Published in final edited form as:

Free Radic Biol Med. 2012 January 15; 52(2): 436–443. doi:10.1016/j.freeradbiomed.2011.10.493.

Simultaneous inhibition of glutathione- and thioredoxin-dependent metabolism is necessary to potentiate 17AAG-induced cancer cell killing via oxidative stress

Peter M. Scarbrough^a, Kranti A. Mapuskar^{a,b}, David M. Mattson^c, David Gius^{d,e}, Walter H. Watson^f, and Douglas R. Spitz^{a,b,*}

^aFree Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA 52240, USA

^bHuman Toxicology Program, Department of Occupational and Environmental Health, The University of Iowa, Iowa City, IA 52240, USA

^cBreast Radiation Oncology Program, Department of Radiation Medicine, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

^dDepartment of Radiation Oncology, Vanderbilt–Ingram Cancer Center, Nashville, TN 37232, USA

^eRadiation Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

^fDivision of Gastroenterology, Hepatology and Nutrition, Department of Medicine, University of Louisville, Louisville, KY 40292, USA

Abstract

17-Allylamino-17-demethoxygeldanamycin (17AAG) is an experimental chemotherapeutic agent believed to form free radicals *in vivo*, and cancer cell resistance to 17AAG is believed to be a thiol-dependent process. Inhibitors of thiol-dependent hydroperoxide metabolism [L-buthionine-S,R-sulfoximine (BSO) and auranofin] were combined with the glucose metabolism inhibitor 2-deoxy-D-glucose (2DG) to determine if 17AAG-mediated cancer cell killing could be enhanced. When 2DG (20 mM, 24 h), BSO (1 mM, 24 h), and auranofin (500 nM, 3 h) were combined with 17AAG, cell killing was significantly enhanced in three human cancer cell lines (PC-3, SUM159, MDA-MB-231). Furthermore, the toxicity of this drug combination was significantly greater in SUM159 human breast cancer cells, relative to HMEC normal human breast epithelial cells. Increases in toxicity seen with this drug combination also correlated with increased glutathione (GSH) and thioredoxin (Trx) oxidation and depletion. Furthermore, treatment with the thiol antioxidant NAC (15 mM, 24 h) was able to significantly protect from drug-induced toxicity and ameliorate GSH oxidation, Trx oxidation, and Trx depletion. These data strongly support the hypothesis that simultaneous inhibition of GSH- and Trx-dependent metabolism is necessary to sensitize human breast and prostate cancer cells to 2DG+17AAG-mediated killing via enhancement of thiol-dependent oxidative stress. These results suggest that simultaneous targeting of both GSH and Trx metabolism could represent an effective strategy for chemosensitization in human cancer cells.

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author at: Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, Iowa City, IA 52240, USA. Fax: +1 319 335 8039. douglas-spitz@uiowa.edu. .

Supplementary materials related to this article can be found online at doi:10.1016/j.freeradbiomed.2011.10.493.

Keywords

Glutathione; Thioredoxin; 2-Deoxyglucose; Auranofin; Thioredoxin reductase; Free radicals

Cancer cells are thought to be under increased metabolic oxidative stress compared to their normal cell counterparts [1]. To date, every cancer cell line tested has shown significantly higher levels of CDCFH₂ and DHE oxidation relative to its normal cell counterpart, which is believed to be indicative of increased steady-state levels of reactive oxygen species (ROS), such as superoxide or hydrogen peroxide, in cancer cells [1]. It is thought that these increased levels of ROS are in part due to dysfunctional mitochondrial metabolism, which has also been commonly observed in cancer cells [1–6]. Increased levels of ROS are believed to contribute to aberrant redox signaling, genomic instability, cell immortalization, and the inability to differentiate, which are all characteristics of the malignant phenotype.

It is thought that cancer cells up-regulate several aspects of hydroperoxide metabolism to compensate for higher levels of oxidative stress, relative to normal cells [7–12]. As evidence of this, metabolic pathways involving glutathione, thioredoxin, and glucose (both glycolysis and pentose-cycle activity) are often elevated in cancer cells relative to normal cells [1,8,13–15]. These pathways are thought to play critical roles in the detoxification of cellular hydroperoxides [1]. Glutathione and thioredoxin are used as cofactors (by glutathione peroxidases or thioredoxin peroxidases) to eliminate cellular hydroperoxides. The pentose phosphate pathway (which utilizes glucose as a substrate) provides NADPH, which is a critical cofactor allowing the disulfide forms of glutathione and thioredoxin to be recycled back to their reduced state (through glutathione reductase and thioredoxin reductase) [16–19]. The hypothesis that glucose metabolism protects cancer cells from endogenous oxidative stress is supported by the observation that glucose deprivation has been found to cause selective clonogenic cell killing in cancer cell populations via increases in hydroperoxide and superoxide levels [1,17,19]. Glucose metabolism via glycolysis may also limit the need to utilize aerobic respiration to provide energy for the cell, which could limit a significant source of endogenous ROS. This hypothesis was supported by a work by Ahmad et al. [17], in which it was shown that Rho0 cells (lacking mitochondrial DNA) were resistant to the oxidative stress and cytotoxicity caused by glucose deprivation. Thus, glucose and hydroperoxide metabolism are thought to work coordinately and play critical roles, during oxidative metabolism, in detoxifying hydroperoxides.

Glucose deprivation is not achievable *in vivo* because the liver provides a source of glucose production through the process of gluconeogenesis [16]. Thus the only way of mimicking glucose deprivation *in vivo* is to use pharmacological agents that inhibit glucose metabolism, such as 2-deoxy-D-glucose (2DG), which is a competitive inhibitor of glucose metabolism. Previous studies have shown that 2DG induces differential cytotoxicity between cancer and normal cells as well as inducing oxidative stress in cancer cells [1,9,11,20,21].

There is a body of evidence showing the capacity for agents that enhance oxidative stress, such as 2DG, to increase cancer cell killing and sensitivity to chemotherapy agents [9–11,20]. Using this biochemical rationale, pharmacological interventions that could effectively inhibit hydroperoxide metabolism in cancer cells for the purpose of sensitizing cancer cells to oxidative stress and chemotherapy-mediated cytotoxicity have been under development, such as the GSH-depleting agent L-buthionine-S,R-sulfoximine (BSO) and the thioredoxin reductase inhibitor auranofin (AUR) [18].

In this study we tested the hypothesis that inhibiting thiol-mediated antioxidant defenses would enhance sensitivity to a chemotherapeutic geldanamycin derivative [17-allylamino-17-demethoxygeldanamycin (17AAG)] [22,23]. In support of this hypothesis, electron paramagnetic resonance spectroscopy has shown that 17AAG can form free radicals *in vivo* and 17AAG resistance correlates with elevated glutathione [12,24,25]. When 2DG was used to inhibit glucose metabolism, some cancer cell lines (MDA-MB-231 and PC-3) but not others (SUM159) were sensitized to clonogenic cell killing by 17AAG. However, when thiol-mediated hydroperoxide metabolism was compromised using both GSH and Trx metabolic inhibitors (BSO and AUR) simultaneously, 2DG + 17AAG toxicity was significantly increased in all cell lines tested. Furthermore, this increased toxicity was accompanied by increased thiol oxidation and depletion, and these changes, as well as the cytotoxicity caused by the drug combination, were attenuated by treatment with the thiol antioxidant *N*-acetylcysteine (NAC). Because sensitization to 2DG + 17AAG-induced cell killing did not occur unless both BSO and AUR were used in combination, the current data support the conclusion that simultaneous disruption of both glutathione and thioredoxin metabolism is necessary to provide an effective and consistent sensitization to 17AAG-induced cancer cell killing by enhancing oxidative stress.

Materials and methods

Cell lines, media, and culture conditions

Hormone-independent human breast cancer cells (MDA-MB-231) were a kind gift from the lab of Dr. Michael Henry from The University of Iowa (Iowa City, IA, USA), originally obtained as a passage 3 culture from the American Type Culture Collection (ATCC; Manassas, VA, USA). Human prostate adenocarcinoma cells (PC-3) were obtained from the ATCC. Each of these three cell lines was grown in RPMI 1640 and 10% fetal bovine serum. Human hormone-independent breast cancer SUM159 cells were obtained from Asterand (Detroit, MI, USA) and were grown in Ham's F12 medium, supplemented with 10 mM Hepes, 10 ng/ml insulin, 50 nM hydrocortisone, and 5% FBS. Normal nonimmortalized human mammary epithelial cells (HMECs) were purchased from Clonetics (East Rutherford, NJ, USA), and the cells were cultured in HuMEC medium, purchased from Invitrogen (Carlsbad, CA, USA; Cat. No. 12752010). All cells were grown and maintained at 37 °C and 21% oxygen. Experiments were not performed on any cell line passed more than 15 times in culture, based on passage number when received from the original source (defined as passage 0). During clonogenic survival assays, the culture media were supplemented with 0.1% gentamycin sulfate.

Drug treatment

Cells were plated and allowed to grow for 48 h, until they reached approximately 70% confluence. The medium on each plate was then changed and the cells were treated with 500 nM 17AAG, 20 mM 2DG, 15 mM NAC, and/or 1 mM BSO for 24 h, in the same medium used to maintain each cell line. For experiments in which the cells were treated with AUR, the cells were treated as above, and 200 or 500 nM AUR was added for the last 15 min or 3 h. For experiments comparing normal and cancer cells, the HuMEC medium was used during the drug treatment interval to avoid any differences in medium composition on drug sensitivity.

Clonogenic survival assay

Both floating and attached cells from the treated dishes were collected. Attached cells were collected using trypsinization. Trypsin (0.25%) was inactivated with medium containing FBS. The cells were then centrifuged, before being resuspended in fresh medium and counted with a Coulter counter. The cells were then plated at a low density, allowed to grow

for 14 days in complete medium, and stained with Coomassie blue; colonies on each plate were counted, and clonogenic cell survival was then determined as previously described [26]. Surviving fraction was calculated as the number of colonies per plate divided by the number of cells initially plated. Normalized surviving fraction was determined by dividing the surviving fraction of treated plates by the surviving fraction of sham-treated control plates.

Glutathione assay

Before the GSH/GSSG assay, cells were scraped in 5% 5-sulfosalicylic acid and frozen. The 5,5'-dithiobis-2-nitrobenzoic acid recycling assay was then used to quantify GSH and GSSG levels in the cell supernatants, as described previously by Griffith [28] and Anderson [27]. Sample data were normalized to protein content, as determined by the bicinchoninic acid protein assay, as per the manufacturer's instructions, using the BCATM protein assay kit "Enhanced Protocol" (Pierce Biotechnology, Rockford, IL, USA).

Thioredoxin redox Western blots

The thioredoxin Western blots were performed as described previously, with minor modifications [29]. Approximately 3,000,000 cells were lysed in G-lysis buffer (50 mM Tris-HCl, pH 8.3, 3 mM EDTA, 6 M guanidine-HCl, 0.5% Triton X-100) containing 50 mM iodoacetic acid (IAA; pH 8.3). For each experiment, control plates, for identifying thioredoxin redox state bands in the Western blot, were also incubated with either 2 mM DL-dithiothreitol (DTT) or 2 mM H₂O₂, for 10 min at room temperature, before incubation with 50 mM IAA. Afterward, the lysate from all cells was incubated in the dark for 30 min, with the IAA. The lysates were then centrifuged in G-25 microspin columns (GE Healthcare). Protein was then quantified from the eluent using the Bradford protein assay, as previously described [30]. Equal amounts of protein were then added to a 15% acrylamide native gel. The gels were run at 100 V constant for approximately 2.5 h. The proteins contained in the gel were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories) using a semidry transfer protocol, as described previously [31]. The nitrocellulose membrane was then washed in PBST (phosphate-buffered saline with 0.1% Tween) and blocked in 5% milk with PBST for 1 h, before being incubated at 4 °C overnight with the primary anti-body, 1:1000 goat anti-hTrx-1 (American Diagnostica) in PBST with 2% bovine serum albumin. The primary antibody was then removed; the blot was washed in PBST for 10 min three times, with constant shaking, before being incubated for 1 h with the secondary antibody [horseradish peroxidase (HRP)-labeled rabbit anti-goat IgG (Santa Cruz Biotechnology, Cat. No. sc2020)]. The blot was then washed again for 10 min three times in PBST before being treated with HRP chemiluminescence detection reagents (Renaissance; NEN). The protein was then visualized by exposing the blot to X-ray film for 2–5 min in a dark room with a film cassette before developing the film. In an alternate method of protein visualization, a Typhoon FLA 7000 (GE Healthcare Life Sciences) imaging system was used to directly detect the fluorescent signal from the blot.

Semiquantitative image analysis

Image files were processed and analyzed using ImageJ software (1.42q; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Briefly, the image was cropped to include only the bands of interest and the immediate surrounding areas of the blot. Background was then subtracted by using a 50-pixel rolling ball algorithm. The integrated density of each band was then measured, and the measurement was subtracted from the measured integrated density of an area of the blot adjacent to the band of interest, containing no other bands. The ratio of oxidized to total thioredoxin was defined as (integrated density of the oxidized thioredoxin band)/[integrated density of the reduced thioredoxin band + integrated density of the oxidized thioredoxin band(s)].

Coomassie blue gel staining and imaging

After the transfer step of the Western blotting protocol, the acrylamide gel was stained in 0.2% Coomassie blue, 7.5% acetic acid, and 50% ethanol for 15 min, with gentle shaking. The gel was then destained with phosphate-buffered saline containing 0.1% Tween 20, with three washes over 2 h. Then, the gel containing the stained protein was imaged using an AlphaImager (Alpha Innotech Corp.).

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 19.0 for Windows (IBM Corp., Somers, NY, USA). Statistically significant differences between the means of different experimental groups were determined by performing a one-way ANOVA, using Tukey's post hoc analysis to test for significance. Statistical significance was defined as $p < 0.05$.

Results

To examine if an inhibitor of glucose metabolism (2DG) could effectively enhance 17AAG toxicity, human breast epithelial cancer cells (MDA-MB-231 and SUM159) as well as human prostate cancer cells (PC-3) were exposed to 500 nM 17AAG + 20 mM 2DG for 24 h and toxicity was measured via a clonogenic cell survival assay. Interestingly, each cell line tested showed a different response profile to the 2DG and 17AAG drug combination (Fig. 1). MDA-MB-231 cells showed what appeared to be additive drug toxicity, and PC-3 cells showed a similar response, but the toxicities did not appear to be additive (Figs. 1A and C). Surprisingly, SUM159 cells appeared to be resistant to the treatment of 2DG and 17AAG (Fig. 1B), both as single agents and in combination. These results encouraged further exploration into mechanisms that could account for the differential susceptibility between the cell lines.

It was hypothesized that, because 2DG suppresses glucose metabolism through glycolysis but does not completely suppress glucose metabolism through the pentose cycle, adding inhibitors of NADPH-dependent hydroperoxide metabolism could further enhance 17AAG toxicity in less sensitive cell lines. Accordingly, cells treated with 2DG + 17AAG were also treated with BSO (an inhibitor of glutamate-cysteine ligase, the rate-limiting enzyme in the synthesis of glutathione) or AUR (an inhibitor of thioredoxin reductase), to limit hydroperoxide metabolism through the glutathione peroxidase and the peroxiredoxin pathways. MDA-MB-231 and PC-3 cells were treated individually with BSO (1 mM, 24 h) or AUR (500 nM, for the last 3 h of drug treatment; Figs. 2A and B). Auranofin was not capable of significantly enhancing 2DG + 17AAG toxicity in either cell line, whereas BSO was able to enhance 2DG + 17AAG toxicity only in MDA-MB-231 cells (Fig. 2A).

In contrast, when BSO and AUR were used in combination, a remarkable sensitization to 2DG + 17AAG-induced cell killing was observed even in the drug-resistant SUM159 cell line (Figs. 3A–C). Furthermore, it was found that, when cells were treated with 15 mM NAC for 24 h during drug treatment, the toxicity from the drug combinations was significantly ameliorated (Figs. 3A–C). Because NAC is a nonspecific thiol antioxidant, these data suggested that BSO and AUR were enhancing the toxicity of the 2DG + 17AAG by causing disruptions to critical cellular thiol pools and enhancing oxidative stress. To examine this hypothesis further, the glutathione and thioredoxin redox couples were examined to determine how the drug treatments were affecting cellular thiol redox status.

In MDA-MB-231 and SUM159 cells, treatment with 2DG + BSO + AUR + 17AAG caused significant decreases in total glutathione content (Figs. 4A and B). PC-3 cells showed a similar pattern of decreased total glutathione content as a function of drug treatment (data

not shown). Interestingly, even though NAC was able to significantly protect cancer cells from 2DG + BSO + AUR + 17AAG toxicity, NAC was not capable of restoring total glutathione levels. This result shows that total glutathione content was not the critical factor determining toxicity. However, in SUM159 cells treated with 2DG + BSO + AUR + 17AAG, NAC was able to significantly suppress the proportion of total GSH in the form of glutathione disulfide, as expressed as the percentage of total cellular glutathione present in the form of glutathione disulfide (%GSSG) (Fig. 4D). Similar results were obtained in MDA-MB-231 cells, but did not reach statistical significance (Fig. 4C). GSSG levels were not reliably detected in PC-3 cells. The ability of NAC treatment to suppress %GSSG as well as suppressing clonogenic cell killing (Figs. 3 and 4) suggests that oxidation of cellular thiol pools may be causally related to cell killing.

Further confirmation for this conclusion was obtained when all three cell lines treated with 2DG + BSO + AUR + 17AAG demonstrated significant increases in the oxidation of thioredoxin-1, which were nearly completely suppressed by NAC treatment (Fig. 5). The drug-induced increases in the ratio of oxidized to total thioredoxin were three- to fivefold higher versus control ratios in every cell line tested. These results, combined with the glutathione data, clearly showed that the combination of 2DG + BSO + AUR + 17AAG caused oxidative stress, as measured by changes in both glutathione and thioredoxin oxidation. These results are also consistent with the recent results from Shan et al. showing that Trx1 may serve as a sensitive biomarker of redox imbalances in prostate cancer [32].

A closer analysis of the thioredoxin redox Western blots from MDA-MB-231 (Fig. 5A, lane 4), SUM159 (Fig. 5B, lane 4), and PC-3 (Fig. 5C, lane 4) also showed what appeared to be a decrease in total thioredoxin levels under the 2DG + BSO + AUR + 17AAG treatment condition, relative to control-treated cells. Coomassie-stained gels suggested that this difference in total amounts of thioredoxin was not due to differences in protein loading (Supplementary Figs. 1A, B, and C). It was therefore hypothesized that either the thioredoxin may be degraded or the thioredoxin was forming mixed-protein disulfide complexes with larger proteins that may not be able to enter the 15% acrylamide native gel used to resolve the oxidized and reduced forms of thioredoxin. To determine if larger mixed-protein disulfide complexes of thioredoxin could be formed during drug treatment, samples from cells treated with 2DG + BSO + AUR + 17AAG were lysed and then treated with DTT to reduce mixed-protein disulfides, before being derivatized with IAA and processed for thioredoxin redox Western blotting (Fig. 6, lane 3). The results showed that DTT was capable of completely reducing the oxidized thioredoxin in the higher molecular weight bands and restored the drug-treated cells to control levels of thioredoxin-1 immunoreactive protein (compare Fig. 6, lanes 1–4). These data strongly support the conclusion that the disappearance of total thioredoxin in the 2DG + BSO + AUR + 17AAG-treated cells was caused by an increase in the formation of higher molecular weight mixed-protein disulfides of thioredoxin. Taken together, the thioredoxin and glutathione data (Figs. 4–6) strongly support the hypothesis that the combination of 2DG + BSO + AUR + 17AAG was effectively causing oxidative stress in cellular thiol pools engaged in hydroperoxide metabolism, which significantly contributed to clonogenic cell killing in all three human cancer cell lines.

To determine if the chemotherapy combinations were preferentially cytotoxic to breast cancer versus normal cells, SUM159 cancer cells and HMEC normal human breast epithelial cells were treated with 20 mM 2DG, 200 nM 17AAG, and 100 μ M BSO for 24 h and then 500 nM AUR was added for the last 15 min of drug treatment (Fig. 7). Before treatment, the medium for each cell line was changed to HuMEC medium, to have identical drug exposure conditions for both cell types. After treatment the cells were assayed for clonogenic survival in their respective maintenance medium preparations. Under these conditions, SUM159 cells

demonstrated significantly greater sensitivity to clonogenic cell killing by the combination of BSO + AUR or 2DG + 17AAG + BSO + AUR, relative to the normal HMECs (Fig. 7). These results provide clear evidence that these drug combinations are selectively cytotoxic to breast cancer cells, compared to normal breast epithelial cells.

Discussion

The data presented in this study support the hypothesis that human cancer cells demonstrate a variety of baseline sensitivities to inhibitors of glucose metabolism (2DG) combined with 17AAG (Fig. 1). Inhibitors of glutathione and thioredoxin metabolism (BSO and AUR), as single agents, were relatively nontoxic in all cancer cell lines (Fig. 2). However, when BSO and AUR were combined, a highly significant sensitization to 2DG + 17AAG-mediated clonogenic cell killing was achieved in all cancer cells tested (Fig. 3). It is especially noteworthy that both thioredoxin and glutathione metabolism needed to be compromised before cancer cell sensitivity was enhanced. This finding suggests that it is necessary for both major intracellular thiol pools involved in hydroperoxide metabolism to be compromised before maximum drug sensitization is observed (Fig. 8). These results also support the hypothesis that when either GSH or Trx metabolism is inhibited, the nontargeted pathway may be able to compensate for the loss of the other pathway.

The combination of 2DG + BSO + AUR + 17AAG was observed to be capable at depleting intracellular glutathione and reduced thioredoxin, while also significantly increasing the oxidation of both glutathione and thioredoxin-1 thiol pools (Figs. 4–6). These changes correlated with the increases in clonogenic cell killing, which were observed in all three human cancer cell lines tested. In a direct test of causality, the drug-induced changes in these thiol pools, as well as the cytotoxicity caused by these drug treatments, were either partially or completely inhibited by treatment with the thiol antioxidant *N*-acetyl-L-cysteine (Fig. 3). These data strongly support a causal connection between disruptions to thiol-mediated hydroperoxide metabolism, oxidative stress, and cancer cell killing by these drug combinations.

The finding that inhibition of both GSH and Trx-1 metabolism was required for maximal sensitization is significant because many times heterogeneity in tumor cell sensitivity to anticancer agents is believed to be a contributing factor to treatment failure. Therefore, the identification of relatively nontoxic and well-tolerated adjuvant strategies for enhancing cancer cell killing is highly desirable, especially in the case of advanced cancers. The results in this report show that simultaneous exposure to BSO and AUR, which individually have been shown to be well-tolerated in humans [18,33], dramatically enhanced cancer cell chemosensitivity to 17AAG in human breast and prostate cancer cells. Furthermore experiments with HMECs and SUM159 cells showed that treatment with 2DG, 17AAG, BSO, and AUR was more toxic to breast cancer cells relative to normal breast epithelial cells, suggesting that this combined modality approach may result in a selective therapeutic advantage. Taken together, these results suggest a potentially important and underexplored biochemical rationale for enhancing the efficacy of chemotherapy for breast and prostate cancer using simultaneous inhibition of both glutathione- and thioredoxin-dependent metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the Radiation and Free Radical Research Core Laboratory at The University of Iowa and Dr. Michel L. McCormick for his assistance in running glutathione assays. The authors also thank Drs. Melissa A. Fath, Andrean L. Simons, and Yueming Zhu, for their helpful discussions during the design of these experiments. This work was supported in part by NIH Grants R01CA133114, R01CA100045, T32CA078586, and P30CA086862 as well as the Department of Radiation Oncology at The University of Iowa.

Abbreviations

17AAG	17-allylamino-17-demethoxygeldanamycin
2DG	2-deoxy-D-glucose
AUR	auranofin
BSO	L-buthionine-(S,R)-sulfoximine
CDCFH₂	5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate
DHE	dihydroethidium
DTT	dithiothreitol
FBS	fetal bovine serum
GSH	glutathione
GSSG	glutathione disulfide
IAA	iodoacetic acid
NAC	N-acetylcysteine
ROS	reactive oxygen species
Trx	thioredoxin

References

- [1]. Aykin-Burns N, Ahman IM, Zhu Y, Oberley LW, Spitz DR. Increased levels of superoxide and H₂O₂ mediate the differential susceptibility of cancer cells versus normal cells to glucose deprivation. *Biochem. J.* 2009; 418:29–37. [PubMed: 18937644]
- [2]. Chiaradonna F, Gaglio D, Vanoni M, Alberghina L. Expression of transforming K-Ras oncogene affects mitochondrial function and morphology in mouse fibroblasts. *Biochim. Biophys. Acta.* 2006; 1757:1338–1356. [PubMed: 16987493]
- [3]. Tororov IN, Todorov GI. Multifactorial nature of high frequency of mitochondrial DNA mutations in somatic mammalian cells. *Biochemistry (Moscow).* 2009; 74:962–970. [PubMed: 19916906]
- [4]. Richter C, Gogvadze V, Laffranchi R, Schlapbach R, Schweizer M, Suter M, Walter P, Yaffee M. Oxidants in mitochondria: from physiology to diseases. *Biochim. Biophys. Acta.* 1995; 1271:67–74. [PubMed: 7599228]
- [5]. Bize IB, Oberley LW, Morris HP. Superoxide dismutase and superoxide radical in Morris hepatomas. *Cancer Res.* 1980; 40:3686–3693. [PubMed: 6254638]
- [6]. Bandy B, Davison AJ. Mitochondrial mutations increase oxidative stress: implications for carcinogenesis. *Free Radic. Biol. Med.* 1990; 8:523–539. [PubMed: 2193852]
- [7]. Liu H, Nishitoh H, Ichijo H, Kyriakis JM. Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol. Cell. Biol.* 2000; 20:2198–2208. [PubMed: 10688666]
- [8]. Corti A, Franzini M, Paolicchi A, Pompella A. Gamma-glutamyltransferase of cancer cells at the crossroads of tumor progression, drug resistance and drug targeting. *Anticancer Res.* 2010; 30:1169–1181. [PubMed: 20530424]

- [9]. Andringa KK, Coleman MC, Aykin-Burns N, Hitchler MJ, Walsh SA, Domann FE, Spitz DR. Inhibition of glutamate cysteine ligase activity sensitizes human breast cancer cells to the toxicity of 2-deoxy-D-glucose. *Cancer Res.* 2005; 66:1605–1610. [PubMed: 16452219]
- [10]. Simons AL, Parsons AD, Foster KA, Orcutt KP, Fath MA, Spitz DR. Inhibition of glutathione and thioredoxin metabolism enhances sensitivity to perfosine in head and neck cancer cells. *J. Oncol.* 2009; 2009:519563. [PubMed: 19746172]
- [11]. Hadzic T, Aykin-Burns N, Zhu Y, Coleman MC, Leick K, Jacobson GM, Spitz DR. Paclitaxel combined with inhibitors of glucose and hydroperoxide metabolism enhances breast cancer cell killing via H₂O₂-mediated oxidative stress. *Free Radic. Biol. Med.* 2010; 48:1024–1033. [PubMed: 20083194]
- [12]. McCollum AK, Teneyck CJ, Sauer BM, Toft DO, Erlichman C. Up-regulation of heat shock protein 27 induces resistance to 17-allylamino-demethoxygeldanamycin through a glutathione-mediated mechanism. *Cancer Res.* 2006; 66:10967–10975. [PubMed: 17108135]
- [13]. Warburg O. On the origin of cancer cells. *Science.* 1956; 132:309–314. [PubMed: 13298683]
- [14]. Weber G. Enzymology of cancer cells (second of two parts). *N. Engl. J. Med.* 1977; 296:541–551. [PubMed: 189189]
- [15]. Franco R, Cidlowski JA. Apoptosis and glutathione: beyond an antioxidant. *Cell Death Differ.* 2009; 16:1303–1314. [PubMed: 19662025]
- [16]. Nelson, DL.; Cox, MM. *Lehninger, Principles of Biochemistry.* 4th edition. Freeman; New York: 2005. p. 543-549.p. 690-704.p. 722
- [17]. Ahmad IM, Aykin-Burns N, Sim JE, Walsh SA, Higashikubo R, Buettner GR, Venkataraman S, Mackey MA, Flanagan SW, Oberley LW, Spitz DR. Mitochondrial O₂•– and H₂O₂ mediate glucose deprivation-induced stress in human cancer cells. *J. Biol. Chem.* 2005; 280:4254–4263. [PubMed: 15561720]
- [18]. Kean WF, Hart L, Buchanan WW. Auranofin. *Br. J. Rheumatol.* 1997; 36:560–572. [PubMed: 9189058]
- [19]. Spitz DR, Sim JE, Ridnour LA, Galoforo SS, Lee YJ. Glucose deprivation-induced oxidative stress in human tumor cells: a fundamental defect in metabolism? *Ann. N. Y. Acad. Sci.* 2000; 899:349–362. [PubMed: 10863552]
- [20]. Simons AL, Ahmad IM, Mattson DM, Dornfeld KJ, Spitz DR. 2-Deoxy-D-glucose combined with cisplatin enhances cytotoxicity via metabolic oxidative stress in human head and neck cancer cell. *Cancer Res.* 2007; 67:3364–3370. [PubMed: 17409446]
- [21]. Nirenberg MW, Hogg JF. Inhibition of anaerobic glycolysis in Ehrlich ascites tumor cells by 2-deoxy-D-glucose. *Cancer Res.* 1958; 18:518–521. [PubMed: 13547043]
- [22]. Grem JL, Morrison G, Guo XD, Agnew E, Takimoto CH, Thomas R, Szabo E, Grochow L, Grollman F, Hamilton JM, Neckers L, Wilson RH. Phase I and pharmacological study of 17-(allylamino)-17-demethoxygeldanamycin in adult patients with solid tumors. *J. Clin. Oncol.* 2005; 23:1885–1893. [PubMed: 15774780]
- [23]. Sharp S, Workman P. Inhibitors of the HSP90 molecular chaperone: current status. *Adv. Cancer Res.* 2006; 95:323–348. [PubMed: 16860662]
- [24]. Neckers L, Kern A, Tsutsumi S. Hsp90 inhibitors disrupt mitochondrial homeostasis in cancer cell. *Chem. Biol.* 2007; 14:1204–1206. [PubMed: 18022558]
- [25]. Samuni Y, Ishii H, Hyodo F, Samuni U, Krishna MC, Goldstein S, Mitchell JB. Reactive oxygen species mediate hepatotoxicity induced by the Hsp90 inhibitor geldanamycin and its analogs. *Free Radic. Biol. Med.* 2010; 48:1559–1563. [PubMed: 20211249]
- [26]. Spitz DR, Malcom R, Roberts R. Cytotoxicity and metabolism of 4-hydroxy-2-nonenal and 2-nonenal in H₂O₂-resistant cell lines: do aldehydic by-products of lipid peroxidation contribute to oxidative stress? *Biochem. J.* 1990; 267:453–459. [PubMed: 2334404]
- [27]. Anderson, ME. *Handbook of Methods for Oxygen Radical Research.* Greenwald, RA., editor. CRC Press., Inc.; Boca Raton, FL: 1985. p. 317-323.
- [28]. Griffith OW. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 1980; 106:207–212. [PubMed: 7416462]

- [29]. Watson WH, Pohl J, Montfort WR, Stuchlik O, Reed MS, Powis G, Jones DP. Redox potential of human thioredoxin 1 and identification of a second dithiol/disulfide motif. *J. Biol. Chem.* 2003; 278:33408–33415. [PubMed: 12816947]
- [30]. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* 1976; 72:248–254. [PubMed: 942051]
- [31]. Abdalla MY, Ahmad IM, Spitz DR, Schmidt WN, Britigan BE. Hepatitis C virus-core and non structural proteins lead to different effects on cellular antioxidant defense. *J. Med. Virol.* 2005; 76:489–497. [PubMed: 15977232]
- [32]. Shan W, Zhong W, Zhao R, Oberley TD. Thioredoxin 1 as a subcellular biomarker of redox imbalance in human prostate cancer progression. *Free Radic. Biol. Med.* 2010; 49:2078–2087. [PubMed: 20955789]
- [33]. Bailey HH. L-S, R-buthionine sulfoximine: historical development and clinical issues. *Chem. Biol. Interact.* 1998; 111–112:239–254.

Fig. 1.

Three human cancer cell lines show different sensitivities to 2DG and 17AAG. (A) MDA-MB-231, (B) SUM159, and (C) PC-3 cancer cells were treated with 20 mM 2DG and 500 nM 17AAG for 24 h before being assayed for clonogenic cell survival. Data from at least three experiments are represented. Data shown represent mean normalized surviving fraction + SD. # $p < 0.05$ versus control treatment, * $p < 0.05$ versus any other treatment condition within the cell line.

Fig. 2.

BSO and AUR as single agents did not cause consistent sensitization to 2DG + 17AAG-mediated cell killing. (A) MDA-MB-231 and (B) PC-3 cells were treated with 20 mM 2DG, 500 nM 17AAG, and 1 mM BSO for 24 h or 500 nM AUR for the last 3 h of exposure, before being assayed for clonogenic cell survival. Data from at least three experiments are represented. Data shown represent mean normalized surviving fraction + SD. Statistical analysis determined that AUR treatment did not significantly alter response in cells treated with or without 2DG + 17AAG, whereas BSO significantly enhanced toxicity only in the MDA-MB-231 cell line. * $p < 0.05$ versus the same treatment without 2DG + 17AAG.

Fig. 3.

The combination of BSO and AUR sensitized cancer cells to 2DG + 17AAG-mediated clonogenic cell killing and this toxicity was inhibited by NAC treatment. (A) MDA-MB-231, (B) SUM159, and (C) PC-3 cells were treated with 20 mM 2DG, 500 nM 17AAG, and 1 mM BSO for 24 h and 500 nM AUR for the last 3 h of drug treatment, before being assayed for clonogenic survival. Data represent mean normalized surviving fraction + SD.

* $p < 0.05$ versus control and # $p < 0.05$ versus the same drug treatment with NAC. Data for MDA-MB-231 and PC-3 cells are from three experiments and for SUM159 from two independent experiments.

Fig. 4.

NAC treatment suppressed glutathione oxidation but was not effective at restoring total glutathione levels in cancer cell lines treated with BSO + AUR. (A) MDA-MB-231 and (B) SUM159 cells were treated with 20 mM 2DG, 500 nM 17AAG, and 1 mM BSO for 24 h and 500 nM AUR for the last 3 h of exposure and then assayed for total glutathione and GSSG content. Data for percentage of glutathione in the form of glutathione disulfide (%GSSG) are also shown for (C) MDA-MB-231 and (D) SUM159 cells. Data represent (A and B) mean fold change in total glutathione (expressed in GSH equivalents) relative to untreated cells + SEM or (C and D) nmol per mg GSSG in GSH equivalents/nmol per mg total glutathione in GSH equivalents $\times$ 100% + SEM. Data from at least three experiments each are represented. * p <0.05 versus control, # p <0.05 versus the same treatment without NAC.

Fig. 5.

2DG, 17AAG, BSO, and AUR treatment significantly increased the ratio of oxidized to total thioredoxin, which was inhibited by NAC. Representative thioredoxin-1 redox Western blots are shown for (A) MDA-MB-231, (B) SUM159, and (C) PC-3 cells treated with 20 mM 2DG, 500 nM 17AAG, and 1 mM BSO for 24 h and 500 nM AUR for the last 3 h of drug treatment. After drug treatment, the cells were scraped directly into G-lysis buffer and then derivatized in 50 mM IAA. 2 mM DTT or 2 mM H₂O₂ was added to control lysed homogenates for 15 min as positive controls for reduced or oxidized thioredoxin, respectively. The Western blots shown have been cropped and minimal adjustments were made to the brightness and contrast settings to facilitate visual resolution and interpretation. Coomassie-stained gels corresponding to each Western blot are shown in Supplementary Fig. 1. Semiquantitative analysis of the redox Western data is shown in (D). Background correction of Western data was performed before image analysis; see Materials and methods. Data represent means [integrated density of oxidized band(s)]/[integrated density of reduced band+integrated density of oxidized band(s)] normalized to control +SEM. *p<0.05 versus control and #p<0.05 versus the same treatment group without NAC. Data from at least three independent experiments are represented. (S-S) denotes oxidation of one set of intramolecular thiols and (S-S)₂ designates oxidation of a second set of intramolecular thiol residues. 2DG, 17AAG, BSO, and AUR treatment significantly increased the ratio of oxidized to total thioredoxin, which was inhibited by NAC.

Fig. 6.

Incubation with DTT before IAA derivatization restores reduced/total levels of thioredoxin-1 after treatment with 2DG, 17AAG, BSO, and AUR. SUM159 cells were treated with 20 mM 2DG, 500 nM 17AAG, and 1 mM BSO for 24 h and 500 nM AUR for the last 3 h of drug treatment. After drug treatment, the cells were scraped directly into G-lysis buffer and then derivatized in 50 mM IAA or incubated 15 min in 2 mM DTT or H₂O₂ before addition of IAA for positive controls. Data from three independent experiments are represented.

Fig. 7.

2DG, 17AAG, BSO, and AUR show differential cell killing between normal and cancerous breast epithelial cells. HMEC and SUM159 cells were cultured as described under Materials and methods. Before drug treatment, the media for both cell lines were replaced with HuMEC medium (Lonza, MEGM BulletKit CC-3151 and CC-4136). The cells were then exposed to 20 mM 2DG and 200 nM 17AAG or 100 μ M BSO for 24 h and 500 nM AUR for the final 15 min of drug treatment or the combination of all four treatments. HMEC clonogenic survival was then assayed in MEBM medium and SUM159 clonogenic survival was assayed in the standard F12 medium used to maintain this cell line (see Materials and methods). Data represent mean normalized surviving fraction + SD. * $p < 0.05$ versus control and # $p < 0.05$ versus the same drug treatment in HMECs. Data for HMECs are from three experiments and for SUM159 from two experiments.

Fig. 8. Theoretical model of how inhibitors of glucose and hydroperoxide metabolism may sensitize cancer cells to oxidative stress-mediated 17AAG-induced clonogenic cell killing. GPx, glutathione peroxidase; Prx, peroxiredoxin; GR, glutathione reductase; ROOH, organic hydroperoxide.