

# Inhibition of glutamine utilization sensitizes lung cancer cells to apigenin-induced apoptosis resulting from metabolic and oxidative stress

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**Abstract.** Recent studies have shown anticancer activity of apigenin by suppressing glucose transporter 1 (GLUT1) expression in cultured cancer cells; however, it is not clear whether apigenin can suppress glucose metabolism in lung cancer cells or sensitize them to inhibition of glutamine utilization-mediated apoptosis through metabolic and oxidative stress. We show that apigenin significantly decreases GLUT1 expression in mice. Furthermore, we demonstrate that apigenin induces growth retardation and apoptosis through metabolic and oxidative stress caused by suppression of glucose utilization in lung cancer cells. The underlying mechanisms were defined that the anticancer effects of apigenin were reversed by ectopic GLUT1 overexpression and galactose supplementation, through activation of pentose phosphate pathway-mediated NADPH generation. Importantly, we showed that severe metabolic stress using a glutaminase inhibitor, compound 968, was involved in the mechanism of sensitization by apigenin. Taken together, the combination of apigenin with inhibitors of glutamine metabolism may provide a promising therapeutic strategy for cancer treatment.

## Introduction

The flavone apigenin (4',5,7,-trihydroxyflavone), is a plant secondary metabolite that occurs widely in numerous fruits.

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*Abbreviations:* PPP, pentose phosphate pathway; GLUT1, glucose transporter 1; ROS, reactive oxygen species; GLS, glutaminase; NAC, N-acetyl-L-cysteine; DCF-DA, 2'-7'-dichlorodihydrofluorescein

*Key words:* apigenin, cancer metabolism, NADPH, oxidative stress, apoptosis

It is a potent anticancer drug that can suppress cancer growth, angiogenesis, and metastasis in multiple types of cancer (1). Mechanistically, several studies have shown that apigenin suppresses the activation of nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B), phosphoinositide 3-kinase (PI3K), epidermal growth factor receptor (EGFR), hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), vascular endothelial growth factor (VEGF), cyclin D1, and vascular cell adhesion molecule 1 (VCAM-1), which play critical roles in the regulation of cell growth, cell cycle, angiogenesis, metastasis, and apoptosis (2). In addition, several studies have shown that apigenin causes accumulation of intracellular reactive oxygen species (ROS) and oxidative stress through the depletion of cellular glutathione (GSH), an important factor in redox balance (3-6). Nevertheless, the precise molecular mechanism by which decreased cellular GSH levels and increased levels of ROS relate to apigenin-mediated apoptosis is not fully understood.

Cancer cells use large amounts of glucose and glutamine to meet the increased energetic and anabolic demands associated with unlimited cell growth and survival (7-9). Increased glucose utilization in cancer cells is achieved primarily by upregulation of the expression of glucose transporter 1 (GLUT1), which is widely expressed in many tumors, including hepatic, pancreatic, breast, brain, lung, colorectal, and cervical cancers (10). Indeed, several reports have shown that GLUT1 inhibitors, such as fasentin, phloretin, and WZB117, suppress growth and induce cell death in cancer cells (11-13). Upregulation of GLUT1 expression is closely associated with chemoresistance, tumor aggressiveness, and decreased survival among patients (14,15). Therefore, downregulation of GLUT1 expression could be a promising therapeutic strategy for cancer treatment.

The pentose phosphate pathway (PPP), which is required for biosynthesis of ribonucleotides and NADPH, can be initiated by glucose-6-phosphate generated by hexokinase as an initial key enzyme in glucose metabolism (16). As a cellular reducing power, NADPH can remove ROS through regulation of the GSH and thioredoxin (TRX) systems that directly scavenge ROS and repair ROS-induced cellular damage (7).

Thus, inhibition of NADPH production has been proposed as a clinical intervention for cancer treatment. Indeed, preclinical studies have shown that inhibition of glucose-6-phosphate dehydrogenase (G6PD), an enzyme that initiates the PPP, is sufficient to decrease cell growth in leukemia, glioblastoma, and lung cancer cells (17).

Glutamine is a critical energy source required to maintain cell growth and survival. Growing cancer cells are commonly dependent upon a supply of glutamine to support the tricarboxylic acid (TCA) cycle. Mechanistically, glutamine can be converted to glutamate and  $\alpha$ -ketoglutarate ( $\alpha$ -KG) via the enzymes glutaminase (GLS) and glutamate dehydrogenase (GDH) (18). Previous studies have shown that inhibition of glutamine metabolism by GLS inhibitors, including 6-diazo-5-oxo-L-norleucine (DON), Bis-2-[5-phenylacetamido-1,2,4-thiadiazol-2-yl] ethyl sulfide (BPTES), and compound 968, or genetic targeting of GLS or GDH using small interfering RNA (siRNA), can suppress cell viability and increase apoptosis in cancer cells. This suggests that inhibition of glutamine utilization might cause cancer cells to become more sensitive to the effects of conventional anticancer drugs (18-20). However, there is no available information addressing synergistic effects of apigenin on targeting cancer specific metabolism, such as glutamine metabolism.

In this study, we examined whether apigenin: i) inhibits GLUT1 expression and glucose utilization, thereby suppressing cell growth and inducing apoptosis; ii) downregulates NADPH and GSH production by inhibition of glucose metabolism, thereby resulting in increased oxidative stress; and iii) sensitizes lung cancer cells to inhibition of glutamine utilization, thereby causing apoptosis and growth retardation.

## Materials and methods

**Reagents and antibodies.** *N*-acetyl-L-cysteine (NAC), apigenin, 2'-7'-dichlorodihydrofluorescein diacetate (DCF-DA) and compound 968 were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Millipore (Billerica, MA, USA). Antibodies recognizing cleaved caspase-9, cleaved caspase-3, PARP, and HK1 (catalog nos. 9502, 9661, 9546, and 2024, respectively) were purchased from Cell Signaling Technology (Danvers, MA, USA). GLUT1 (sc-7903), GLS1 (156876) and  $\beta$ -tubulin (sc-9104) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Abcam (Cambridge, MA, USA).

**Cell culture, plasmids, shRNA, and generation of stable cell lines.** Lung cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 25 mM glucose. The lentiviral vector pLenti-GIII-CMV-SLC2A1, expressing human GLUT1 was obtained from Applied Biological Materials Inc. (Richmond, BC, Canada). A short hairpin RNA construct targeting GLS1 was purchased from Sigma-Aldrich. The hairpin sequence was: 5'-CCGGG CCTGAAGCAGTTCGAAATACTCGAGTATTTCGAAC TGCTTCAGGGCTTTTTG-3'. Lentiviral particles were produced in HEK293T cells, harvested 48 h after transfection, and then used to infect lung cancer cells. Infected lung cancer cells were selected by growth on media containing 2  $\mu$ g/ml of puromycin (Invitrogen, Carlsbad, CA, USA) for 6 days.

**Western blotting.** Cells were lysed in buffer containing 1% IGEPAL, 150 mM NaCl, 50 mM Tris-HCl (pH 7.9), 10 mM NaF, 0.1 mM EDTA, and protease inhibitor cocktail. Protein samples were separated by SDS-PAGE, and the separated proteins were then transferred to a PVDF membrane (Millipore). Membranes were incubated in the presence of primary antibodies (1:1,000) at 4°C overnight, and HRP-conjugated secondary antibodies (1:10,000) were incubated for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECL) Prime kit (GE Healthcare, Pittsburgh, PA, USA).

**ROS measurement.** To measure ROS levels, cells were incubated with Hank's balanced salt solution (HBSS), containing 30  $\mu$ M DCF-DA, for 30 min. After incubation, cells were detached and resuspended in phosphate-buffered saline (PBS) in preparation for fluorescence-activated cell sorting (FACS) analysis.

**RNA isolation and RT-qPCR.** Total RNA was extracted from cells using TRIzol (Invitrogen), and 2  $\mu$ g of total RNA was used for cDNA synthesis using a high capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The sequences of the PCR primers (5'-3') were: GAGAGTTTCATCGGAGAGCC and CAGCGAGAATCGG CTACAG for HK1; GGCATTGATGACTCCAGTGTT and ATGGAGCCCAGCAGCAA for GLUT1; CTTGGGACAG CAGCCTTAAT and CAAGCTGGACGTTAAAGGGA for PGK1; AGCCCTTCTCCATCTCCTT and AACCATGAC CAAGTGCAGAA for HK2; TACAGGCACAGTCGCAG AGT and CACTTCCTGGATGCTTGCTG for ALDOA; CCTGGCATGGATCTTGAGAA and TACGTTACCT CGGTGTCTG for ENO1.

**Glucose consumption, ATP, and lactate production assays.** As previously described (9), lactate and glucose levels in cultured media were measured using lactate colorimetric assay kit and glucose colorimetric assay kit, respectively (BioVision, USA). Briefly, cells were cultured in DMEM without phenol red for 24 h, and then cultured media were mixed with assay solution. To measure intracellular ATP levels, cells were cultured in 6-well plates, and cell lysates were mixed with reaction buffer. ATP levels in the cell lysates were then determined using a luciferin-luciferase-based assay kit (Invitrogen), used according to the manufacturer's instructions. All values were normalized relative to cellular protein concentration.

**Glutathione and NADPH assays.** To prepare samples for determination of glutathione levels, cells were cultured in 6-well tissue culture dishes, and then cell lysates were obtained in extraction buffer without dithiothreitol (DTT) or  $\beta$ -mercaptoethanol. The levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using a Glutathione Colorimetric Detection kit (BioVision) and an OxiSelect Glutathione Assay kit (Cell Biolabs), respectively, as previously described (21). The levels of NADPH in cultured cells were measured using a NADPH quantitation kit (BioVision). Briefly, cell lysates were extracted in NADPH extraction buffer, and then NADPH levels were determined by measurement of absorption at a wavelength of 450 nm.

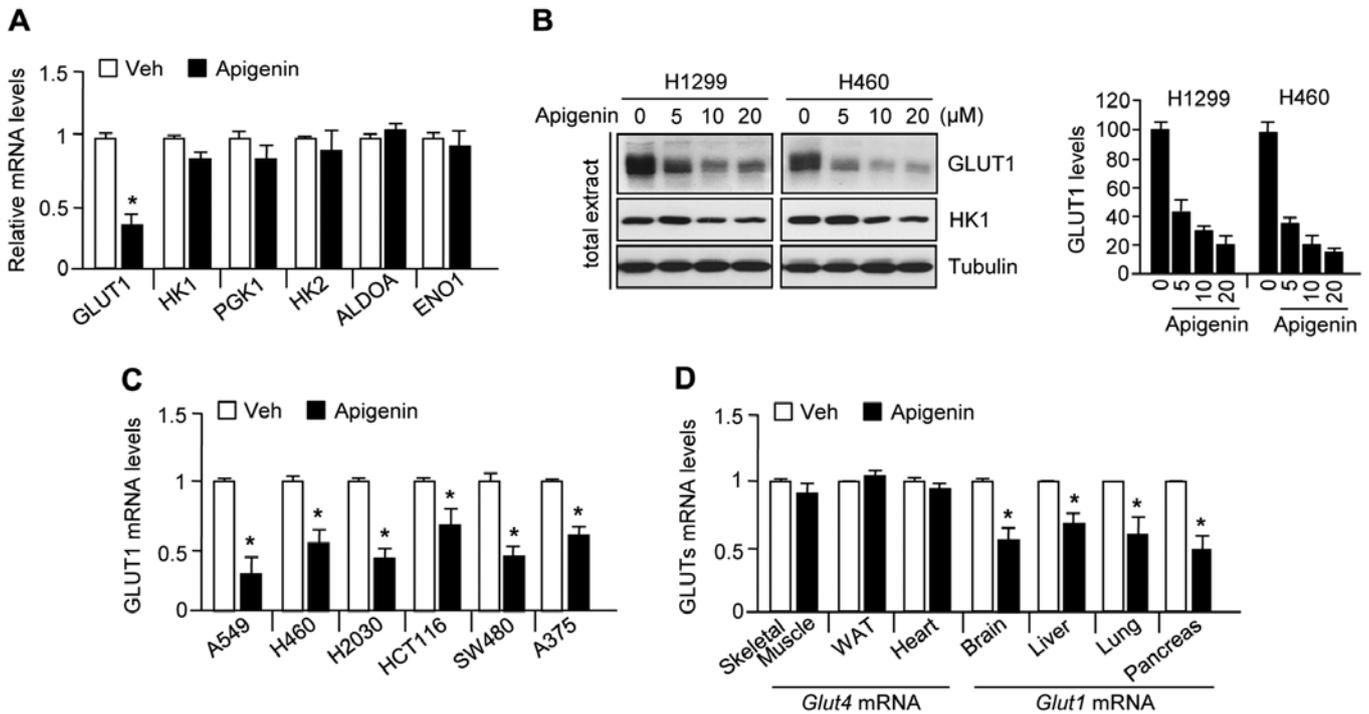


Figure 1. Apigenin decreases GLUT1 mRNA and protein levels in several cancer cell lines. (A) H1299 (lung carcinoma) cells were incubated in the absence or presence of 10  $\mu$ M apigenin for 24 h, and then glycolytic gene expression levels were assessed by quantitative RT-PCR. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p$ <0.05. (B) GLUT1 protein levels were determined by immunoblotting (n=3) and quantified by ImageJ. (C) GLUT1 mRNA levels were measured by quantitative RT-PCR in A549 (lung carcinoma), H460 (lung carcinoma), H2030 (lung adenocarcinoma), HCT116 (colorectal carcinoma), SW480 (colorectal adenocarcinoma), and A375 (melanoma). (D) Mice were injected daily with apigenin (1 mg/kg) or DMSO for 10 days. GLUT1 and GLUT4 mRNA levels in the liver, lung, brain, pancreas, skeletal muscle, white adipose, and heart were measured by quantitative RT-PCR. Values represent mean  $\pm$  SD (n=6); \* $p$ <0.05.

**Clonogenic cell survival assays.** To measure clonogenic cell survival,  $1 \times 10^4$  lung cancer cells were seeded in 6-well tissue culture dishes, and incubated for 7 days. After incubation, cells were fixed in 10% paraformaldehyde, and then stained with crystal violet for 10 min.

**Apoptosis assays.** A kit utilizing Annexin V-FITC and propidium iodide (BD Biosciences, San Jose, CA, USA) was used to quantify the numbers of apoptotic cells. Briefly, cells were detached from the culture dishes, washed with cold PBS, and then incubated with anti-Annexin V-FITC antibody and propidium iodide. Apoptotic cell numbers were determined by FACS analysis.

**Statistical analysis.** All data were analyzed using the unpaired Student's t-test for two experimental comparisons and one-way ANOVA with Tukey post-test for multiple comparisons. Data are represented as means  $\pm$  standard deviations (SD). A  $p$ -value <0.05 was considered statistically significant.

## Results

**Apigenin reduces GLUT1 expression levels.** Because it has been reported that apigenin exerts an anticancer effect through suppression of GLUT1 expression (22-25), we examined whether apigenin also downregulates gene expression related to glycolysis, including GLUT1, HK1, PGK1, HK2, ALDOA, and ENO1. The results were consistent with those of

previous reports (22-25) that apigenin significantly decreased GLUT1 mRNA (Fig. 1A) and protein (Fig. 1B) levels, but did not decrease the levels of other glycolytic enzymes in H1299 or H460 lung carcinoma cells. In addition, GLUT1 mRNA levels were also decreased by apigenin treatment in other cancer cell lines, including A549 (lung carcinoma), H460 (lung carcinoma), H2030 (lung adenocarcinoma), HCT116 (colorectal carcinoma), SW480 (colorectal adenocarcinoma), and A375 (melanoma) (Fig. 1C). To elucidate whether apigenin suppresses GLUT1 expression in mice, we measured GLUT1 and GLUT4 expression levels in skeletal muscle, white adipose tissue, the heart, brain, liver, lung, and pancreas obtained from apigenin-treated mice. Fig. 1D shows that apigenin significantly suppressed GLUT1 but not GLUT4 expression in the brain, liver, lung, and pancreas. These results indicate that apigenin decreases GLUT1 mRNA and protein levels in various human cancer cell lines, and in mice. All experiments were performed according to the guidelines of the Animal Care and Use Committee, Konkuk University, Korea.

**Apigenin causes cellular metabolic and oxidative stress.** Cancer cells use large amounts of glucose to generate glycolytic intermediates and end products, including lactate, ATP, nucleotides, lipids, amino acids, and NADPH, all of which contribute to rapid cell proliferation (8). Therefore, we investigated whether apigenin can block glucose utilization in cancer cells. Interestingly, glucose consumption, lactate production, and ATP production were all strongly decreased by apigenin

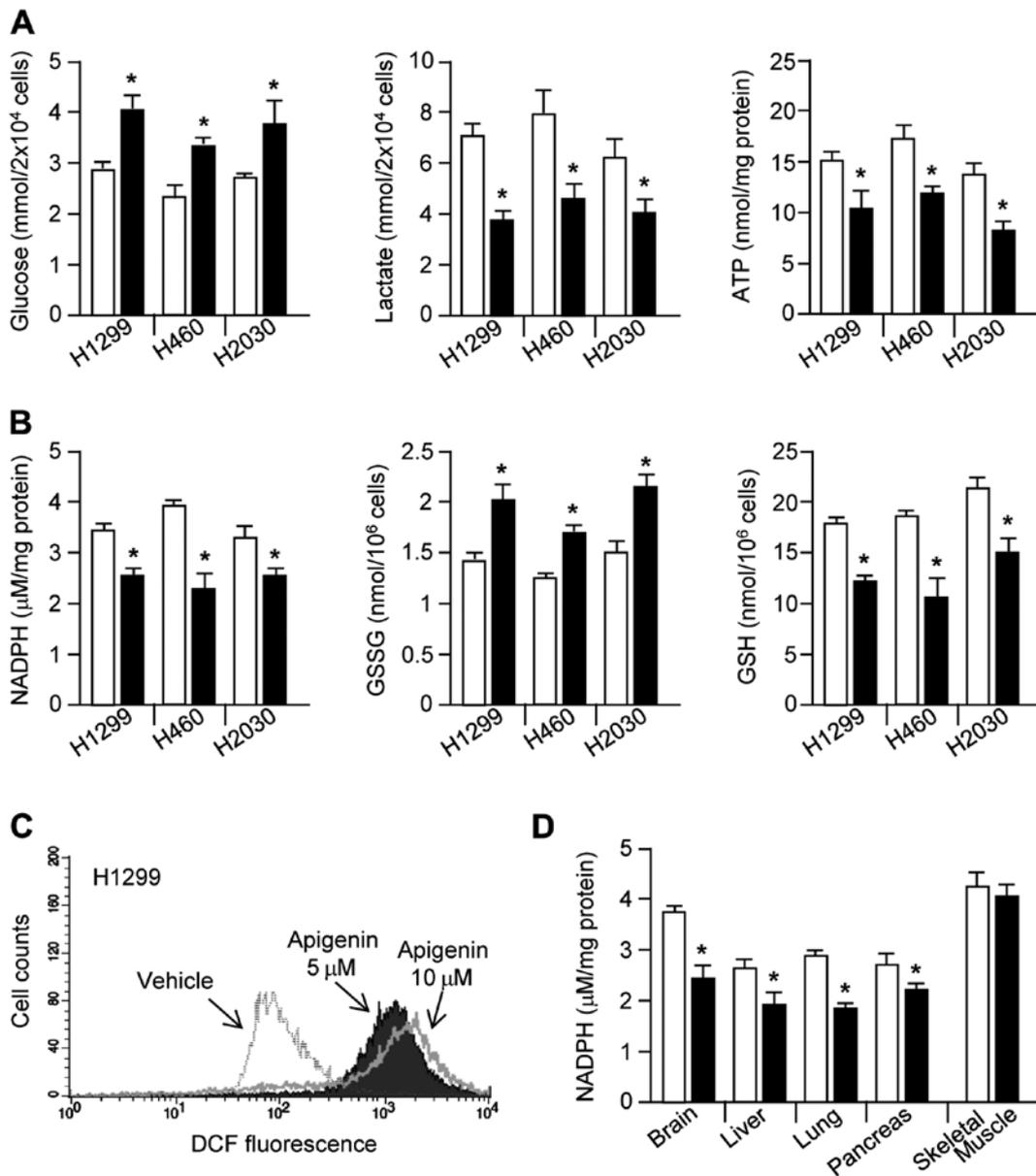


Figure 2. Apigenin suppresses aerobic glycolysis and induces oxidative stress. (A) Glucose consumption, lactate production, and ATP production were measured in lung cancer cell lines treated with 10  $\mu$ M apigenin. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05. (B) Intracellular NADPH, GSSG, and GSH levels were measured in lung cancer cell lines treated with 10  $\mu$ M of apigenin. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05. (C) Intracellular ROS levels were measured using the oxidation-sensitive probe, DCF-DA, in H1299 lung cancer cells treated with 10  $\mu$ M apigenin. (D) NADPH levels in tissues from mice introduced with 1 mg/kg apigenin. Values represent mean  $\pm$  SD ( $n$ =6); \* $p$ <0.05.

treatment (Fig. 2A). Glucose-6-phosphate, which is produced from glucose, can be shunted into the oxidative branch of the PPP by glucose-6-phosphate dehydrogenase, thereby generating NADPH and promoting redox homeostasis (26,27). Here, we demonstrated that NADPH generation by the oxidative branch of the PPP is decreased by apigenin treatment. Fig. 2B shows that, in apigenin-treated lung cancer cells, NADPH and GSH levels were significantly decreased, whereas GSSG levels were increased. Since NADPH maintains redox homeostasis through generation of GSH, which is necessary for the elimination of hydrogen peroxide ( $H_2O_2$ ) by glutathione peroxidase (GPx) (27), we measured intracellular ROS levels. As we had hypothesized, intracellular ROS levels were strongly increased in apigenin-treated H1299 cells (Fig. 2C). Fig. 2D shows

significantly decreased NADPH levels in the brain, liver, lung, and pancreatic tissues derived from apigenin-treated mice. These results suggest that apigenin induces oxidative stress through destruction of glucose utilization-mediated redox homeostasis.

*Apigenin causes oxidative stress leading to apoptosis.* Because apoptotic signal transduction cascades involving caspase-9, -3 and PARP cleavage can be activated by increased ROS levels (9,28), we examined whether apigenin could likewise increase apoptosis through this pathway. Fig. 3A shows that apoptotic cell numbers were increased by  $\sim$ 30% in apigenin-treated H1299, H460, and H2030 lung cancer cells. Furthermore, in H1299 and H460 cells, apigenin increased caspase-9, -3 and

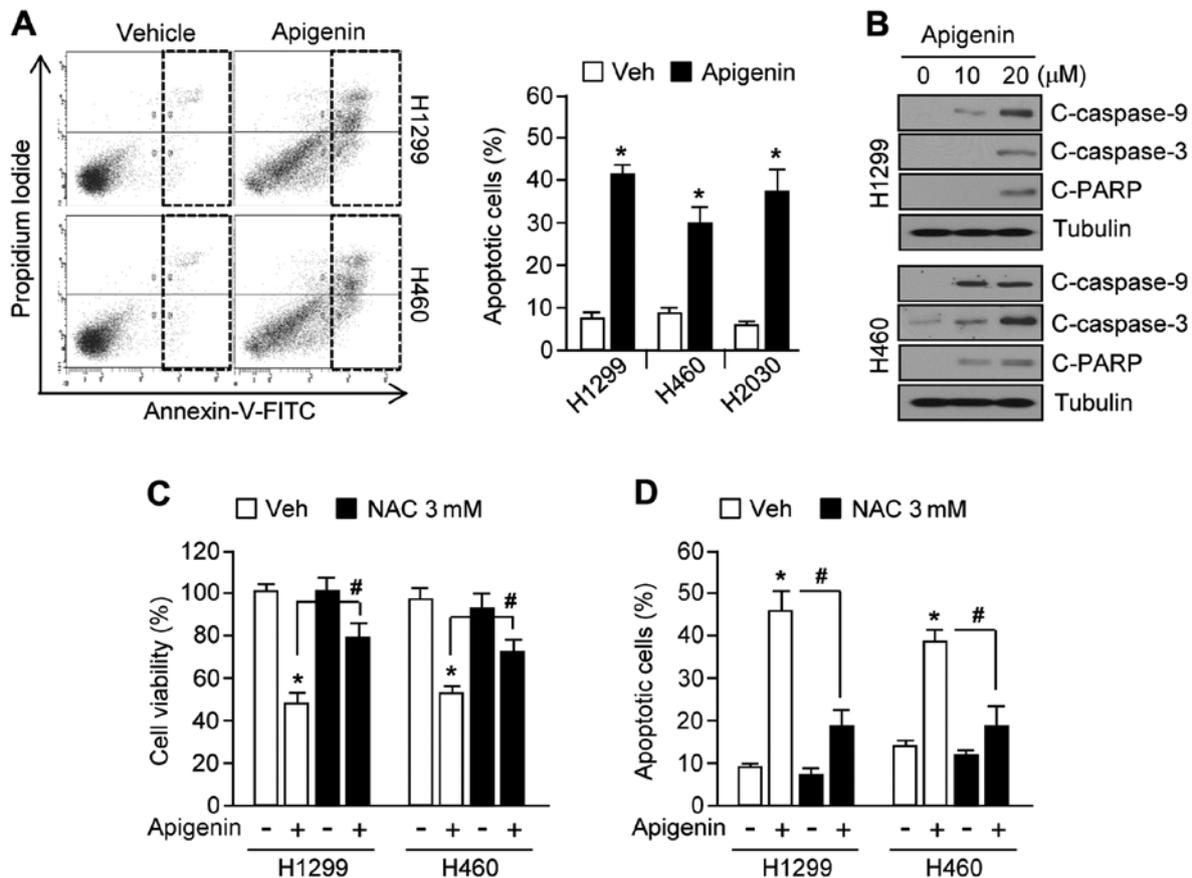


Figure 3. NAC treatment attenuates the apigenin-dependent induction of apoptosis through inhibition of caspase-9, -3 and PARP activation. (A) Apoptosis was measured in H1299 and H460 lung cancer cell cultures treated with 10  $\mu$ M apigenin. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p$ <0.05. (B) Activation of caspase-9, -3 and PARP was detected by immunoblotting. (C) Cell viability was measured in H1299 and H460 lung cancer cell cultures treated with 10  $\mu$ M apigenin in the absence or presence of NAC. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p$ <0.05 and # $p$ <0.05. (D) Apoptosis was measured in H1299 and H460 lung cancer cell cultures treated with 10  $\mu$ M apigenin in the absence or presence of NAC. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p$ <0.05, # $p$ <0.05.

PARP cleavage in a dose-dependent manner (Fig. 3B). In addition, apigenin-induced apoptosis may be mediated by elevated ROS levels in H1299 and H460 cells, because treatment with the antioxidant, *N*-acetyl-L-cysteine (NAC), significantly rescued cell viability (Fig. 3C), and suppressed cell apoptosis (Fig. 3D). Taken together, these results show that accumulation of ROS mediates apigenin-induced apoptosis.

*Galactose supplementation confers resistance to apigenin-induced apoptosis by re-activating antioxidant capacity.* Because galactose supports NADPH production and cancer cell proliferation largely through its metabolism in the pentose phosphate pathway (26,29), we examined whether galactose supplementation could attenuate apigenin-mediated oxidative stress. Interestingly, the decreased levels of NADPH and GSH in apigenin-treated H1299 cells were strongly reversed in the presence of 20 mM galactose (Fig. 4A). Moreover, galactose decreased ROS levels by ~50% (Fig. 4B). Fig. 4C shows that galactose increased cell viability by ~40% in apigenin-treated H1299 and H460 cells. Consistent with these results, the numbers of apoptotic cells were also markedly decreased by ~20% in the presence of apigenin with galactose supplemented cells (Fig. 4D). Fig. 4E shows that apigenin-mediated activation of caspase-9, -3 and PARP apoptotic pathways (left panel)

and decreased clonogenic cell survival (right panel) were completely blocked by 20 mM galactose in H1299 lung cancer cells. Together, these results indicate that galactose confers resistance to apigenin-mediated apoptosis by activating NADPH production through the pentose phosphate pathway.

*Cancer cells expressing high levels of GLUT1 are resistant to apigenin-induced apoptosis through metabolic compensation of glucose utilization.* Because apigenin induces cancer cell death by suppressing GLUT1 expression and glucose utilization in multiple types of cancer cell lines, we examined whether GLUT1-overexpressing cancer cells could confer resistance to apigenin-induced apoptosis. For this purpose, we generated H1299 cell stably expressing a GLUT1 lentiviral vector, or an empty vector (Fig. 5A). Fig. 5B shows that, even in the presence of apigenin, glucose consumption increased dramatically in GLUT1-overexpressing H1299 cells compared to cells harboring the empty vector. Furthermore, GLUT1 overexpression produced high levels of lactate in apigenin-treated H1299 cells compared to levels of lactate in cells carrying the empty vector. To determine whether GLUT1 overexpression could block apigenin-mediated suppression of NADPH and GSH production, we measured intracellular NADPH and GSSG levels in GLUT1-overexpressing H1299

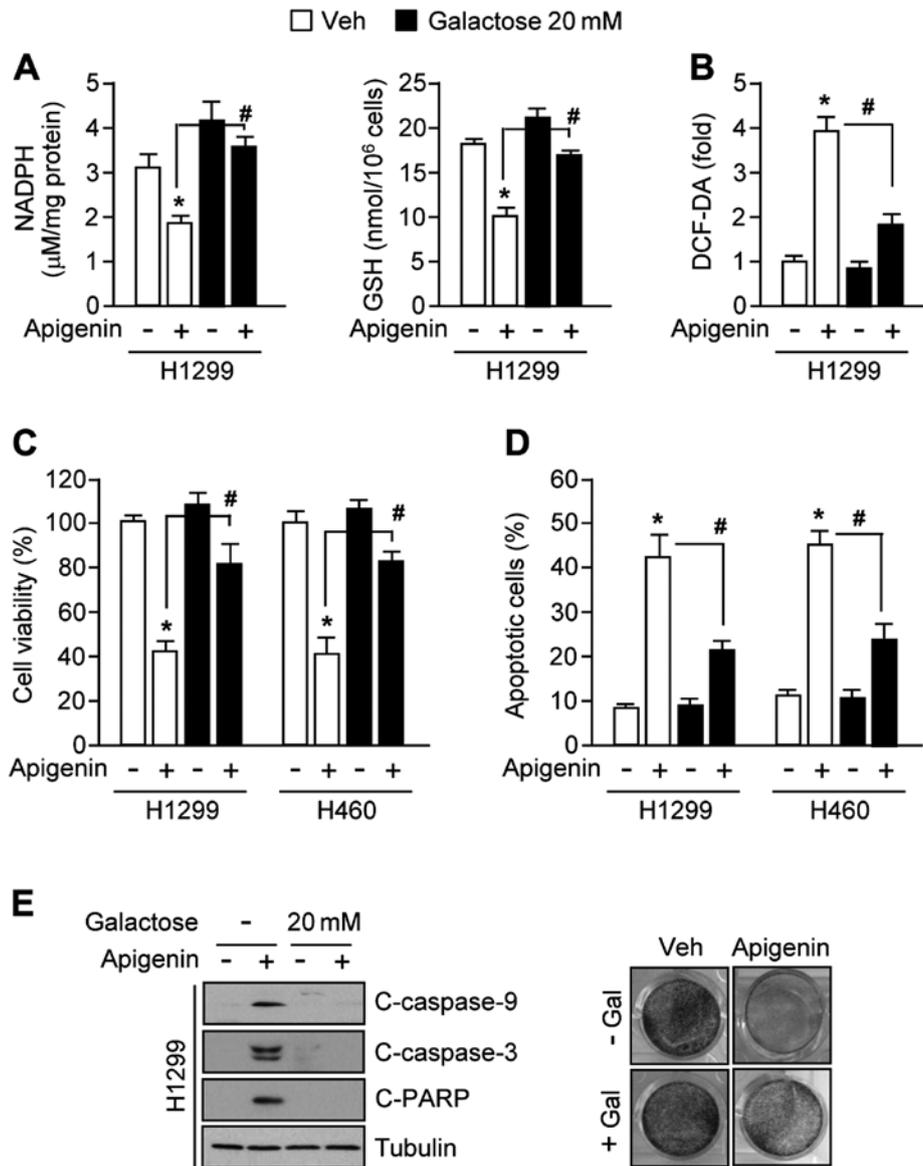


Figure 4. Galactose supplementation confers resistance to apigenin-induced apoptosis. (A) Intracellular NADPH and GSH levels were measured in H1299 lung cancer cell cultures treated with  $10 \mu\text{M}$  apigenin in the absence or presence of 20 mM of galactose. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p < 0.05$ , # $p < 0.05$ . (B) Intracellular ROS levels. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p < 0.05$ , # $p < 0.05$ . (C) Cell viability was measured in H1299 and H460 lung cancer cell cultures treated with  $10 \mu\text{M}$  apigenin in the absence or presence of 20 mM of galactose. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p < 0.05$ , # $p < 0.05$ . (D) Apoptosis was measured in H1299 and H460 lung cancer cell cultures treated with  $10 \mu\text{M}$  apigenin in the absence or presence of 20 mM of galactose. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p < 0.05$ , # $p < 0.05$ . (E) Cleaved caspase-9, -3 and PARP were detected by immunoblotting in apigenin-treated H1299 lung cancer cells in the absence or presence of 20 mM of galactose. Clonogenic cell survival was evaluated in apigenin-treated H1299 lung cancer cells in the absence or presence of 20 mM of galactose.

cells in the presence or absence of apigenin. Interestingly, the effects of apigenin on NADPH and GSH levels were significantly rescued by GLUT1 overexpression (Fig. 5C). Furthermore, GLUT1 overexpression attenuated the effect of apigenin on ROS, resulting in a clear decrease in ROS levels of  $\sim 50\%$  (Fig. 5D). Because increased oxidative stress is a critical step for apigenin-mediated apoptosis, we examined whether GLUT1-overexpressing cells were resistant to apigenin-mediated cell death. Fig. 5E shows that  $50 \mu\text{M}$  apigenin was required to decrease cell viability in GLUT1-overexpressing cells, while  $10 \mu\text{M}$  apigenin was sufficient to decrease cell viability in cells harboring the empty vector. Fig. 5F shows that  $30 \mu\text{M}$  apigenin induced 20% of the cells to undergo apoptosis

in GLUT1-overexpressing H1299 cells. In contrast,  $30 \mu\text{M}$  apigenin induced 50% of cells harboring the empty vector to undergo apoptosis. Furthermore, GLUT1 overexpression dramatically reversed the apigenin-mediated suppression of clonogenic cell survival (Fig. 5G, left panel) and attenuated the activation of caspase-9 and caspase-3 in H1299 cells (Fig. 5G, right panel). These results suggest that GLUT1 overexpression causes resistance to apigenin-mediated apoptosis through suppression of metabolic and oxidative stress.

*Inhibition of glutamine utilization sensitizes apigenin-induced apoptosis through exacerbation of metabolic stress.* In cancer cells, glutamine is a nutrient critical for promotion of

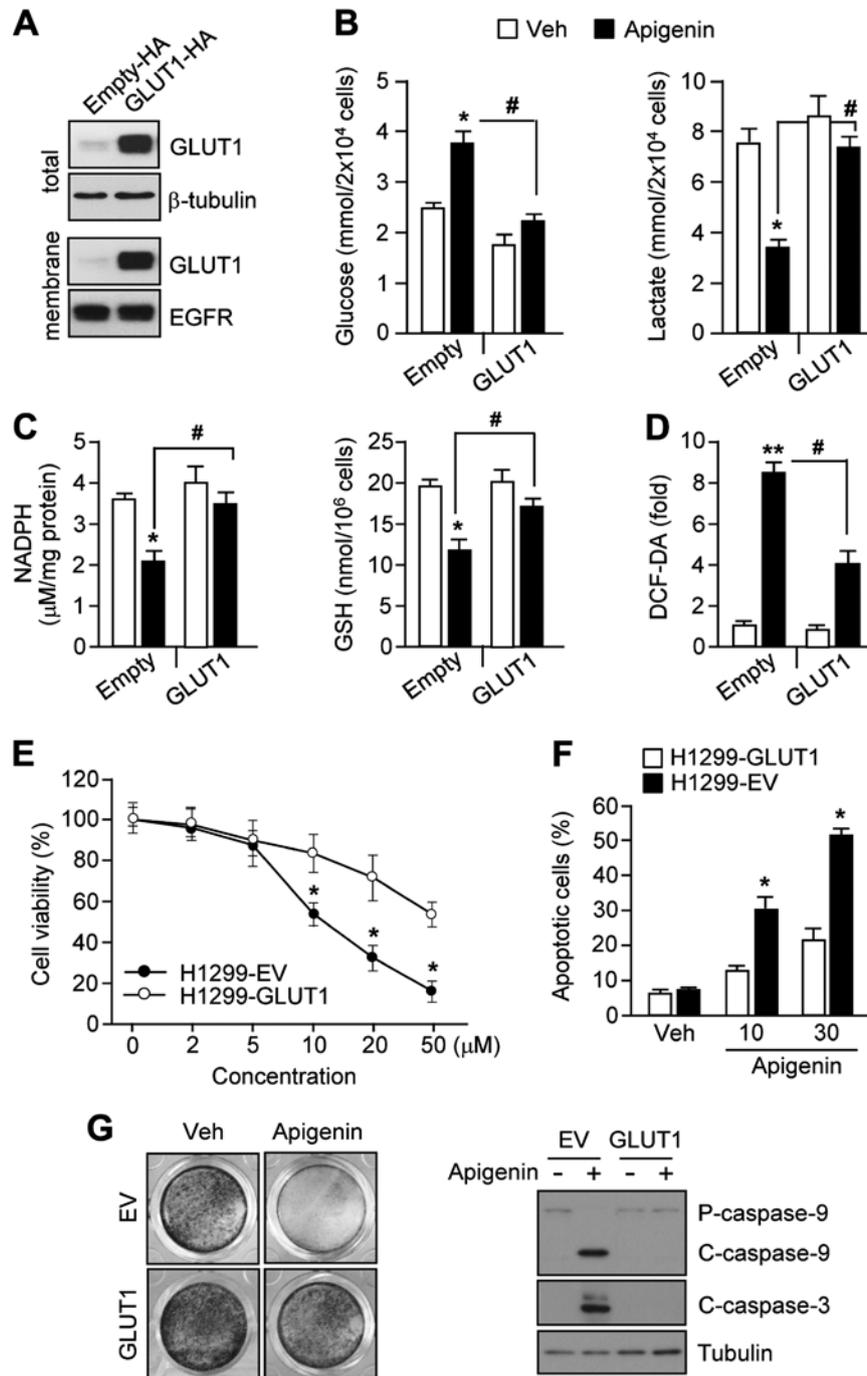


Figure 5. GLUT1 overexpression confers resistance to apigenin-induced apoptosis. (A) Ectopically expressed GLUT1 was detected by immunoblotting. (B) Glucose consumption and lactate production were measured in apigenin-treated H1299 lung cancer cells harboring either the empty vector or the GLUT1 expression vector. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p$ <0.05, # $p$ <0.05. (C) Intracellular NADPH and GSH levels were determined in apigenin-treated H1299 lung cancer cells harboring either the empty vector or the GLUT1 expression vector. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05, # $p$ <0.05. (D) Intracellular ROS levels. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \*\* $p$ <0.01, # $p$ <0.05. (E) Cell viability was determined in apigenin-treated H1299 lung cancer cells harboring either the empty vector or the GLUT1 expression vector. Note the dose-dependent effects of apigenin treatment. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05. (F) Apoptotic cell numbers were determined, after treatment at the indicated concentrations of apigenin, in H1299 lung cancer cells harboring either the empty vector or the GLUT1 expression vector. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05. (G) Clonogenic cell survival was performed after treatment with 10  $\mu$ M apigenin in H1299 cell cultures harboring either the empty vector or the GLUT1 expression vector. The cleaved caspase-9 and caspase-3 proteins were detected by immunoblotting.

tumor growth and survival (30,31). Thus, inhibitors targeting glutamine utilization have shown potential as anticancer drugs (18-20). We therefore hypothesized that inhibition of glutamine

utilization might sensitize cancer cells to apigenin-mediated cell death, and that the mechanism would involve suppression of the major pathways of glucose and glutamine metabolism

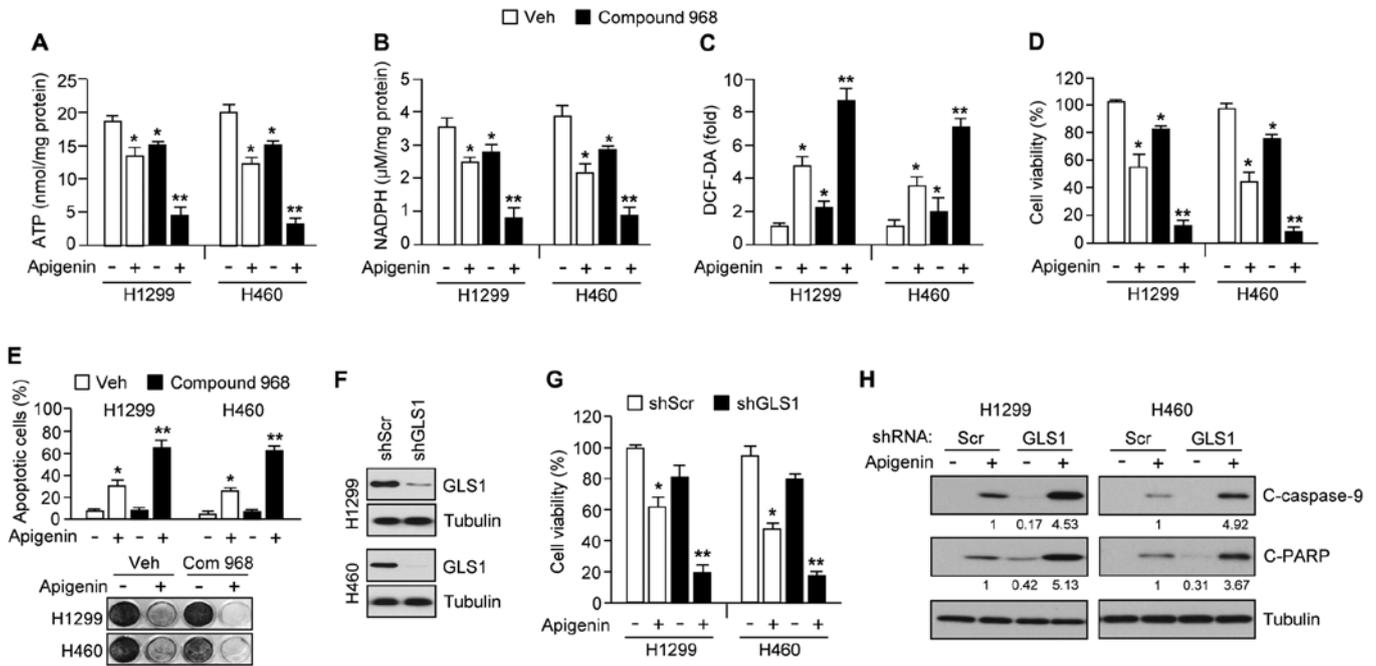


Figure 6. Inhibition of glutamine metabolism sensitizes lung cancer cells to apigenin-induced apoptosis. (A) Intracellular ATP levels were determined in H1299 and H460 lung cancer cells treated with 10  $\mu$ M apigenin in the absence or presence of 5  $\mu$ M compound 968. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05, \*\* $p$ <0.01. (B) NADPH levels were determined in H1299 and H460 lung cancer cells treated with 10  $\mu$ M apigenin in the absence or presence of 5  $\mu$ M compound 968. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p$ <0.05, \*\* $p$ <0.01. (C) Intracellular ROS levels were measured in H1299 and H460 lung cancer cells treated with 10  $\mu$ M apigenin in the absence or presence of 5  $\mu$ M compound 968. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05, \*\* $p$ <0.01. (D) Cell viability was determined in H1299 and H460 lung cancer cell cultures treated with 10  $\mu$ M apigenin in the absence or presence of 5  $\mu$ M compound 968. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05, \*\* $p$ <0.01. (E) Apoptotic cell numbers were determined in H1299 and H460 lung cancer cell cultures treated with 10  $\mu$ M apigenin in the absence or presence of 5  $\mu$ M compound 968. Values represent mean  $\pm$  SD of three independent experiments performed in duplicate; \* $p$ <0.05, \*\* $p$ <0.01. (F) GLS1 protein levels in H1299 and H460 cells were determined by immunoblotting. (G) Cell viability was determined in H1299 and H460 lung cancer cells expressing either GLS1 shRNA or scrambled shRNA in absence or presence of apigenin. Values represent mean  $\pm$  SD of three independent experiments performed in triplicate; \* $p$ <0.05, \*\* $p$ <0.01. (H) Cleaved caspase-9 and PARP were detected by immunoblotting in H1299 and H460 lung cancer cells expressing GLS1 shRNA or scrambled shRNA in the absence or presence of apigenin ( $n$ =3). Protein levels were quantified by ImageJ.

required for tumor growth and survival. In support of this idea, Fig. 6A shows that compound 968, a GLS inhibitor, synergized with apigenin, resulting in an  $\sim$ 75% reduction of ATP levels in H1299 and H460 cells. Because glutamine utilization supports NADPH production needed for redox control (31), we additionally examined whether combination treatment of apigenin and compound 968 is sufficient to increase severe oxidative stress by decreasing NADPH and increasing intracellular ROS levels in cancer cells. Here, we found that compound 968 hugely decreased NADPH (Fig. 6B) and increased intracellular ROS levels (Fig. 6C) in the presence of apigenin. Furthermore, apigenin-treated cells, compound 968 exacerbated the reduction in cell viability (Fig. 6D), increased the induction of apoptosis (Fig. 6E, upper panel), and reduced clonogenic cell survival (Fig. 6E, bottom panel). In addition, we used a short hairpin RNA (shRNA) to knock down GLS1 expression by >70% in H1299 and H460 cell (Fig. 6F). Fig. 6G shows that apigenin caused a large decrease in cell viability of  $\sim$ 80% in GLS1 knocked-down H1299 and H460 cells. Consistent with this, caspase-9 and PARP were more strongly activated in these cells (Fig. 6H), suggesting that decreased GLS1 expression or activity would make cancer cells more susceptible to apigenin-mediated apoptosis. Taken together, these results indicate that inhibition of glutamine utilization should sensitize lung cancer cells to apigenin-mediated apoptosis.

## Discussion

In recent years, apigenin, which is abundant in common fruits and vegetables, has shown considerable promise for development as an anticancer and chemopreventive agent (2,3,6). In fact, several recent reports have shown that apigenin is capable of selectively suppressing growth and inducing apoptosis in cancer cells by affecting the activities of protein tyrosine kinases, including epidermal growth factor receptor (EGFR), Src tyrosine kinase, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (32-36). It has been reported that apigenin suppresses glucose uptake by a mechanism involving decreased GLUT1 expression, leading to increased apoptosis and chemosensitivity in pancreatic carcinoma, laryngeal carcinoma, and head and neck cancer (22-25). However, there is no previous evidence to indicate that a combination of apigenin with other inhibitors of cancer metabolism would show a synergistic effect in the suppression of cancer growth and survival. In this study, we observed that downregulation of GLUT1 by apigenin led to suppression of glucose utilization through decreased glycolysis and pentose phosphate metabolism, thereby suppressing the generation of ATP, macromolecules, and NADPH required for cancer cell growth, proliferation, and survival. Furthermore, our findings indicate

that ectopic overexpression of GLUT1, combined with galactose supplementation to enhance PPP-mediated NADPH and biomass generation, confers resistance to apigenin-mediated apoptosis in several lung cancer cell lines. GLUT1 is upregulated in multiple types of cancer, and is closely correlated with cancer grade, radio-resistance, and chemo-resistance (10,37). Indeed, several GLUT1 inhibitors, including WZB117 and fasentin, have been developed as anticancer drugs (12,13). Thus, apigenin treatment might provide a selective anticancer strategy, acting through suppression of GLUT1-mediated cancer growth and survival.

Growing cancer cells use large amounts of glucose and glutamine to meet the bioenergetics and biosynthetic demands of increased cell growth and survival (7,8). It is becoming clear that altered metabolic pathways in cancer cells, such as aerobic glycolysis, fatty acid synthesis, and glutamine utilization are closely linked to therapeutic resistance in cancer treatment (7,8,26). Therefore, treatment of cancer using combinations of chemotherapeutic drugs and selective cancer metabolism inhibitors could represent a promising strategy to overcome chemoresistance. Indeed, several reports have shown that inhibition of glycolysis enhances the susceptibility of lung cancer, multiple myeloma, and breast cancer to apoptosis during treatment with anticancer drugs, such as cisplatin, doxorubicin, and trastuzumab (18). Previous reports have shown that GLS inhibitors, such as CB-839 and compound 968, can suppress cancer growth and survival *in vitro* and *in vivo* (20,38). However, these studies did not address whether the combined inhibition of glucose and glutamine utilization might represent a promising strategy for cancer treatment. In this study, we demonstrated that the combined suppression of glucose and glutamine utilization, using a combination of apigenin and compound 968, markedly decreased cell viability and increased apoptosis in lung cancer cells. Thus, our findings suggest that the simultaneous inhibition of multiple cancer metabolic pathways may provide a promising strategy for cancer treatment.

Mechanistically, increased ROS is involved in apigenin-induced apoptosis in prostate and colorectal cancer cells (3,6). However, the molecular mechanism by which apigenin increases intracellular ROS levels is not well understood. Because suppression of glucose utilization is reported to induce oxidative stress through inhibition of the PPP and NADPH generation (27), we hypothesized that apigenin might induce oxidative stress by the same mechanism. Indeed, we found that apigenin significantly decreased glucose utilization through suppression of GLUT1 expression, and consequently decreased NADPH production, which led to increased ROS levels. In addition, activation of NADPH production by galactose supplementation, which can be entered into PPP, significantly reversed the apigenin-induced ROS accumulation. These results therefore suggest a possible mechanism by which apigenin induces oxidative stress through inhibition of glucose utilization and PPP-mediated NADPH production.

In conclusion, the major findings are that: i) apigenin decreases glucose utilization, via glycolysis and the PPP, through suppression of GLUT1 expression, thereby resulting in the inhibition of cancer cell growth and survival; ii) activation of glucose utilization and the PPP by GLUT1 overexpression and galactose supplementation confer resistance to apigenin-induced apoptosis by compensating NADPH production,

which suppresses ROS accumulation; iii) targeting glutamine utilization sensitizes lung cancer cells to apigenin-induced apoptosis under conditions of severe metabolic stress. These results may provide a promising therapeutic strategy for cancer treatment.

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