

Synergistic Effects of Metformin Treatment in Combination with Gefitinib, a Selective EGFR Tyrosine Kinase Inhibitor, in LKB1 Wild-type NSCLC Cell Lines

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Abstract

Purpose: EGF receptor (EGFR) tyrosine kinase inhibitors (TKI) have been found to be effective against lung cancer, but clinical resistance to these agents has developed as their usage has increased. Metformin is a widely used antidiabetic drug and also displays significant growth-inhibitory and proapoptotic effects in several cancer models, alone or in combination with chemotherapeutic drugs.

Experimental Design: The effects of gefitinib, a selective EGFR-TKI, and metformin on a panel of non-small cell lung cancer (NSCLC) cell lines were assessed by using MTT, bromide assay, flow cytometry, anchorage-independent growth, coimmunoprecipitation, and Western blot analysis.

Results: The combination of metformin with gefitinib induced a strong antiproliferative and proapoptotic effect in NSCLC cell lines that harbored wild-type *LKB1* gene. Treatment with metformin as single agent, however, induced an activation and phosphorylation of mitogen-activated protein kinase (MAPK) through an increased C-RAF/B-RAF heterodimerization. The inhibition of EGFR phosphorylation and of downstream signaling by adding gefitinib to metformin treatment abrogated this phenomenon and induced a strong apoptotic effect *in vitro* and *in vivo*.

Conclusions: Metformin and gefitinib are synergistic in LKB1 wild-type NSCLC cells. However, further studies are required to investigate better the effect of metformin action on the RAS/RAF/MAPK pathway and the best context in which to use metformin in combination with molecular targeted agents. *Clin Cancer Res*; 19(13): 3508–19. ©2013 AACR.

Introduction

Non-small cell lung cancer (NSCLC) is the major cause of cancer-related deaths worldwide (1). Platinum-based combination regimens offer a modest but significant survival advantage to patients with NSCLCs with advanced or metastatic disease, although most patients eventually experience disease progression (2).

Advances in the understanding of the molecular biology of cancer have enabled the discovery of several potential

molecular targets with the development of novel targeted therapies. The EGF receptor (EGFR) is involved in the development and the progression of several human cancers including NSCLCs. Two EGFR tyrosine kinase inhibitors (TKI), gefitinib (ZD1839, Iressa) and erlotinib (OSI774, Tarceva), represent the first examples of molecularly targeted agents developed in the treatment of NSCLCs and are, currently, used in the management of patients with advanced NSCLCs who progressed on previous chemotherapy or in the treatment of those patients harboring specific activating somatic EGFR gene mutations (3).

Despite impressive clinical successes with different kinase-targeted therapies, most, if not all, patients with cancer with an initially responsive disease eventually experience relapse as a result of acquired drug resistance to these agents. Consequently, it is critical to define the mechanisms by which drug resistance develops for the identification of therapeutic strategies to avoid and/or to overcome cancer cell acquired resistance (4).

Metformin (*N,N'*-dimethylbiguanide) belongs to the biguanide class of oral hypoglycemic agents and is a widely used antidiabetic drug now prescribed to almost 120 million people in the world for the treatment of type II diabetes

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Translational Relevance

The combination of metformin with gefitinib, a molecular targeted agent blocking the EGF receptor (EGFR), has been shown to be particularly synergistic, *in vitro* and *in vivo*, in those non-small cell lung cancer (NSCLC) cell lines harboring an LKB1 wild-type gene. In addition to its antiproliferative effects in NSCLC cell lines, we observed also an increase in the level of activated phosphorylated mitogen-activated protein kinase (MAPK) after metformin single-agent treatment, which is mediated by an increased heterodimerization between C-RAF and B-RAF. This could be therapeutically relevant, as we have shown that while exerting antiproliferative and proapoptotic effects, single-agent metformin treatment could enhance pro-proliferating signals through the RAS/RAF/MAPK pathway. At the same time, treatment with both metformin and gefitinib can avoid aberrant signals through the RAS/RAF/MAPK pathway and could explain, at least in part, the mechanism of such significant synergism. These findings open new possibilities for the treatment of patients whose cancers become refractory to initially effective molecular targeted agents, such as anti-EGFR. Future perspective studies are required in patients with NSCLCs to investigate better the effect of metformin action on the RAS/RAF/MAPK pathway and the best context in which to use metformin in combination with molecular targeted agents.

(5). Metformin also displays significant growth-inhibitory and proapoptotic effects in several cancer models, alone (6–9) or in combination with chemotherapeutic drugs (10, 11). A recent study showed that metformin prevents tobacco-induced carcinogenesis in mice with 72% decrease in tumor burden (12).

The effects of metformin on cancer cell proliferation have been associated with AMPK activation, reduced mTOR signaling, and protein synthesis (13, 14).

The aim of the present work was to examine the effects of a combined treatment of metformin with gefitinib, a selective EGFR-TKI on NSCLC cell lines. For this purpose, we have used a panel of human NSCLC cell lines with a defined spectrum of sensitivity to the inhibition of EGFR by gefitinib, including also an *in vitro* model of acquired resistance to gefitinib developed from the sensitive human CALU-3 lung cancer cells (15).

Materials and Methods

Cell lines, drugs, and chemicals

The human NSCLC H1299, H1975, A549, H460, GLC82, H460, and CALU-3 cell lines were provided by the American Type Culture Collection and maintained in RPMI-1640 supplemented with 10% FBS (Life Technologies) in a humidified atmosphere with 5% CO₂. CALU-3 GEF-R is

a cell line obtained *in vitro* as previously described (15). Briefly, over a period of 12 months, human CALU-3 lung adenocarcinoma cells were continuously exposed to increasing concentrations of gefitinib. The starting dose was the dose causing the inhibition of 50% of cancer cell growth (IC₅₀; gefitinib, 1 μmol/L). The drug dose was progressively increased to 15 μmol/L in approximately 2 months, to 20 μmol/L after other 2 months, to 25 μmol/L after additional 2 months, and, finally, to 30 μmol/L for a total of 12 months. The established resistant cancer cell lines were then maintained in continuous culture with the maximally achieved dose of each TKI that allowed cellular proliferation (30 μmol/L for each drug).

Gefitinib was provided by AstraZeneca; metformin was purchased from Sigma-Aldrich. Primary antibodies against p-EGFR (Tyr1068), EGFR, p-MAPK44/42 (Thr202/Tyr204), MAPK44/42, p-AKT (Ser473), AKT, C-RAF, BRAF, AMPK, p-AMPK (thr172), S6, p-S6 (ser 235-236), P70S6K, p-P70S6K (Thr389/421), 4EBP1, p-4EBP1 (Thr37/46), acetyl-CoA carboxylase (ACC), p-ACC (Ser79), and β-actin were obtained from Cell Signaling Technology. All other chemicals were purchased from Sigma-Aldrich.

Cell proliferation assays

Cancer cells were seeded in 96-well plates and were treated with different doses of gefitinib, metformin or both for 72 hours. Cell proliferation was measured with the MTT assay. The IC₅₀ values were determined by interpolation from the dose–response curves. Results represent the median of 3 separate experiments each conducted in quadruplicate. The results of the combined treatment were analyzed according to the method of Chou and Talalay by using the CalcuSyn software program (Biosoft; ref. 16). The resulting combination index (CI) is a quantitative measure of the degree of interaction between different drugs. If CI = 1, it denotes additivity; if CI > 1, it denotes antagonism; and if CI < 1, it denotes synergism.

DNA synthesis was measured by 5-bromo-2'-deoxyuridine (BrdUrd) labeling and detection kit (Roche Diagnostics). Cells were seeded onto glass coverslips and treated with metformin for 72 hours. Then, cells were incubated for 1 hour with BrdUrd (10 mmol/L) and fixed. Coverslips were incubated with anti-BrdUrd and secondary fluorescein-conjugated antibody. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2, Zeiss) interfaced with the image analyzer software KS300. Cell nuclei were counterstained with Hoechst. The average results ± SD of 3 independent experiments in which at least 500 cells were counted are shown.

Western blotting

Following treatment, cancer cells were lysed with Tween-20 lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonylfluoride, and 10 μg/mL of leupeptin and aprotinin) and sonicated. Equal amounts of protein were analyzed by SDS-PAGE. Thereafter, proteins were

transferred to nitrocellulose membranes and analyzed by specific primary antibodies, as indicated in the experiment. Proteins were detected via incubation with horseradish peroxidase-conjugated secondary antibodies and ECL chemiluminescence detection system.

For the siRNA transfection, NSCLC cells in the logarithmic growth phase in 6-well plates (5×10^5 cells/well) were transfected with 10 μ L of 20 μ mol/L LKB1 siRNA (Si-LKB1 Qiagen SI02665383) or control scrambled siRNA (Dharmacon Research) using Lipofectamine 2000 (Invitrogen), according to the protocol of the manufacturer. LKB1 DNA transfection (Myc-DDK-tagged STK11) was conducted using Lipofectamine LTX according to the manufacturer's specifications.

Immunoprecipitations were done using 1 mg protein from the total cell lysates and 1 μ g mouse monoclonal anti-C-RAF antibody, mouse monoclonal anti-B-RAF antibody, or healthy preimmune serum anti-mouse for the negative control and by incubating overnight at 4°C. The immunocomplexes were precipitated with protein-G agarose (Pharmacia-LKB Biotechnology). The immunoprecipitates were resolved on 6% SDS-PAGE gels, followed by Western blotting as described elsewhere (17).

Growth in soft agar

Cells (10^4 cells per well) were suspended in 0.5 mL 0.3% Difco Noble agar (Difco) supplemented with complete culture medium. This suspension was layered over 0.5-mL 0.8% agar-medium base layer in 24-multiwell cluster dishes (Becton Dickinson) and treated with different concentrations of metformin, gefitinib, or both in combination. The medium was changed daily during this period, at the end of which, tumor cell colonies measuring at least 80 μ m were counted under using a dissection microscope. Assays were conducted in triplicate.

Assessment of apoptosis

Apoptosis was detected by flow cytometry via the examination of altered plasma membrane phospholipid packing by lipophilic dye Annexin V as described elsewhere (18). Briefly, treated cells were harvested by trypsin, washed twice with PBS, and then resuspended in binding buffer at a concentration of 1×10^6 cells/mL according to the manufacturer's instruction. Thereafter, 5 μ L of Annexin V-FITC and 5 μ L of propidium iodide were added into 100 μ L of cell suspension and incubated for 30 minutes at room temperature in the dark. After adding 400 μ L of binding buffer, labeled cells were counted by flow cytometry within 30 minutes. All early apoptotic cells (Annexin V-positive, propidium iodide-negative), necrotic/late apoptotic cells (double positive), as well as living cells (double negative) were detected by FACSCalibur flow cytometer and subsequently analyzed by Cell Quest software (Becton Dickinson). Argon laser excitation wavelength was 488 nm, whereas emission data were acquired at wavelength 530 nm (FL-1 channel) for fluorescein isothiocyanate (FITC) and 670 nm (FL-3 c3 channel) for propidium iodide.

Tumor xenografts in nude mice

Four- to 6-week old female balb/c athymic (*nu+ / nu+*) mice were purchased from Charles River Laboratories. The research protocol was approved and mice were maintained in accordance with the Institutional Guidelines of the Second University of Naples Animal Care and Use Committee. Mice were acclimatized for 1 week before being injected with cancer cells and injected subcutaneously with 10^7 H1299 and CALU-3 GEF-R cells that had been resuspended in 200 μ L of Matrigel (Collaborative Biomedical Products). When established tumors of approximately 75 mm³ in diameter were detected, mice were left untreated or treated with oral administrations of metformin (200 mg/mL metformin diluted in drinking water and present throughout the experiment), gefitinib (150 mg/kg daily orally by gavage), or both for the indicated time periods. Each treatment group consisted of 10 mice. Tumor volume was measured using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. Tumor tissues were collected from the xenografts and analyzed by Western blotting for the expression and activation of EGFR, AMPK, mitogen-activated protein kinase (MAPK), and S6.

Statistical analysis

The Student *t* test was used to evaluate the statistical significance of the results. All *P* values represent 2-sided tests of statistical significance. All analyses were conducted with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software).

Results

Effect of metformin alone and in combination with gefitinib on NSCLC proliferation and anchorage-independent colony-forming ability

To evaluate the antiproliferative effects of different doses of metformin, alone and in combination with gefitinib, we did an MTT assay on a panel of 7 NSCLC cell lines with different sensitivity to gefitinib. Treatment with metformin, ranging from 0.1 to 20 mmol/L as single agent for 72 hours, resulted in a dose-dependent inhibition of growth with an IC₅₀ value between 2 and 2.5 mmol/L in H1299, GLC82, H1975, CALU-3, and CALU-3 GEF-R cell lines. H460 and A549 cell lines displayed a limited metformin-induced inhibition of cell proliferation (Table 1A), with an IC₅₀ over 20 mmol/L. Treatment with gefitinib alone led to an inhibition of CALU-3 and GLC82 cell proliferation, with an IC₅₀ of 2 μ mol/L, according to previous reports (15). H1299, H460, H1975, CALU-3 GEF-R, and A549 cancer cell lines showed a limited sensitivity to gefitinib treatment with about 80% to 90% cells surviving at 5 μ mol/L dose of EGFR inhibitor (Table 1A).

We then assessed the growth-inhibitory effects of combinations of metformin with gefitinib. As shown in Table 1B and Supplementary Fig. S1, a 72-hour exposure to metformin and gefitinib resulted in a clear synergism in CALU-3, CALU-3 GEF-R, H1975, H1299, and GLC82, with a CI between 0.001 and 0.1 (Table 1B and Supplementary Fig. S1). Of interest, the synergism was evident also in H1975,

Table 1. Effects of metformin, gefitinib, and their combination on NSCLC cell lines

| Cancer cell line | Metformin IC ₅₀ , mmol/L | Gefitinib IC ₅₀ , μmol/L | |
|---|-------------------------------------|-------------------------------------|------------|
| A. Evaluation of the cell growth-inhibitory effects of metformin or gefitinib given as single agents | | | |
| CALU-3 | 1 | 2 | |
| CALU-3 GEF-R | 2 | >10 | |
| H1299 | 2.5 | >10 | |
| GLC82 | 1.5 | 2 | |
| A549 | >20 | >10 | |
| H460 | >20 | >10 | |
| H1975 | 1.5 | >10 | |
| Cancer cell line | CI at fa50 | CI at fa75 | CI at fa90 |
| B. Evaluation of the growth-inhibitory effects by the combined treatment with metformin and gefitinib | | | |
| CALU-3 | 0.001 | 0.005 | 0.11 |
| CALU-3 GEF-R | 0.039 | 0.038 | 0.037 |
| H1299 | 0.001 | 0.005 | 0.065 |
| GLC82 | 0.001 | 0.007 | 0.012 |
| A549 | 1.4 | 1.5 | 1.3 |
| H460 | 1.7 | 2 | 1.4 |
| H1975 | 0.001 | 0.005 | 0.04 |

NOTE: A. The evaluation of the cell growth-inhibitory effects of metformin or gefitinib, given as single agents, was done by MTT bromide assay. IC₅₀ values for each drug were calculated by carrying out dose–response experiments. Cells were treated for 72 hours with metformin (0.01–20 mmol/L) or with gefitinib (0.01–20 μmol/L) for 72 hours. B. Evaluation of the growth-inhibitory effects by the combined treatment with metformin and gefitinib using an MTT bromide assay. Treatment combinations and sequences are described in Materials and Methods. CI values were calculated according to the Chou and Talalay mathematical model for drug interactions using the CalcuSyn software for different fractions affected (fa). CI is a quantitative measure of the degree of interaction between different drugs. If CI = 1, it denotes additivity; if CI > 1, it denotes antagonism; if 1 < CI < 0.7, it denotes slight synergism; if CI = 0.7–0.3, it denotes synergism; if CI < 0.3, it denotes strong synergism. Results are the median of 3 independent experiments, each done in 8 replicate wells for experimental point.

CALU-3 GEF-R, and H1299 cell lines, which are strongly resistant to the EGFR inhibition, indicating that metformin can revert resistance to gefitinib in some cancer cell lines. In contrast, no synergistic effect was observed by the combination of metformin and gefitinib in A549 and H460 cell lines. However, as metformin is known to disrupt mitochondrial respiration, which may affect the MTT assay results, we measured inhibition of cell proliferation also by using the BrdUrd incorporation. To this end, we used the IC₅₀ dose of metformin 2.5 mmol/L alone and in combination with gefitinib. Results confirmed a strongest inhibitory efficacy by adding metformin to gefitinib in H1975, CALU-3, CALU-3 GEF-R, H1299, and GLC82 cell lines (Fig. 1A).

We then tested the effects of metformin alone and in combination with gefitinib on anchorage-independent colony formation of NSCLC cells. As shown in Fig. 1B, CALU-3, CALU-3 GEF-R, H1299, and GLC82 cells showed greater sensitivity to single-agent metformin treatment at dose of 5 mmol/L than A549 and H460, and sensitivity was even greater when metformin was combined with 1 μmol/L gefitinib, with the reduction of the anchorage-independent colony forming ability to less than 10% of untreated control cells.

Effects on apoptosis by combined treatment of metformin and gefitinib

We further asked whether the increased antiproliferative effect induced by metformin alone and in combination with gefitinib would be the result of an increased apoptosis. Therefore, we analyzed the induction of apoptosis in CALU-3, CALU-3 GEF-R, and in H1299 human NSCLC cell lines after 72-hour treatment with metformin as single agent or in combination with gefitinib. As shown in Fig. 1C, flow cytometric analysis of CALU-3 cells revealed that 19% of gefitinib-treated cells and 21% [95% confidence interval, 14.3%–28.6%, $P < 0.05$] of metformin-treated cells underwent apoptosis. In contrast, combined treatment with both gefitinib and metformin significantly enhanced the apoptotic cell percentage to 65% (95% confidence interval, 55.4%–75.8%; $P < 0.001$; Fig. 1B). Similar results were observed in CALU-3 GEF-R and H1299 cells, with respectively a 5% and 4% apoptotic rate in gefitinib-treated cells, 20% and 26% in metformin-treated cells, and 68% and 78% of apoptotic cells after the combined treatment with gefitinib and metformin ($P < 0.001$; Fig. 1B). These results were confirmed by Western blot analysis: metformin single agent was able to induce the cleavage of the 113-kDa PARP to the 89-kDa fragment in all tested cell lines. Single-agent gefitinib

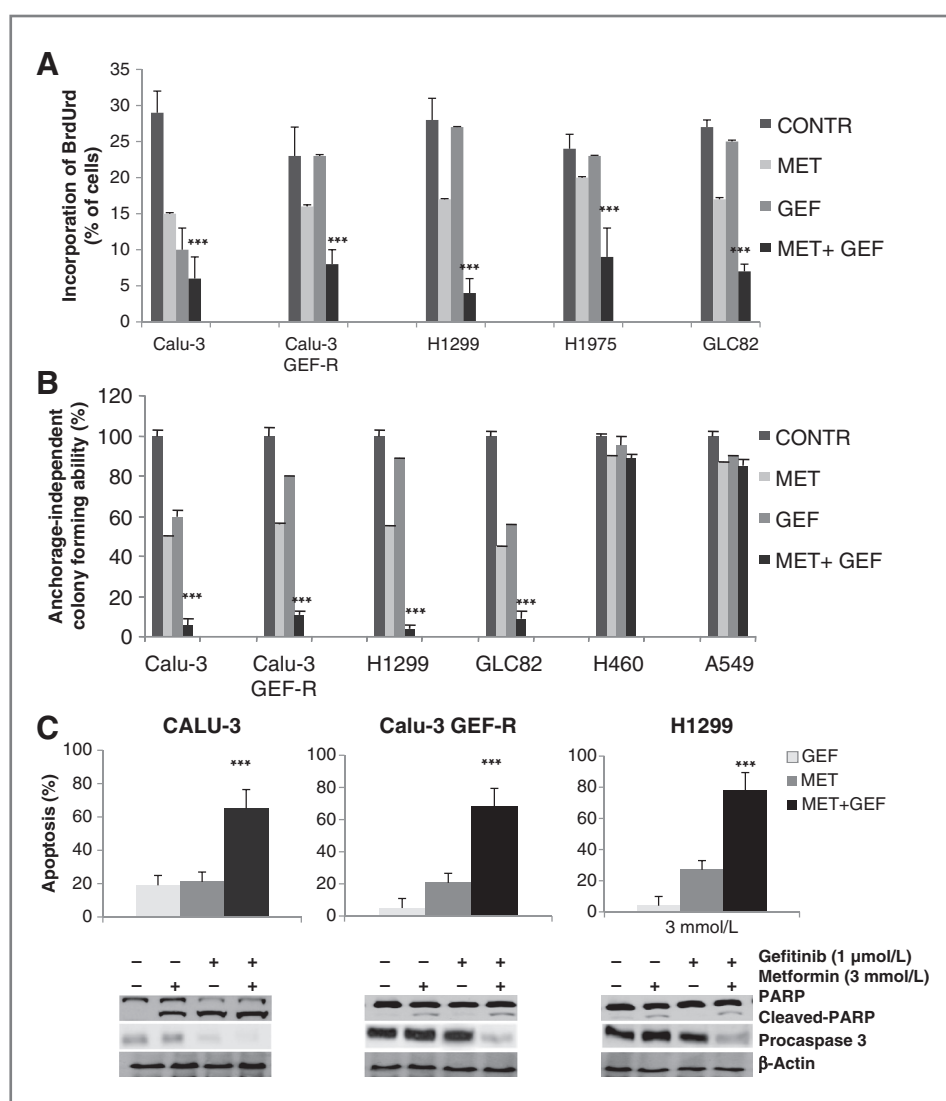


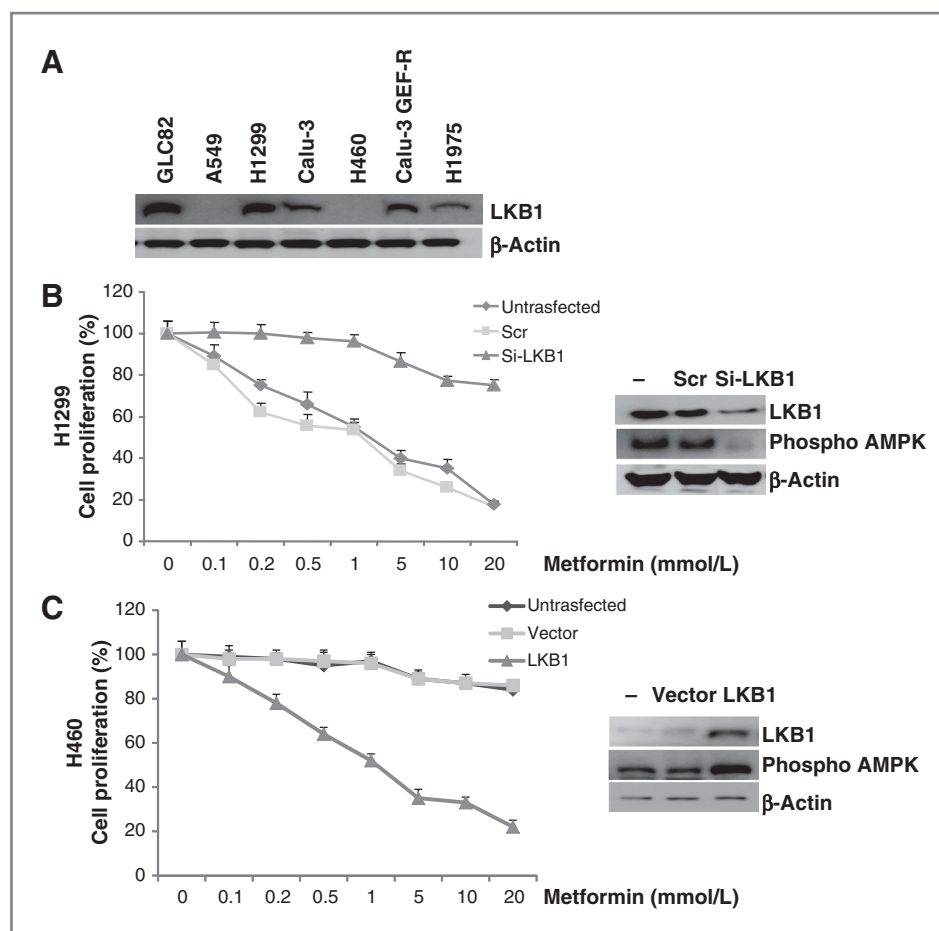
Figure 1. A, effect of metformin (MET) alone and in combination with gefitinib (GEF) on cell proliferation, on anchorage-independent growth ability of NSCLC cell lines, and on the induction of apoptosis in CALU-3, CALU-3 GEF-R, and H1299 cell lines. Cells were treated with metformin, gefitinib, and combination of both. A, cell proliferation was measured by BrdUrd incorporation assay. BrdUrd was added for 1 hour, and cells were processed for immunofluorescence with anti-BrdUrd. Cell nuclei were counterstained with Hoechst. The average results \pm SD of 3 independent experiments in which at least 500 cells were counted are shown. B, anchorage-independent growth efficiency was assessed by counting colonies consisting of more than 50 cells after 10 days of growth. Independent experiments were repeated three times. Columns, mean of 3 identical wells of a single representative experiment; bars, upper 95% confidence interval; ***, $P < 0.001$ for comparisons between cells treated with drug combination and cells treated with single agent. C, apoptosis was evaluated as described in Materials and Methods with Annexin V staining in CALU-3, CALU-3 GEF-R, and H1299 cancer cells, which were treated with metformin (3 mmol/L) or with gefitinib (1 μ mol/L) or both. Columns, mean of 3 identical wells of a single representative experiment; bars, top 95% confidence interval; ***, $P < 0.001$ for comparisons between cells treated with drug combination and cells treated with single agent. Bottom, Western blotting analysis of PARP and caspase-3 cleavage following treatment with metformin alone or with gefitinib. Western blotting on β -actin is included as a loading control.

induced an increased cleavage of procaspase-3 only in CALU-3 cell line. However, the combination of metformin and gefitinib was accompanied by a sustained cleavage of procaspase-3 and 113-kDa PARP in CALU-3, CALU-E GEF-R, and H1299 cell lines (Fig. 1C). Similar data were obtained with GLC82 cancer cell lines (data not shown). These findings suggest that metformin blocks proliferation and/or survival mechanisms in NSCLC cancer cells in which EGFR is blocked by gefitinib or is no longer the principal driver of growth.

Role of LKB1 as mediator of metformin anticancer activity

We therefore asked what could be the reason of the different response to metformin treatment among the NSCLC cell lines examined. Metformin interferes with the energetic metabolism of the cell by disrupting mitochondrial respiration, leading to an increase in the intracellular ratio of AMP:ATP, which in turn leads to activation of AMPK by LKB1 (19). Thus, loss of function of LKB1 can reduce the sensibility to metformin. Somatic inactivation of *LKB1* gene

Figure 2. Role of LKB1 in the prediction of a benefit from metformin treatment. **A**, Western blotting of LKB1 in our panel of NSCLC cell lines. Western blotting on β -actin is included as a loading control. **B**, MTT cell proliferation assays in human H1299 cancer cell transfected with scramble (scr) or LKB1 siRNA (si-LKB1), with the indicated concentrations of metformin for 3 days. Results represent the median of 3 separate experiments each carried out in quadruplicate. Right, Western blotting for evaluation of LKB1 and phospho-AMPK after transfection. **C**, MTT cell proliferation assays in human H460 cancer cell transfected with a vector or LKB1 expression plasmid with the indicated concentrations of metformin for 3 days. Bottom, Western blotting for evaluation of LKB1 and phospho-AMPK after transfection.



is a common event in NSCLCs, especially in lung adenocarcinoma cells (20). Previous reports show that among the panel of NSCLC cell lines, A549 and H460 are deficient in LKB1 expression (20). We confirmed and extended these results by conducting Western blot analysis for LKB1 protein expression. In Fig. 2A the levels of LKB1 protein in the panel of NSCLC cell lines tested are reported. LKB1 protein in A549 and in H460 cells was not detectable. To confirm a role of LKB1 as mediator of metformin anti proliferative activity, we then tested the effects of metformin on H1299 NSCLC cell line, in which LKB1 expression was inhibited by siRNA transfection. As shown in Fig. 2B, a significant reduction in the antiproliferative effects of metformin on H1299 cells transfected with an siRNA for LKB1 was observed, suggesting a role for LKB1 as a mediator of efficacy of metformin activity in NSCLC cells. The same experiment was carried out with another siRNA for LKB1 with similar results (data not shown). In addition, we confirmed by Western blotting (Fig. 2B) the reduced metformin-mediated AMPK activation by knockdown of LKB1. To determine whether LKB1 expression may resume sensitivity to metformin, H460 cells were transiently transfected with an LKB1 expression plasmid. Twenty-four hours after transfection, treatment with metformin led to a marked decrease of H460 cell proliferation (Fig. 2C).

Effects on intracellular signaling pathways following metformin treatment

To further elucidate the mechanism responsible for the cell growth-inhibitory effects of metformin, we next evaluated the effects of metformin on the expression of selected proteins and their activated forms, known to be important steps in the prosurvival and proliferation pathways, in CALU-3, CALU-3 GEF-R (Fig. 3A), and A549 cells. Furthermore, as metformin activates AMPK, we sought to examine whether pathways known to be influenced by AMPK activity might be affected in human tumor cells by metformin treatment. AMPK acts as a metabolic checkpoint inhibiting cellular growth. The most thoroughly described mechanism by which AMPK regulates cell growth is through the suppression of the mTORC1 pathway. mTOR exerts its effects by phosphorylating the eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and via the ribosomal protein S6 kinase (S6K, formerly known as p70s6K; ref. 21). As the activation of AMPK correlates tightly with phosphorylation at Thr-172 (pAMPK α), we assessed the activation of AMPK by determining phosphorylation AMPK α and its primary downstream targeting enzyme, ACC, using immunoblots with specific phospho-Thr-172 and phospho-Ser-79 antibodies, respectively.

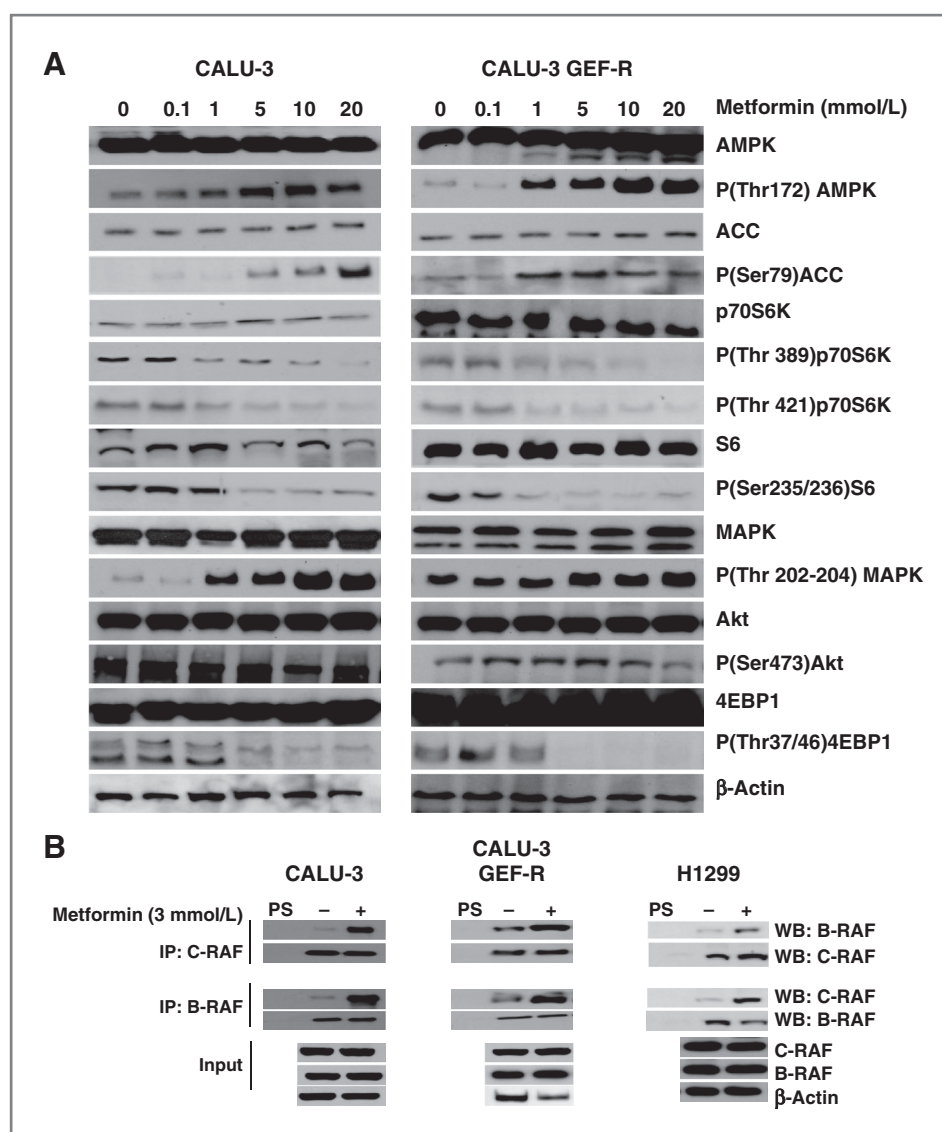


Figure 3. Effects on the downstream pathway by metformin treatment. A, Western blotting of AMPK, ACC, MAPK, Akt, p70S6K, S6, and 4EBP1 activation following treatment with the indicated concentration of metformin. β -Actin was included as a loading control. B, coimmunoprecipitation was done for the interaction between B-RAF and C-RAF. Whole-cell extracts from CALU-3, CALU-3 GEF-R, and H1299 cells untreated or treated with metformin (3 mmol/L) for 72 hours were immunoprecipitated (IP) with anti-C-RAF or anti-B-RAF antibodies. The immunoprecipitates were subjected to Western blot analysis (WB) with indicated antibodies. Control immunoprecipitation was done using control mouse preimmune serum (PS).

Treatment of CALU-3 and CALU-3 GEF-R cells with metformin (from 0.1 to 20 mmol/L) for 72 hours induced increased levels of activated phosphorylated AMPK without affecting total amount of AMPK protein (Fig. 3A). We tested also the ACC phosphorylation, which is a standard indicator of AMPK activity and resulted increased by metformin treatment. A significant decrease in the level of activated phosphorylated P70S6K and its principal target, S6, were evident after metformin treatment in a dose-dependent manner. Similar reduction was observed on the phosphorylation status of 4EBP1 at Thr37/46. In contrast, total amounts of P70S6K, 4EBP1, and S6 were not changed.

As the activated, phosphorylated forms of AKT and MAPK are key intracellular mediators of growth factor-activated cell survival and proliferation signals, investigating the activation state of these molecular pathways may be of interest in the understanding the mechanism of action of metformin in NSCLC cancer cells. Metformin treatment, at

different dose levels, did not affect the protein levels of MAPK and AKT (Fig. 3A). While metformin treatment did not influence the activation status of AKT, it caused an unexpected increase in the levels of activated phosphorylated MAPK (p-MAPK) in CALU-3 and CALU-3 GEF-R cells. Similar results were obtained in H1299 and GLC82 (data not shown). We then asked what could be the mechanism of the metformin-induced activation of MAPK. The activation of AMPK represses protein synthesis, resulting in the inhibition of cell growth. Repression of protein synthesis is accomplished by AMPK-induced phosphorylation and activation of TSC2 and consequently inhibition of Rheb (RAS homolog enriched in brain)/mTORC1 signaling (22). Rheb, a member of the Ras/Rap/Ral subfamily of RAS proteins, is known to inhibits B-RAF activity, and recently, it has been shown to inhibit also the kinase activity of C-RAF, and, more importantly, the heterodimerization of B-RAF and C-RAF (23). The B-RAF

and C-RAF heterodimerization is a crucial event for the RAS-dependent activation of MAPK. Therefore, the metformin-induced activation of AMPK could inactivate Rheb and therefore enhance the BRAF and C-RAF association. To this end, we carried out coimmunoprecipitation experiments. C-RAF immunoprecipitates from CALU-3, CALU-3 GEF-R, and H1299 cells treated with 3 mmol/L metformin for 72 hours showed greater B-RAF binding compared with untreated cells (Fig. 3B). Control immunoprecipitates using preimmune serum exhibited no immunoreactive band. Similarly, B-RAF immunoprecipitates from metformin-treated CALU-3, CALU-3 GEF-R, and H1299 cells exhibited greater levels of C-RAF binding than untreated cells did (Fig. 3B). These results suggest that metformin induces physical interaction between C-RAF and B-RAF that could be responsible for the activation of MAPK after metformin treatment.

The effect of metformin was tested also on A549 and H460 cell lines, and no changes in the level of activated phosphorylated AMPK, P70S6k, 4EBP1, p-AKT, and p-MAPK were observed (Supplementary Fig. S2).

Taken together, these results suggest that activation of AMPK and inhibition of mTOR downstream signals are important to predict sensitivity to metformin treatment.

Effects on the intracellular signaling pathways following treatment with metformin and gefitinib

To determine whether the synergistic growth inhibition effects obtained by the combination of metformin and the EGFR-TKI, gefitinib, was due to a more effective inhibition of EGFR activation and the intracellular signaling through MAPK and/or AKT, Western blot analyses were done on protein extracts from CALU-3 and CALU-3 GEF-R NSCLC cells that were treated with 3 mmol/L of metformin, 1 μ mol/L gefitinib, or with combinations of both metformin and gefitinib. Treatment was conducted for 72 hours.

We selected these cells as they represent a model of sensitivity (CALU-3) and resistance (CALU-3 GEF-R) to treatment with anti-EGFR drugs as single agent. Figure 4 illustrates that gefitinib treatment, as single agent, although causing a decrease on EGFR phosphorylation, had a very little effect in inhibiting downstream mitogenic and pro-survival signals in CALU-3 GEF-R, as shown by the levels of activated phosphorylated AKT and MAPK and by little or no effect on the activation of p70S6k and S6.

Treatment with metformin in combination with gefitinib resulted in a more pronounced decrease in the levels of protein phosphorylation (p-MAPK, p-AKT). The combined treatment also affected the mTOR signaling as suggested by the sustained inhibition of the phosphorylation of S6 and of p70S6K.

Effects of the combined treatment with metformin and gefitinib on H1299 and CALU-3 GEF-R tumor xenografts

We finally investigated the *in vivo* antitumor activity of metformin in nude mice bearing H1299 or CALU-3 GEF-R cells that were grown subcutaneously as tumor xeno-

grafts. Treatment with metformin or gefitinib, as single agents, caused a slight decrease in tumor size as compared with control untreated mice. For example, at day 35 from the starting of treatment, the mean tumor volume in mice bearing H1299 tumor xenografts and treated with metformin and gefitinib were 78% and 70%, respectively, as compared with control untreated mice. Treatment with the combination of metformin and gefitinib induced a significant reduction in tumor growth (Fig. 5A). In this respect, at day 35 from the starting of treatment, the mean tumor volumes in the combination treatment group in mice bearing H1299 tumor xenografts ranged between 27% and 40%, as compared with control untreated mice. Similar results were reported for mice bearing CALU-3 GEF-R tumor xenografts (Fig. 5B). During our experiments, no obvious side effects were observed in mice treated with metformin. We then studied the effects of gefitinib, metformin, and their combination on the activation of AMPK, EGFR, MAPK, and S6 *in vivo*. According to Western blot analysis of total protein extracts harvested from the H1299 xenograft tumor tissues, 2 samples for each arm of treatment, the levels of pAMPK were increased by metformin. Interestingly, metformin treatment activated MAPK. In addition, combined treatment with gefitinib and metformin induced marked decrease in the levels of phosphoS6 (Fig. 5C).

Discussion

The correlation between diabetes and increased cancer risk has been supposed since 1910, by G.D. Maynard (24), and 100 years later, a Joint Conference of the American Diabetes Association and the American Cancer Society led to a Consensus Report that clearly indicated an association

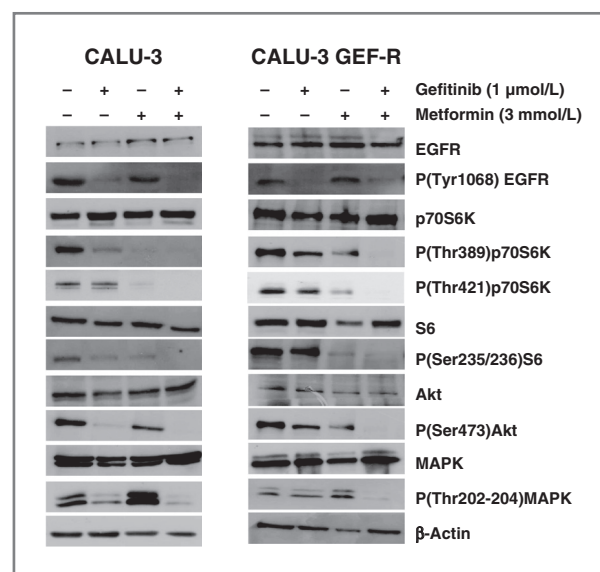


Figure 4. Effects on the downstream pathway by combined treatment of metformin and gefitinib. Western blotting of EGFR, MAPK, AKT p70S6K, and S6 activation following treatment with the indicated concentration of metformin and gefitinib in CALU-3 and CALU-3 GEF-R cell lines. β -Actin was included as a loading control.

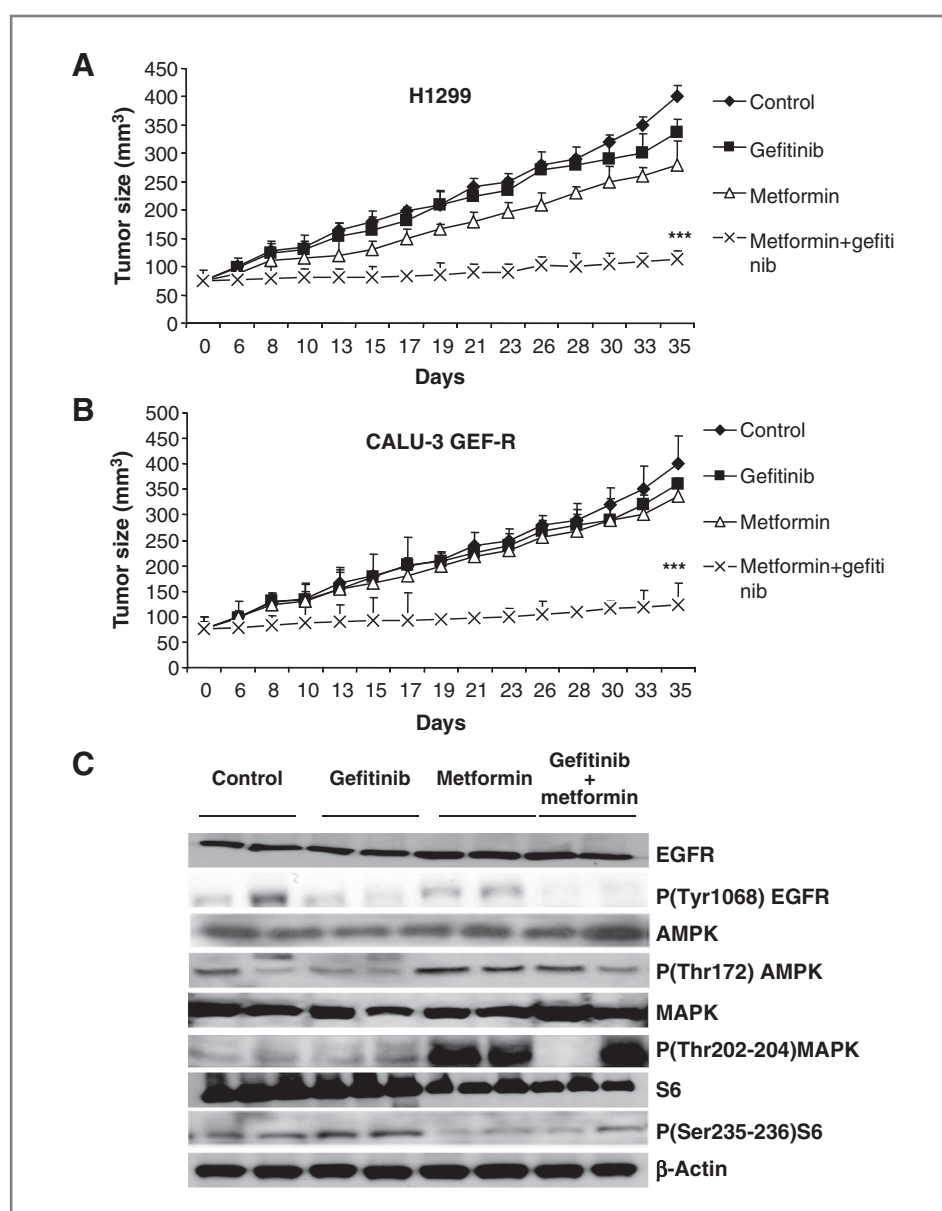


Figure 5. Effects of the combination treatment of metformin and gefitinib on NSCLC tumor xenografts. Athymic nude mice were injected subcutaneously into the dorsal flank with 10^7 H1299 (A) or with CALU-3 GEF-R (B) cancer cells. After 7 to 10 days (average tumor size, 75 mm³), mice were treated as indicated in Materials and Methods for 5 weeks. Briefly, mice were left untreated or treated with oral administrations of metformin (200 mg/mL metformin diluted in drinking water and present throughout the experiment), gefitinib (150 mg/kg daily orally by gavage), or both for the indicated time periods. Each treatment group consisted of 10 mice. Data represent the average (\pm SD). Student *t* test was used to compare tumor sizes among different treatment groups at day 35 following the start of treatment. Metformin and gefitinib versus single-agent treatment (2-sided $P < 0.001$). C, Western blotting for the expression of AMPK, MAPK, and S6 activation following treatment with gefitinib, metformin, or both on protein extracts obtained from harvested tumors. β -Actin was included as a loading control

between diabetes and the incidence of cancers (25). Since the first report of Evans and colleagues (5), a series of studies have suggested that metformin is associated with a reduced risk of cancer, as compared with other antidiabetic treatments (sulfonylurea, insulin) in diabetic patients (26–29).

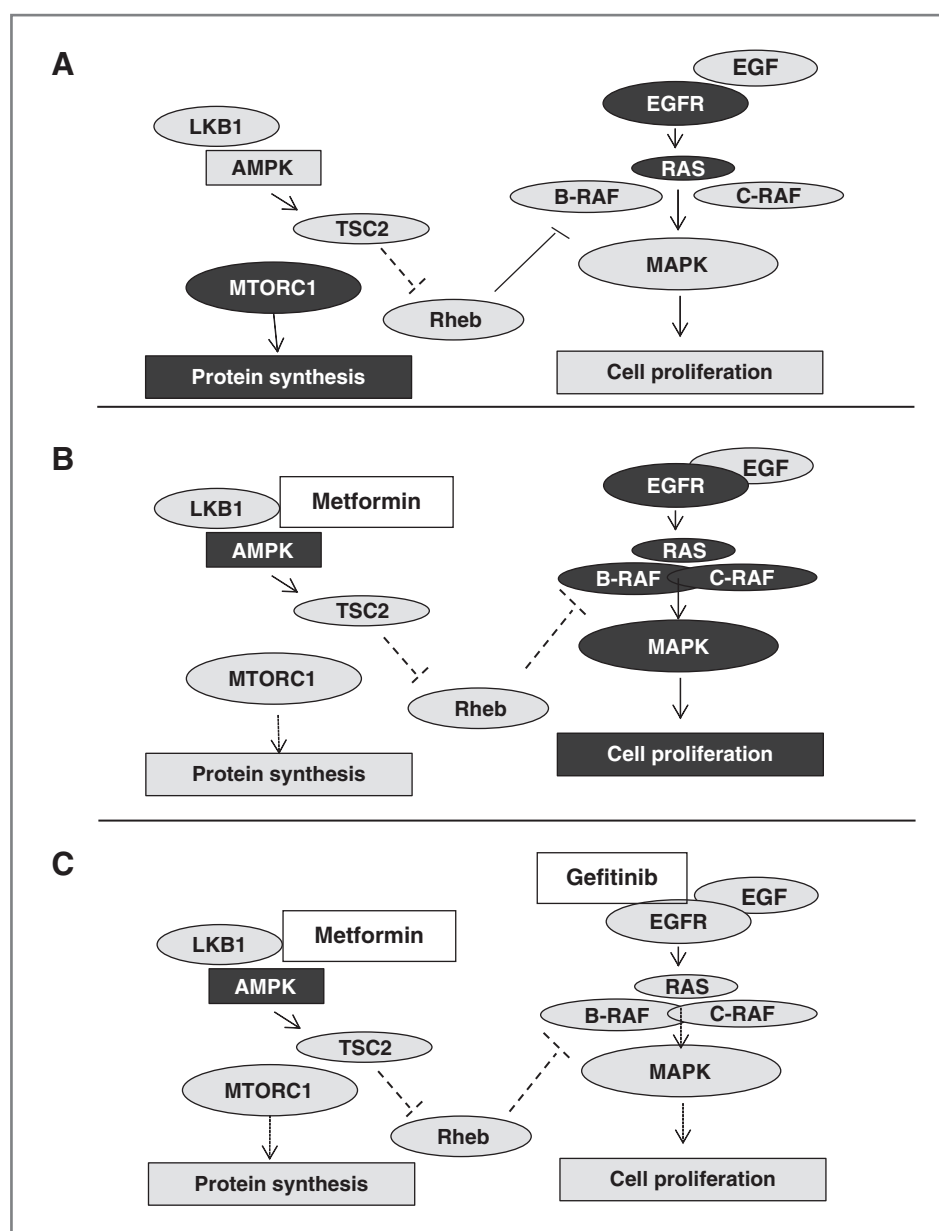
Metformin is a widely used antidiabetic drug now prescribed to almost 120 million people in the world for the treatment of type II diabetes. At the cellular level, metformin interferes with the energetic metabolism of the cell by disrupting mitochondrial respiration, leading to an increase in the intracellular ratio of AMP:ATP, which in turn leads to activation of AMPK by the LKB1 (19). Metformin-mediated AMPK activation leads to an inhibition of mTOR signaling, a reduction in phosphorylation of its major downstream effectors, the 4E-BPs and p70S6Ks, and an inhibition of global protein synthesis and proliferation in a number of

different cancer cell lines (6–10). Several reports established a direct action of metformin on cancer cells with a significant and consistent antiproliferative action, *in vitro* and *in vivo* (6–10). In addition, previous reports showed an adjuvant role of metformin for other chemotherapeutic drugs, such as paclitaxel, carboplatin, and doxorubicin, however, without describing any mechanism to explain such synergism (10).

In this study, we have evaluated for the first time the effects of metformin and of the combination of metformin and gefitinib, a selective EGFR-TKI, in NSCLC cell lines and showed a significant potentiating of the antiproliferative and proapoptotic activity both *in vitro* and *in vivo*.

Although we and others (6–10, 30) used doses of metformin higher than that observed in diabetic patients, where the metformin plasma concentration is between

Figure 6. Schematic model of intracellular effects following metformin, gefitinib, or combined treatment of metformin and gefitinib on NSCLC cell lines. The activity of each protein is represented according to the colors: light gray for low activity and dark gray for high activity. The relative level of interaction between the components is indicated by the type of lines of the arrows: dashed lines for weak interaction, solid lines for strong interaction. A, under basal conditions, EGF activates EGFR and downstream pathway. B, in metformin-treated cells, AMPK is activated and inhibits mTORC1-driven protein synthesis. In addition, AMPK activates TSC2 and consequently inhibits Rheb. Inhibition of Rheb enhances the B-RAF and C-RAF association, thus potentiating signaling from RAS to MAPK. C, combined treatment with metformin and gefitinib resulted in a pronounced inhibition of MAPK and mTOR signaling, leading to inhibition of cell proliferation and protein synthesis.



6 and 30 $\mu\text{mol/L}$ (http://www.rxlist.com/cgi/generic/fortamet_cp.htm), the *in vivo* experiment showed results similar to those obtained *in vitro* without any evident toxicity for animals, suggesting that the events showed *in vitro* can be translated *in vivo*.

In addition, it has been shown that the positive charge of metformin could promote its accumulation within the mitochondrial matrix by 1,000-fold (>20 mmol/L). Indeed, metformin accumulates in tissues at concentrations several fold higher than those in blood (31–33), indicating that concentrations of metformin similar to those used in pre-clinical models (1–10 mmol/L) might be attained also during cancer treatment.

We further showed that this synergism is strictly dependent on LKB1 mutation status. Indeed, Algire and collea-

gues (34) reported that cancer cells lacking LKB1 protein expression do not respond to metformin *in vitro*, suggesting that metformin triggers various cellular responses, which may vary depending on cancer origin. Somatic LKB1 gene alterations are rare in sporadic cancers. However, a few reports have shown the presence of somatic LKB1 gene alterations in a subset of lung cancers. In particular, LKB1 genetic alterations have been frequently detected in NSCLCs as compared with small cell lung cancers and are significantly more frequent in cancer cell lines with KRAS mutations, occurring in a subset of poorly differentiated lung adenocarcinoma that appear to correlate with smoking males (20).

In the present study, we observed also an increase in the level of activated phosphorylated MAPK after metformin

single-agent treatment (Fig. 3). Metformin is known to activate the phosphorylation of AMPK, thus activating TSC2 and consequently inhibiting Rheb/mTORC1 signaling (22). A recent study (23) showed a role of Rheb as inhibitor of C- and B-RAF kinase activity and their heterodimerization. Therefore, we supposed that the metformin-induced activation of AMPK could enhance the C-RAF/B-RAF dimerization through a downregulation of Rheb. Indeed, the results of the present study suggest that metformin treatment increases such association, thus potentiating the signaling from RAS to MAPK. This could be therapeutically relevant, as we have shown that, while exerting antiproliferative and proapoptotic effects, single-agent metformin treatment could enhance pro-proliferating signals through the RAS/RAF/MAPK pathway, that could in turn induce cell proliferation in those cell lines with constitutively activating RAS mutations (Fig. 6). The activation of MAPK by metformin treatment, which is reported for the first time in this work, is the new scenario that we have to consider when testing metformin as anticancer agent. In our cell model, the activation of MAPK is not enough strong to counteract the inhibitory effects of metformin on cell proliferation and survival. However, we have to ask what could happen in a system with a mutated and constitutively active RAS. This consideration opens new possibilities of treatment combination first of all, metformin and MEK inhibitors.

At the same time, treatment with both metformin and gefitinib can avoid aberrant signals through the RAS/RAF/

MAPK pathway and could explain, at least in part, the mechanism of such significant synergism.

Future perspective studies are required in patients with NSCLCs to investigate better the effect of metformin action on the RAS/RAF/MAPK pathway and the best context in which to use metformin in combination with molecular targeted agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Morgillo, D. Vitagliano, C.M. Della Corte, E. Martinelli, T. Troiani

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