Chapter 2
The Role of Akt Pathway Signaling in Glucose Metabolism and Metabolic Oxidative Stress

Andrean L. Simons, Kevin P. Orcutt, Joshua M. Madsen, Peter M. Scarbrough, and Douglas R. Spitz

Abstract Glucose metabolism plays an important role in hydroperoxide detoxification and the inhibition of glucose metabolism has been shown to increase prooxidant production and cytotoxicity in cancer cells. Increased Akt pathway signaling has been shown to be directly correlated with increased rates of glucose metabolism observed in cancer cells versus normal cells. These observations have led to the proposal that inhibition of Akt signaling would inhibit glycolysis and increase hydroperoxide production which would preferentially kill tumor cells versus normal cells via oxidative stress. The current study shows that inhibition of the Akt pathway inhibits glucose consumption and induces parameters indicative of oxidative stress such as glutathione disulfide (%GSSG) and thioredoxin reductase (TR) activity in human head and neck cancer (HNSCC) cells. A theoretical model to explain the results is presented and implications for the use of Akt pathway inhibitors in combination with glycolytic inhibitors and/or manipulations that increase prooxidant production are discussed.

A.L. Simons, PhD (✉)
Department of Pathology, Holden Comprehensive Cancer Center, The University of Iowa, 200 Hawkins Drive, Iowa City, IA 52242, USA

Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, B180 Medical Laboratories, Iowa City, IA 52242, USA
e-mail: andrean-simons@uiowa.edu

K.P. Orcutt, MD • J.M. Madsen, PhD • P.M. Scarbrough, PhD • D.R. Spitz, PhD
Free Radical and Radiation Biology Program, Department of Radiation Oncology, Holden Comprehensive Cancer Center, The University of Iowa, B180 Medical Laboratories, Iowa City, IA 52242, USA

D.R. Spitz et al. (eds.), *Oxidative Stress in Cancer Biology and Therapy*,
Oxidative Stress in Applied Basic Research and Clinical Practice,
DOI 10.1007/978-1-61779-397-4_2,

2.1 Introduction

Glucose metabolism plays an important role in hydroperoxide detoxification and the inhibition of glucose metabolism has been shown to increase prooxidant production and cytotoxicity in cancer cells. Increased Akt pathway signaling has been shown to be directly correlated with increased rates of glucose metabolism observed in cancer cells versus normal cells. These observations have led to the proposal that inhibition of Akt signaling would inhibit glycolysis and increase hydroperoxide production which would preferentially kill tumor cells versus normal cells via oxidative stress. The current preliminary studies explore how the inhibition of the Akt pathway would disrupt glucose consumption and induce parameters indicative of oxidative stress such as glutathione disulfide (%GSSG) and thioredoxin reductase (TR) activity in human head and neck cancer (HNSCC) cells. A theoretical model to explain the results is presented and implications for the use of Akt pathway inhibitors in combination with glycolytic inhibitors and/or manipulations that increase prooxidant production are discussed.

2.2 Upregulation of Glucose Metabolism in Cancer Cells

Cancer cells exhibit increased glucose metabolism and pentose phosphate cycle activity compared to normal untransformed cells [1–3]. The most consistent of these observations is that cancer cells metabolize glucose into pyruvate producing excess lactate even though the supply of oxygen is adequate to support mitochondrial respiration [1–3]. Warburg referred to this phenomenon as aerobic glycolysis [1]. He hypothesized that these metabolic abnormalities were due to "damage" to the tumor cell respiratory mechanism (now known to be mitochondria) and cancer cells compensated for this defect by increasing glycolysis [1]. Warburg originally proposed that cancer cells upregulated glycolysis to compensate for a deficit in ATP production due to compromised oxidative phosphorylation and hypoxic microenvironments [1]. However, conflicting evidence regarding Warburg's hypothesis involving ATP deficits in transformed cells has limited the utility of these concepts in enhancing cancer therapy.

2.3 Glucose Metabolism and Detoxification of Hydroperoxides

Several studies have focused on alternative mechanisms contributing to the Warburg effect [4–7]. Of particular interest is glycolysis' role in the detoxification of hydroperoxides. Glycolysis plays an important role as a source of electrons for energy metabolism in cells under normal steady-state conditions. However, glucose metabolism also plays a major role in the detoxification of hydroperoxides produced as byproducts of O_2 metabolism [8–12]. The major pathways of glucose

metabolism following the formation of glucose 6-phosphate (via the action of hexokinase) include glycolysis and the pentose phosphate cycle [13]. Glycolysis results in the formation of pyruvate (PYR) and the pentose phosphate pathway results in the regeneration of NADPH from NADP+ [13]. PYR, in addition to being a substrate for energy metabolism via the tricarboxylic acid (TCA) cycle and mitochondrial oxidative phosphorylation, has been shown to scavenge H_2O_2 and other hydroperoxides directly [8, 11, 12]. NADPH, by virtue of being the source of reducing equivalents for the glutathione and thioredoxin antioxidant system, has also been shown to participate in the detoxification of H_2O_2 and organic hydroperoxides [14, 15]. Therefore, glucose metabolism appears to be integrally related to the detoxification of intracellular hydroperoxides. In fact, increasing glucose concentrations up to 10–20 mM in tissue culture media has been shown to render cells resistant to H_2O_2-induced cytotoxicity [10].

Given that glucose metabolism appears to be involved with the detoxification of intracellular hydroperoxides and other investigators have suggested that cancer cells demonstrate increased intracellular hydroperoxide production [16], we proposed tumor cells may increase the metabolism of glucose to compensate for increased intracellular hydroperoxides caused by a defect in mitochondrial respiration [17]. Furthermore, we proposed that therapeutic interventions designed to inhibit glucose metabolism and hydroperoxide detoxification combined with manipulations that increase prooxidant production would preferentially kill tumor cells versus normal cells via oxidative stress [17, 18].

2.4 PI3K/Akt Signaling Pathway

The Akt pathway has garnered significant interest in HNSCC research given that increased Akt activity accounts for up to 60% of all HNSCC [19, 20]. Akt is a member of a serine-threonine specific kinase family in mammalian cells [21–24] and Akt is able to activate and deactivate many downstream targets involved in key cellular processes such as cell growth, survival, cell cycle progression, and metabolism [25, 26].

Signaling through the Akt pathway can start at the cell membrane from the binding of ligands with their receptor, which include human epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2/neu, or ErbB-2) [27]. The receptors are activated which then activate phosphoinositide 3-kinase (PI3K), which converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [28, 29]. Akt and phosphoinositide-dependent kinase 1 (PDK1) are recruited to the plasma membrane by binding to PIP_3 via their pleckstrin homology (PH) domains [30]. After localizing to the membrane, Akt is phosphorylated at Thr 308 and Ser 473 by PDK1 and PDK2 also known as mammalian target of rapamycin complex 2 (mTORC2), respectively [30]. Activated Akt can then go on to activate or deactivate many downstream proteins which control cell proliferation and survival. Akt activity is negatively regulated by

the tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10), which acts as a phosphatase to dephosphorylate PIP_3 back to PIP_2 [31]. PTEN inhibits the ability of Akt to be localized to the cell membrane [32–34]. Without this localization, Akt activation decreases, along with all the other downstream pathways that depend on Akt for activation [32–34].

2.5 Role of Akt Signaling in Glycolysis

Akt hyperactivation is believed to be associated with increased rates of glucose metabolism observed in tumor cells [35–37]. Akt signaling exerts a direct influence on glycolysis in cancer cells by several mechanisms. Akt has been shown to regulate the localization of the glucose transporter GLUT1 to the plasma membrane [38–42] and regulate hexokinase expression, activity, and mitochondrial interaction [43–45]. In addition, Akt may indirectly activate the glycolysis rate-controlling enzyme phosphofructokinase-1 (PFK1) by directly phosphorylating phosphofructokinase-2 (PFK2) [46], which produces the product, fructose-2.6-bisphosphate (Fru-1,6-P2), which is the most potent allosteric activator of PFK1. In support of these findings, the activity of Akt was found to be correlated to the degree of glycolysis in glioblastoma cells, i.e., the higher the Akt activity, the higher the rate of glycolysis [36]. In addition, activation of Akt signaling in glioblastoma cells previously without constitutive Akt activity stimulated a high level of aerobic glycolysis without increasing oxygen consumption [36].

From these observations, it is clear that Akt activation causes an increase in glycolysis in cancer cells, and this may be the key step in the metabolic transformation of cells to increase glycolysis (originally observed by Warburg) in the development of cancer. This was supported by work showing that lymphoma cells with mitochondrial respiration defects lead to activation of the Akt pathway but not in the parental cells lacking mitochondrial respiration defects [47]. Therefore, the increased glycolytic rates observed by Warburg in cancer cells exhibiting mitochondrial respiration malfunction compared to normal cells may involve activation of the Akt pathway.

2.6 Role of EGFR Signaling in Glycolysis

There is also a strong relationship between EGFR and glucose metabolism. High concentrations of epidermal growth factor (EGF) have been shown to increase glucose consumption and cause selective cytotoxicity in MDA-468 human breast cancer cells, a cancer cell line over expressing EGFR, compared to MCF-7 breast cancer cells, which have a very low expression of EGFR [48]. EGFR may additionally influence with glucose metabolism as it has been found that the physical presence of EGFR within the membrane stabilizes the sodium glucose cotransporter (SGLT1) that may cause increased glucose consumption by EGFR over expression independent of EGFR signaling initiation [49].

2.7 Role of Glucose Metabolism and Oxidative Stress in Akt Pathway Signaling

Since glucose metabolism appears to play an important role in hydroperoxide detoxification then therapeutic interventions designed to inhibit glucose metabolism would be expected to increase prooxidant production and cytotoxicity in cancer cells. Furthermore, if increased Akt pathway signaling is correlated with increased rates of glucose metabolism observed in cancer cells versus normal cells, then the inhibition of Akt pathway signaling would be expected to inhibit glycolysis and increase hydroperoxide production which would preferentially kill tumor cells versus normal cells via oxidative stress. Based on these assumptions, the combination of Akt pathway inhibitors with glycolytic inhibitors and/or manipulations that increase prooxidant production should further and preferentially cause cytotoxicity in cancer cells, with minimal to no toxicity to normal cells.

The preliminary studies presented here were designed to determine:

1. If inhibition of EGFR, PI3K, and Akt signaling inhibited glucose consumption.
2. If inhibition of EGFR, PI3K, and Akt signaling induced oxidative stress.
3. If the glycolytic inhibitor, 2-deoxyglucose (2DG), would enhance the cytotoxicity induced by Akt pathway inhibitors via metabolic oxidative stress.

To accomplish these goals, we performed select experiments in FaDu and Cal-27 human head and neck squamous carcinoma cells.

2.7.1 Materials and Methods

2.7.1.1 Cells and Culture Conditions

FaDu and Cal-27 human head and neck squamous cell carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cultures were maintained in 5% CO_2 and humidified in a 37°C incubator.

2.7.1.2 Drug Treatment

2-Deoxy-D-glucose (2DG), *N*-acetyl cysteine (NAC), LY294002 (LY), and L-buthionine-[S,R]-sulfoximine (BSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Perifosine (PER) was obtained from Cayman Chemical (Ann Arbor, MI). Erlotinib (ERL) was obtained from OSI Pharmaceuticals (Long Island, NY). All drugs were used without further purification. Drugs were added to cells at a final concentration of 20 mM 2DG, 20 mM NAC, 5 μM LY, 1.0 mM BSO, 5 μM PER,

and 10 μM ERL. 2DG and BSO were dissolved in PBS. NAC was dissolved in 1 M sodium bicarbonate (pH 7.0). LY, ERL, and PER were each dissolved in DMSO then diluted with 0.9% sodium chloride (Hospira, Lake Forest, IL). The required volume was added directly to complete cell culture media on cells to achieve the desired final concentrations. All cells were placed in a 37°C incubator and harvested at the time points indicated.

2.7.1.3 Glucose Consumption

FaDu cells (100,000) were plated and grown for 24 h prior to treatment with 20 mM 2DG, 5 μM LY (LY5), 5 μM PER, or 10 μM EDRL for 24 h. Glucose levels were measured on 20 μL samples with an ACCU-CHEK® Aviva glucometer (Roche; Mannheim, Germany) before and after 24 h of drug treatment.

2.7.1.4 Glutathione Assay

Following treatment, medium was collected and centrifuged to harvest floating cells, and attached cells were scrape harvested in ice-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. Cell pellets were thawed and homogenized in 50 mM PO_4 buffer (pH 7.8) containing 1.34 mmol/L diethylenetriaminepentaacetic acid (DETAPAC) buffer. Total glutathione content was determined by the method of Anderson [50]. GSH and GSSG were distinguished by the addition of 2 μL of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 μL of sample followed by incubation for 1 h and assayed as described previously [51]. All glutathione determinations were normalized to the protein content of whole homogenates using the method of Lowry et al. [52].

2.7.1.5 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 mL trypsin–EDTA (CellGro, Herndon, VA) and inactivated with DMEM containing 10% FBS (Hyclone). The cells were diluted and counted using a Coulter counter. Cells were plated at low density (300–1,000 per plate), and clones were allowed to grow in a humidified 5% CO_2, 37°C environment for 14 days in complete medium, and in the presence of 0.1% gentamicin. Cells were fixed with 70% ethanol and stained with Coomassie blue for analysis of clonogenic cell survival as previously described [53]. Individual assays were performed with multiple dilutions with at least four cloning dishes per data point.

2.7.1.6 Thioredoxin Reductase Activity Assay

Thioredoxin reductase (TR) activity was determined spectrophotometrically using the method of Holmgren and Bjornstedt [54]. Enzymatic activity was determined by subtracting the time-dependent increase in absorbance at 412 nm in the presence of TR activity inhibitor aurothioglucose from total activity. One unit of activity was defined as 1 μM TNB formed/(min mg protein). Protein concentrations were determined by the Lowry assay [52].

2.7.1.7 NADPH Measurements

Treated cells were washed with PBS and scrape harvested in PBS at 4°C. After centrifugation at $200 \times g$ for 5 min, cell pellets were resuspended in extraction buffer containing 0.1 M Tris–HCl (pH 8.0), 0.01 M EDTA, and 0.05% (v/v) Triton X-100. The cell suspension was sonicated at a duty cycle of 34% (Sonics Vibracell, VC750) in ice water. The solution was centrifuged at $2{,}300 \times g$ for 5 min. The supernatants were collected and analyzed immediately for NADPH as described previously [55]. Results were obtained by comparison with a standard curve using genuine NADPH and normalized per milligram of cellular protein.

2.7.1.8 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, one-way ANOVA with Bonferroni posttests were performed. Two-way ANOVA was used to determine differences over different time points and treatment groups. Error bars represent ±1 standard deviation. All statistical analysis was performed at the $p < 0.05$ level of significance.

2.7.2 Results

2.7.2.1 Effect of Akt Pathway Inhibitors on Glucose Metabolism

In order to determine if Akt pathway inhibitors would affect glucose metabolism in cancer cells, we used the PI3K inhibitor LY294002 (LY), the Akt inhibitor perifosine (PER), and the EGFR inhibitor Erlotinib (ERL) and compared their effects on glucose consumption to that of the glycolytic inhibitor 2-deoxyglucose (2DG) in FaDu human head and neck cancer cells (Fig. 2.1). We found that 5 μM LY (LY5), 5 μM PER, and 10 μM ERL were all able to inhibit glucose consumption in FaDu cells compared to untreated control cells over a 24 h period (Fig. 2.1). We have also duplicated these results in other head and neck cancer cell lines such as Cal-27,

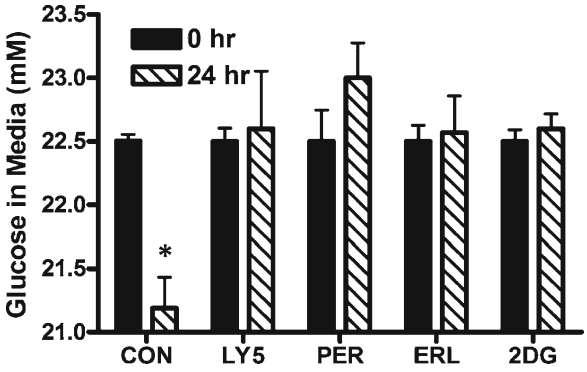

Fig. 2.1 Glucose consumption in FaDu cells. 100,000 FaDu cells were plated and grown for 24 h prior to treatment with 5 μM LY294002 (LY5), 5 μM perifosine (PER), 10 μM Erlotinib (ERL), or 20 mM 2-deoxyglucose (2DG) for 24 h. Glucose levels were measured with a glucometer before and after 24 h of drug treatment. *Error bars* represent ±1 SD of at least $N = 3$ experiments. *$p < 0.05$ versus time 0 h

SCC-25, and SQ20B cells (data not shown). These results suggest that EGFR/PI3K/Akt pathway inhibitors do in fact inhibit glucose consumption and they do so just as well as 2DG, suggesting a possible role of glucose metabolism in all 3 of these pathways (EGFR, PI3K and Akt).

2.7.2.2 Effect of Akt Pathway Inhibitors on Oxidative Stress Parameters

We examined if oxidative stress could be contributing to the effects of EGFR/PI3K/Akt pathway inhibitors by measuring glutathione (GSH/GSSG) levels in the cells. The GSH/GSSG redox couple represents a major small molecular weight thiol-disulfide redox buffer in cells [56]. The amount of total GSH that was oxidized (GSSG) was used to calculate the percentage of GSSG (%GSSG). Consequently, an increase in %GSSG is believed to signify a shift toward a more highly oxidizing intracellular environment indicative of oxidative stress [56]. We analyzed %GSSG levels in FaDu cells after treatment with LY5, PER, ERL, and 2DG for 24 h. 2DG has previously been shown to disrupt glutathione metabolism and induce an increase in %GSSG in head and neck cancer cells and we were able to repeat those findings here (Fig. 2.2a, 18). We additionally observed that all agents tested induced a significant increase in %GSSG, with ERL inducing the greatest increase compared to untreated control cells (Fig. 2.2a). These results suggest that thiol oxidation was induced in these cells in response to EGFR/PI3K/Akt pathway inhibitors (Fig. 2.2a). To further investigate this phenomenon, we pretreated the cells for 1 h with 20 mM of the thiol antioxidant NAC. NAC has been previously shown to increase GSH production in FaDu cells and may function by enhancing GSH-mediated hydroperoxide metabolism [18]. NAC in combination with each agent tested, suppressed the

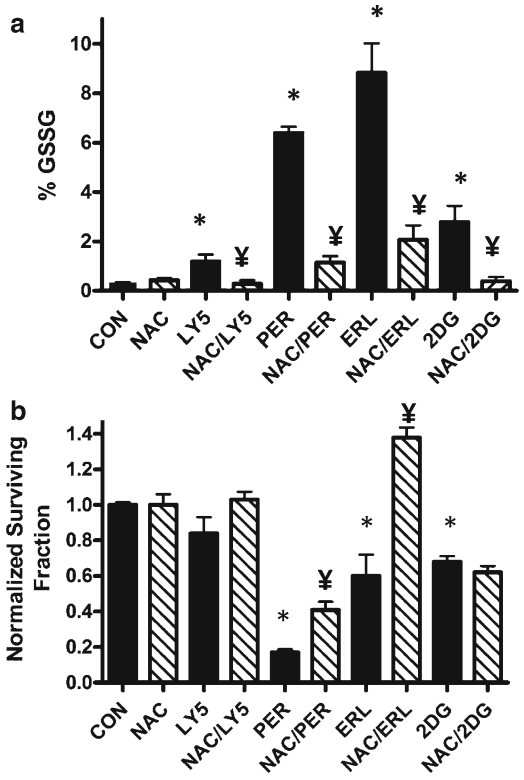

Fig. 2.2 Effect of LY294002 (LY5) on percentage glutathione disulfide (**a**) and cell survival (**b**) in FaDu head and neck cancer cells. FaDu cells were treated with 5 μM LY294002 (LY5), 5 μM perifosine (PER), 10 μM Erlotinib (ERL), or 20 mM 2-deoxyglucose (2DG) for 24 h then harvested for glutathione analysis using the spectrophotometric recycling assay (**a**) or plated for clonogenic survival (**b**). Clonogenic cell survival data were normalized to control (CON). *Error bars* represent ±1 SD of $N=4$–6 experiments performed on different days with at least two cloning dishes taken from one treatment dish. *$p<0.05$ versus control (CON), ¥$p<0.05$ versus respective treatment without NAC

increase in %GSSG induced by the EGFR/PI3K/Akt pathway inhibitors and 2DG (Fig. 2.2a) in FaDu cells further supporting the role of thiol oxidation in the mechanism of Akt inhibitors.

2.7.2.3 Effect of Akt Inhibitors on Survival

To investigate the effect of EGFR/PI3K/Akt inhibitors on FaDu cell growth, cells were analyzed for clonogenic survival after 24 h of treatment with LY5, PER, ERL, and 2DG. We observed that LY5 caused a slight decrease in survival but PER, ERL, and 2DG significantly decreased survival in FaDu cells compared to control ($p<0.01$, Fig. 2.2b). These results show that EGFR/PI3K/Akt inhibitors are able to induce varying degrees of cytotoxicity in FaDu head and neck cancer cells. When we analyzed the effect of NAC on the cytotoxicity induced by these agents, NAC partially but significantly rescued the cytotoxicity induced by PER, and completely rescued the cytotoxicity induced by LY5 and ERL (Fig. 2.2b). NAC was not able to rescue 2DG-induced cytotoxicity in FaDu cells, but we have shown in other studies that antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT)

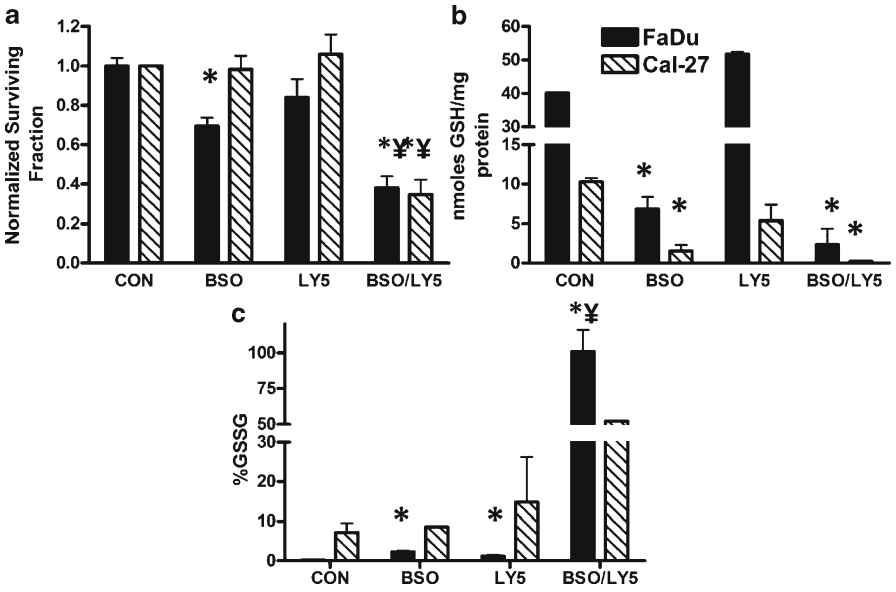

Fig. 2.3 Effect of buthionine sulfoximine (BSO) on LY294002 (LY5)-induced toxicity (**a**), total glutathione (**b**), and percentage of glutathione disulfide (**c**) in FaDu head and neck cancer cells. (**a**) FaDu cells were treated with 5 μM LY5 for 24 h with or without treatment with 1 mM BSO for 1 h before and during LY5 exposure. Clonogenic cell survival data were normalized to control (CON). *Error bars* represent ±1 SD of $N=3$ experiments performed on different days with at least four cloning dishes taken from one treatment dish. (**b**, **c**) Cells were treated as stated above and harvested for total glutathione (GSH) levels (**b**) and percentage glutathione disulfide (%GSSG) levels (**c**) using the spectrophotometric recycling assay. *Error bars* represent ± 1 SD of $N=3$ experiments. *$p<0.001$ versus control; ¥$p<0.05$ versus respective treatment without BSO

were able to completely rescue 2DG-induced cytotoxicity [18]. It is also worth mentioning that pretreating FaDu cells with exogenous glucose prior to drug treatment was also able to rescue the cytotoxicity induced by LY5, PER, ERL, and 2DG (data not shown). Taken together, Fig. 2.2 supports the hypothesis that the EGFR/PI3K/Akt inhibitors induce disruptions in thiol metabolism consistent with oxidative stress, which was reversed by NAC, and EGFR/PI3K/Akt inhibitor-induced cytotoxicity in FaDu cells may be due in part to increases in oxidative stress and the inhibition of glucose metabolism.

2.7.2.4 PER-Induced Cytotoxicity Is Enhanced by Buthionine Sulfoximine

To further probe the involvement of thiol metabolism in the mechanism of Akt pathway inhibition, an inhibitor of GSH synthesis, BSO, was used in combination with LY294002 in FaDu and Cal-27 cells. Pretreatment of cells with 1 mM BSO sensitized both cell lines to LY294002 (Fig. 2.3a), which was associated with

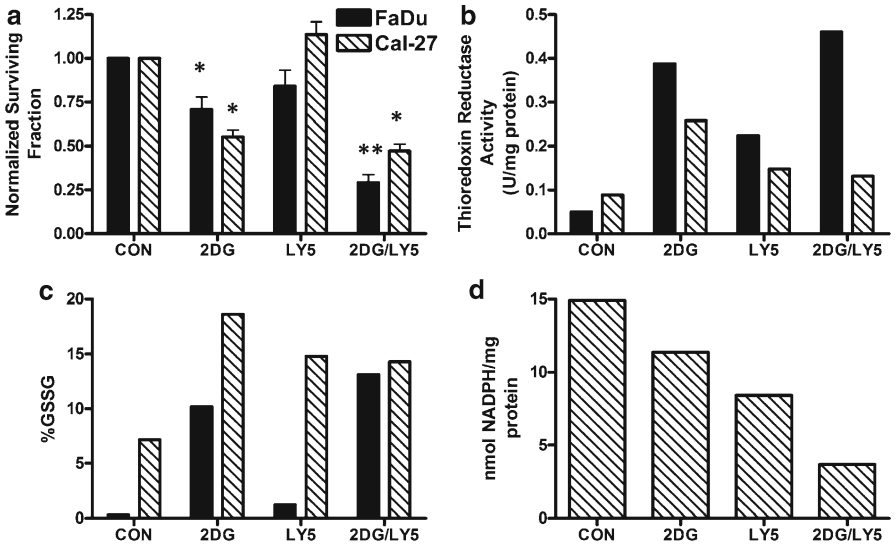

Fig. 2.4 Effect of 2-deoxyglucose (2DG) in combination with LY294002 (LY5) on cytotoxicity (**a**), thioredoxin reductase activity (**b**), percentage glutathione disulfide levels (%GSSG) levels (**c**), and NADPH levels (**d**) in FaDu and Cal-27 head and neck cancer cells. Cells were treated with 5 μM LY5 and/or 20 mM 2DG for 24 h with or without treatment with 20 mM NAC for 1 h before and during LY5 exposure. Clonogenic survival data were normalized to control (CON). *Error bars* represent ±1 SD of $N=3$ experiments performed on different days with at least two cloning dishes taken from one treatment dish. *$p<0.05$ versus control; **$p<0.001$ versus 2DG or LY5

depletion of GSH (Fig. 2.3b) and significant increases in %GSSG (Fig. 2.3c). These results suggest that BSO is enhancing LY294002-induced oxidative stress by limiting hydroperoxide metabolism through the GSH antioxidant system. Overall, the results in Figs. 2.2 and 2.3 strongly suggest that LY294002 is inducing oxidative stress via disruptions in thiol metabolism in our head and neck cancer cell model.

2.7.2.5 2DG-Induced Sensitization to LY294002

Since we observed that both 2DG and LY294002 (LY5) inhibited glucose consumption and increased thiol oxidation, we next determined the effects of 2DG in combination with LY5 in FaDu and Cal-27 cells. The cells were treated with 20 mM 2DG in combination with LY5 for 24 h and then analyzed for clonogenicity (Fig. 2.4a), %GSSG (Fig. 2.4c), thioredoxin reductase (TR, Fig. 2.4b), and NADPH levels (Fig. 2.4d). LY5 caused no significant decrease in clonogenicity as a single agent in either cell line but significant sensitization was observed with 2DG in combination with LY5 compared to either agent alone in FaDu cells (Fig. 2.4a). In addition, %GSSG increased profoundly when treated with 2DG in combination with LY5 (Fig. 2.4c), which suggests that 2DG sensitized FaDu cells to LY5 by increasing

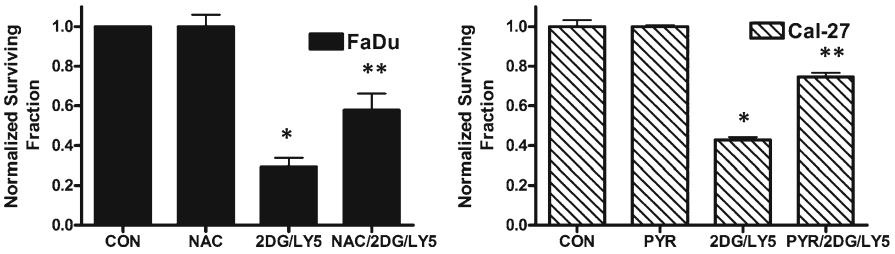

Fig. 2.5 Effect of *N*-acetylcysteine (NAC) and pyruvate (PYR) on 2-deoxyglucose (2DG) and LY294002 (LY5)-induced cytotoxicity in FaDu (**a**) and Cal-27 (**b**) human head and neck cancer cells. Cells were treated with 5 μM LY5 and/or 20 mM 2DG for 24 h with or without treatment with 20 mM NAC (**a**) or 1 mM PYR (**b**) for 1 h before and during LY5 exposure. Clonogenic survival data were normalized to control (CON). *Error bars* represent ± 1 SD of $N = 3$ experiments performed on different days with at least two cloning dishes taken from one treatment dish. *$p < 0.05$ versus control; **$p < 0.001$ versus 2DG + LY5

thiol oxidation. Since the thioredoxin (TRX) antioxidant system is another major antioxidant system in the cell [54], we analyzed TR activity to determine if the TRX system was a target in 2DG + LY5-induced cytotoxicity. TR catalyzes the reduction of TRX using NADPH as a reducing agent [54]. We observed a dramatic increase in TR activity in response to 2DG and 2DG + LY5 (Fig. 2.4b) suggesting that TR activity was being increased to counteract the increase in oxidative stress induced by 2DG + LY5 in FaDu cells.

Although 2DG only slightly enhanced sensitivity of Cal-27 cells to LY5, 2DG as a single agent was very effective at causing cytotoxicity, while inducing %GSSG and TR activity in Cal-27 cells compared to FaDu cells (Fig. 2.4a). Furthermore, NADPH levels in Cal-27 cells were significantly decreased in response to 2DG and/or LY5 suggesting that in addition to glucose metabolism being inhibited, pentose phosphate cycle activity was also being inhibited by 2DG and/or LY5 (Fig. 2.4d).

To follow up on these observations, we determined if the cell killing seen with 2DG in combination with LY5 could be mediated by oxidative stress in both FaDu and Cal-27 cells. The cells were treated with 20 mM 2DG and/or 5 μM LY5 for 24 h with or without 20 mM NAC, for 1 h before and during 2DG and LY5 exposure. We show in Fig. 2.5a that NAC partially but significantly protected FaDu cells from the cytotoxicity associated with 2DG + LY5. Treatment of Cal-27 cells with NAC was cytotoxic to these cells and we therefore pretreated the cells with the antioxidant pyruvate (PYR) which is also a byproduct of glycolysis. PYR was able to significantly protect Cal-27 cells from 2DG + LY5-induced cytotoxicity (Fig. 2.5b).

The overall data support the hypothesis that inhibition of glycolysis with 2DG or Akt pathway inhibitors induced metabolic oxidative stress in head and neck cancer cells. Furthermore, since the thiol antioxidant NAC and PYR inhibited the cytotoxicity associated with 2DG + LY5, these results suggest that a causal relationship exists between Akt pathway inhibition, inhibition of glucose metabolism, metabolic oxidative stress, and enhanced cancer cell killing.

2.8 EGFR/PI3K/Akt Pathway in Tumorigenesis

Alterations in the Akt pathway can lead to abnormal cell signaling, leading to cell proliferation, differentiation, survival, and/or migration. The result of such uncontrolled cell signaling promotes the acquisition of a cancerous phenotype. Although Akt gene mutations are rare in human cancer, several studies have shown Akt amplifications in human ovarian, pancreas, breast, and gastric malignant tumors [57–60]. It is possible that Akt gene amplification may lead to increased response to ambient levels of growth factors. However, gene amplification of PI3K has been reported in glioblastoma, human ovarian, cervical, and gastric cancers [27, 57, 61–65] and also represents about 40% of early genomic aberrations observed in HNSCC [20].

PTEN, the negative regulator of Akt activation, has been shown to be frequently deleted or mutated in a wide variety of human tumors notably in glioblastoma, prostate, endometrial cancers, and HNSCC [27, 65–74]. PTEN acts as a tumor suppressor gene, and in addition to p53, it is one of the most important tumor suppressor proteins [75, 76]. Over expression of PTEN has been shown to inhibit cell growth and enhance apoptosis in different cancer types and is also thought to be a prognostic indicator of clinical outcome [77].

Alterations upstream of the Akt pathway are also able to lead to Akt activation. For example, receptor tyrosine kinases (RTKs), the initiating signaling elements of the Akt pathway, are highly activated and dysregulated due to over expression, truncation, or mutation in certain cancer types [78–80]. Of particular interest are the members of the EGFR family (also known as Erb B tyrosine kinase receptors), which include EGFR (ErbB-1), HER2/neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4), which have been linked to the development and growth of up to 90% of HNSCC tumors [81]. EGFR, which exists on the cell surface, may be activated by binding of specific ligands such as EGF and transforming growth factor (TGFα). Upon activation, EGFR transitions from an inactive monomeric form to an active homodimer. EGFR may also dimerize with other EGFR family members to form an activated homodimer [78]. ErbB-2 containing heterodimers are potent activators of the Akt pathway, and tumors overexpressing ErbB-2 show constitutive Akt activity [78].

EGFR gene amplification is one of the predominant mechanisms leading to EGFR overexpression in tumors [82]. Other mechanisms of EGFR overexpression include increased activation of EGFR with its ligands, most notably TGFα, which is frequently observed in HNSCC, and the expression of mutant forms of EGFR. As a result, EGFR overexpression leads to an increase in its kinase activity by spontaneous dimerization and the activation of downstream signaling pathways which include the Akt pathway [82–86]. In addition, EGFR overexpression has been shown to be correlated with decreased sensitivity to chemotherapy and radiation, and increased risk of recurrence [87].

Resistance to many therapeutic agents may be associated with Akt hyperactivity, as seen, for example, with EGFR signaling inhibitors and in the case of head and neck cancer, cisplatin [88, 89]. Thus, inhibition of the Akt pathway is an attractive

therapeutic strategy and has potential in monotherapy and in combination with existing therapies. The preliminary studies presented here demonstrate that the mechanism of action of various agents that result in the inhibition of Akt pathway signaling (i.e., PI3K, Akt and EGFR inhibitors) involves inhibition of glucose metabolism and the induction of metabolic oxidative stress in head and neck cancer cells. In our studies, we used the PI3K inhibitor LY5 (LY), the Akt inhibitor perifosine (PER), and the EGFR inhibitor Erlotinib (ERL) and compared their effects on glucose consumption to that of the well-known glycolytic inhibitor 2DG in FaDu human head and neck cancer cells.

2.9 Effect of EGFR/PI3K/Akt Pathway Inhibitors on Glucose Metabolism

LY is a derivative of the flavonoid quercetin and is a commonly used pharmacological inhibitor of PI3K in vitro [31, 90]. LY acts by targeting the ATP binding site in the p110 catalytic subunit of PI3K which in most cases results in the inhibition of Akt activity [31, 90]. We found that 5 μM LY (LY5) was able to inhibit glucose consumption in FaDu cells compared to untreated control cells over a 24 h period (Fig. 2.1). In support of these findings, LY was found to decrease hexokinase activity in murine blastocysts [91] and inhibit GLUT1 mRNA and protein expression in human pancreatic cancer cells [92], both of which may be the mechanisms of action responsible for our observed results.

PER is a synthetic alkylphospholipid which inhibits translocation of Akt to the cell membrane preventing Akt's activation [93–96]. PER is the most developed and best characterized Akt inhibitor to date and has shown in phase I trials to be tolerable with the dose-limiting toxicity being gastrointestinal toxicity [97–102]. PER as a single agent has shown favorable responses in patients with advanced soft tissue sarcomas [99] and Waldenstrom macroglobulinemia [103]. However, responses to PER in patients with common solid tumors have been disappointing and have not justified the further investigation of PER as a single agent. Inhibition of Akt with 5 μM PER was also able to inhibit glucose consumption in FaDu cells compared to control cells (Fig. 2.1). As mentioned before, Akt is able to induce the expression of hexokinase, which catalyzes the first step of glycolysis and is able to activate the most important rate-controlling enzyme in glycolysis – phosphofructokinase-1 [43–46]. Therefore, Akt seems to have multiple mechanisms for controlling glucose metabolism in cells.

Erlotinib is a small molecule tyrosine kinase inhibitor of EGFR [104, 105]. Erlotinib binds the intercellular ATP binding domain and prevents the autophosphorylation of EGFR and subsequent signal progression [104, 105]. Since Akt could be activated by EGFR signaling, we proposed that inhibition of EGFR with Erlotinib would inhibit signaling down the Akt pathway, thus inhibiting glucose metabolism. Figure 2.1 shows 10 μM ERL was indeed capable of inhibiting glucose consumption in FaDu cells during 24 h of exposure. Previous results have shown that EGFR

is a stabilizer of an active glucose transporter, SGLT1, and activation of EGFR in the epithelium of intestine led to active transport of glucose [49]. Although ERL does inhibit Akt expression in FaDu cells (data not shown), it remains to be determined if ERL inhibited glucose consumption in our Fadu cells by the destabilization of SGLT1 or by the inhibition of Akt. We have duplicated these results in other head and neck cancer cell lines such as Cal-27, SCC-25, and SQ20B cells (data not shown) all of which over express Akt and EGFR.

These results all suggest that EGFR, PI3K, and Akt inhibitors do in fact inhibit glucose metabolism comparably to 2DG, which is a commonly used glycolytic inhibitor. More importantly, based on these results, increased Akt pathway signaling may have a significant role in the Warburg effect and this phenomenon may be exploited to selectively target cancer cells for the purpose of enhancing radio- and chemo-sensitivity in cancer therapy.

2.10 Redox Regulation of the Akt Pathway

Intracellular redox status plays a vital role in the reversible activation and inactivation of the Akt pathway [47, 106–109]. For example, moderate levels of ROS activate Akt pathway signaling and promote cell survival, but high or chronic oxidative stress inhibits this pathway resulting in apoptosis [47, 106–109]. Activation of the Akt pathway occurs mainly through the oxidative inactivation of Cys-dependent phosphatases (CDPs) or the direct oxidation of pathway kinases [106, 107]. For example, the phosphatase PTEN, the main phosphatase involved in the negative regulation of the Akt pathway, has been shown to be inactivated by oxidation by both H_2O_2 and nitrosylation, posttranslational modifications which would hyperactivate the Akt signaling pathway [107, 110]. Akt is also directly activated by oxidative stimuli. H_2O_2 and peroxynitrite treatments have both been shown to promote Akt activity via posttranslational modification of Akt [107, 111].

Since cancer cells are under increased metabolic oxidative stress compared to normal cells and the Akt pathway may be activated for survival under these oxidizing conditions, we proposed that inhibition of the Akt pathway would increase oxidative stress to such an extent that would render cancer cells sensitive to further increases in oxidative stress.

We investigated the effects of LY, PER, ERL, and 2DG on oxidative stress by analyzing glutathione (GSH/GSSG) levels. LY, PER, and ERL induced significant increases in %GSSG in FaDu cells compared to control cells (Fig. 2.2a) which indicated an increase in oxidative stress and suggests that inhibition of Akt may be involved in increasing oxidative stress. These results also implicate the role of glycolytic inhibition as a mechanism of action in Akt-induced oxidative stress, since 2DG was also able to induce %GSSG (Fig. 2.2a). To further support this idea, the thiol antioxidant NAC was able to completely suppress the increase in %GSSG induced by all three agents (Fig. 2.2a). In addition, NAC was able to significantly reverse the cytotoxicity induced by LY, PER, and ERL in FaDu cells suggesting that

increased oxidative stress was responsible for the cytotoxicity induced by these agents (Fig. 2.2a). NAC was not able to rescue 2DG-induced cytotoxicity in FaDu cells (Fig. 2.2b), but we have shown in other studies that antioxidant enzymes such as SOD and catalase (CAT) were able to rescue 2DG-induced cytotoxicity [18]. It is also worth mentioning that pretreating FaDu cells with exogenous glucose or pyruvate prior to drug treatment was also able to rescue the cytotoxicity induced by LY5, PER, ERL, and 2DG (data not shown), suggesting that inhibition of glucose metabolism and thiol oxidation are involved in the mechanism of action of Akt inhibitors.

To further probe the role of thiol metabolism in the effects of Akt inhibitors, we used BSO, an inhibitor of glutamate cysteine ligase, which is believed to be the rate-limiting enzyme in the synthesis of GSH [112, 113]. Previous studies in our laboratory have shown that BSO significantly depleted GSH pools in breast and head and neck cancer cells while sensitizing cancer cells to chemotherapy agents [18, 114]. BSO has also been used in clinical trials for cancer therapy to enhance the cytotoxicity of chemotherapeutic agents [115]. In the present studies, BSO was found to significantly increase the cytotoxicity induced by LY in FaDu and Cal-27 cells (Fig. 2.3a). As expected, BSO significantly decreased total GSH levels (Fig. 2.3b) and increased %GSSG as a single agent and in combination with PER (Fig. 2.3c), which suggests that inhibition of GSH synthesis further enhanced the oxidative stress induced by LY and further sensitized these cells to the toxicity of LY in FaDu and Cal-27 cells.

2.11 Role of Akt Signaling and FOXO Proteins on Glycolysis

The results in Figs. 2.1–2.3 all suggest that inhibition of glucose metabolism and increased oxidative stress are involved in the inhibition of the Akt pathway. Although Akt inhibition has been shown to have direct effects on glucose metabolism by disrupting glucose transport and inhibiting enzymes involved in glycolysis, it is possible that downstream substrates of Akt may also exert an effect on glucose metabolism. For example, Akt pathway activation results in the cytoplasmic accumulation of the forkhead box O (FoxO) family of transcription factors, FOXO1 and FOXO3a, occluding these factors from the nuclear genes that they activate [116]. FOXO1 has been shown to stimulate expression of gluconeogenic genes (i.e., phosphoenolpyruvate carboxykinase [PEPCK] and glucose-6-phosphatase) and suppress expression of genes involved in glycolysis (i.e., glucokinase) in liver cells [117]. In addition, some of the genes that FOXO3a regulates include antioxidant enzyme genes such as MnSOD, CAT, Peroxiredoxin, Sesn3, and iNOS [111]. Since FOXO1 is involved in the suppression of glycolysis and Akt inhibits FOXO1 activity, then inhibition of Akt may stimulate FOXO1 activity, which would then suppress the expression of enzymes involved in glycolysis. In addition, inhibition of Akt and activation of FOXO3a would increase expression of target antioxidant enzymes which may be in response to the oxidative stress induced by the inhibition of Akt.

Taken together, it is clear that in addition to increased metabolic oxidative stress, disruptions in glucose metabolism are directly and indirectly involved in the inhibition of Akt pathway signaling.

2.12 Effect of LY5 in Combination with 2-Deoxyglucose

The glucose analogue 2DG is a well known and clinically relevant inhibitor of glucose metabolism [118, 119]. We hypothesized that 2DG in combination with LY may act to inhibit critical aspects of thiol-mediated hydroperoxide metabolism leading to increased steady-state levels of ROS and enhanced tumor cell killing via metabolic oxidative stress. 2DG is a clinically relevant analogue of glucose that competes with glucose for uptake and entry into glucose metabolic pathways [119–122]. 2DG can therefore create a drug-induced state of glucose deprivation, although it does not completely inhibit the regeneration of NADPH from NADP+ because it is a substrate for glucose-6-phosphate dehydrogenase [122]. Inhibition of glucose metabolism has been observed in animals administered 2DG without toxicity until high levels (>2 g/kg body weight) were achieved [120], and 2DG has been shown to be tolerable in humans when administered up to 200 mg/kg [123].

We predicted that the combination of 2DG and LY would have an additive and possibly synergistic effect on clonogenic cell killing in FaDu and Cal-27 human head and neck squamous carcinoma cells by enhancing metabolic oxidative stress. Accordingly, treatment with 2DG has been shown to induce cytotoxicity, significant increases in prooxidant production, and profound disruptions in thiol metabolism in head and neck cancer, colon, breast, cervical, and prostate cancer cells, suggesting that oxidative stress was involved with the mechanism of action [5, 17, 55, 114, 124, 125].

In the current study, we found that the combination of 2DG and LY showed at least additive (and possibly more than additive) cell killing in FaDu cells compared with 2DG or LY alone (Fig. 2.4a). However, Cal-27 cells were more resistant to LY, and 2DG + LY-induced cell killing was only slightly increased compared to 2DG alone (Fig. 2.4a). These observations in Cal-27 cells suggest that 2DG is more cytotoxic in this cell line compared to FaDu and that the cytotoxicity we see with 2DG + LY in Cal-27 is mostly due to 2DG (Fig. 2.4a).

The increase in %GSSG induced by 2DG + LY (Fig. 2.4c) compared to control in both cell lines suggests that oxidative stress is involved. 2DG alone seemed to cause the greatest increase in %GSSG in Cal-27 cells which again indicates that 2DG is more effective in this cell line (Fig. 2.4b). Nevertheless, we believe that 2DG and the combination of 2DG + LY cause an increase in steady-state levels of hydroperoxides and this increase exceeds the metabolic capabilities of the glutathione system to maintain glutathione in the reduced form.

To further determine the role of 2DG and LY on hydroperoxide metabolism, we investigated the thioredoxin (TRX) antioxidant system. The TRX system is a highly conserved, ubiquitous system composed of thioredoxin reductase (TR), thioredoxin

(TRX), thioredoxin peroxidases (a.k.a. peroxiredoxins) and NADPH [126]. The TRX system plays an important role in the redox regulation of multiple intracellular processes and resistance to cytotoxic agents that induce oxidative stress [127, 128]. TrxRed is a selenocysteine-containing protein that catalyzes the reduction of Trx using NADPH as a reducing agent [126]. TrxRed has been shown to initiate signaling pathways in response to oxidative stress that play a role in protecting the cell from oxidative stress and is therefore believed to be a potential target for cytotoxic agents that induce oxidative stress [127, 129, 130].

We observed that 2DG and LY alone and in combination increased TR activity in both cell lines, with the greatest effects observed in FaDu cells (Fig. 2.4b). Again 2DG alone was more effective in Cal-27 cells by showing the greatest increase in TR activity compared to the other treatment groups (Fig. 2.4b). These results suggest that TR activity may be increased in response to the oxidative stress induced by 2DG and/or Akt pathway inhibitors. In addition, inhibition of glucose metabolism with 2DG and/or Akt pathway inhibitors may induce oxidative stress by disrupting glutathione and thioredoxin metabolism.

We next measured NADPH levels in Cal-27 cells since NADPH is the source of reducing equivalents for the glutathione and thioredoxin antioxidant systems [9, 12, 15]. We observed that 2DG and/or LY greatly decreased NADPH levels with 2DG + LY causing the greatest decrease in NADPH compared to the other treatment groups (Fig. 2.4d). The results in Fig. 2.4 suggest that inhibition of glucose metabolism with 2DG and LY inhibited the pentose phosphate cycle resulting in decreased NADPH, increased %GSSG, and increased TR activity.

Finally, to further demonstrate that oxidative stress was involved in 2DG + LY-induced cytotoxicity, we show that NAC significantly protected FaDu cells from 2DG + LY-induced cytotoxicity (Fig. 2.5a). Since NAC was very toxic in Cal-27 cells in the presence of 2DG, we treated these cells with pyruvate (PYR). Pyruvate, in addition to being a substrate for energy metabolism via the TCA cycle and mitochondrial oxidative phosphorylation, has been shown to scavenge H_2O_2 and other hydroperoxides directly [8, 11, 12]. We observed that PYR did protect Cal-27 cells from 2DG + LY-induced cytotoxicity (Fig. 2.5b). This protection with PYR has also been observed in other head and neck cancer cells (unpublished data).

2.13 Rationale for Glucose Consumption and Oxidative Stress in Akt Signaling

Overall, the data provided here suggest that inhibition of glucose metabolism and increased intracellular oxidative stress contributes to the toxicity of Akt pathway inhibitors, which is similar to the mechanism of actions observed for 2DG. The results also suggest that manipulating intracellular redox levels (i.e., glutathione) may affect cellular responses to LY and other Akt inhibitors. The scheme shown in Fig. 2.6 illustrates some of the hypothetical relationships between glucose metabolism,

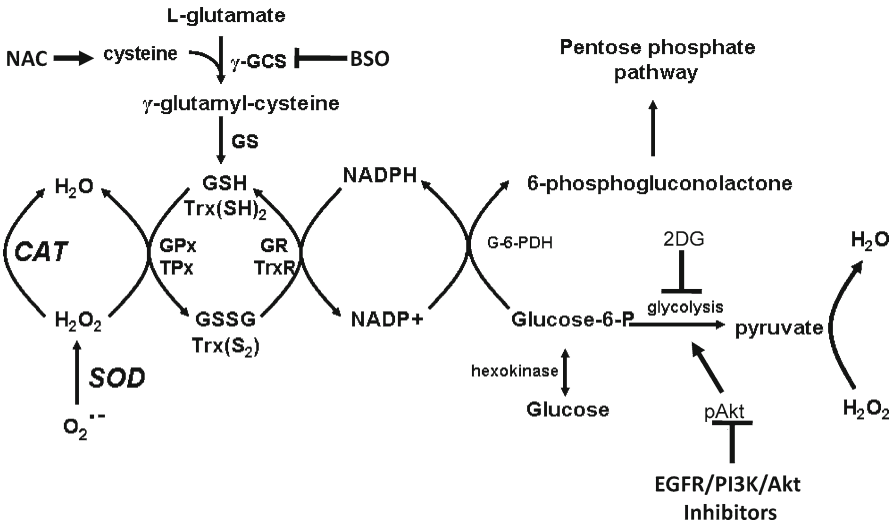

Fig. 2.6 Hypothetical biochemical rationale to explain the role that Akt plays in glucose metabolism and hydrogen peroxide metabolism. The activated form of Akt (pAkt) activates glycolysis by direct and indirect mechanisms thereby increasing glucose transport into the cell and hexokinase activity allowing for the phosphorylation of glucose to glucose-6-phosphate (Glucose-6-P). Glucose-6-phosphate continues into glycolysis to form pyruvate, a known scavenger of hydrogen peroxide (H_2O_2). Glucose-6-P proceeds through the first step in the pentose phosphate cycle via glucose-6-phosphate dehydrogenase (G-6-PDH) to 6-phosphogluconolactone leading to the generation of NADPH from NADP+. NADPH is a source of reducing equivalents for the glutathione system consisting of GSH, GSSG, glutathione peroxidase (GPx), and glutathione reductase (GR) and the thioredoxin system consisting of reduced thioredoxin ($Trx(SH)_2$), thioredoxin disulfide ($Trx(S_2)$), thioredoxin peroxidase (TPx), and thioredoxin reductase (TrxR). The glutathione and thioredoxin systems participate in the detoxification of H_2O_2 and organic hydroperoxides. 2-Deoxy-D-glucose (2DG) competes with glucose for uptake into the cell and phosphorylation by hexokinase into 2-deoxy-D-glucose-6-phosphate (2DG-6-P). 2DG-6-P is unable to continue down the glycolytic pathway but is able to proceed through the first step in the pentose phosphate cycle via G-6-PDH to 6-phospho-2-deoxygluconolactone. 6-Phospho-2-deoxygluconolactone cannot go further in the pentose phosphate cycle. Agents that inhibit Akt signaling (i.e., EGFR, PI3K, or Akt inhibitors) are also able to inhibit glycolysis. Superoxide dismutase (*SOD*) converts superoxide ($O_2^{\bullet-}$) to H_2O_2 which is converted to H_2O by catalase (*CAT*) or GPx. *N*-Acetyl-cysteine (*NAC*) provides cysteine which reacts with L-glutamate catalyzed by glutamate cysteine ligase (GCL, inhibited by L-buthionine-[S,R]-sulfoximine [*BSO*]) to form ã-glutamyl-cysteine. Glutathione synthetase (GS) converts ã-glutamyl-cysteine into GSH

ROS metabolism, Akt pathway inhibitors, and antioxidants suggested by the results of the current study. Inhibiting glucose metabolism with Akt pathway inhibitors and/or 2DG in cancer cells is hypothesized to limit the production of pyruvate and the regeneration of NADPH leading to increased steady-state levels of H_2O_2 and hydroperoxides from metabolic sources resulting in cytotoxicity. BSO is thought to further enhance the toxicity of Akt pathway inhibitors by inhibiting the synthesis of GSH that is required for GSH peroxidases and GSH transferases, both of which are believed to protect against oxidative stress. Finally, the antioxidants NAC and PYR

are able to protect against LY and 2DG+LY by acting to augment small molecular weight intracellular thiols that are capable of scavenging toxic species and by directly scavenging H_2O_2 and other hydroperoxides directly, respectively.

Overall, the results of this study support the hypothesis that Akt pathway inhibitors disrupt glucose metabolism and induce metabolic oxidative stress in head and neck cancer cells. These data also strongly support the potential therapeutic use of Akt pathway inhibitors in combination with chemotherapeutic agents that increase prooxidant production, as well as the new biochemical rationale shown in Fig. 2.6 for combining Akt pathway inhibitors and inhibitors of glucose and hydroperoxide metabolism for enhancing the cytotoxicity of anticancer agents thought to cause injury via oxidative stress.

References

1. Warburg O (1956) On the origin of cancer cells. Science 123:309–314
2. Weber G (1977) Enzymology of cancer cells (first of 2 parts). New Engl J Med 296:486–492
3. Weber G (1977) Enzymology of cancer cells (second of 2 parts). New Engl J Med 296:541–551
4. Galoforo SS, Berns CM, Erdos G et al (1996) Hypoglycemia-induced AP-1 transcription factor and basic fibroblast growth factor gene expression in multidrug resistant human breast carcinoma MCF-7/ADR cells. Mol Cell Biochem 155:163–171
5. Lee YJ, Galoforo SS, Berns CM et al (1997) Glucose deprivation-induced cytotoxicity in drug resistant human breast carcinoma MCF-7/ADR cells: role of c-myc and bcl-2 in apoptotic cell death. J Cell Sci 110:681–686
6. Gupta AK, Lee YJ, Galoforo SS et al (1997) Differential effect of glucose deprivation on MAPK activation in drug sensitive human breast carcinoma MCF-7 and multidrug resistant MCF-7/ADR cells. Mol Cell Biochem 170:23–30
7. Liu X, Gupta AK, Corry PM et al (1997) Hypoglycemia-induced c-Jun phosphorylation is mediated by c-Jun N-terminal kinase 1 and Lyn kinase in drug resistant human breast carcinoma MCF-7/ADR cells. J Biol Chem 272:11690–11693
8. Nath KA, Ngo EO, Hebbel RP et al (1995) α-Ketoacids scavenge H_2O_2 in vitro and in vivo and reduce menadione-induced DNA injury and cytotoxicity. Am J Physiol 268:227–236
9. Tuttle SW, Varnes ME, Mitchell JB et al (1992) Sensitivity to chemical oxidants and radiation in CHO cell lines deficient in oxidative pentose cycle activity. Int J Radiat Oncol Biol Phys 22:671–675
10. Averill-Bates DA, Przybytkowski E (1994) The role of glucose in cellular defences against cytotoxicity of hydrogen peroxide in Chinese hamster ovary cells. Arch Biochem Biophys 312:52–58
11. Wang X, Perez E, Liu R et al (2007) Pyruvate protects mitochondria from oxidative stress in human neuroblastoma SK-N-SH cells. Brain Res 1132:1–9
12. Das UN (2006) Pyruvate is an endogenous anti-inflammatory and anti-oxidant molecule. Med Sci Monit 12:79–84
13. Lehninger AL (1975) Biochemistry: the molecular basis of cell structure and function, 2nd edn. Worth Publishers, New York
14. Berggren MI, Husbeck B, Samulitis B et al (2001) Thioredoxin peroxidase-1 (peroxiredoxin-1) is increased in thioredoxin-1 transfected cells and results in enhanced protection against apoptosis caused by hydrogen peroxide but not by other agents including dexamethasone, etoposide, and doxorubicin. Arch Biochem Biophys 392:103–109

15. Nomura K, Imai H, Koumura T et al (1999) Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. J Biol Chem 274:29294–29302
16. Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 51:794–798
17. Spitz DR, Sim JE, Ridnour LA et al (2000) Glucose deprivation-induced oxidative stress in human tumor cells. A fundamental defect in metabolism? Ann N Y Acad Sci 899:349–362
18. Simons AL, Ahmad IM, Mattson DM et al (2007) 2-Deoxy-D-glucose (2DG) combined with cisplatin enhances cytotoxicity via metabolic oxidative stress in human head and neck cancer cells. Cancer Res 67:3364–3370
19. Amornphimoltham P, Sriuranpong V, Patel V et al (2004) Persistent activation of the Akt pathway in head and neck squamous cell carcinoma: a potential target for UCN-01. Clin Cancer Res 10:4029–4037
20. Pedrero JM, Carracedo DG, Pinto CM et al (2005) Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma. Int J Cancer 114:242–248
21. Jones PF, Jakubowicz T, Hemmings BA (1991) Molecular cloning of a second form of rac protein kinase. Cell Regul 2:1001–1009
22. Cheng JQ, Godwin AK, Bellacosa A et al (1992) AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc Natl Acad Sci USA 89:9267–9271
23. Brodbeck D, Cron P, Hemmings BA (1999) A human protein kinase B with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. J Biol Chem 274:9133–9136
24. Coffer PJ, Woodgett JR (1991) Molecular cloning and characterization of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur J Biochem 201:475–481
25. Brazil DP, Hemmings BA (2001) Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci 26:657–664
26. Song G, Ouyang G, Bao S (2005) The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 9:59–71
27. Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2:489–501
28. Katso R, Okkenhaug K, Ahmadi K et al (2001) Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol 17:615–675
29. Vanhaesebroeck B, Waterfield MD (1999) Signaling by distinct classes of phosphoinositide 3-quinces. Exp Cell Res 253:239–254
30. Alessi DR, Cohen P (1998) Mechanism of activation and function of protein kinase B. Curr Opin Genet Dev 8:55–62
31. Sanchez-Margalet V, Goldfine ID, Vlahos CJ et al (1994) Role of phosphatidylinositol-3-kinase in insulin receptor signaling: studies with inhibitor, LY294002. Biochem Biophys Res Commun 204:446–452
32. Myers MP, Tonks NK (1997) PTEN: sometimes taking it off can be better than putting it on. Am J Hum Genet 61:1234–3128
33. Cantley LC, Neel BG (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 96:4240–4245
34. Maehama T, Dixon JE (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273: 13375–13378
35. Kohn AD, Summers SA, Birnbaum MJ et al (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271:31372–31378

36. Elstrom RL, Bauer DE, Buzzai M et al (2004) Akt stimulates aerobic glycolysis in cancer cells. Cancer Res 64:3892–3899
37. Robey RB, Hay N (2009) Is Akt the "Warburg kinase"? – Akt-energy metabolism interactions and oncogenesis. Semin Cancer Biol 19:25–31
38. Kim DI, Lim SK, Park MJ et al (2007) The involvement of phosphatidylinositol 3-kinase/Akt signaling in high glucose-induced downregulation of GLUT-1 expression in ARPE cells. Life Sci 80:626–632
39. Samih N, Hovsepian S, Aouani A et al (2000) Glut-1 translocation in FRTL-5 thyroid cells: role of phosphatidylinositol 3-kinase and N-glycosylation. Endocrinology 141:4146–4155
40. Clarke JF, Young PW, Yonezawa K et al (1994) Inhibition of the translocation of GLUT1 and GLUT4 in 3 T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. Biochem J 300:631–635
41. Okada T, Kawano Y, Sakakibara T et al (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and anti-lipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. J Biol Chem 269:3568–3573
42. Barthel A, Okino ST, Liao J et al (1999) Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. J Biol Chem 274:20281–20286
43. Miyamoto S, Murphy AN, Brown JH (2008) Akt mediates mitochondrial protection incardiomyocytes through phosphorylation of mitochondrial hexokinase-II. Cell Death Differ 15:521–529
44. Aubert-Foucher E, Font B, Gautheron DC (1984) Rabbit heart mitochondrial hexokinase: solubilization and general properties. Arch Biochem Biophys 232:391–399
45. Vander Heiden MG, Plas DR, Rathmell JC et al (2001) Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol Cell Biol 21:5899–5912
46. Deprez J, Vertommen D, Alessi DR et al (1997) Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J Biol Chem 272:17269–17275
47. Pelicano H, Xu RH, Du M et al (2006) Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechanism. J Cell Biol 175:913–923
48. Kaplan O, Jaroszewski JW, Faustino PJ et al (1990) Toxicity and effects of epidermal growth factor on glucose metabolism of MDA-468 human breast cancer cells. J Biol Chem 265:13641–13649
49. Weihua Z, Tsan R, Huang WC et al (2008) Survival of cancer cells is maintained by EGFR independent of its kinase activity. Cancer Cell 13:385–393
50. Anderson ME (1985) Handbook of methods for oxygen radical research. CRC, Florida
51. Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106:207–212
52. Lowry OH, Rosebrough NJ, Farr AL et al (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
53. Spitz D, Malcolm R, Robert R (1990) Cytotoxicity and metabolism of 4-hydroxy-2-nonenol and 2-nonenol in H_2O_2-resistant cell lines. Do aldehydic by-products of lipid peroxidation contribute to oxidative stress? Biochem J 267:453–459
54. Holmgren A, Bjornstedt M (1995) Thioredoxin and thioredoxin reductase. Methods Enzymol 252:199–208
55. Ahmad IM, Aykin-Burns N, Sim JE et al (2005) Mitochondrial superoxide and hydrogen peroxide mediate glucose deprivation-induced cytotoxicity and oxidative stress in human cancer cells. J Biol Chem 280:4254–4263
56. Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30:1191–1212
57. Knobbe CB, Reifenberger G (2003) Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3- kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol 13:507–518
58. Bellacosa A, de Feo D, Godwin AK et al (1995) Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer 64:280–285

59. Cheng JQ, Ruggeri B, Klein WM et al (1996) Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc Natl Acad Sci USA 93:3636–3641
60. Staal SP (1987) Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci USA 84:5034–5037
61. Samuels Y, Wang Z, Bardelli A et al (2004) High frequency of mutations of the PIK3CA gene in human cancers. Science 304:554
62. Shayasteh L, Lu Y, Kuo WL et al (1999) PI3CA is implicated as an oncogene in ovarian cancer. Nat Genet 21:99–102
63. Ma YY, Wei SJ, Lin YC et al (2000) PIK3CA as an oncogene in cervical cancer. Oncogene 19:2739–2744
64. Woenckhaus J, Steger K, Werner E et al (2002) Genomic gain of PIK3CA and increased expression of p110alpha are associated with progression of dysplasia into invasive squamous cell carcinoma. J Pathol 198:335–342
65. Byun DS, Cho K, Ryu BK et al (2003) Frequent monoallelic deletion of PTEN and its reciprocal association with PIK3CA amplification in gastric carcinoma. Int J Cancer 104:318–327
66. Nassif NT, Lobo GP, Wu X et al (2004) PTEN mutations are common in sporadic microsatellite stable colorectal cancer. Oncogene 23:617–628
67. Frisk T, Foukakis T, Dwight T et al (2002) Silencing of the PTEN tumor-suppressor gene in anaplastic thyroid cancer. Genes Chromosomes Cancer 35:74–80
68. Garcia JM, Silva JM, Dominguez G et al (1999) Allelic loss of the PTEN region (10q23) in breast carcinomas of poor pathophenotype. Breast Cancer Res Treat 57:237–243
69. Wang DS, Rieger-Christ K, Latini JM et al (2000) Molecular analysis of PTEN and MXI1 in primary bladder carcinoma. Int J Cancer 88:620–625
70. Ittmann MM (1998) Chromosome 10 alterations in prostate adenocarcinoma. Oncol Rep 5:1329–1335
71. An HJ, Logani S, Isacson C et al (2004) Molecular characterization of uterine clear cell carcinoma. Mod Pathol 17:530–537
72. Rasheed BK, Wiltshire RN, Bigner SH et al (1999) Molecular pathogenesis of malignant gliomas. Curr Opin Oncol 11:162–167
73. Saito T, Oda Y, Kawaguchi K et al (2004) PTEN and other tumor suppressor gene mutations as secondary genetic alterations in synovial sarcoma. Oncol Rep 11:1011–1015
74. Goel A, Arnold CN, Niedzwiecki D et al (2004) Frequent inactivation of PTEN by promoter hypermathylation in microsatellite instablity-high sporadic colorectal cancers. Cancer Res 64:3014–3021
75. Li J, Yen C, Liaw D et al (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943–1947
76. Steck PA, Pershouse MA, Jasser SA et al (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 15:356–362
77. Chu EC, Tarnawski AS (2004) PTEN regulatory functions in tumor suppression and cell biology. Med Sci Monit 10:235–241
78. Olayioye MA, Neve RM, Lane HA et al (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 19:3159–3167
79. Siegel PM, Ryan ED, Cardiff RD et al (1999) Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer. EMBO J 18:2149–2164
80. Zhou BP, Hu MC, Miller SA et al (2000) HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. J Biol Chem 275:8027–8031
81. Grandis JR, Tweardy DJ (1993) Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer. Cancer Res 53:3579–3584

82. Rodrigo JP, Ramos S, Lazo PS et al (1996) Amplification of ERBB oncogenes in squamous cell carcinomas of the head and neck. Eur J Cancer 32A:2004–2010
83. Grandis J, Sok J (2004) Signaling through the epidermal growth factor receptor during the development of malignancy. Pharmacol Ther 102:37–46
84. Kalyankrishna S, Grandis J (2006) Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol 24:2666–2672
85. Rogers S, Harrington K, Rhys Evans P et al (2005) Biological significance of c-erbB family oncogenes in head and neck cancer. Cancer Metastasis Rev 24:47–69
86. Bei R, Budillon A, Masuelli L et al (2004) Frequent overexpression of multiple ErbB receptors by head and neck squamous cell carcinoma contrasts with rare antibody immunity in patients. J Pathol 204:317–325
87. Lo H, Hung M (2007) Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. Br J Cancer 96(Suppl):16–20
88. Bianco R, Shin I, Ritter CA et al (2003) Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. Oncogene 22:2812–2822
89. She QB, Solit D, Basso A et al (2003) Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3′-kinase/Akt pathway signaling. Clin Cancer Res 9:4340–4346
90. Vlahos CJ, Matter WF, Hui KY et al (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269:5241–5248
91. Riley JK, Carayannopoulos MO, Wyman AH et al (2006) Phosphatidylinositol 3-kinase activity is critical for glucose metabolism and embryo survival in murine blastocysts. J Biol Chem 281:6010–6019
92. Melstrom LG, Salabat MR, Ding XZ et al (2008) Apigenin inhibits the GLUT-1 glucose transporter and the phosphoinositide 3-kinase/Akt pathway in human pancreatic cancer cells. Pancreas 37:426–431
93. Hilgard P, Klenner T, Stekar J et al (1997) D-21266, a new heterocyclic alkylphospholipid with antitumour activity. Eur J Cancer 33:442–446
94. Maly K, Uberall F, Schubert C et al (1995) Interference of new alkylphospholipid analogues with mitogenic signal transduction. Anticancer Drug Des 10:411–425
95. Berkovic D (1998) Cytotoxic etherphospholipid analogues. Gen Pharmacol 31:511–517
96. Kondapaka SB, Singh SS, Dasmahapatra GP et al (2003) Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Mol Cancer Ther 2:1093–1103
97. Crul M, Rosing H, de Klerk GJ et al (2002) Phase I and pharmacological study of daily oral administration of perifosine (D-21266) in patients with advanced solid tumours. Eur J Cancer 38:1615–1621
98. Van Ummersen L, Binger K, Volkman J et al (2004) A phase I trial of perifosine (NSC 639966) on a loading dose/maintenance dose schedule in patients with advanced cancer. Clin Cancer Res 10:7450–7456
99. Bailey HH, Mahoney MR, Ettinger DS et al (2006) Phase II study of daily oral perifosine in patients with advanced soft tissue sarcoma. Cancer 107:2462–2467
100. Argiris A, Cohen E, Karrison T et al (2006) A phase II trial of perifosine, an oral alkylphospholipid, in recurrent or metastatic head and neck cancer. Cancer Biol Ther 5:766–770
101. Knowling M, Blackstein M, Tozer R et al (2006) A phase II study of perifosine (D-21226) in patients with previously untreated metastatic or locally advanced soft tissue sarcoma: A National Cancer Institute of Canada Clinical Trials Group trial. Invest New Drugs 24:435–439
102. Leighl NB, Dent S, Clemons M et al (2008) A Phase 2 study of perifosine in advanced or metastatic breast cancer. Breast Cancer Res Treat 108:87–92
103. Leleu X, Gay J, Roccaro AM et al (2009) Update on therapeutic options in Waldenström macroglobulinemia. Eur J Haematol 82:1–12

104. Perez-Soler R, Chachoua A, Hammond LA et al (2004) Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. J Clin Oncol 22:3238–3247
105. Bulgaru AM, Mani S, Goel S et al (2003) Erlotinib (Tarceva): a promising drug targeting epidermal growth factor receptor tyrosine kinase. Expert Rev Anticancer Ther 3:269–279
106. Leslie NR, Bennett D, Lindsay YE et al (2003) Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J 22:5501–5510
107. Leslie NR (2006) The redox regulation of PI 3-kinase-dependent signaling. Antioxid Redox Signal 8:1765–1774
108. Kaneki M, Shimizu N, Yamada D et al (2007) Nitrosative stress and pathogenesis of insulin resistance. Antioxid Redox Signal 9:319–329
109. Yasukawa T, Tokunaga E, Ota H et al (2005) S-nitrosylation-dependent inactivation of Akt/protein kinase B in insulin resistance. J Biol Chem 280:7511–7518
110. Clerkin JS, Naughton R, Quiney C et al (2008) Mechanisms of ROS modulated cell survival during carcinogenesis. Cancer Lett 266:30–36
111. Nogueira V, Park Y, Chen CC et al (2008) Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. Cancer Cell 14:458–470
112. Spitz DR, Kinter MT, Roberts RJ (1995) The contribution of increased glutathione content to mechanisms of oxidative stress resistance in hydrogen peroxide resistant hamster fibroblasts. J Cell Physiol 165:600–609
113. Arrick BA, Griffith OW, Cerami A (1981) Inhibition of glutathione synthesis as a chemotherapeutic strategy for trypanosomiasis. J Exp Med 153:720–725
114. Andringa KK, Coleman MC, Aykin-Burns N et al (2006) Inhibition of glutamate cysteine ligase (GCL) activity sensitizes human breast cancer cells to the toxicity of 2-deoxy-D-glucose. Cancer Res 66:1605–1610
115. Bailey HH (1998) L-S, R-buthionine sulfoximine: historical development and clinical issues. Chem Biol Interact 111:39–54
116. Greer EL, Brunet A (2005) FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene 24:7410–7425
117. Zhang W, Patil S, Chauhan B et al (2006) FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. J Biol Chem 281:10105–10117
118. Laszlo J, Humphreys SR, Goldin A (1960) Effects of glucose analogues (2-deoxy-D-glucose, 2-deoxy-D-galactose) on experimental tumors. J Natl Cancer Inst 24:267–280
119. Shenoy MA, Singh BB (1985) Non-nitro radiation sensitizers. Int J Radiat Biol 48:315–326
120. Landau BR, Lubs HA (1958) Animal responses to 2- deoxyglucose administration. Proc Soc Exp Biol Med 99:124–127
121. Dwarkanath BS, Zolzer F, Chandana S et al (2001) Heterogeneity in 2-deoxy-D-glucose-induced modifications in energetics and radiation responses of human tumor cell lines. Int J Radiat Oncol Biol Phys 50:1051–1061
122. Suzuki M, O'Dea JD, Suzuki T et al (1993) 2-Deoxyglucose as a substrate for glutathione regeneration in human and ruminant red blood cells. Comp Biochem Physiol B 75:195–197
123. Mohanti BK, Rath GK, Anantha N et al (1996) Improving cancer radiotherapy with 2-deoxy-D-glucose: phase I/II clinical trials on human cerebral gliomas. Int J Radiat Oncol Biol Phys 35:103–111
124. Blackburn RV, Spitz DR, Liu X et al (1999) Metabolic oxidative stress activates signal transduction and gene expression during glucose deprivation in human tumor cells. Free Radic Biol Med 26:419–430
125. Lin X, Zhang F, Bradbury CM et al (2003) 2-Deoxy-D-Glucose-induced cytotoxicity and radiosensitization in tumor cells is mediated via disruptions in thiol metabolism. Cancer Res 63:3413–3417
126. Mustacich D, Powis G (2007) Thioredoxin reductase. Biochem J 346:1–8

127. Becker K, Gromer S, Schirmer RH et al (2000) Thioredoxin reductase as a pathophysiological factor and drug target. Eur J Biochem 267:6118–6125
128. Powis G, Kirkpatrick DL, Angulo M et al (1998) Thioredoxin redox control of cell growth and death and the effects of inhibitors. Chem Biol Interact 112:23–34
129. Smart DK, Ortiz KL, Mattson D et al (2004) Thioredoxin reductase as a potential molecular target for anticancer agents that induce oxidative stress. Cancer Res 64:6716–6724
130. Nguyen P, Awwad RT, Smart DD et al (2006) Thioredoxin reductase as a novel molecular target for cancer therapy. Cancer Lett 236:164–174

http://www.springer.com/978-1-61779-396-7

Oxidative Stress in Cancer Biology and Therapy
Spitz, D.R.; Dornfeld, K.J.; Krishnan, K.; Gius, D. (Eds.)
2012, XVIII, 462 p., Hardcover
ISBN: 978-1-61779-396-7
A product of Humana Press