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 epidermal growth factor receptor (EGFR) T790M is the most common mechanism of resistance to reversible EGFR tyrosine kinase inhibitors (TKIs) in non-small cell lung cancer (NSCLC) patients 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 mammalian target of rapamycin (mTOR). The alteration of the AMPK/mTOR signaling pathway by the inhibition of glucose metabolism induced specific downregulation of Mcl-1, a member of anti-apoptotic Bcl-2 family, through the translational control. The enhancement of afatinib sensitivity by 2DG was confirmed in in vivo PC9-GR xenograft model.

In conclusion, this study examined whether the inhibition of glucose metabolism using 2DG enhances the sensitivity to afatinib in NSCLC cells with EGFR T790M through the regulation of 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.
INTRODUCTION

The epidermal growth factor 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 known to have a strong oncogenic potential (3, 4).

First-generation EGFR tyrosine kinase inhibitors (TKIs) 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 against 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 anti-tumor activity in NSCLC with the EGFR T790M in vitro and in vivo. Based on these results, afatinib is expected to be a standard therapeutic option for NSCLC patients with EGFR T790M (8-10). However, afatinib were over 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 demonstrated 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 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). Mammalian target of rapamycin (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) (24). Previous studies demonstrated 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 2DG enhances the 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 (Manassas, VA) 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. The both cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. 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 (Logan, UT).

Reagents and antibodies

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

Cell viability assay

After incubation with drugs for 72 h, 0.5 mg/ml of MTT (Amresco, Solon, OH)
was added to the medium. Formazan crystals in viable cells were solubilized with 100 μl DMSO. The optical density of the MTT formazan product was read at 565 nm on a microplate reader. All experiments were performed in triplicate.

**Analysis of cell death using annexin V/propidium iodide (PI) staining**

Annexin V/PI double staining was used according to the manufacturer's instructions (BD Pharmigen, San Diego, CA). Briefly, cells were incubated with annexin V/PI in 1X binding buffer for 15 min and then analyzed by flow cytometry (BD Biosciences, San Jose, CA). Data were processed using WinMDI 2.9 software (Salk Institute, La Jolla, CA).

**Intracellular ATP assay**

Intracellular ATP was determined with an ATP colorimetric assay kit according to the manufacturer's instructions (Abcam, Cambridge, MA). Briefly, after centrifugation (13,000 rpm, 5 min, 4 °C), cell lysates were incubated with ATP reaction mixture for 30 min. 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, Mountain View, CA). Briefly, after centrifugation (13,000 rpm, 15 min, 4 °C), cell culture medium were diluted in lactate
assay buffer and mixed with lactate reaction mixture for 30 min. 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). Transfections were performed with Lipofectamine\textsuperscript{TM}2000 reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly, cells were transfected with 1.5 µg/well of DNA for 6 h with transfection reagent and replaced with fresh growth medium. After 24 h, cells were treated with drugs for further experiments. 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, Richmond, CA). Membranes were blocked with 5% skim milk and incubated with the appropriate primary antibody at 4 °C overnight. Proteins were detected using HRP-conjugated secondary antibodies and ECL chemiluminescence detection system (Amersham-Pharmacia Biotech, Buckinghamshire, England).

**Methyl\textsuperscript{7}-GTP sepharose 4B pull-down assay**

450 µg of cell lysates was incubated with 50 µL of methyl\textsuperscript{7}-GTP sepharose 4B
beads (Amersham Biosciences, Piscataway, NJ) for 2 h at 4 °C. The beads were washed and then boiled in 2X sample buffer. After SDS-PAGE resolution, the association of 4E-BP1 with eIF4E was detected by Western blot.

**qRT-PCR**

qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the SYBR Green detection protocol. The primers used for real-time PCR are as follows: Mcl-1, (F) 5’-GGACATCAAAAAACGAAGACG-3’; (R) 5’-GCAGCTTTCTTGGTTTATGG-3’; Bcl-2, (F) 5’-ATGTGTGTGGAGAGCGTCAAC-3’; (R) 5’-TGAGCAGAGTCCTCCAGGACGCGCTCAAC-3’; β-actin, (F) 5’-CTGGAACGG-TGAAGGTGACA -3’; (R) 5’-AAGGGACTTCTCTGTAACATGCA -3’.

**Xenograft studies**

Female athymic BALB-c/nu mice were obtained from Orient Bio (Orient Bio, Seoul, Korea) at 5-6 weeks of age. All mice were handled in accordance with the Animal Research Committee’s Guidelines at Yonsei University College of Medicine and all facilities are approved by AAALAC (association of assessment and accreditation of laboratory animal care). Mice were injected subcutaneously with PC9-GR cells (5X10^6). When tumor volumes reached approximately 70 mm^3, mice were randomly allocated into groups of six animals to receive either vehicle control, afatinib alone, 2DG alone or afatinib and 2DG together. Afatinib was suspended in 0.5% (w/v) methylcellulose containing 0.4% Tween 80 and administered orally by gavage at 5 mg/kg on a once daily dosing schedule. 2DG was dissolved in saline and administered
by intraperitoneal injection at a daily dose of 500 mg/kg. Tumor size was measured every 2 days using calipers. The average tumor volume in each group was expressed in mm$^3$ and calculated according to the equation for a prolate spheroid: tumor volume = 0.523 x (large diameter) x (small diameter)$^2$.

**Immunohistochemistry (IHC)**

Sacrificed tumors were fixed, embedded in paraffin and sectioned (4 μm). Tissue sections were deparaffinized, soaked in ethanol, and incubated in 3% H$_2$O$_2$ for 10 min after microwave treatment in 0.01 mol/L sodium citrate buffer (pH 6.0). After incubation in 1% BSA in PBS for 10 min, 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. Student’s t-test was performed to determine statistically significant differences between groups, and a $P$ value <0.05 was considered statistically significant.
RESULTS

Inhibition of glycolysis enhances afatinib sensitivity in NSCLC cells with EGFR T790M mutations.

We first determined if inhibition of glucose metabolism could enhance afatinib-induced cytotoxicity using MTT assay. To block glycolysis, we used 2-deoxy-D-glucose (2DG). 2DG is a non-metabolizable form of glucose and known as a blocker of the first rate-limiting step in glycolysis (29). Structures of afatinib and 2DG are depicted in Fig. 1A. Treatment of 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 of 2DG also increased cell death induced by afatinib (Fig. 1C).

Since cancer cells but not normal cells are strictly dependent on glycolysis for their energy supply, we tested if the inhibition of glycolysis induces selective cancer cell cytotoxicity. As shown in supplementary Fig. 1, in NSCLC cells including H1975 and PC9-GR, cell growth was inhibited in a time-dependent manner by treatment of 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 of 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 if 2DG treatment blocked glycolysis, we performed an intracellular ATP assay and lactate (a product of aerobic glycolysis) assay. Treatment of 2DG alone led to marked reduction in both intracellular ATP level and lactate production 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 2B). 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 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 if 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 constitutive Akt activation by the forced expression of myr-AKT abrogated the inhibitory effect of afatinib alone and the combinatorial 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 if 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. 2A). 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. 2B), suggesting the mediation of AMPK activation in the enhanced cytotoxicity of these 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. 2C). 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 anti-apoptotic 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 anti-apoptotic 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 while Bcl-2 or Bcl-xL levels were not affected by the treatment with 2DG and afatinib, alone or in combination (supplementary Fig. 2D). To determine if the maintenance of Mcl-1 is
important for cell survival, we examined whether Mcl-1 knockdown using Mcl-1 targeting small interfering RNA (siMcl-1) reduces cell viability in H1975 and PC9-GR cells. As shown in supplementary Fig. 3A, efficient Mcl-1 knockdown was demonstrated 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. 3B). 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. 2E, 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. 2F). In addition, treatment with rapamycin, an mTOR inhibitor, reduced Mcl-1 levels in H1975 and PC9-GR cells (Fig. 4F and supplementary Fig. 2G). 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 anti-apoptotic members.
Mcl-1 is downregulated through a translational mechanism upon glycolysis inhibition by combined treatment with 2DG and afatinib.

As shown in supplementary Fig. 4, 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. Since it is well known that the central mechanism of Mcl-1 regulation is ubiquitin-mediated proteosomal 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 treatment 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. 5A). 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 (CHX), an inhibitor of protein biosynthesis (Fig. 5B and supplementary Fig. 5B). 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. 5C, 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. 5D, 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 to Mcl-1. Taken together, these results indicate that among
Bcl-2 family, Mcl-1 is specifically downregulated at translational level by combined
treatment of 2DG and afatinib.

The addition of 2DG synergistically enhances anti-tumor activity of afatinib in
PC9-GR xenograft models.

To examine the anti-tumor 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 anti-tumor activity. Notably, the combination of 2DG with afatinib resulted in significant tumor regression (Fig. 6A). The synergistic anti-tumor activity of the combined treatment of 2DG and afatinib was also confirmed by IHC staining for PCNA, a marker for cell proliferation (Fig. 6B). Consistent with \textit{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 \textit{in vitro} and \textit{in vivo} experiments suggest that glucose metabolism is an attractive therapeutic target for enhancement of afatinib susceptibility in NSCLC with the EGFR T790M mutation.
DISCUSSION

In this study, we identified that glycolysis inhibition by treatment of 2DG potentiates the sensitivity to afatinib in NSCLC cells harboring EGFR T790M mutation. The combined treatment of 2DG and afatinib altered AMPK-mTOR pathway through the induction of metabolic stress. Interestingly, we demonstrated that, upon glycolysis inhibition, the AMPK-mTOR pathway controlled Mcl-1 levels neither through transcriptional nor through 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 NSCLC with secondary EGFR T790M.

Given that the acquisition of EGFR T790M is a main mechanism of acquired resistance to reversible EGFR TKIs in NSCLC patients 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 anti-tumor activity in NSCLC 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 demonstrate 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 ~50% of acquired
resistance mechanism to reversible EGFR TKIs, a considerable number of the patients enrolled in the study might have the EGFR T790M. These results suggest that the development of new therapeutic options is needed to improve the efficacy of afatinib in patients with the EGFR T790M. Herein, we firstly found that the use of 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 NSCLC 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 the 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 demonstrated 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 to
controls. These data are consistent with previous report by Maschek and colleagues (17). 2DG is a glucose analogue that competes with glucose for cellular uptake (41). In \textit{in vitro} study, 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 tumor region in \textit{in vivo} settings. Nonetheless, combined treatment with 2DG and afatinib in \textit{in vivo} xenograft model showed more potent anti-tumor activity than that in \textit{in vitro}. Although cancer cells exhibit increased glycolysis and depend more on this pathway for ATP generation, the inhibition of glycolysis alone is not be sufficient to effectively kill the malignant cells, like monotherapy with glycolysis inhibitors including 2DG do not show anti-tumor activity in \textit{in vivo} xenograft studies (17, 42). It has been suggested that ATP depletion should reach certain thresholds in order to trigger cell death by apoptosis or necrosis (43). Therefore, combination therapies with glycolysis inhibitor and drugs which block enzymes regulating glycolysis pathway are expected to be much more effective. Since afatinib inhibits Akt that regulates the activity of phosphofructokinase II, co-treatment of 2DG can enhance anti-tumor activity of afatinib. Plus, since hypoxic cells rely on solely on glycolysis pathway for ATP production, tumor cells in hypoxic inner tumor of \textit{in vivo} xenograft model can be more severely affected by the combined treatment of 2DG and afatinib compared to 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 anti-tumor activity in \textit{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 anti-apoptotic 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 downregulation of Mcl-1 (33, 34). Consistently, we found that Mcl-1-specific downregulation at 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 anti-apoptotic 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 a 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 NSCLC after progression on reversible EGFR TKIs. Here we demonstrated the inhibition of glucose metabolism by 2DG improves the efficacy of afatinib through the downregulation 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.
REFERENCES


et al. PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. Cancer research. 2007;67:11924-32.


41. Brown J. Effects of 2-deoxyglucose on carbohydrate metabolism: review of the
literature and studies in the rat. Metabolism. 1962;11:1098-112.


FIGURE LEGENDS

Figure 1. 2DG sensitizes NSCLC cells with EGFR T790M to afatinib. A, Structures of 2-deoxy-D-glucose (2DG) and afatinib (BIBW2992). B, Cells were incubated with indicated drugs for 72 h. Cell viability was determined by MTT assay. * P < 0.05; ** P < 0.01; *** P < 0.001. C, Cells were treated with 2 mM 2DG, 100 nM afatinib, or combination of two agents for 24h. Dead cells were assessed by annexin V/PI staining and FACS analysis.

Figure 2. Combined treatment of 2DG and afatinib synergistically inhibits glycolytic metabolism. A and B, Cells were treated with 2 mM 2DG, 100 nM afatinib, or combined treatment of two agents. After 48 h, ATP or lactate level was measured. ** P < 0.01; *** P < 0.001.

Figure 3. Afatinib blocks ATP production via the inhibition of Akt activity. A and B, At 24 h post-transfection of mock or myr-Akt vectors, cells were treated with 2 mM 2DG, 100 nM afatinib, or combination with two agents. After 48 h, cells were harvested for ATP production assay or Western blot analysis.

Figure 4. Cell cytotoxicity by combined treatment with 2DG and afatinib is mediated through the regulation of AMPK/mTOR/Mcl-1 signaling pathway. A, Cells were treated with 2 mM 2DG, 100 nM afatinib, or combination with two agents for 24 h, and then harvested. B, Cells were pretreated with 1 μM compound C for 1 h, and then incubated with 2 mM 2DG, 100 nM afatinib, combination with two agents for 72 h. C, After
treatment with AICAR for 72 h, cell viability was determined by MTT assay. 

_D_ Cell were pretreated with 1 μM compound C for 1 h, and then incubated with 2 mM 2DG, 100 nM afatinib, or combination with two agents for 24 h. 

_E and F_, Cells were treated with AICAR or rapamycin for 24 h and then harvested. *** _P_ < 0.001.

**Figure 5.** Downregulation of Mcl-1 by the combined treatment of 2DG and afatinib is occurred through the blockade of cellular translation. 

_A and B_, Cell were treated with 2 mM 2DG, 100 nM afatinib, or combination with two agents for 24 h, and then treated with 20 μM MG132 for 3 h or 100 μg/ml cycloheximide (CHX) for 1 h. Quantification of Mcl-1 was normalized to β-actin and results were expressed as ratio of Mcl-1 level to control (non-treated). 

_C_, Cells were pretreated with 1 μM compound C for 1 h and further incubated with 2 mM 2DG, 100 nM afatinib, or the combination of two agents for 24 h. After methyl7-GTP pull down assay, the association between 4E-BP1 and eIF4E was revealed by Western blot analysis. Total cell lysates (TCL) were used as input control. 

_D_, H1975 was pretreated with 1 μM compound C for 1 h and further incubated with 2 mM 2DG, 100 nM afatinib, or the combination of two agents for 24 h. Cell lysates were fractionated in SDS-PAGE gel and phospho- and total p70S6K were immunoblotted. β-actin was used as a loading control.

**Figure 6.** 2DG increases the anti-tumor activity by afatinib in PC9-GR tumor xenograft model. 

_A_, Mice bearing PC9-GR xenografts were administrated daily with indicated drugs. Data represent mean ± SE. *** _P_ < 0.001 vs control; ### _P_ < 0.001 vs 2DG; +++ _P_ < 0.001 vs afatinib. 

_B_, 5 days after drug treatment, tumors were sacrificed for IHC analysis.
Figure 1

A

2-Deoxy-D-Glucose (2DG)

Afatinib (BIBW2992)

B

H1975

Cell viability (% of control)

2DG (mM)

0 1 2 5

DMSO

10 nM afatinib

100 nM afatinib

PC9-GR

Cell viability (% of control)

2DG (mM)

0 1 2 5

DMSO

10 nM afatinib

100 nM afatinib

C

CON 2DG Afatinib 2DG + Afatinib

H1975

Propidium iodide

Annexin V

PC9-GR
Figure 2

A

H1975

PC9-GR

ATP (% of control)

Control 2DG Afatinib 2DG + Afatinib

Control 2DG Afatinib 2DG + Afatinib

**

B

H1975

PC9-GR

Lactate (% of control)

Control 2DG Afatinib 2DG + Afatinib

Control 2DG Afatinib 2DG + Afatinib

**

***
Figure 3

A  

H1975  

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<td>β-actin</td>
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B  

PC9-GR  

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Figure 5

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Relative Mcl-1 levels

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Relative Mcl-1 levels

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M7-GTP sepharose

4E-BP1

eIF4E

D

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TCL

4E-BP1

eIF4E

β-actin

p-p 70S6K

p 70S6K

β-actin
Figure 6

A

![Graph showing tumor volume over days for different treatments.](graph)

B

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Molecular Cancer Therapeutics

Glycolysis inhibition sensitizes non-small cell lung cancer with T790M mutation to irreversible EGFR inhibitors via translational suppression of Mcl-1 by AMPK activation

Sun Mi Kim, Mi Ran Yun, Yun Kyoung Hong, et al.

Mol Cancer Ther Published OnlineFirst July 24, 2013.

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