Cancer Therapy: Preclinical

Metformin Sensitizes EGFR-TKI–Resistant Human Lung Cancer Cells In Vitro and In Vivo through Inhibition of IL-6 Signaling and EMT Reversal

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Abstract

Purpose: The EGF receptor tyrosine kinase inhibitors (EGFR-TKI) have become a standard therapy in patients with EGFR-activating mutations. Unfortunately, acquired resistance eventually limits the clinical effects and application of EGFR-TKIs. Studies have shown that suppression of epithelial–mesenchymal transition (EMT) and the interleukin (IL)-6/STAT3 pathway may abrogate this acquired mechanism of drug resistance of TKIs. This study aims to investigate the effect of metformin on sensitizing EGFR-TKI–resistant human lung cancer cells in vitro and in vivo through inhibition of IL-6 signaling and EMT reversal.

Experimental Design: The effect of metformin on reversing TKI resistance was examined in vitro and in vivo using MTT, BrdUrd incorporation assay, invasion assay, flow cytometry analysis, immunostaining, Western blot analysis, and xenograft implantation.

Results: In this study, metformin, a widely used antidiabetic agent, effectively increased the sensitivity of TKI-resistant lung cancer cells to erlotinib or gefitinib. Metformin reversed EMT and decreased IL-6 signaling activation in TKI-resistant cells, while adding IL-6 to those cells bypassed the anti-TKI-resistance effect of metformin. Furthermore, overexpression or addition of IL-6 to TKI-sensitive cells induced TKI resistance, which could be overcome by metformin. Finally, metformin-based combinatorial therapy effectively blocked tumor growth in xenografts with TKI-resistant cancer cells, which was associated with decreased IL-6 secretion and expression, EMT reversal, and decreased IL-6–signaling activation in vivo.

Conclusion: Metformin, generally considered nontoxic and remarkably inexpensive, might be used in combination with TKIs in patients with non–small cell lung cancer, harboring EGFR mutations to overcome TKI resistance and prolong survival. Clin Cancer Res; 20(10); 2714–26. ©2014 AACR.

Introduction

Reversible small-molecule EGF receptor tyrosine kinase inhibitors (EGFR-TKI), including gefitinib (Iressa) and erlotinib (Tarceva), have shown dramatic therapeutic efficacy in patients with non–small cell lung cancer (NSCLC) with EGFR-activating mutations, and have been recommended as the standard first-line therapy in these patients (1, 2). However, despite excellent initial clinical responses, nearly all responding patients eventually develop drug resistance after a median period of about 10 months (3). Thus, innovative treatment strategies are urgently needed to overcome therapeutic resistance to EGFR-TKIs to improve the survival of patients with NSCLC.

Molecular mechanisms underlying acquired TKI resistance are still not fully understood. Two principal mechanisms accounting for approximately 50% of acquired resistance to TKIs in lung cancer are secondary mutations of a threonine-to-methionine substitution at amino acid position 790 (T790M) of EGFR and amplification of the N-methyl-N0-nitro-N-nitroso-guanidine (MNNG) HOS transforming gene (MET) oncogene (3, 4). Various other molecular mechanisms are also involved, including epithelial–mesenchymal transition (EMT). EMT is a process during which cells undergo morphologic changes from epithelial phenotype to mesenchymal phenotype, resulting in enhanced motility and increased invasion, proliferation, and metastasis of cancer cells (3). EMT has been associated with the sensitivity not only to conventional chemotherapies in several types of cancer (6, 7), but to EGFR-TKIs in lung cancer cells, xenografts, and patients (8). Targeting EMT may reverse
Inhibitors (9). TGF-β is a major driving force of the EMT and resistance in EGFR-TKI-resistant cell lines and xenograft models. Our data suggest that metformin could effectively overcome TKI resistance both in vitro and in vivo by inhibiting the interleukin-6 signaling pathway and reverting epithelial–mesenchymal transition. Thus, in patients with NSCLC with EGFR mutations, metformin might be used in combination with TKIs to delay or overcome TKI resistance. We have already successfully started a clinical trial (http://www.clinicaltrials.gov/ct2/show/NCT01864681) to observe the benefits of metformin in combination with gefitinib to treat advanced patients with NSCLC with EGFR mutations. This study provides the preclinical evidence that metformin has promising potential to be developed as a novel anticancer agent.

**Translational Relevance**

Although patients with non–small cell lung cancer (NSCLC) with EGFR mutations show an excellent initial response to EGFR receptor tyrosine kinase inhibitors (EGFR-TKI), their acquired resistance inevitably arises and has no effective treatment yet. In this study, we investigated whether metformin could reverse TKI resistance in TKI-resistant cell lines and xenograft models. Our data suggest that metformin could effectively overcome TKI resistance both in vitro and in vivo by inhibiting the interleukin-6 signaling pathway and reverting epithelial–mesenchymal transition. Thus, in patients with NSCLC with EGFR mutations, metformin might be used in combination with TKIs to delay or overcome TKI resistance. We have already successfully started a clinical trial (http://www.clinicaltrials.gov/ct2/show/NCT01864681) to observe the benefits of metformin in combination with gefitinib to treat advanced patients with NSCLC with EGFR mutations. This study provides the preclinical evidence that metformin has promising potential to be developed as a novel anticancer agent.

Here, we show that metformin in combination with gefitinib or erlotinib has a synergistic inhibitory effect on the proliferation, migration, and invasion of cell lines resistant to TKIs. The effect of metformin against TKI resistance is attributed to its ability to reverse EMT and decrease IL-6 signaling activation. In addition, this combinational therapy reduces tumor masses much more effectively than gefitinib or metformin alone in a xenograft mouse model, and this synergistic interaction is associated with the ability of metformin to decrease the activation of STAT3 and AKT and reverse EMT. We thus provide rationale and experimental evidence for the combined use of metformin and TKIs to overcome TKI resistance in patients with NSCLC with EGFR mutations.

**Materials and Methods**

**Cell lines and reagents**

Gefitinib (Iressa) was purchased from Tocris Bioscience and erlotinib (Tarceva) from Cayman Chemical. Both drugs were prepared in dimethyl sulfoxide (DMSO) to obtain a stock solution of 10 mmol/L. Metformin (Sigma) was dissolved in deionized water and stored at -20°C. The recombinant human IL-6 (rHIL-6) was purchased from PeproTech. Erlotinib-sensitive H1650 cells and erlotinib-resistant H1650-M3 cells were kindly provided by Dr. R. Sordella from Cold Spring Harbor Laboratory. Gefitinib-sensitive PC-9 cells and gefitinib-resistant PC-9GR cells were gifted by Prof. J. Xu and Dr. M. Liu from Guangzhou Medical University (China). H1975 cells were provided by the American Type Culture Collection. All the cells were cultured in RPMI-1640 medium (HyClone) with Earle’s salts supplemented with 10% FBS (Gibco). 2 mmol/L l-glutamine solution (Gibco), 100 U/mL penicillin (HyClone), and 100 µg/mL streptomycin (HyClone), at 37°C, with 5% CO2, and 90% humidity.

**Cell growth, invasion, and migration assays**

The cytotoxic effects of gefitinib or erlotinib plus metformin were determined by the MTT dye reduction method and BrdUrd incorporation assay (10). Cell invasion was measured using 24-well 6.5-mm-diameter inserts (8.0-µm pore size; Corning Incorporated). The relative cell invasion index was calculated as reported (18). Cell migration was evaluated using single-cell tracking assay as described earlier (19). Cell apoptosis was analyzed by flow cytometry. For more details, please refer to the Supplementary Materials and Methods.

**Animal experiments**

For xenograft implantation, a total of 2 × 10^6 PC-9GR or PC-9 cells were injected subcutaneously into the back next to the left forelimb of 6-week-old female BALB/c-a nu mice (Laboratory Animal Center of Third Military Medical University, Chongqing, China), all of which developed tumors with a size of ~30 mm^3 within 5 to 7 days. The mice were then randomly assigned to 4 groups (8 mice/group) with or without oral administration of 1 mg/mL metformin, or 250 mg/L gefitinib, or both, in drinking water. Tumor volume...
was calculated as (length × width^2)/2 and measured twice a week (Fig. 5A). The animals were kept in individual ventilated cages in compliance with institutional guidelines. All animal protocols were approved by the Ethics Committee of the Third Military Medical University. After 4 weeks, tumor-bearing mice were sacrificed, and tumors were harvested, fixed with 4% paraformaldehyde, and embedded in paraffin. To assess survival, the animals were monitored for 90 days until being euthanized. The distribution of survival percentages over time was estimated using the Kaplan–Meier method.

Statistical analysis
All data are presented as mean ± SEM. Statistical analyses were carried out using the unpaired, 2-tailed Student t test and statistical significance was assumed at a value of P < 0.05. Kaplan–Meier curves were compared using the log-rank test.

For details of the Materials and Methods, please refer to the Supplementary Materials and Methods.

Results

Metformin resensitizes EGFR-TKI–resistant human lung cancer cells in vitro

We first performed MTT assays to determine whether metformin could enhance the inhibitory effects of TKIs on the growth of TKI-resistant lung cancer cell lines, PC-9GR and H1650-M3. PC-9 cells were highly sensitive to gefitinib (Fig. 1A), whereas PC-9GR cells were highly resistant to it (Fig. 1B). Treatment with 5 mmol/L metformin resensitized PC-9GR to gefitinib (Fig. 1B and Supplementary Fig. S1). Similarly, H1650-M3 cells, which were resistant to erlotinib treatment, displayed enhanced sensitivity to erlotinib after metformin treatment (Fig. 1C and Supplementary Fig. S1). Treatment with 5 mmol/L metformin alone for 48 hours slightly decreased viability of PC-9GR cells and H1650-M3 cells (Fig. 1D).

As metformin disrupts mitochondrial respiration, which may affect the results of MTT assay, we then applied BrdUrd incorporation assay to measure cell proliferation to avoid any nonspecific effects of metformin. Our results confirmed that metformin in combination with gefitinib resulted in robust inhibition of cell proliferation in PC-9GR and another well-established resistant cell line, H1975 cells (Fig. 1E and Supplementary Fig. S2). To confirm that metformin can be uptaken by the cell lines used in this study, we next examined the expression of organic cation transporter 1 (OCT1), the transporter required for uptake of metformin, in PC-9, PC-9GR, H1650, and H1650-M3 cells. Results showed that these cell lines expressed high levels of OCT1, suggesting that metformin can be uptaken by those cell lines (Supplementary Fig. S3).

To further determine whether metformin in combination with TKIs has a better inhibitory effect on tumor cell invasion and migration than metformin or TKIs alone, we performed transwell assay and single-cell tracking assay. Both the invasion and motility of resistant PC-9GR cells were increased as compared with those of parental PC-9 cells. Gefitinib treatment alone (IC_{25}^{th}) had little effect on invasion or motility. Interestingly, metformin alone was able to decrease the invasion ability and migration rate of PC-9GR cells, and could further enhance these effects when combined with gefitinib (Fig. 1F and G). The same finding was observed in H1650-M3 cells treated with metformin, or erlotinib, or both (Supplementary Fig. S4). We next analyzed the induction of apoptosis in PC-9GR cells treated with metformin alone or in combination with gefitinib. Flow cytometric analysis revealed that metformin alone enhanced the apoptosis of PC-9GR cells, and the combination therapy further augmented this effect (Fig. 1H). Taken together, these in vitro data suggest that the combined use of metformin and gefitinib resensitizes resistant cells to TKIs and overcomes the acquired TKI resistance in these cells.

Metformin reverses EMT in TKI-resistant lung cancer cells

Knowing that EMT is extensively correlated with therapeutic resistance to EGFR-TKIs (20), we next examined whether metformin could reverse EMT in TKI-resistant cell lines. Typical epithelial morphology and expression of E-cadherin, an epithelial marker, was observed in PC-9 cells and H1650 cells, whereas mesenchymal morphology and high expression of Vimentin (a marker of mesenchymal phenotype) and SNAIL (a key regulator of EMT) were observed in PC-9GR and H1650-M3 cells. Metformin treatment induced a transition from spindle-like to epithelial-like morphology, as evidenced by the upregulation of E-cadherin and downregulation of Vimentin and SNAIL in both resistant cell lines (Fig. 2A–D and Supplementary Fig. S5). Western blot analysis results further demonstrated that metformin effectively increased E-cadherin expression while suppressed Vimentin and SNAIL expression in the absence or presence of TKIs as indicated (Fig. 2E and Supplementary Fig. S6). Based on these findings, we conclude that metformin reverses EMT in TKI-resistant lung cancer cells.

Metformin decreases IL-6 signaling activation in TKI-resistant lung cancer cell lines

To identify the molecular mechanisms of overcoming acquired TKI resistance by metformin, we next examined the effect of metformin on IL-6 activation in TKI-resistant cell lines, which was reported to be the key mechanism underlying TKI resistance and the promoter of the EMT process (10). We first performed ELISA analysis and real-time PCR analysis, finding higher levels of protein secretion and gene transcription of IL-6 in both resistant cell lines. Metformin treatment significantly decreased IL-6 protein secretion and gene expression in both resistant cell lines (Fig. 2F and Supplementary Fig. S7). STAT3 and AKT, the key components of IL-6 signaling, were highly phosphorylated in both resistant cell lines. Metformin alone effectively downregulated STAT3 and AKT activation in PC-9GR cells and STAT3 activation in H1650-M3 cells, but it had little effect on AKT activation in H1650-M3 cells. Exposure
Metformin overcomes TKI resistance in TKI-resistant human lung cancer cells. A and B, metformin increased the sensitivity of gefitinib-resistant cells to gefitinib. Cell viability of parental PC-9 and resistant PC-9GR cells treated with the indicated doses of gefitinib for 48 hours were assessed with the MTT method. C, parental H1650 and H1650-M3 cells were treated with the indicated doses of erlotinib for 48 hours. Cell viability, assessed by the MTT method, was expressed as % of control for each time point. D, treatment with 5 mmol/L metformin alone for 48 hours slightly decreased the viability of both PC-9GR and H1650-M3 cells. The data shown represent the mean value of the percentage of viable cells ± SEM (*, P < 0.05 and †, P < 0.01 compared with the control). E, metformin (5 mmol/L) and gefitinib (IC2548h) synergistically inhibited the proliferation of PC-9GR cells, as determined by BrdUrd incorporation assay. *, P < 0.01 compared with control; †, P < 0.01 compared with metformin alone. Scale bars, 50 μm. F, metformin (5 mmol/L) and gefitinib (IC2548h) synergistically inhibited the invasiveness of PC-9GR cells. Scale bars: 100 μm. *, P < 0.01 compared with that of PC-9; †, P < 0.05 compared with PC-9GR; ‡, P < 0.05 compared with metformin treatment alone. G, metformin treatment decreased single cell motility. Each trace represents the movement of a single cell within an hour, with starting point set at (0, 0). H, metformin in combination with gefitinib enhanced apoptosis of PC-9GR cells. Images are representative of 3 independent experiments. *, P < 0.01 compared with control; †, P < 0.05 compared with metformin treatment. Met, metformin; Gef, gefitinib; DAPI, 4′,6-diamidino-2-phenylindole.
to gefitinib or erlotinib slightly enhanced STAT3 and AKT phosphorylation, whereas metformin in combination with either gefitinib or erlotinib decreased activation of STAT3 and AKT in PC-9GR and STAT3 activation in H1650-M3 cell lines, respectively (Fig. 2E and Supplementary Fig. S6). We then investigated the possible mechanism by which metformin reduces STAT3 and AKT activation. Metformin disrupts mitochondrial respiration, leading to an increase in the intracellular AMP:ATP ratio, and resulting in the activation of AMPK by LKB1. Thus, we examined activation of AMPK and acetyl coA carboxylase (ACC, a standard indicator of AMPK activity) under metformin treatment. Levels of phosphorylated AMPK and ACC were significantly decreased in resistant cell lines, whereas metformin alone or in combination with either gefitinib or erlotinib significantly increased the activation of AMPK and ACC (Fig. 2E and Supplementary Fig. S6). In addition, all the cell lines used in this study expressed LKB1 (Supplementary Fig. S3). These results suggest that the inhibition of the IL-6 signaling pathway may represent the key mechanism by which metformin overcomes TKI resistance.

Metformin overcomes IL-6–induced TKI resistance in TKI-sensitive lung cancer cells

Given that the stimulation of TKI-sensitive cells by IL-6 could directly decrease erlotinib sensitivity (10), we then investigated whether metformin could overcome IL-6–induced TKI resistance. It was found that 48-hour culture
Metformin reverses IL-6–induced TKI resistance, EMT, and IL-6 signaling activation. A, metformin reversed IL-6–induced gefitinib resistance in parental PC-9 cells. Parental PC-9 cells (untreated, or treated with 10 ng/mL IL-6 or IL-6 plus 5 mmol/L metformin) were incubated with gefitinib at the indicated concentrations. Cell viability was assessed with the MTT method after 48-hour treatment. B, metformin decreased IL-6–enhanced invasiveness of parental PC-9 cells. Scale bars, 100 μm. †, P < 0.01 compared with no treatment group; †, P < 0.01, compared with IL-6 treatment group. C, metformin reversed IL-6–induced EMT in parental PC-9 cells. Morphology of PC-9 cells with different treatments was shown by phase-contrast images. Expression of E-cadherin and Vimentin was determined by immunofluorescence staining. Cells were counter-stained with 4′,6-diamidino-2-phenylindole. Scale bars, 100 μm for phase-contrast images and 20 μm for immunofluorescence images. D, metformin decreased IL-6 signaling activation in IL-6–stimulated parental PC-9 cells. Whole cell protein lysates from PC-9 cells with different treatments were immunoblotted with antibodies as indicated, and β-actin was used to confirm equal gel loading. Similar results were obtained in 3 independent experiments. E, quantification of blots in D. †, P < 0.05 and †, P < 0.01, compared with PC-9 cells, respectively; †, P < 0.05 and †, P < 0.01, compared with IL-6 treatment, respectively. Met, metformin; E-cad, E-cadherin; Vim, Vimentin.

in IL-6–containing medium was able to decrease the sensitivity of PC-9 cells to gefitinib. Interestingly, metformin addition restored the sensitivity of PC-9 cells to gefitinib (Fig. 3A). Metformin alone increased sensitivity of PC-9 cells to gefitinib, especially at low level of gefitinib (0.005 mmol/L), although the difference of IC_{50} value was not significant when compared with the control. Similarly, overexpression of IL-6 in PC-9 cells (named PC-9psb cells) also resulted in decreased sensitivity to gefitinib, whereas metformin treatment restored gefitinib sensitivity (Supplementary Fig. S8). When assessing the effects of IL-6 activation on tumor cell invasion, we found that the addition of IL-6 enhanced invasiveness of PC-9 cells. In contrast, metformin inhibited invasion of PC-9 cells induced by IL-6 (Fig. 3B). Taken together, these results suggest that IL-6 is sufficient to decrease gefitinib cytotoxicity, which can be reverted by metformin.

Next, we examined EMT and IL-6 activation in PC-9 cells treated with IL-6 or IL-6 plus metformin. IL-6 treatment induced EMT in those sensitive cells, as characterized by spindle-shaped morphology, the loss of E-cadherin and the expression of EMT markers Vimentin and SNAIL (Fig. 3C–E). In addition, exposure to IL-6 resulted in significant phosphorylation of its downstream molecules STAT3 and AKT, but inhibited the activation of AMPK and ACC (Fig. 3D and E). Metformin significantly reverted EMT, diminished phosphorylation of STAT3 and AKT, and enhanced activation of AMPK and ACC (Fig. 3F).
3C–E). In summary, our data indicate that the activation of STAT3 and AKT by IL-6 is sufficient to induce gefitinib resistance, acquisition of mesenchymal-like features and invasion ability, whereas metformin could successfully restore gefitinib sensitivity, reverse EMT, and decrease STAT3 and AKT phosphorylation.

**Metformin overcomes TKI resistance by inhibiting IL-6 signaling activation**

To establish a causal link between metformin treatment, IL-6 signaling inhibition, and enhanced TKI response, we increased IL-6 signaling in metformin-pretreated TKI-resistant cells by adding IL-6 to the culture medium. Further addition of IL-6 significantly diminished the response of metformin-pretreated PC-9GR cells to gefitinib (Fig. 4A) and the response of metformin-pretreated H1650-M3 cells to erlotinib (Supplementary Fig. S9A). In addition, further exposure to IL-6 enhanced the invasiveness of PC-9GR (Fig. 4B) and H1650-M3 cells (Supplementary Fig. S9B), decreased the expression of E-cadherin and increased the expression of Vimentin and SNAIL (Fig. 4C–E and Supplementary Fig. S9C–S9E). IL-6 also restored and enhanced STAT3 and AKT phosphorylation, while inhibited the activation of AMPK and ACC in both cell lines (Fig. 4D and E and Supplementary Fig. S9D and S9E). Taken together, these findings suggest that metformin overcomes TKI resistance by inhibiting IL-6 signaling activation.
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Metformin plus gefitinib potentiates gefitinib-induced antitumor activity in PC-9GR/mouse xenografts

We next tested the possibility that the metformin-based combinational therapy is more effective in xenografts established with PC-9GR cells. Treatment with metformin slightly slowed down the tumor growth in xenografts. Gefitinib alone resulted in xenograft shrinkage, similar to the result obtained by metformin. Importantly, in accord with the above results in cell lines, a synergic effect of metformin in combination with gefitinib was observed upon co-administration of gefitinib and metformin, which further reduced the tumor size in this model (Fig. 5B). After 4 weeks of drug administration when the animals were sacrificed, the combination therapy caused a 58% decrease in tumor volume as compared with the control group (P < 0.01), whereas gefitinib alone and metformin alone both caused a 32% decrease (P < 0.05 for both as compared with the control; Fig. 5C). During the experiments, no obvious weight loss was observed in mice treated with metformin, gefitinib, or both (Supplementary Fig. S10). Besides, no significant reduction in serum insulin levels or glucose levels was observed with metformin treatment (Supplementary Fig. S11). A similar combinational effect of metformin and gefitinib was observed in xenografts established with TKI-sensitive PC-9 cells. Gefitinib alone resulted in significant tumor shrinkage in PC-9 xenografts, whereas metformin in combination with gefitinib further reduced the tumor sizes (P < 0.05 as compared with gefitinib alone; Supplementary Fig. S12). We then evaluated the survival of PC-9GR xenografts treated with gefitinib, or metformin, or both. Log-rank test showed that metformin or gefitinib alone did not prolong the survival significantly as compared with the control group (P = 0.07 and 0.14, respectively). Met, metformin; Gef, gefitinib.

The combinational effect of metformin with gefitinib in mouse xenografts is associated with EMT reversal and inhibition of IL-6 signaling

To clarify the underlying reason why combination of metformin and gefitinib is more effective than either drug alone, we next analyzed EMT and components of the IL-6 signaling pathway in the context of metformin-based combinational therapy in PC-9GR xenografts. Immunofluorescence staining showed high expression of Vimentin and low expression of E-cadherin in the control and gefitinib alone-
treated groups, indicating that EMT occurred in both groups. Metformin treatment alone or in combination with gefitinib increased E-cadherin expression and decreased Vimentin expression (Fig. 6A). These results were confirmed by Western blot analysis (Fig. 6D). Similarly, in PC-9 xenografts, metformin in combination with gefitinib increased the expression of E-cadherin and decreased that of Vimentin when compared with the control (Supplementary Fig. S13). Next, we examined IL-6 secretion and expression in PC-9GR xenografts. Using ELISA analysis, we detected higher levels of IL-6 in peripheral blood in the control groups. Gefitinib treatment alone did not decrease IL-6 secretion significantly as compared with the control group. IL-6 levels were significantly decreased in both metformin alone-treated and the metformin + gefitinib groups (P < 0.05 for both, as compared with the control
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Metformin on xenografts, the dose of metformin varies in vivo previous cancer treatment, as discussed elsewhere earlier (17). In 10 mmol/L might be attained in tumor tissues during higher than that in blood, so that a concentration of 1 to 10 mmol/L might be attained in tumor tissues during cancer treatment, as discussed elsewhere earlier (17). In previous in vivo experiments testing the antitumor effect of metformin on xenografts, the dose of metformin varies from 200 μg/mL (14) to 200 mg/mL (17), and it is diluted in drinking water, present throughout the experiment. In this study, we used 1 mg/mL metformin, corresponding to 75 mg/kg as reported by Iliopoulos and colleagues (14). The well-established Reagan–Shaw method (29) shows the human equivalent dose (mg/kg) = animal dose (mg/kg) × animal Km/human Km, where Km values are based on body surface area. For a 60 kg human adult, Km is 37, whereas for a 20-g mouse, it is 3. Thus, the human equivalent of the murine dose of 75 mg/kg is 365 mg in an adult of 60 kg, which is much less than the maximum metformin dose of 2,550 mg/day recommended by the Food and Drug Administration. Thus, the in vivo dose of metformin in this study is within a therapeutic range in humans.

Discussion

Human NSCLCs with activating EGFR mutations show an excellent response to treatment with EGFR-TKIs, such as gefitinib and erlotinib. However, nearly all patients succumb to relapse because of drug resistance, for which no effective therapy is available at present. Thus, novel strategies are urgently needed to delay or overcome acquired resistance to EGFR-TKIs. In this study, we have demonstrated that metformin can effectively overcome drug resistance to erlotinib and gefitinib. Furthermore, metformin plus gefitinib significantly decrease tumor growth in vivo and prolong animal survival.

Clinical implications of metformin plus TKIs to overcome drug resistance

Several epidemiological studies have indicated that metformin uptake can lower the risk of several types of cancer in patients with diabetes (21–23). The patients with diabetes with breast cancer receiving metformin and neoadjuvant chemotherapy had a higher pathologic complete response rate than did those not receiving metformin (24). In mouse xenografts, metformin exerted comparable effects on tumor regression when it was combined with a 4-fold reduced dose of doxorubicin that is not effective as a monotherapy (14). Metformin inhibited the proliferation of NSCLC (25) and breast cancer cell lines (26), and blocked transformation in an inducible model system (27, 28). These reports, together with our findings that metformin significantly enhances the effect of erlotinib and gefitinib on TKI-resistant cell lines in vitro and in vivo, suggest that metformin has the promising potential to be used as a novel anticancer agent.

The dose of metformin we used in in vitro experiments is higher than that used in diabetic patients. However, as mentioned by Iliopoulos and colleagues (14), metformin accumulates in tissues at the concentration several-fold higher than that in blood, so that a concentration of 1 to 10 mmol/L might be attained in tumor tissues during cancer treatment, as discussed elsewhere earlier (17). Metformin significantly increases the activation of AMPK and ACC (Fig. 6D and Supplementary Figs. S14 and S15). The expression of phosphorylated AKT and STAT3 was further confirmed by immunohistochemistry (Supplementary Fig. S16). These results suggest that the therapeutic advantage of the combination of metformin with gefitinib is associated with its ability to reverse EMT and inhibit IL-6 signaling in vivo.

Mechanistic implications

Understanding the molecular mechanisms underlying the ability of metformin to overcome TKI resistance is pivotal to develop it as a novel agent to treat patients with NSCLC. EGFR TKI treatment resulted in STAT3 activation, which was caused by IL-6 in an autocrine manner (11). Inhibiting IL-6/STAT3 suppressed cancer cell growth and enhanced the sensitivity to anticancer drugs (30). However, AKT phosphorylation was upregulated in gefitinib-resistant NSCLC cells, and maintenance of PI3K/AKT pathway signaling was associated with therapeutic resistance to EGFR-TKIs. As IL-6 is a major activator of the JAK/STAT3 and PI3K/AKT pathways (31), inhibiting its signaling has emerged as a possible solution to the problem of EGFR-TKI resistance.

In this study, we observed robust activation of the IL-6 signaling pathway in TKI-resistant cells when compared with their parental sensitive cells. In addition, IL-6 overexpression or addition in TKI-sensitive PC-9 cells resulted in gefitinib resistance, accompanied by increased phosphorylation of STAT3 and AKT. Metformin treatment effectively overcomes TKI resistance in established TKI-resistant cell lines, IL-6 stimulated PC-9 cells, and IL-6 overexpressing PC-9 cells. Furthermore, adding IL-6 into metformin-pretreated TKI resistant cell lines abolished metformin’s effect, restored TKI resistance, and reactivated the IL-6 signaling pathway. Thus, our data provide compelling evidence that metformin overcomes acquired resistance to molecular-targeted therapies by inhibiting IL-6 signaling pathways (Supplementary Fig. S17).

Besides the inhibitory effect on the IL-6 signaling pathway, metformin’s ability to reverse EMT may also play an important role in overcoming TKI resistance. EMT has been associated with resistance to EGFR inhibitor treatment in NSCLCs (20). AXL, an EMT marker, was found to be upregulated in patients with NSCLC with acquired resistance to EGFR-TKI treatment, and AXL activation is a major cause of EGFR-TKI resistance (32). In this study, we demonstrated that metformin reversed EMT in TKI-resistant lung cancer cell lines and IL-6–stimulated PC-9 cells. We also found that metformin inhibited the IL-6/STAT3 pathway, whereas IL-6 is capable of inducing...
EMT in cancer cells (33). Thus, we conclude that EMT reversal by metformin is associated with its ability to overcome TKI resistance.

**Metformin may disrupt the evil axis of TGF-β/IL-6, EMT, cancer stem cells and drug resistance**

Cancer stem cells (CSCs), which constitute a small portion of neoplastic cells, are hypothesized to be critical initiators of cancers and mediate resistance to conventional antitumor therapies (34). Lung CSCs have also been successfully isolated from lung cancer cell lines based on SP phenotypes (low Hoechst 33342 staining pattern; ref. 35), and from primary patient tumors (36). These lung CSCs are resistant to most conventional drugs currently used to treat patients with lung cancer. However, lung cancer cells surviving conventional or targeted therapies exhibited several CSC features, such as strong clonogenic capacity, self-renewal, and high tumorigenicity (37, 38). Interestingly, the 2 inflammation-associated cytokines, IL-6, and TGF-β1 are capable of inducing EMT in human breast cancer cells, resulting in generating cells with stem-cell properties (39, 40). Indeed, EMT–CSCs–drug resistance has been proposed to be an emerging axis of evil in the war against cancer (41). In addition, as shown in this study, TKI-resistant lung cancer cells displayed EMT features and increased activation of IL-6 signaling pathways. Besides, trastuzumab-refractory CSC populations were found to be significantly enriched in the expression of mesenchymal markers, and an increased secretion of TGF-β (42). Thus, TGF-β/IL-6, EMT, CSCs, and drug resistance are strongly correlated with each other to maintain the TKI resistance in lung cancer.

In this study, we found that metformin could inhibit IL-6 secretion, decrease IL-6 signaling activation and reverse EMT. It was also reported that metformin could reverse TGF-β-induced EMT (15). In addition, metformin treatment inhibited the growth of breast CSCs (13), ovarian CSCs (43), pancreatic CSCs (44), and thyroid CSCs (45), possibly by transcriptionally repressing the stem cell property EMT (46). Taken together, metformin emerges as a new therapeutic option to inhibit TGF-β/IL-6 activation, reverse EMT, kill CSCs, and then overcome TKI resistance, thus disrupting this evil axis in the war against cancer (Supplementary Fig. S18). Given the important role of CSCs in TKI resistance, we are currently examining the effect of metformin on killing CSCs in TKI-resistant lung cancer cells.

**Metformin may alleviate TKI-induced interstitial pneumonia**

Acute interstitial pneumonia is one of the serious adverse effects of TKI treatment (47). IL-6 is known to induce interstitial pneumonia (48). TKI treatment activates AP-1 in lung cancer cells and promotes IL-6 secretion, which further increases the expression of collagen and α-actin, markers for fibrosis (49). In this study, we provide sound evidence that metformin can inhibit the IL-6/STAT3 axis. Also, it has been reported that TGF-β is an important inducer of pulmonary fibrosis (50), whereas metformin can significantly inhibit TGF-β signaling (15). Accordingly, the combination of metformin and EGFR-TKIs may be more effective and safer for patients, because this treatment may not only overcome TKI resistance but prevent or alleviate the development of acute interstitial pneumonia.

Therapeutic resistance to EGFR-TKIs is almost inevitable in patients with activating EGFR mutations who initially respond well to therapy. Management of TKI resistance has become the focus of research to lengthen overall survival of these patients. In this study, we have proven for the first time that metformin overcomes TKI resistance in vitro and in vivo by inhibiting the IL-6 signaling pathway and reverting EMT. In addition, we have successfully started a clinical trial (registered in Clinicaltrials.gov: http://www.clinicaltrials.gov/ct2/show/NCT01864681) to observe the clinical benefits of metformin in combination with gefitinib to treat patients with advanced NSCLC with EGFR mutations. Our future research aims at providing more solid evidence to develop metformin in combination with TKIs as a new therapeutic approach to prolonging the survival of patients with NSCLC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** L. Li, Y. He

**Development of methodology:** R. Han

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** L. Li, R. Han, H. Xiao, C. Lin, Y. Wang, H. Liu, K. Li, H. Chen, F. Sun

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L. Li, R. Han, H. Xiao, C. Lin, Y. Wang, Z. Yang

**Writing, review, and/or revision of the manuscript:** L. Li, Y. He

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** H. Xiao, K. Li, H. Chen, F. Sun, J. Jiang

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