

# 2-Deoxyglucose induces Akt phosphorylation via a mechanism independent of LKB1/AMP-activated protein kinase signaling activation or glycolysis inhibition

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## Abstract

The compound 2-deoxyglucose (2-DG) enhances chemotherapy/radiotherapy in cell lines and animal models, prompting two phase I clinical trials with this cancer therapeutic. Although its mechanism of action has not been fully elucidated, it is hypothesized that the molecular basis of 2-DG activity is related to glycolysis inhibition. Here, we report that 2-DG induced Akt phosphorylation at Thr<sup>308</sup> and Ser<sup>473</sup> as early as 15 min post-treatment. These phosphorylation events required phosphatidylinositol-3-kinase activity but were not related to LKB1/AMP-activated protein kinase signaling, the inhibition of glycolysis or epidermal growth factor receptor signaling. The 2-DG-mediated Akt phosphorylation also led to the phosphorylation of Akt downstream targets, such as Foxo3a, GSK3 $\beta$ , and Chk1. Because the functional consequence of Akt activation includes chemotherapy/radiotherapy resistance, our data suggested that the combination of phosphatidylinositol-3-kinase/Akt inhibitory agents in 2-DG-based chemotherapy/radiotherapy may result in enhanced therapeutic efficacy. [Mol Cancer Ther 2008;7(4):809–17]

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## Introduction

2-Deoxyglucose (2-DG) is best known as an inhibitor of glucose metabolism (1). Inside a cell, it is converted to phosphorylated 2-DG (2-DG-P) by hexokinase, the first and the rate-limiting enzyme in glycolysis. However, 2-DG-P cannot be metabolized by the second enzyme in glycolysis, phosphoglucose isomerase (2). This leads to trapping and accumulation of 2-DG-P, which competitively inhibits hexokinase at the rate-limiting step of glycolysis. A direct consequence of 2-DG treatment is intracellular ATP depletion (3, 4). Because one of the primary hallmarks of cancer is that tumor cells are more dependent on their aerobic glycolytic metabolism to generate ATP (Warburg effect; ref. 5), 2-DG was evaluated as a single agent for cancer therapy. Whereas 2-DG is cytotoxic *in vitro*, *in vivo* studies with xenografts indicated that 2-DG treatment did not enhance tumor cell killing as a single agent (6).

The inhibition of glycolysis by 2-DG treatment has indirect effects on various signaling pathways. For example, glucose withdrawal and 2-DG treatment inhibit mammalian target of rapamycin (mTOR) signaling (7), which is mediated by LKB1/AMP-activated protein kinase (AMPK) signaling, an energetic stress-sensing signaling pathway. Specifically, the inhibition of glycolysis by 2-DG treatment leads to a decrease in intracellular ATP concentration but an increase in intracellular AMP concentration. AMP can bind to AMPK and alter AMPK conformation resulting in AMPK activation by its upstream regulator, LKB1, via phosphorylation of AMPK at Thr<sup>172</sup> (8–10). Activated AMPK then phosphorylates TSC2, activates its GAP activity, and inhibits Rheb-mediated mTOR (raptor) activation (11, 12). In addition, the inhibition of glycolysis by 2-DG also induces oxidative stress and disrupts thiol metabolism (13, 14), suggesting that metabolic oxidative stress caused by reactive oxygen species may be related to 2-DG-induced cytotoxicity in transformed cells.

In this study, we discovered that 2-DG treatment led to Akt phosphorylation at Thr<sup>308</sup> and Ser<sup>473</sup>, both of which are required for Akt activation (15). The purpose of this study is to determine whether 2-DG-induced Akt activation is a consequence of LKB1/AMPK signaling activation, glycolysis inhibition, or phosphatidylinositol-3-kinase (PI3K) activation and whether this activation has functional consequences on downstream Akt targets. Furthermore, we explore the possibility if the inhibition of Akt activity enhances 2-DG-mediated cell growth inhibition.

## Materials and Methods

### Reagents and Antibodies

2-DG, ATP, bromopyruvic acid, 6-aminonicotinamide, oxythiamine chloride, sodium arsenate dibasic heptahydrate, sodium oxamate, and sodium fluoride were purchased from Sigma-Aldrich. Compound C (an inhibitor of AMPK; 10 mmol/L) solution was purchased from Calbiochem. Erlotinib [an epidermal growth factor receptor (EGFR) inhibitor] and LY294002 (a PI3K inhibitor) were purchased from LC Laboratories and dissolved in DMSO at a concentration of 10 mmol/L. Perifosine was supplied by Keryx Biopharmaceuticals. This agent was dissolved in PBS and stored at  $-20^{\circ}\text{C}$ . Mouse monoclonal antibody against LKB1 was purchased from Abcam. Antibody against phospho-Foxo3a (p-Foxo3a<sup>T32</sup>) was purchased from Upstate. Antibodies against total AMPK, phospho-AMPK $\alpha$  (p-AMPK<sup>T172</sup>), total Akt, phospho-Akt (p-Akt<sup>T308</sup>, p-Akt<sup>S473</sup>), phospho-p70S6K (p-S6K<sup>T389</sup>), phospho-GSK3 $\beta$  (p-GSK3 $\beta$ <sup>S9</sup>), and Chk1 were purchased from Cell Signaling Technology. Rabbit polyclonal anti-actin antibody was purchased from Sigma-Aldrich.

### Cell Lines and Cell Cultures

HeLa, HCT116, DLD1, MDA-MB-435, T-47D, MCF-7, H1299, H1792, H460, A549, H157, and H23 cell lines were purchased from the American Type Culture Collection and propagated according to the conditions recommended by American Type Culture Collection. Among these cell lines, LKB1 gene mutations were present in H460, A549, H157, and H23 cells (16).

### Western Blot Analysis

The procedures for preparation of whole-cell protein lysates and for Western blotting were done as described previously (16). Briefly, whole-cell protein lysates (20  $\mu\text{g}$ ) of total protein were electrophoresed through 10% denaturing polyacrylamide gels and transferred to the polyvinylidene difluoride membrane (Bio-Rad) using a semidry blotting technique according to the manufacturer's protocol. The membranes were blocked with 5% nonfat milk and probed with antibodies against the specified proteins. The same blots were used for probing phosphospecific antibodies and antibodies against total protein. Actin was used as loading controls.

### Small Interfering RNA Treatment

LKB1 small interfering RNA (siRNA) duplexes were purchased from Dharmacon. Lamin A/C siControl was used to control for any nonspecific off-target effects of siRNA transfection. The sequence of LKB1 siRNA was 5'-GGACUGACGUGUAGAACAATT-3'. Specifically, H1299 and HCT116 cells were grown to 60% to 70% confluence in six-well plates, Oligofectamine reagent (Invitrogen) was incubated with Opti-MEM1 reduced serum medium for 10 min, and a mixture of siRNA was then added. After incubation for 15 min at room temperature, the mixture was diluted with medium and added to each well. siRNA (200 pmol) was used per well in 2 mL medium. To improve gene silencing, we transfected the same cells twice with the same siRNA. Twenty-four hours after the second transfection, cells were washed and resuspended in new culture

medium in the presence or absence of 2-DG for desired length of time. Total cell lysates were used for Western blot analysis as described above.

### Immunofluorescence Analysis

Immunofluorescence of HeLa cells were done on cells plated onto circular glass coverslips placed into 24-well plates 24 h before treatment with 2-DG (25 mmol/L) and UV exposure (254 nm). The UV light source was provided from a 6 W UVP handheld UV lamp emitting 254 and 365 nm wavelengths (Fisher Scientific). Cells were fixed using freshly prepared fixative containing 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.4% Triton X-100 in PHEMO buffer [PIPES (0.068 mol/L), HEPES (0.025 mol/L), EGTA $\text{Na}_2$  (0.015 mol/L),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (0.003 mol/L), and DMSO (10% v/v), pH adjusted to 6.8] for 10 min at room temperature. Chk1 primary antibody was used at 1:100 (Cell Signaling Technology) with an overnight incubation at  $4^{\circ}\text{C}$  followed by a goat Alexa Fluor 488 secondary antibody used at 1:500 (Invitrogen) for 1 h at room temperature. The nucleus was stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) using 300 nmol/L at room temperature for 10 min before mounting in Gel Mount mounting medium (Biomedex). Fixed and immunofluorescently stained cells were imaged using a Zeiss LSM510 Meta (Zeiss) laser scanning confocal system configured to a Zeiss Axioplan 2 upright microscope with a 63xO (NA 1.4) plan-apochromat objective.

### Cell Growth Inhibition Assay

Cell growth inhibition was determined by the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instruction.

### ATP Luciferase Assay

Intracellular ATP concentration was measured by the ENLITEN ATP Assay System Bioluminescence Detection Kit (Promega) according to the manufacturer's instruction. In brief, HeLa cells were plated into a six-well plate at a density of  $2 \times 10^5$  per well. Cells were treated with either 25 mmol/L 2-DG or 50  $\mu\text{mol/L}$  3-bromopyruvate (3-BrPA) for 4 h the next day. Cells were washed twice with PBS, and intracellular ATP was extracted by cold 2% trichloroacetic acid and neutralized by adding Tris-acetate buffer [0.1 mol/L Tris, 2 mmol/L EDTA (pH 7.75)]. Sample (50  $\mu\text{L}$ ) was added to a tube containing 100  $\mu\text{L}$  rL/L reagent, and ATP concentration was then measured by luminometer against an ATP standard curve. Reactions were carried out in triplicates and ATP concentrations were expressed as percentage of untreated controls.

## Results

### 2-DG Treatment Led to Akt Phosphorylation

LKB1/AMPK are involved in sensing energy stress, and we have shown previously that 2-DG treatment leads to the phosphorylation of Thr<sup>172</sup> on AMPK $\alpha$  subunit in LKB1 wild-type cells in 15 min (16). Unexpectedly, we also observed the phosphorylation of Akt after 2-DG treatment in various cancer cell lines, such as HCT116, HeLa, T-47D, and several non-small cell lung cancer (NSCLC) cell lines

(Figs. 1 and 2A). The only exception is MCF-7, where minimal Akt phosphorylation was detected. The lack of Akt phosphorylation after 2-DG treatment in MCF-7 was reported previously (17) but is specific to MCF-7, as most cancer cell lines evaluated in our system had robust Akt phosphorylation after 2-DG treatment.

2-DG-induced Akt phosphorylation was detected 15 min after the addition of 2-DG in culture medium and was observed with 2.5 mmol/L 2-DG treatment in cell lines, such as H1792 and H1299 (Fig. 1B). Such phosphorylation also occurred on both Ser<sup>473</sup> and Thr<sup>308</sup> (Fig. 1C), both of which were necessary for Akt to activate its downstream targets (15).

### 2-DG-Induced Akt Phosphorylation Is Independent of LKB1/AMPK Signaling

The inhibition of mTOR (raptor) by rapamycin was recently shown to induce Akt phosphorylation (18, 19). Because 2-DG treatment leads to the activation of LKB1/

AMPK signaling, which can inhibit mTOR (raptor), we determined whether 2-DG-induced Akt phosphorylation requires LKB1/AMPK signaling. First, 2-DG-induced Akt phosphorylation was evaluated in various cancer cell lines with or without LKB1 biallelic inactivations (20), and the phosphorylation of AMPK at Thr<sup>172</sup> was used to evaluate the functional status of LKB1 protein. As expected, 2-DG treatment induced AMPK phosphorylation only in LKB1 wild-type H1299, H1792, and Calu-1 NSCLC cells as well as HCT116 colorectal cancer cells but not in LKB1 mutant cell lines, such as A549, H460, H157, and H23 (Fig. 2A). In contrast, Akt phosphorylation was induced by 2-DG treatment in all eight cell lines, suggesting that 2-DG-induced Akt phosphorylation is independent of LKB1 function.

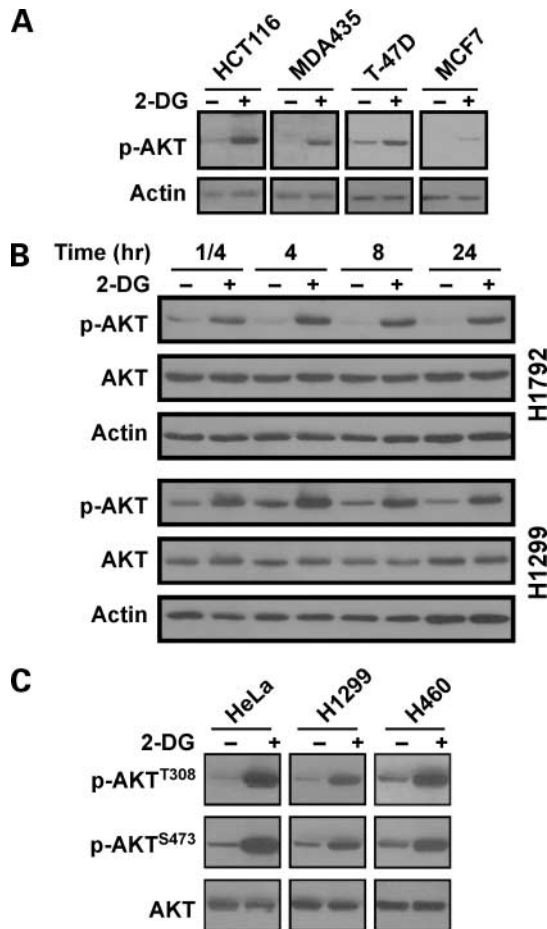
To definitively evaluate the role of LKB1 in 2-DG-induced Akt phosphorylation, LKB1 siRNA was transiently transfected into LKB1 wild-type cell lines, H1299 and HCT116, to deplete LKB1 expression, and transient depletion of LKB1 by RNA interference did not alter the induction of Akt phosphorylation by 2-DG in either cell lines (Fig. 2B).

mTOR is involved in the regulation of translation machinery, and one of the direct downstream substrates of mTOR is S6 kinase (S6K), which is phosphorylated by activated mTOR at Thr<sup>389</sup>. The inhibition of mTOR signaling by rapamycin, for example, led to a decrease in phosphorylated S6K (18). In H1299 cells, 2-DG treatment resulted not only an increase in AMPK phosphorylation but also a decrease in S6K phosphorylation (Fig. 2C), which was consistent with the notion that 2-DG-induced activation of LKB1/AMPK signaling inhibited mTOR activity.

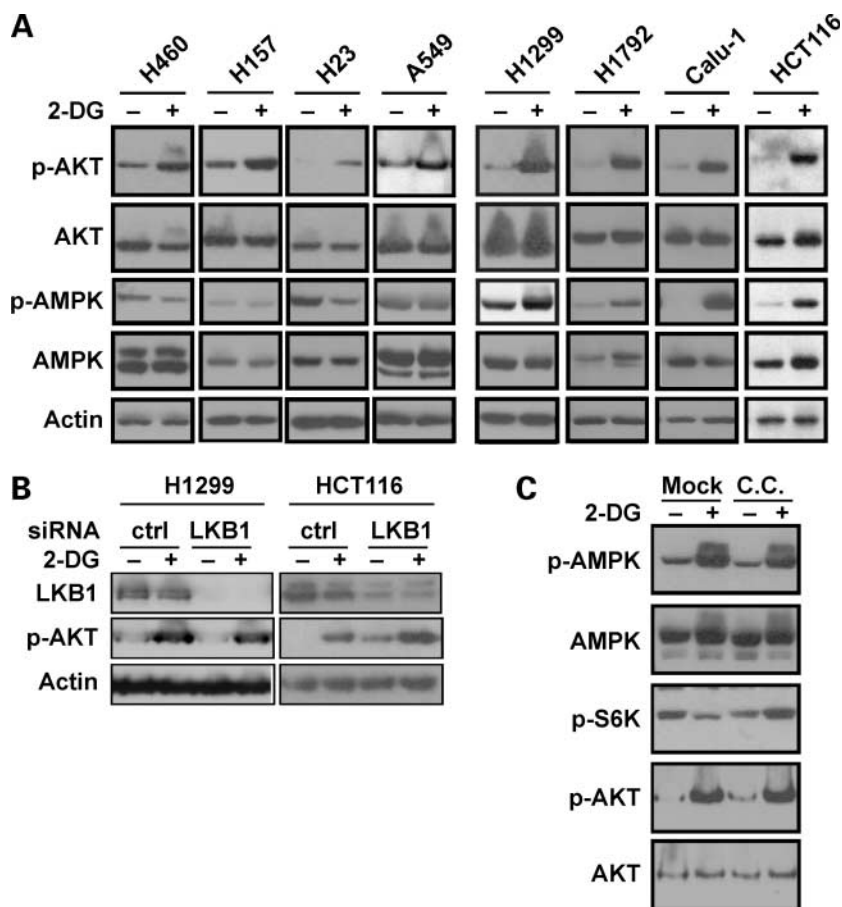
Compound C is small molecule developed by Merck that has been shown to be able to inhibit AMPK activity (21). H1299 cells were pretreated with 10  $\mu$ mol/L compound C for 30 min before 2-DG treatment, and this treatment did not affect AMPK phosphorylation at Thr<sup>172</sup> but alleviated S6K dephosphorylation after 2-DG treatment, indicating that LKB1/AMPK signaling was blocked in the presence of this chemical. In contrast, 2-DG-induced Akt phosphorylation was not affected by compound C, further confirming the notion that 2-DG-induced Akt phosphorylation is independent of LKB1/AMPK signaling activation.

### 2-DG-Induced Akt Phosphorylation Is Independent of Glycolysis Inhibition

2-DG is metabolized by hexokinase to 2-DG-P, and intracellular accumulation of 2-DG-P competitively inhibits hexokinase. We also evaluated another inhibitor of hexokinase, 3-BrPA, which directly inhibits hexokinase (22). Although this chemical can induce Akt phosphorylation in H1299, it failed to do so in HeLa cells, suggesting that in HeLa cells 2-DG-induced Akt phosphorylation was not dependent on hexokinase inhibition (Fig. 3A). We also evaluated intracellular ATP concentrations with a luciferase assay in HeLa cells after either 2-DG or 3-BrPA treatment. Similar to previous report (23), intracellular ATP level was decreased by 76% after 25 mmol/L 2-DG treatment and by 98% after 50  $\mu$ mol/L 3-BrPA treatment in



**Figure 1.** Induction of Akt phosphorylation by 2-DG treatment. **A**, HCT116, MDA-MB-435, T-47D, and MCF-7 were treated with 25 mmol/L 2-DG for 24 h, and Akt phosphorylation was detected by anti-p-Akt<sup>S473</sup> antibody. Actin was used as loading controls. **B**, a time course of Akt phosphorylation at Ser<sup>473</sup> with 2.5 mmol/L 2-DG treatment in H1299 and H1792 cell lines. **C**, 2-DG-induced Akt phosphorylation at both Thr<sup>308</sup> and Ser<sup>473</sup> in HeLa, H1299, and H469 cell lines.



**Figure 2.** 2-DG-induced Akt phosphorylation is independent of LKB1/AMPK-mediated regulation of mTOR. **A**, cancer cell lines with either mutant (H460, H157, H23, and A549) or wild-type LKB1 (H1299, H1792, Calu-1, and HCT116) were treated with 25 mmol/L 2-DG for 4 h. Anti-p-Akt<sup>S473</sup> antibody was used to detect Akt phosphorylation, and anti-p-AMPK<sup>T172</sup> was used to detect AMPK phosphorylation. Total Akt, total AMPK, and actin were used as loading controls. **B**, LKB1 knockdown with siRNA did not affect 2-DG-induced Akt activation. siRNA against LKB1 was used to down-regulate LKB1 expression in H1299 and HCT116, and LKB1 antibody was used to evaluate the extent of LKB1 down-regulation. Cells were treated with 25 mmol/L 2-DG for 24 h, and Akt phosphorylation was assessed with anti-p-Akt<sup>S473</sup> antibody. **C**, compound C can block 2-DG-mediated S6K dephosphorylation but not 2-DG-induced Akt phosphorylation. H1299 cells were pretreated with 10  $\mu$ mol/L compound C for 30 min before 2-DG treatment. Cell lysates were collected 24 h after 2-DG treatment, and phosphorylation of AMPK, S6K, and Akt was assessed with anti-p-AMPK<sup>S172</sup>, S6K<sup>T389</sup>, and Akt<sup>S473</sup> antibodies using total AMPK and Akt as loading controls.

our experimental setting (Fig. 3B). As 3-BrPA failed to induce Akt phosphorylation in HeLa cells (Fig. 3A), 2-DG-induced Akt phosphorylation was not caused by the depletion of intracellular ATP. We also evaluated inhibitors to other enzymes in the glycolytic pathway. For example, oxxythiamine chloride is an inhibitor of transketolase-like-1 (24), but it also failed to induce Akt phosphorylation in HeLa cells (Fig. 3A). The remaining glycolytic inhibitors tested in our study failed to induce Akt phosphorylation in either H1299 or HeLa cell lines. Therefore, 2-DG was the only compound that consistently induced Akt phosphorylation in both cell lines.

An indirect consequence of glycolysis inhibition is the increase in metabolic oxidative stress (25, 26), and 2-DG treatment was also found to disrupt thiol metabolism by causing decreases in intracellular total glutathione content (14). As the effect of 2-DG on thiol metabolism can be blocked by the thiol antioxidant *N*-acetylcysteine (NAC; ref. 14), we determined whether the inclusion of NAC in culture medium was capable of blocking 2-DG-induced Akt phosphorylation. NAC treatment failed to alter 2-DG-induced Akt phosphorylation in H460 and H1299 cells, suggesting that the alteration of metabolic oxidative stress was not responsible for 2-DG-induced Akt phosphorylation in H1299 and H460 cells. NAC did partially block 2-DG-induced Akt phosphorylation in HeLa cells.

### 2-DG-Induced Akt Phosphorylation Is Mediated through PI3K but Not EGFR Signaling

We next determined whether 2-DG induced Akt phosphorylation via EGFR signaling. H1650 NSCLC cells contained a deletion in exon 19 of the *EGFR* gene (DelE746A750), an activation mutation that resulted in constitutive activation of Akt phosphorylation (27). Inhibition of this mutant EGFR by erlotinib resulted in a significant decrease of Akt phosphorylation as expected (Fig. 4A). On the other hand, the inhibition of PI3K function by a chemical inhibitor, LY294002, only led to a slight decrease in Akt phosphorylation in H1650 cells. This observation is similar to a previous study that compared the effect of gefitinib and LY294002 on Akt phosphorylation in NSCLC cells with EGFR mutations (28). In combination, these data suggest that activated EGFR mutation stimulated Akt phosphorylation mostly via the Ras-mitogen-activated protein kinase pathway but not PI3K pathways in H1650 cells. 2-DG treatment induced Akt phosphorylation in H1650 cells in the presence of erlotinib, indicating that 2-DG-induced Akt phosphorylation is independent of EGFR function (Fig. 4A).

To determine whether 2-DG-induced Akt phosphorylation is mediated by PI3K, we first evaluated the effect of LY294002 in H520 NSCLC cells, which contained 5- to 10-fold gene amplification of PIK3CA gene and had

elevated endogenous Akt phosphorylation (Fig. 4B; ref. 29). In contrast to H1650 cells, erlotinib did not affect Akt phosphorylation but LY294002 significantly reduced Akt phosphorylation in H520 cells. Furthermore, LY294002 was capable of blocking 2-DG-induced Akt phosphorylation in H520 cells, suggesting that 2-DG-induced Akt phosphorylation is dependent on PI3K function. To determine whether LY294002 was capable of blocking 2-DG-mediated Akt phosphorylation in other cancer cell lines, H1299, H1792, HCT116, and DLD1 cells were pretreated with or without LY294002 for 30 min before 2-DG treatment. In the absence of LY294002, 2-DG treatment led to increased Akt phosphorylation. In the presence of LY294002, basal level of Akt phosphorylation was abolished, and 2-DG treatment failed to increase Akt phosphorylation level (Fig. 4C). Compound C was used as a control in this experiment, and it failed to alter 2-DG-induced Akt phosphorylation as expected. These results suggest that PI3K activity is required for 2-DG-induced Akt phosphorylation.

#### 2-DG-Induced Akt Activation Led to Foxo3a, GSK3 $\beta$ , and Chk1 Phosphorylation

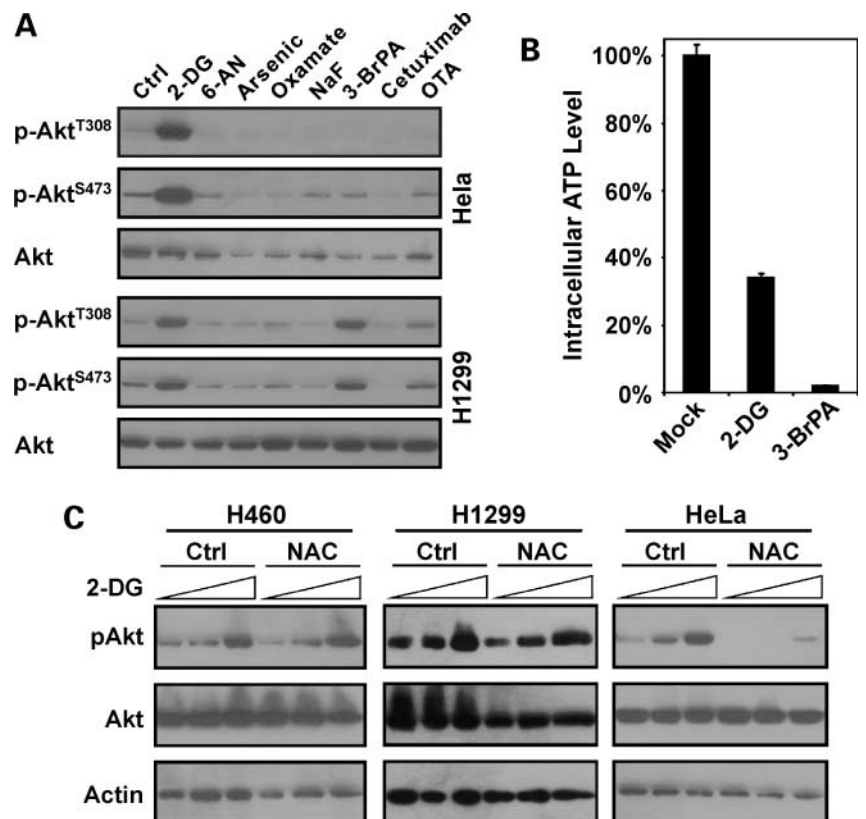
Activated Akt has many downstream targets and we evaluated the phosphorylation of Foxo3a at Thr<sup>32</sup> and GSK3 $\beta$  at Ser<sup>9</sup> with phosphospecific antibodies. Foxo3a phosphorylation was detected in HCT116, MDA-MB-435, H1792, and H1299 after 2-DG treatment (Fig. 5A and B), and GSK3 $\beta$  phosphorylation was also detected in these cell lines, except H1299. We observed a slight increase in Akt

phosphorylation in MCF-7 cells and a corresponding increase in Foxo3a phosphorylation after 2-DG treatment. However, GSK3 $\beta$  phosphorylation was not altered in MCF-7 cells with similar treatment.

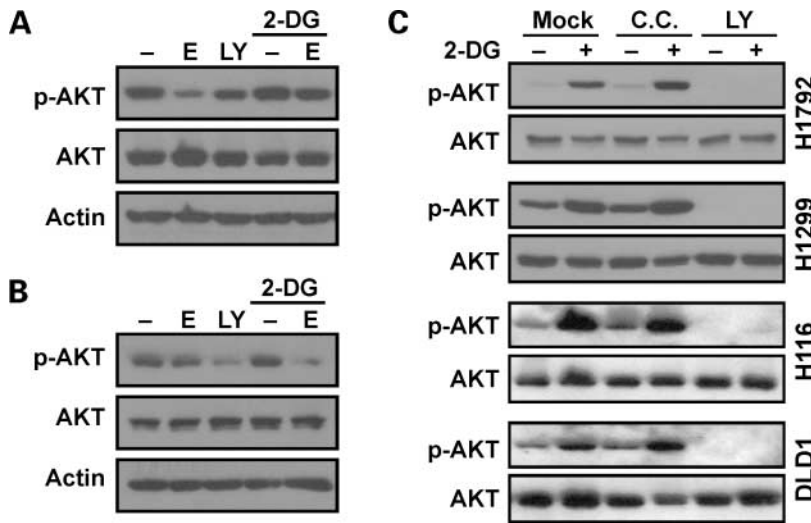
Another phosphorylation target site of activated Akt is Ser<sup>280</sup> of Chk1. Chk1 is a G<sub>2</sub>-M DNA damage checkpoint protein that is translocated into the nucleus when exposed to a DNA-damaging agent, such as UV light. Nuclear translocation of Chk1 can be inhibited by Akt phosphorylation, such that Chk1 remains in the cytoplasm and its G<sub>2</sub>-M checkpoint function is abolished (30, 31). To determine whether 2-DG-mediated Akt phosphorylation inhibits Chk1 function by preventing Chk1 nuclear induction, HeLa cells were exposed to UV light in the presence of 2-DG. In control untreated cells, Chk1 remained in the cytoplasm as when cells were treated with 2-DG alone. Exposing HeLa cells to UV light resulted in Chk1 nuclear localization within 7 h as expected (Fig. 5C). In contrast, preincubation of UV-exposed cells with 2-DG inhibited Chk1 nuclear accumulation, indicating that 2-DG treatment blocked DNA damage-induced Chk1 nuclear translocation.

#### Inhibition of Akt Enhances 2-DG-Mediated Growth Inhibition

The inhibition of glycolysis by 2-DG negatively affects cell growth (32) but activation of Akt enhanced survival (33); therefore, 2-DG-mediated growth inhibition may be partially offset by 2-DG-mediated Akt activation. To investigate this possibility, we evaluated whether the



**Figure 3.** 2-DG-mediated Akt phosphorylation was not due to the inhibition of glycolysis. **A**, 2-DG was the only glycolytic inhibitor that induced Akt phosphorylation in different cell lines. HeLa and H1299 cells were treated with 25 mmol/L 2-DG, 5  $\mu$ mol/L 6-aminonicotinamide (6-AN), 0.1 mmol/L sodium arsenate, 1 mg/ml sodium oxamate, 5 mmol/L sodium fluoride, 50  $\mu$ mol/L 3-BrPA, 10 nmol/L cetuximab, or 20 mmol/L oxythiamine chloride for 4 h and cell lysates were probed with indicated antibodies. **B**, 2-DG-induced Akt phosphorylation was not related to ATP depletion. HeLa cells were treated with either 25 mmol/L 2-DG or 50  $\mu$ mol/L 3-BrPA for 4 h, and intracellular ATP concentrations were measured by ATP luciferase assay. Reactions were carried out in triplicates and ATP concentrations were expressed as percentage of untreated controls. **C**, effect of NAC pretreatment on 2-DG-induced Akt phosphorylation. H460, H1299, and HeLa cells were pretreated with 30  $\mu$ mol/L NAC for 30 min before the addition of 0, 2.5, and 25 mmol/L 2-DG. Cell lysates were collected 4 h after 2-DG treatment and probed with anti-Akt<sup>S473</sup> antibody using total Akt and actin as loading controls.



**Figure 4.** 2-DG-induced Akt phosphorylation can be inhibited by LY294002. **A**, H1650 cells were pretreated with either 25  $\mu\text{mol/L}$  erlotinib (*E*) or 10  $\mu\text{mol/L}$  LY294002 (*LY*) for 30 min before the addition of 25 mmol/L 2-DG. Cells were harvested 4 h after 2-DG treatment. **B**, H520 cells were pretreated with either 25  $\mu\text{mol/L}$  erlotinib or 10  $\mu\text{mol/L}$  LY294002 for 30 min before the addition of 25 mmol/L 2-DG. Cells were harvested 4 h after 2-DG treatment. **C**, H1792, H1299, HCT116, and DLD1 cell lines were pretreated with either 10  $\mu\text{mol/L}$  LY294002 or 10  $\mu\text{mol/L}$  compound C (*C.C.*) for 30 min before addition of 25 mmol/L 2-DG. Cells were harvested 24 h after 2-DG treatment and subjected to preparation of whole-cell protein lysates. Cell lysates were probed with anti-Akt<sup>S473</sup> antibody using either total Akt or actin as loading controls in Western blot analysis.

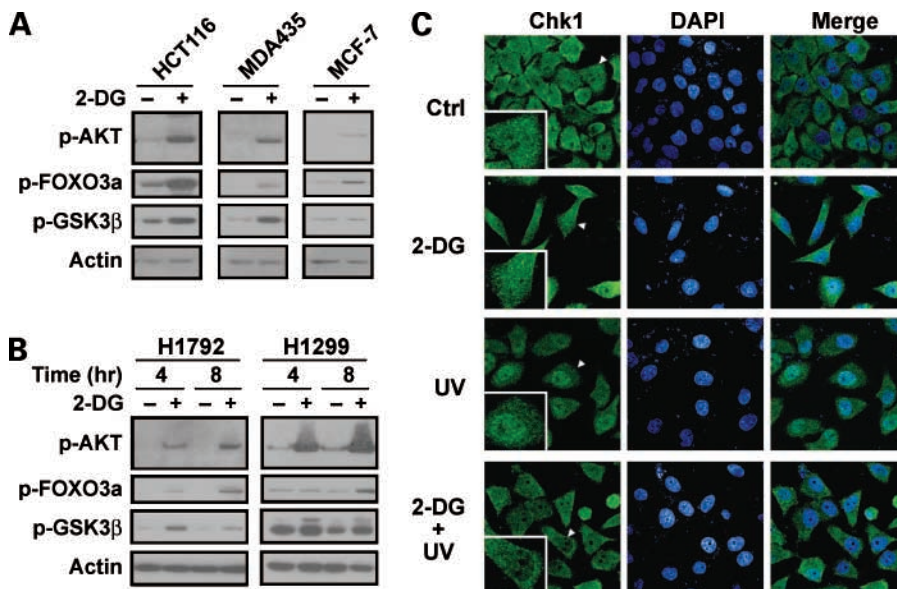
inhibition of Akt can enhance 2-DG-mediated growth inhibition. We showed previously that 10  $\mu\text{mol/L}$  LY294002 is sufficient to block 2-DG-induced Akt activation (Fig. 4), and this treatment only led to 23% growth inhibition after 24 h of treatment in H1299 cells (Fig. 6A). The presence of either 0.25 or 2.5 mmol/L 2-DG alone in the medium also led to 19% or 35% growth inhibition, respectively. The combination 2-DG and LY294002, however, significantly enhanced the growth inhibitory effect (Fig. 6A).

Akt function is also inhibited by an alkyl phospholipid, perifosine, and Akt inhibition is necessary for perifosine-induced apoptosis (34). We showed that the presence of 5  $\mu\text{mol/L}$  perifosine was sufficient to inhibit Akt function in most cancer cell lines and the inclusion of 5  $\mu\text{mol/L}$  perifosine in the culture medium did not lead to significant

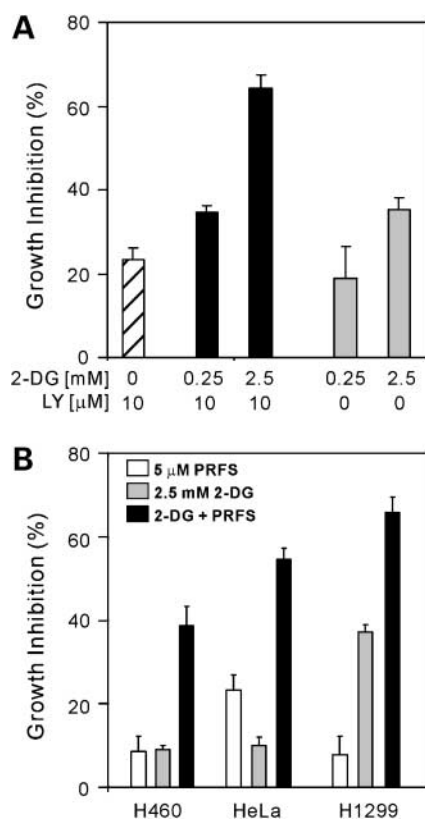
alterations in cell growth (Fig. 6B). This compound, however, significantly enhanced 2-DG-mediated growth inhibition compared with 2.5 mmol/L 2-DG alone in H460, HeLa, and H1299 cell lines, suggesting that inhibition of 2-DG-induced Akt activation by perifosine also enhanced 2-DG-mediated growth inhibition.

## Discussion

In this study, we showed that 2-DG treatment induced Akt phosphorylation. Akt phosphorylation can be detected as early as 15 min and can be sustained for at least 24 h in the presence of 2-DG. More importantly, 2-DG-induced Akt phosphorylation was observed in many cancer epithelial cell lines of different tissue origins, implying the presence of a common mechanism. Rapamycin inhibits



**Figure 5.** Phosphorylation of Foxo3a, GSK3 $\beta$ , and Chk1 by 2-DG-induced Akt activation. **A**, HCT116, MDA-MB-435, and MCF-7 cell lines were treated with 25 mmol/L 2-DG for 24 h before the collection of cell lysates that were analyzed by Western blot analysis for indicated proteins. **B**, H1792 and H1299 cell lines were treated with 25 mmol/L 2-DG for 4 and 8 h, and cell lysates were analyzed for indicated proteins. **C**, HeLa cells were treated with either 25 mmol/L 2-DG alone or UV exposure alone. In addition, HeLa cells were pretreated with 25 mmol/L 2-DG for 30 min before the exposure to UV irradiation. Chk1 cellular localization was analyzed by immunofluorescence, and 4',6'-diamidino-2-phenylindole staining was used to determine the nuclear localization.



**Figure 6.** Combination of 2-DG and Akt inactivating agents enhances growth inhibition. **A**, H1299 cells were seeded in 96-well plates and treated with the indicated concentration of 2-DG, 10  $\mu$ mol/L LY294002 alone, and their respective combination. Plates were subjected to MTS assay after 24 h. **B**, H460, HeLa, and H1299 cells were treated with 2.5 mmol/L 2-DG alone, 5  $\mu$ mol/L perifosine (PRFS) alone, and their respective combination. Plates were subjected to MTS assay after 48 h. Reactions were carried out in quadruples. Bars, 1 SD.

mTOR (raptor), and treatment of cancer cell lines with this compound has recently been shown to induce in Akt phosphorylation (18, 19). Because 2-DG treatment activates LKB1/AMPK signaling, which subsequently inhibits mTOR (raptor), we determined whether LKB1 function was involved in this process. Three lines of evidence, however, refuted a role of LKB1 in 2-DG-mediated Akt phosphorylation. First, Akt phosphorylation was observed in all NSCLC cell lines with either wild-type or inactivated mutant LKB1 (Fig. 2A). Second, inactivation of LKB1 by RNA interference did not alter 2-DG-induced phosphorylation of Akt (Fig. 2B). Third, AMPK is a downstream target of LKB1, and inhibition of AMPK by compound C alleviated mTOR inhibition by 2-DG but did not affect Akt phosphorylation (Fig. 2C). Therefore, neither the activation of LKB1/AMPK signaling nor the suppression of mTOR activity plays a role in 2-DG-induced Akt phosphorylation.

Akt activation has been shown to stimulate aerobic glycolysis (35), but it is unknown whether the inhibition of glycolysis can lead to Akt activation. The mechanistic

basis of 2-DG-mediated inhibition of glycolysis has been elucidated over a decade ago. Specifically, 2-DG-P is a competitive inhibitor of hexokinase, and 2-DG treatment targeted this rate-limiting enzyme in glycolysis. 3-BrPA is a direct inhibitor of hexokinase (22), and both 2-DG and 3-BrPA treatment resulted in intracellular ATP depletion as a consequence of glycolysis inhibition (Fig. 3B). However, 3-BrPA failed to induce Akt phosphorylation in HeLa cells, suggesting that the inhibition of hexokinase or the depletion of intracellular ATP concentration is not related to 2-DG-induced Akt phosphorylation. We also targeted other enzymes in the glycolytic pathways by inhibiting 6-phospho-D-gluconate:NADP oxidoreductase (6-PG dehydrogenase) with 6-aminonicotinamide (36), pyruvate dehydrogenase with arsenic (37), lactate dehydrogenase with sodium oxamate (38), transketolase-like-1 with oxythiamine, and enolase with sodium fluoride (39), yet most of them did not induce Akt phosphorylation, suggesting that the inhibition of glycolysis is not linked to Akt activation.

2-DG treatment was also shown to increase metabolic oxidative stress (14, 25, 26). Such stress can generate reactive oxygen species, which are capable of inducing Akt phosphorylation (40, 41). The effect of 2-DG on metabolic oxidative stress and thiol metabolism can be blocked by the thiol antioxidant NAC (14). Although NAC treatment reduced 2-DG-induced Akt phosphorylation in HeLa cells, this reagent failed to alter 2-DG-induced Akt phosphorylation in H1299 and H460 cells, implying that the alteration of metabolic oxidative stress is not related to 2-DG-induced Akt phosphorylation in H1299 and H460 cells.

To explore the possibility that 2-DG treatment leads to the production of an autocrine factor that is activating this pathway through EGFR signaling, we evaluated 2-DG-induced Akt phosphorylation in H1650 NSCLC cells, which contains an EGFR activation mutation. Erlotinib was capable of inhibiting EGFR-mediated Akt phosphorylation but failed to block 2-DG-induced Akt phosphorylation (Fig. 3). Hence, 2-DG-induced Akt phosphorylation was not initiated by EGFR signaling.

2-DG-induced Akt phosphorylation was observed at both Thr<sup>308</sup> and Ser<sup>473</sup> residues of Akt. Phosphorylation of Thr<sup>308</sup> at the catalytic T-loop is mediated by PDK1 (42), and phosphorylation of Ser<sup>473</sup> at the COOH-terminal hydrophobic motif has been shown recently to be mediated by the SIN1-ricor-mTOR complex (43). The presence of the PI3K inhibitor, LY294002, eliminated 2-DG-induced Akt phosphorylation at both phosphorylation sites, suggesting that PI3K activity is required for this process. This was consistent with previous observations that the phosphorylations at these two sites were dependent on PI3K-generated phosphatidylinositol-3,4,5-trisphosphate (44). LY294002 has been reported to be able to inhibit 2-DG uptake by inactivating PI3K (45, 46). Hence, LY294002 treatment may eliminate intracellular 2-DG accumulation, thus inhibiting 2-DG-induced Akt phosphorylation. Alternatively, 2-DG may directly activate PI3K or other upstream regulators of PI3K to activate Akt, and the presence of LY294002 prevents Akt phosphorylation by

inhibiting PI3K. In either case, the elucidation of such mechanism requires further investigation.

The phosphorylation of Ser<sup>473</sup> and Thr<sup>308</sup> has been linked to the activation of Akt protein function (47). Activated Akt has been shown previously to phosphorylate Foxo3a at Thr<sup>32</sup>, GSK3 $\beta$  at Ser<sup>9</sup>, and Chk1 at Ser<sup>280</sup>. Indeed, in our experimental system, 2-DG-induced Akt phosphorylation can lead to the phosphorylation of these target sites in many cancer cell lines, indicating that 2-DG treatment activated Akt function. The phosphorylation of these downstream proteins has been shown to inhibit their functions in inducing apoptosis, causing cell cycle arrests, and establishing DNA damage checkpoint (48). Consequently, Akt activation is often associated with cell survival and resistance to cancer therapy mostly due to its effects on these downstream targets. In this context, 2-DG-induced Akt activation will have an adverse side effect in 2-DG-based therapy.

Although 2-DG is not effective as a single agent *in vivo*, it was able to enhance chemosensitivity/radiosensitivity of cancer cells in combination therapies. *In vitro* studies with cancer cell lines indicated that 2-DG treatment can enhance ionizing radiation (14, 49–52) and carboplatin treatments (53). In addition, 2-DG enhanced anticancer activity of Adriamycin and paclitaxel in mice xenograft models bearing human tumors (6). A phase I/II clinical trial in India indicated that up to 5 mmol/L 2-DG can be detected in the blood of a patient following oral administration of 2-DG (54). The therapeutic potential of 2-DG has prompted enough interest in the United States, and there is an ongoing phase one clinical trial for this compound (ClinicalTrials.gov identifier: NCT00247403). Nonetheless, it is unknown to what extent is 2-DG enhanced chemosensitivity/radiosensitivity counteracted by 2-DG-induced Akt activation because the activation of Akt has been known to enhance chemoresistance of cancer cells. Our data indicated that 2-DG-mediated growth inhibition can be enhanced by inhibitors to either PI3K or Akt, and there is a potential that 2-DG-based chemotherapy/radiotherapy may be further improved with PI3K or Akt inhibitors.

In summary, we discovered that Akt activation is a novel consequence of 2-DG treatment but is unrelated to LKB1/AMPK signaling or the glycolysis inhibition. Because LY294002 can inhibit 2-DG-mediated Akt activation and enhance 2-DG-mediated growth inhibition, the inclusion of PI3K or Akt inhibitors may improve the therapeutic efficacy of 2-DG-based chemotherapy/radiotherapy.

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