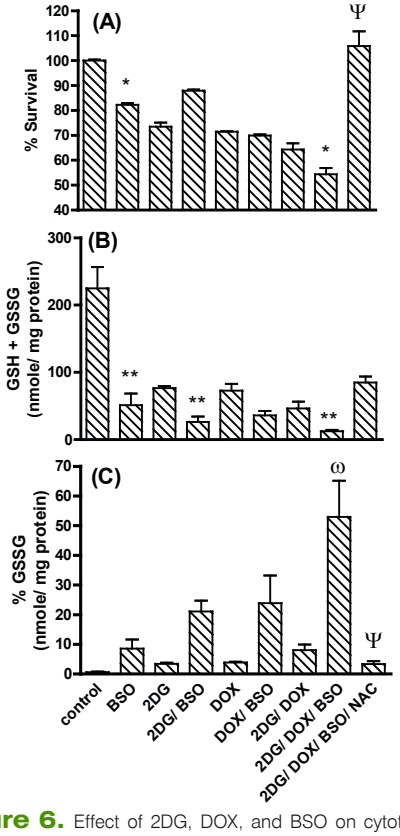

Figure 6. Effect of 2DG, DOX, and BSO on cytotoxicity (A), total glutathione (B), and %GSSG levels (C) in T47D cells. In (A), cells were treated with 20 mM 2DG and/or 0.1 mM DOX for 24 h with or without treatment with 1 mM BSO for 2 h before and during 2DG and DOX exposure. Cell survival data were normalized to control. Columns, mean of N=4 experiments; bars, SEM. In (B) and (C), cells were treated as stated above and harvested for glutathione analysis using the spectrophotometric recycling assay. *P<0.01 *versus* respective treatment without BSO; Ψ, P<0.001 *versus* 2DG/DOX/BSO; **P<0.05 *versus* respective treatment without BSO; ω, P<0.001 *versus* respective controls.

[1-3]. The most pronounced and almost universal metabolic disruptions appear to involve metabolism of glucose and the loss of regulation between glycolytic metabolism and respiration [1-3]. In general, cancer cells exhibit increased glycolysis and pentose phosphate cycle activity, while demonstrating only slightly reduced rates of respiration [1-3]. Initially these metabolic differences were thought to arise as a result of "damage" to the respiratory mechanism and tumor cells were thought to compensate for this defect by increasing glycolysis.

But, if cancer cells (relative to normal cells) increase glucose metabolism – to form pyruvate and NADPH as a compensatory mechanism in response to ROS formed as byproducts of oxidative energy metabolism – then inhibition of glucose metabolism would be expected to sensitize cancer cells to agents that increase levels of hydroperoxides (*i.e.*, ionizing radiation and chemotherapy agents such a quinones known to redox cycle and produce ROS, [29]). Although it is not possible to deprive cells of glucose *in vivo*, it is possible to treat tumor bearing animals and humans with 2DG, a relatively non-toxic analog of glucose that competes with glucose for uptake *via* the glucose transporters as well as being phosphorylated by hexokinase at the entry point to glycolysis. Competition between 2DG and glucose is thought to cause inhibition of glucose metabolism, thereby creating a chemically induced state of glucose deprivation. Although there are reports that the phosphorylated form of 2DG (2DG-6-P) can proceed through the first step in the pentose cycle (Glucose 6-phosphate dehydrogenase) leading to the regeneration of one molecule of NADPH [30], 2DG-6-P appears to be incapable of further metabolism in the pentose cycle as well as incapable of metabolism to pyruvate. Administration of 2DG to mice has been shown to be an effective way to inhibit glucose metabolism without causing toxicity until very high levels are achieved ($LD_{50} \geq 2$ g/kg body weight) [31] and to be tolerable in humans when administered up to 200 mg/kg [32]. Furthermore, for more than 20 years, 2DG treatment was known to sensitize tumor cells to the cytotoxic effects of ionizing radiation both *in vitro* and *in vivo* [10,20,33]. This has led to phase I/II clinical trials in humans testing 2DG as an adjuvant to radiation therapy [33]. Therefore using 2DG as an inhibitor of glucose metabolism *in vivo* may provide a very effective addition to multi-modality cancer therapies designed to limit hydroperoxide metabolism for the purpose of enhancing radio- and chemo-sensitivity in human cancers. In the current study, we have shown that 2DG inhibited T47D cell growth over the 48-h exposure period (Figure 2A) while killing 27% of cells after a 24-h exposure period (Figure 3A), while no effect of 2DG was seen in slowly growing MCF-7 cells (Figure 4A). These findings support previous studies, showing 2DG-induced growth inhibition and cytotoxicity in MDA-MB231 cells [32], and FaDu cells [34], accompanied by increases in parameters indicative of oxidative stress and these effects were enhanced with BSO treatment. Furthermore, our results were consistent with a previous study by Singh *et al*. (1999) showing that the growth rate of rapidly dividing DU145 prostate cancer cells depend on high levels of glucose consumption, whereas the growth rate of relatively slow-growing LNCaP cells are much less dependent on glucose [35]. They found a direct correlation between glycolytic capacity and degree of growth inhibition in response to glucose deprivation for these two cell lines. Their results are also consistent with earlier studies that showed a direct relationship between glycolytic capacity and growth rate for several rat hepatoma tumors [3,36], and lend further support to the hypothesis that high glucose consumption is required for rapid proliferation of most, if not all, cancer cells [37]. Given that glucose metabolism appears to be involved with the detoxification of intracellular hydroperoxides and other authors have suggested that tumor cells demonstrate increased intracellular hydroperoxide production [38], we propose that the extent to which tumor cells increase their metabolism of glucose is predictive of tumor susceptibility to glucose deprivation-induced cytotoxicity and oxidative stress. Therefore, when deprived of glucose using inhibitors of glycolytic metabolism (*i.e.* 2DG), tumor cells with high glucose utilization will be more sensitive to cell death resulting from respiratory dependent metabolic oxidative stress than tumor cells with low glucose utilization and normal cells. We hypothesize that the reason for this is because cancer cells with high glucose utilization generate more $O_2^{\bullet -}$ and H_2O_2 from their mitochondrial electron transport chains. Recently, other researchers have shown that 2DG increased the efficacy of DOX and Pacitaxel in human osteosarcoma and non-small cell lung cancers *in vivo* [39]. This suggests that 2DG may potentially increase the efficacy of standard chemotherapeutic drugs. Therefore, inhibitors of glycolytic metabolism (2DG) combined with inhibitors of hydroperoxide detoxification (BSO) could further enhance DOX mediated anti-tumor effects *via* metabolic oxidative stress involving production of ROS in rapidly dividing T47D breast cancer cells.

Doxorubicin is a quinone chemotherapeutic agent that is known to be metabolized to the semi-quinone intermediate that is thought to be capable of causing damage directly, or redox cycling to produce superoxide and hydrogen peroxide capable of causing

oxidative stress [40]. Recently 2DG was shown to enhance the anti-tumor activity of DOX *in vivo* but the mechanism for this interaction was not clearly defined [39].

Doxorubicin has been suggested to be effective in prostate, breast, and colon cancer therapy [41,42]. However, there are many chronic side effects that limit the amount of DOX that can be given to a patient, the most detrimental side effect is the cardiomyopathy [15,18,19,40]. Therefore, decreasing the amount of DOX given to patients by using combination therapy 2DG and BSO that enhances tumor toxicity would be a highly desirable outcome of therapy.

In the current study, we found that the combination of 2DG and DOX showed a significant cell killing in rapidly dividing T47D cells compared with 2DG or DOX alone (Figure 3A), while no effect was seen in slowly growing MCF-7 cells (Figure 4A). The increase in %GSSG induced by 2DG and DOX in T47D cells (Figure 3C) suggests that oxidative stress is involved. We believe that the combination of 2DG and DOX causes an increase in steady state levels of hydroperoxides and this increase exceeds the metabolic capabilities of the glutathione peroxidase and glutathione reductase system to maintain glutathione in the reduced form. To further support this idea, the thiol antioxidant NAC was able to inhibit the increase in %GSSG (Figure 3C) and inhibit the cytotoxicity induced by 2DG and DOX (Figure 3A). Because NAC caused significant increases in total glutathione in 2DG- and DOX-treated T47D cells (Figure 3B), NAC may function by increasing intracellular thiol pools necessary for counteracting the effects of 2DG and DOX.

Although our data suggest that disruptions in thiol metabolism are involved with the toxicity of 2DG and DOX alone, this mechanism may not be the only mechanism of cytotoxicity in cells treated with 2DG or DOX alone in this model system. However, the increase in MDA levels (secondary products of lipid peroxidation) (Figure 5A) suggests that lipid peroxidation contribute to the mechanism of 2DG and/or DOX induced cytotoxicity. Lipid peroxidation is one of the major outcomes of free radical-mediated injury to tissue. Peroxidation of lipids can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction.

BSO is a relatively specific inhibitor of γ-glutamylcysteine synthetase – the rate-limiting enzyme in the synthesis of glutathione. BSO has been shown to sensitize to Adriamycin, and is currently being used as such in clinical trials [43]. Administration of BSO leads to decreased GSH levels in virtually all tissues including developing embryos, and a marked GSH depletion is associated with tissue damage [44-46]. Additionally, pretreatment with BSO enhances the toxicity of radiation and drugs [32,34,45,47]. In the current study, BSO significantly increased the cytotoxicity induced by 2DG and DOX on T47D (Figure 6A). As expected, BSO significantly decreased total GSH levels when combined with 2DG or 2DG and DOX (Figure 6B). When BSO was combined with 2DG and DOX, oxidative stress was enhanced, as evidenced by increases in %GSSG (Figure 6C). Furthermore, NAC significantly inhibited the cytotoxicity and the increase in %GSSG induced by 2DG/DOX/BSO (Figure 6A and C). These results support the hypothesis that metabolic oxidative stress significantly contributes to the interaction of 2DG/DOX/BSO in killing rapidly dividing T47D cells in this cell culture model. The metabolic disruption and increased dependency of cancer cells on glycolysis for ROS detoxification is an attractive target to gain therapeutic advantage to kill cancer cells while sparing normal cells. 2DG is clinically relevant competitor of glucose, thereby creating a chemically induced state of glucose deprivation [10]. 2DG inhibits glucose metabolism in animals and is not toxic to animals except at very high levels (>2 g/kg body weight) [31] and is tolerable in humans at up to 200 mg/kg [33]. Interestingly, other researchers have shown that 2DG increased the efficacy of DOX and Pacitaxel in human osteosarcoma and non-small cell lung cancers *in vivo* [39]. These data also strongly support the potential therapeutic use of 2DG in combination with DOX and BSO to treat tumor cells with high glucose utilization.

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