Glycolysis Inhibition Sensitizes Non–Small Cell Lung Cancer with T790M Mutation to Irreversible EGFR Inhibitors via Translational Suppression of Mcl-1 by AMPK Activation

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Abstract

The secondary EGF receptor (EGFR) T790M is the most common mechanism of resistance to reversible EGFR-tyrosine kinase inhibitors (TKI) in patients with non–small cell lung cancer (NSCLC) with activating EGFR mutations. Although afatinib (BIBW2992), a second-generation irreversible EGFR-TKI, was expected to overcome the acquired resistance, it showed limited efficacy in a recent phase III clinical study. In this study, we found that the inhibition of glycolysis using 2-deoxy-D-glucose (2DG) improves the efficacy of afatinib in H1975 and PC9-GR NSCLC cells with EGFR T790M. Treatment with the combination of 2DG and afatinib induced intracellular ATP depletion in both H1975 and PC9-GR cells, resulting in activation of AMP-activated protein kinase (AMPK). AMPK activation played a central role in the cytotoxicity of the combined treatment with 2DG and afatinib through the inhibition of mTOR. The alteration of the AMPK/mTOR signaling pathway by the inhibition of glucose metabolism induced specific downregulation of Mcl-1, a member of the antiapoptotic Bcl-2 family, through translational control. The enhancement of afatinib sensitivity by 2DG was confirmed in the in vivo PC9-GR xenograft model. In conclusion, this study examined whether the inhibition of glucose metabolism using 2DG enhances sensitivity to afatinib in NSCLC cells with EGFR T790M through the regulation of the AMPK/mTOR/Mcl-1 signaling pathway. These data suggest that the combined use of an inhibitor of glucose metabolism and afatinib is a potential therapeutic strategy for the treatment of patients with acquired resistance to reversible EGFR-TKIs due to secondary EGFR T790M.

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Introduction

The EGF receptor (EGFR), a member of the HER family of receptor tyrosine kinases, mediates cell proliferation, angiogenesis, invasion, and metastasis (1, 2). Aberrant expression of EGFR is frequently observed in multiple tumor types and is known to have a strong oncogenic potential (3, 4).

First-generation EGFR-tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib reversibly bind to the ATP cleft within the EGFR kinase domain to block autophosphorylation of EGFR (5). Although these EGFR-TKIs were shown to be effective in patients with advanced non–small cell lung cancer (NSCLC) harboring EGFR-activating mutations such as small in-frame deletions in exon 19 or the L858R missense mutation in exon 21, patients almost always develop resistance to these agents, most commonly through the acquisition of a secondary T790M mutation in EGFR exon 20 (6). To date, there is no standard therapeutic option for patients with acquired resistance to reversible EGFR TKIs due to acquisition of EGFR T790M (7).

Afatinib (BIBW2992) is one of the second-generation irreversible EGFR-TKIs. In recent preclinical studies, afatinib was shown to have antitumor activity in NSCLCs with the EGFR T790M in vitro and in vivo. On the basis of these results, afatinib is expected to be a standard therapeutic option for patients with NSCLCs with EGFR T790M (8–10). However, afatinib was more than 100-fold less potent in NSCLC cells harboring EGFR T790M mutation than in NSCLC cells with activating EGFR mutation (11). It also showed limited efficacy in a recent...
phase III clinical study suggesting the necessity of developing a new strategy to improve the efficacy of afatinib (12).

In 1924, Otto Warburg proposed that most cancer cells preferentially use glycolysis to generate ATP rather than undergo oxidative phosphorylation (OXPHOS), regardless of the availability of oxygen (13). Because ATP production through aerobic glycolysis is less effective than that through OXPHOS, cancer cells maintain a high rate of glycolysis to generate sufficient ATPs for rapid cell proliferation (14). Increased aerobic glycolysis has been recently discussed as a potential hallmark of cancer and is considered a possible therapeutic target for treatment of cancers (14, 15). Indeed, recent studies showed that targeting glycolysis induces cell death and sensitizes cancer cells to chemotherapeutic agents or radiotherapy in different types of cancer (16–21). To date, there is no published study showing that targeting glycolysis potentiates the sensitivity of NSCLC cells to EGFR TKIs.

AMP-activated protein kinase (AMPK) is the major energy sensor kinase and is activated by the increase of the intracellular AMP/ATP ratio, which is a good indicator of energetic stress. The critical function of AMPK is to phosphorylate a number of downstream targets that switch metabolism of the cell toward catabolic instead of biosynthetic pathways (22, 23). mTOR, one of the targets of AMPK, is known to promote cell growth and proliferation through the regulation of protein translation by direct interaction with p70 ribosomal S6 kinase (p70-S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1; ref. 24). Previous studies showed that AMPK activation or mTOR inhibition mediates cellular cytotoxicity in a variety of cancer types (25–27).

Herein, we examined whether the inhibition of glycolysis using 2-deoxy-d-glucose (2DG) enhances sensitivity to afatinib in NSCLC cells with EGFR T790M. Combined treatment with 2DG and afatinib showed significant anti-tumor activity through the downregulation of Mcl-1 via the alteration of the AMPK/mTOR signaling pathway in those cells. These data suggest that the combined use of an inhibitor of glycolysis and afatinib is a potential therapeutic strategy for the treatment of patients with acquired resistance to reversible EGFR-TKIs due to secondary EGFR T790M.

Materials and Methods

Cell culture

The NCI-H1975 cells (EGFR L858R/T790M) were purchased from the American Type Culture Collection and were not authenticated. The PC9-GR cells (EGFR delE746_A750/T790M) were provided by Lee JC (Korea Institute of Radiological and Medical Science, Seoul, Republic of Korea). Existence of EGFR T790M mutation in PC9-GR cells was identified by direct sequencing. Both cells were maintained in RPMI-1640 supplemented with 10% FBS. Culture methods for normal human bronchial epithelial (NHBE) cells and MRC5 can be found in Supplementary Methods. Cell culture media and supplements were obtained from HyClone.

Reagents and antibodies

Afatinib was provided by Boehringer Ingelheim Pharma (Boehringer Ingelheim Pharma GmbH & Co KG). AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside) and cycloheximide were obtained from Sigma. Anti-β-actin antibody was purchased from Santa Cruz Biotechnology, and all other antibodies were purchased from Cell Signaling. All other reagents were purchased from Calbiochem.

Cell viability assay

After incubation with drugs for 72 hours, 0.5 mg/mL of MTT (Amresco) was added to the medium. Formazan crystals in viable cells were solubilized with 100 μL dimethyl sulfoxide (DMSO). The optical density of the MTT formazan product was read at 565 nm on a microplate reader. All experiments were carried out in triplicate.

Analysis of cell death using Annexin V/propidium iodide staining

 Annexin V/propidium iodide (PI) double staining was used according to the manufacturer’s instructions (BD Pharmigen). Briefly, cells were incubated with Annexin V/PI in 1× binding buffer for 15 minutes and then analyzed by flow cytometry (BD Biosciences). Data were processed using WinMDI 2.9 software (Salk Institute).

Intracellular ATP assay

Intracellular ATP was determined with an ATP colorimetric assay kit according to the manufacturer’s instructions (Abcam). Briefly, after centrifugation (13,000 rpm, 5 minutes, 4°C), cell lysates were incubated with ATP reaction mixture for 30 minutes. The optical density of the mixture in each well was read at 570 nm on a microplate reader. The ATP concentration was calculated from standard curve and normalized against cell numbers.

Lactate production assay

Lactate production was measured with a lactate assay kit according to the manufacturer’s instructions (Biovision). Briefly, after centrifugation (13,000 rpm, 15 minutes, 4°C), cell culture media were diluted in lactate assay buffer and mixed with lactate reaction mixture for 30 minutes. The optical density of the mixture in each well was read at 570 nm on a microplate reader. The lactate concentration was calculated from a standard curve and normalized against cell numbers.

Transient transfection

pUseAkt-CA (myristoylated constitutively active Akt) plasmid was kindly obtained from Lee JC (Korea Institute of Radiological and Medical Science, Seoul, Republic of Korea). The presence of endogenous Akt was inhibited with 20 μM AICAR for 1 hour before transfection. Transient transfection was performed using Lipofectamine 2000 (Invitrogen).
of Radiological and Medical Science, Seoul, Republic of Korea). Transfections were carried out with Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen). Briefly, cells were transfected with 1.5 μg/well of DNA for 6 hours with transfection reagent and replaced with fresh growth medium. After 24 hours, cells were treated with drugs for further experiments. The method for siRNA transfection can be found in Supplementary Methods.

Western blot analysis
Cell lysates were prepared as previously described (28). Equal amounts of protein were fractionated by SDS-PAGE and then transferred onto a nitrocellulose membrane (BioRAD). Membranes were blocked with 5% skim milk and incubated with the appropriate primary antibody at 4°C overnight. Proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL chemiluminescence detection system (Amersham-Pharmacia Biotech).

Quantitative reverse transcription PCR
Quantitative reverse transcription PCR (qRT-PCR) was carried out on a 7500 Real-Time PCR System (Applied Biosystems) using the SYBR Green detection protocol. The primers used for real-time PCR are as follows: Bcl-2, (F) 5'ACTGTGTGTGGATGCACCACAACC-3'; (R) 5'-TGAGCAGATGCTTCTGAGCC-3'; β-actin, (F) 5'-CTGGAACGGTGTTTAA-3'; (R) 5'-GCAGCTTATACATTGCA-3'.

Methyl7-GTP sepharose 4B pull-down assay
About 450 μg of cell lysates was incubated with 50 μL of methyl7-GTP sepharose 4B beads (Amersham Biosciences) for 2 hours at 4°C. The beads were washed and then boiled in 2× sample buffer. After SDS-PAGE resolution, the association of 4E-BP1 with eIF4E was detected by Western blotting.

Immunohistochemistry
Sacrificed tumors were fixed, embedded in paraffin, and sectioned (4 μm). Tissue sections were deparaffinized, soaked in ethanol, and incubated in 3% H2O2 for 10 minutes after microwave treatment in 0.01 mol/L sodium citrate buffer (pH 6.0). After incubation in 1% bovine serum albumin (BSA) in PBS for 10 minutes, sections were incubated overnight at 4°C with a monoclonal mouse anti-PCNA (1:300 dilution), a monoclonal rabbit anti-p-AMPKα-T172 (1:100 dilution), a monoclonal rabbit anti-p-mTOR-S2448 (1:100 dilution), and a monoclonal rabbit anti-Mcl-1 (1:100 dilution). After incubation with peroxidase-conjugated secondary antibody, peroxidase activity was revealed using diaminobenzidine.

Statistical analysis
In vitro results are expressed as mean ± SD and in vivo results are expressed as mean ± SE. The Student t test was conducted to determine statistically significant differences between groups, and P < 0.05 was considered statistically significant.

Results
Inhibition of glycolysis enhances afatinib sensitivity in NSCLC cells with EGFR T790M mutations
We first determined whether inhibition of glucose metabolism could enhance afatinib-induced cytotoxicity using MTT assay. To block glycolysis, we used 2DG. 2DG is a nonmetabolizable form of glucose and is known as a blocker of the first rate-limiting step in glycolysis (29). Structures of afatinib and 2DG are depicted in Fig. 1A. Treatment with 2DG decreased cell viability in a dose-dependent manner and significantly enhanced sensitivity to afatinib in both H1975 and PC9-GR cells (Fig. 1B). Treatment with 2DG also increased cell death induced by afatinib (Fig. 1C).

As cancer cells, but not normal cells, are strictly dependent on glycolysis for their energy supply, we tested whether the inhibition of glycolysis induces selective cancer cell cytotoxicity. As shown in Supplementary Fig. S1, in NSCLC cells, including H1975 and PC9-GR, cell growth was inhibited in a time-dependent manner by treatment with 2DG or afatinib alone. Combined treatment with both agents markedly inhibited cell growth in a time-dependent manner and decreased cell numbers below those on day 0 (control), indicating cell death. In contrast to cancer cells, cell growth in normal cells, including NHBE and MRC5, was slightly inhibited in a time-dependent manner by treatment with 2DG or afatinib alone. The combined inhibitory effect on cell growth was much less than that observed in cancer cells. These data suggest that the combination of 2DG and afatinib has cancer cell–selective cytotoxicity.
Combined treatment of 2DG and afatinib hampers cancer cell metabolism and induces ATP depletion

To verify whether 2DG treatment blocked glycolysis, we conducted an intracellular ATP assay and lactate (a product of aerobic glycolysis) assay. Treatment of 2DG alone led to marked reduction in both intracellular ATP content and lactate levels in both H1975 and PC9-GR cells. Interestingly, the treatment of afatinib alone also decreased intracellular ATP content and lactate levels in both cells. Combined treatment of 2DG and afatinib markedly induced ATP depletion and reduced lactate production (Fig. 2A and B). These data show that the combination of 2DG and afatinib effectively inhibits glucose metabolism in both H1975 and PC9-GR cells.

Next, we examined how afatinib interferes with the glucose metabolism in cancer cells. Several lines of evidence are suggesting that the PI3K/Akt signaling pathway positively regulates glycolysis (30–32). Afatinib is well known to have an inhibitory effect on Akt activity through the blockade of the ErbB family (11). Therefore, we tested whether Akt is involved in afatinib-induced inhibition of glycolysis. As shown in Fig. 3A and B, Akt was markedly inactivated by treatment of afatinib alone but not 2DG alone in both H1975 and PC9-GR cells. Induction of constitutative Akt activation by the forced expression of myr-AKT abrogated the inhibitory effect of afatinib alone and the combinational treatment of 2DG and afatinib on Akt activation and ATP production in both cells. These results suggest that blockade of glycolysis by afatinib is mediated through the inhibition of Akt activity.

Cytotoxicity by the combination of 2DG and afatinib is mediated by the regulation of AMPK/mTOR/Mcl-1 signaling pathway

AMPK is known to be activated by stimuli that increase the cellular AMP/ATP ratio (22, 23). Therefore,
we examined whether ATP depletion induced by 2DG and afatinib could activate AMPK. In both H1975 and PC9-GR cells, the combination of 2DG and afatinib induced marked activation of AMPK (Fig. 4A and Supplementary Fig. S2A). The treatment with compound C, an AMPK inhibitor, prevented the reduction of cell viability induced by combined treatment with 2DG and afatinib in H1975 and PC9-GR cells (Fig. 4B and Supplementary Fig. S2B), suggesting the mediation of AMPK activation in the enhanced cytotoxicity of this combination. To confirm the possibility that AMPK activation mediates cytotoxicity of these tumor cells, we used AICAR, an AMPK activator. In H1975 and PC9-GR cells, treatment with AICAR significantly decreased cell viability in a dose-dependent manner (Fig. 4C and Supplementary Fig. S2C). These results show that the combination of 2DG and afatinib induces cytotoxicity through AMPK activation in EGFR T790M–harboring NSCLC cells.

Several reports have suggested that the maintenance of antiapoptotic Bcl-2 family proteins is critical for survival under metabolic stress (33, 34). We therefore examined whether the combined treatment with 2DG and afatinib affects the expression levels of antiapoptotic Bcl-2 family proteins such as Mcl-1, Bcl-2, and Bcl-xL. Treatment with 2DG alone or afatinib alone decreased Mcl-1 levels, and a combination of 2DG and afatinib synergistically reduced Mcl-1 levels in PC9-GR cells, whereas Bcl-2 or Bcl-xL levels were not affected by the treatment with 2DG and afatinib, alone or in combination (Supplementary Fig. S2D). To determine whether the maintenance of Mcl-1 is important for cell survival, we examined whether Mcl-1 knockdown using Mcl-1 targeting siRNA (siMcl-1) reduces cell viability in H1975 and PC9-GR cells. As shown in Supplementary Fig. S3A, efficient Mcl-1 knockdown was shown by Western blot analysis. An MTT assay showed that treatment with siMcl-1 alone induced a significant decrease in cell growth, similar to that observed for treatment with the combination of 2DG and afatinib, in both H1975 and PC9-GR cells (Supplementary Fig. S3B). These data suggest that Mcl-1 downregulation by the combined treatment of 2DG and afatinib is critical for the growth inhibition of EGFR T790M–harboring NSCLC cells.
Next, we examined whether Mcl-1 downregulation is mediated via alteration of the AMPK/mTOR signaling pathway upon the treatment of H1975 and PC9-GR cells with 2DG and afatinib. As shown in Fig. 4D and Supplementary Fig. S2E, combined treatment of 2DG and afatinib synergistically induced AMPK activation and Mcl-1 downregulation in both cells. In addition, we found that mTOR inhibition was accompanied upon the combination of 2DG and afatinib. Both mTOR inhibition and Mcl-1 downregulation by 2DG and afatinib were dramatically restored in the presence of compound C. In both cancer cells, AICAR treatment inhibited mTOR and decreased Mcl-1 levels in a dose-dependent manner (Fig. 4E and Supplementary Fig. S2F). In addition, treatment with rapamycin, an mTOR inhibitor, reduced Mcl-1 levels in H1975 and PC9-GR cells (Fig. 4F and Supplementary Fig. S2G). These findings indicate that AMPK activation and mTOR inhibition upon glycolysis block by combined treatment of 2DG and afatinib results in the downregulation of Mcl-1, but no other Bcl-2, antiapoptotic members.

**Mcl-1 is downregulated through a translational mechanism upon glycolysis inhibition by combined treatment with 2DG and afatinib**

As shown in Supplementary Fig. S4, Mcl-1 mRNA level was not affected by the treatment with 2DG or afatinib, alone or in combination, indicating that Mcl-1 levels were not regulated at the transcriptional level. As it is well known that the central mechanism of Mcl-1 regulation is ubiquitin-mediated proteasomal degradation (35, 36), we therefore monitored Mcl-1 protein levels in the presence of MG132, a proteasome inhibitor. In both H1975 and PC9-GR cells, the relative changes of Mcl-1 levels after MG132 treatment were not different among the treatments with 2DG or afatinib, alone or in combination, although it seemed that Mcl-1 protein level in the combination treatment of both reagents was lower than in alone treatment with MG132 (Fig. 5A and Supplementary Fig. S5A). Also, the half-life of Mcl-1 by treatment of 2DG, afatinib, or the combination of two agents was not accelerated in the presence of cycloheximide, an inhibitor of protein synthesis.
Taken together, these results indicate that the significant reduction of Mcl-1 levels by the combination treatment of 2DG and afatinib was neither due to transcriptional nor due to posttranslational regulation.

Next, we further examined whether Mcl-1 downregulation by 2DG and afatinib was controlled at the translational level in H1975 and PC9-GR cells. The possibility of Mcl-1 downregulation by translational inhibition was supported by following experiment using methyl 7-GTP sepharose 4B beads, which resemble the 5' cap structure of mRNA. As shown in Fig. 5C and Supplementary Fig. S5C, the methyl 7-GTP pull-down assay showed that binding of the translational suppressor 4E-BP1 was significantly increased by treatment with the combination of 2DG and afatinib and markedly decreased by the addition of compound C. In addition, p70S6K, which regulates translation initiation factors and ribosome biosynthesis (37, 38), was almost completely inhibited by the combined treatment of both drugs and the blockade of AMPK by compound C partially restored the activity of p70S6K (Fig. 5D). These results suggest that translational repression by the combination of 2DG and afatinib occurs cooperatively via the translational inhibition of 4E-BP1 and downregulation of p70S6K activity in AMPK-dependent manner. To verify that the inhibition of glycolysis specifically blocks Mcl-1 translation, we monitored the polysome distribution of Mcl-1 upon the treatment with 2DG or afatinib, alone or in combination in PC9-GR cells. As shown in Supplementary Fig. S5D, the amount of Mcl-1 mRNA associated with polysomes was markedly reduced by the combination of 2DG and afatinib. Under the same condition, the
polysome distribution of a control mRNA (β-actin) or Bcl-2 was largely unaffected compared with Mcl-1. Taken together, these results indicate that among the Bcl-2 family, Mcl-1 is specifically downregulated at the translational level by combined treatment of 2DG and afatinib.

The addition of 2DG synergistically enhances antitumor activity of afatinib in PC9-GR xenograft models

To examine the antitumor activity of combination therapy with 2DG and afatinib, athymic nude mice bearing PC9-GR implanted xenografts were treated with control, 2DG, afatinib, or a combination of both agents. Afatinib monotherapy for 30 days delayed tumor growth compared with control. 2DG monotherapy did not show significant antitumor activity. Notably, the combination of 2DG with afatinib resulted in significant tumor regression (Fig. 6A). Consistent with in vitro observations, staining for p-AMPK was clearly enhanced, whereas staining for mTOR and Mcl-1 was markedly reduced upon combined administration of 2DG and afatinib. Taken together, our data obtained by both in vitro and in vivo experiments suggest that glucose metabolism is an attractive therapeutic target for enhancement of afatinib susceptibility in NSCLCs with the EGFR T790M mutation.

Discussion

In this study, we identified that glycolysis inhibition by treatment of 2DG potentiates sensitivity to afatinib in NSCLC cells harboring EGFR T790M mutation. The combined treatment of 2DG and afatinib altered the AMPK/mTOR pathway through the induction of metabolic stress. Interestingly, we showed that upon glycolysis inhibition, the AMPK/mTOR pathway controlled Mcl-1 levels neither through a transcriptional nor through a posttranslational mechanism but rather by controlling its translation.
Therefore, our results show a novel mechanism for the sensitization to irreversible EGFR-TKIs linking glucose metabolism to Mcl-1 downregulation. In addition, this study provides a rationale for the combined use of an inhibitor of glucose metabolism with irreversible EGFR-TKIs in the treatment of NSCLCs with secondary EGFR T790M.

Given that the acquisition of EGFR T790M is a main mechanism of acquired resistance to reversible EGFR-TKIs in patients with NSCLCs with activating EGFR mutations, it is important to develop new therapeutic strategies to overcome the EGFR T790M-mediated acquired resistance (7). Because afatinib showed a strong preclinical antitumor activity in NSCLCs harboring EGFR T790M, it was expected to overcome EGFR T790M-mediated acquired resistance in the clinic (8–10). Disappointingly, a recent phase III study of afatinib failed to show overall survival benefit in patients with acquired resistance to reversible EGFR-TKIs (12). The population of the study was enriched for patients who were sensitive to reversible EGFR-TKIs, indicating that a significant proportion of the enrolled patients originally harbored the activating EGFR mutation. Given that the EGFR T790M mutation accounts for about 50% of acquired resistance mechanism to reversible EGFR-TKIs, a considerable number of the patients enrolled in the study might have EGFR T790M. These results suggest that the development of new

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Figure 6. 2DG increases the antitumor activity by afatinib in PC9-GR tumor xenograft model. A, indicated drugs were administered daily to mice bearing PC9-GR xenografts. Data represent mean ± SE. **; *; P < 0.001 versus control; ###; *; P < 0.001 versus 2DG; ++++, *; P < 0.001 versus afatinib. B, five days after drug treatment, tumors were sacrificed for IHC analysis.
therapeutic options is needed to improve the efficacy of afatinib in patients with EGFR T790M. Herein, we first found that the use of a glycolysis inhibitor, 2DG, sensitizes NSCLC cells harboring EGFR T790M to afatinib through AMPK-dependent Mcl-2 downregulation, suggesting that targeting of glycolysis is an effective therapeutic option to overcome the limited efficacy of afatinib in NSCLCs with EGFR T790M.

In our study, enhanced cell cytotoxicity by the combined treatment of 2DG and afatinib was mediated by the reduction of intracellular ATP production, resulting in AMPK activation and mTOR inhibition. Interestingly enough, afatinib alone decreased intracellular ATP in both H1975 and PC9-GR cells through the inactivation of Akt. Akt has been known to regulate glycolysis at multiple steps of glucose metabolism. Akt increases glucose uptake through translocation of glucose transporter from the cytoplasm into the plasma membrane (39). Furthermore, Akt regulates the activities of phosphofructokinase II involved in the glycolytic pathway (40). Akt activation also increases glycolysis through the activation of hexokinase, a key rate-limiting step (30, 31). Moreover, it was shown that Akt activation switches the metabolism of cancer cells from mitochondrial oxidative phosphorylation to aerobic glycolysis, thus increasing the dependency on aerobic glycolysis for persistent growth and survival (32). To our knowledge, this is the first report to date showing that EGFR-TKIs hamper glycolysis leading to reduction of ATP production.

Herein, although 2DG alone decreased cell growth in both H1975 and PC9-GR cells in vitro, 2DG monotherapy did not affect tumor growth in vivo compared with controls. These data are consistent with a previous report by Maschek and colleagues (17). 2DG is a glucose analogue that competes with glucose for cellular uptake (41). In in vitro studies, the addition of 2DG is sufficient to inhibit glucose metabolism due to the limited availability of glucose in culture media. In contrast, glucose is continuously supplied from the blood circulation to the tumor region in vivo settings. Nonetheless, combined treatment with 2DG and afatinib in the in vivo xenograft model showed more potent antitumor activity than that in in vitro. Although cancer cells exhibit increased glycolysis and depend more on this pathway for ATP generation, the inhibition of glycolysis alone is not sufficient to effectively kill the malignant cells, like monotherapy with glycolysis inhibitors including 2DG do not show antitumor activity in in vivo xenograft studies (17, 42). It has been suggested that ATP depletion should reach certain thresholds to trigger cell death by apoptosis or necrosis (43). Therefore, combination therapies with a glycolysis inhibitor and drugs that block enzymes regulating the glycolysis pathway are expected to be much more effective. As afatinib inhibits Akt, which regulates the activity of phosphofructokinase II, cotreatment of 2DG can enhance the antitumor activity of afatinib. Plus, as hypoxic cells rely solely on the glycolysis pathway for ATP production, tumor cells in the hypoxic inner tumor of an in vivo xenograft model can be more severely affected by the combined treatment of 2DG and afatinib compared with tumor cells cultivated at normoxia. Several studies show that glycolysis inhibitors are particularly effective against tumor cells under hypoxic conditions (44). Although 2DG monotherapy did not show antitumor activity in in vivo study, combined treatment with 2DG and afatinib resulted in potent antitumor activity, suggesting that this combination therapy could be more effective than afatinib alone in a clinical setting.

Bcl-2 family proteins are known to be key regulators of apoptotic cell death. Overexpression of antiapoptotic Bcl-2 family members such as Bcl-2, Bcl-xl, and Mcl-1 has been identified in a number of cancer types and is therefore considered as a therapeutic target for cancer treatment (45, 46). Recent reports have shown that apoptosis by metabolic stress is mediated by the down-regulation of Mcl-1 (33, 34). Consistently, we found that Mcl-1-specific downregulation at the translational level by the inhibition of mTOR played a key role in metabolic stress–induced cytotoxicity upon the combined treatment of 2DG and afatinib in NSCLC cells with EGFR T790M. Although mTOR regulates general protein synthesis through repression of 4E-BP1 (47), why did the combined treatment of 2DG and afatinib specifically downregulate the translation of Mcl-1, among Bcl-2 family members in our study? Several groups reported that, once translated, Mcl-1 has a faster turnover rate than other antiapoptotic Bcl-2 family members due to the rapid degradation through the ubiquitin-dependent pathway (35, 36, 48, 49). To consistently maintain the basal protein level, Mcl-1 should have a rapid translation. For that reason, the blockade of translation by mTOR inhibition could induce selective downregulation of Mcl-1 among Bcl-2 family members. Consistent with our data, recent studies showed that Mcl-1 is specifically regulated at the translational level in an mTOR-dependent manner, suggesting that Mcl-1 might play a critical role in cell cytotoxicity induced by diverse stimuli leading to inactivation of mTOR (33, 50).

In conclusion, the emergence of EGFR T790M mutation–mediated acquired resistance poses the greatest unmet medical need in patients with NSCLCs after progression on reversible EGFR-TKIs. Here, we showed that the inhibition of glucose metabolism by 2DG improves the efficacy of afatinib through the down-regulation of Mcl-1 via the alteration of the AMPK/mTOR signaling pathway in NSCLC cells with EGFR T790M. These data suggest that combined treatment with an inhibitor of glucose metabolism and afatinib is a potential therapeutic strategy for treatment of patients with acquired resistance to reversible EGFR-TKIs due to secondary EGFR T790M.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Inhibition of Glycolysis Enhances Sensitivity to Afatinib

Authors’ Contributions

Conception and design: S.M. Kim, J.H. Kim, B.C. Cho
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Kim, M.R. Yun
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M. Kim, B.C. Cho
Writing, review, and/or revision of the manuscript: S.M. Kim, F. Solca, J.H. Kim, H.-J. Kim, B.C. Cho

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. Kim, Y.K. Hong
Study supervision: H.-J. Kim, B.C. Cho

References


### Molecular Cancer Therapeutics

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