Cancer Therapy: Preclinical

Transient PI3K Inhibition Induces Apoptosis and Overcomes HGF-Mediated Resistance to EGFR-TKIs in EGFR Mutant Lung Cancer

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Abstract

Purpose: Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, show favorable response to EGFR mutant lung cancer. However, the responders acquire resistance almost without exception. We recently reported that hepatocyte growth factor (HGF) induces EGFR-TKI resistance by activating MET that restores downstream mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK)1/2 and phosphoinositide 3-kinase (PI3K)/Akt signaling. The purpose of this study was to determine whether inhibition of PI3K, a downstream molecule of both EGFR and MET, could overcome HGF-mediated EGFR-TKI resistance in EGFR mutant lung cancer cells PC-9 and HCC827.

Experimental Design: We explored therapeutic effect of a class I PI3K inhibitor PI-103 on HGF-induced EGFR-TKI resistance in vitro and in vivo.

Results: Unlike gefitinib or erlotinib, continuous exposure with PI-103 inhibited proliferation of PC-9 and HCC827 cells, even in the presence of HGF. On the other hand, in gefitinib-resistant xenograft model by using PC-9 cells mixed with HGF high producing fibroblasts, PI-103 monotherapy did not inhibit tumor growth. However, PI-103 combined with gefitinib successfully regressed gefitinib-resistant tumor. In vitro experiments by considering short half-life of PI-103 reveal that transient exposure of PI-103 combined with gefitinib caused sustained inhibition of Akt phosphorylation, but not ERK1/2 phosphorylation, resulting in induction of tumor cell apoptosis even in the presence of HGF.

Conclusions: These results indicate that transient blockade of PI3K/Akt pathway by PI-103 and gefitinib could overcome HGF-mediated resistance to EGFR-TKIs by inducing apoptosis in EGFR mutant lung cancer. Clin Cancer Res; 17(8); 2260–9. ©2011 AACR.

Introduction

Lung cancer is one of the most prevalent malignancies and the leading cause of cancer-related death worldwide. Non–small cell lung cancer (NSCLC) accounts for nearly 80% of lung cancer cases. Substantial efforts are being made to identify the optimal target for NSCLC therapy. The tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, have been shown to inhibit epidermal growth factor receptor (EGFR)-mediated downstream pathways, including mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK)1/2 and phosphoinositide 3-kinase (PI3K)/Akt, and to show favorable activity in NSCLC patients with mutant EGFR (1). Recent phase III clinical trials showed that patients with EGFR mutant NSCLC had superior outcomes with gefitinib treatment, compared with standard first-line cytotoxic chemotherapy (2–3). However, the patients develop acquired resistance to EGFR-TKIs almost without exceptions within a couple of years (4). In addition, 20% to 25% patients with EGFR activating mutations show intrinsic resistance to EGFR-TKIs.

Two genetically conferred mechanisms—T790M second mutation in EGFR (4–5) and the MET gene amplification (6)—have been well reported to induce the acquired resistance to EGFR-TKIs in EGFR mutant lung cancer. Recently, we identified a third mechanism, hepatocyte growth factor (HGF)-induced resistance. It induces EGFR-TKI resistance by activating MET that restores phosphorylation of downstream MAPK/ERK1/2 and PI3K/Akt pathways (7–8). This is not a genetically conferred mechanism and may be involved in both intrinsic resistance and acquired resistance to EGFR-TKIs in EGFR mutant lung cancer (7). Although HGF is reported to be produced predominantly
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Translational Relevance

The acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) is one of the most serious problems on the management of EGFR mutant lung cancer. We recently reported the novel mechanism that hepatocyte growth factor (HGF) induces EGFR-TKI resistance by activating MET that restores phosphorylation of downstream mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK)1/2 and phosphoinositide 3-kinase (PI3K)/Akt pathways.

In this study, we showed that transient blockade of PI3K/Akt pathway by PI3K inhibitor and gefitinib could overcome HGF-mediated resistance to EGFR-TKIs by inducing apoptosis in both in vitro and in vivo models. Our findings indicate usefulness of double blockade of EGFR and PI3K, and further postulate the need to develop PI3K inhibitor analogues with more suitable pharamcokinetics and metabolic profiles for more successful therapy of EGFR mutant lung cancer.

Several strategies are available to block HGF-MET-mediated signaling, including ligand (HGF) blockade, MET tyrosine kinase inhibition, and inhibition of downstream molecules (PI3K/Akt, MAPK/ERK; ref. 11). PI3Ks are responsible for the generation of 3-phosphorylated inositides, including the important second messenger PtIns(3,4,5)P3 (antiphosphatidylinositol 3,4,5-triphosphate), resulting in activation of signal transduction pathways in many physiologic process (12). PI3Ks are divided into 3 classes on the basis of their primary structures and in vitro substrate specificity (13), with class I PI3Ks being the most well characterized. Class I PI3Ks can be further subdivided into class IA (p110α, p110β, and p110δ) and class IB (p110γ) according to their structure and interaction with p85 and p55 regulatory subunits. Class IA PI3Ks, each composed of a p85 regulatory subunit and a p110 catalytic subunit, are the most widely involved in cancer (14). The major effector of PI3K in cancer is Akt, a serine–threonin kinase that is directly activated in response to PI3K (13–14). Recent studies indicate that the PI3K/Akt pathway plays crucial roles in resistance to various types of TKIs, including EGFR TKIs (6, 15–17). Accordingly, a large numbers of PI3K inhibitors are being developed (18).

We sought to determine whether inhibition of PI3K signaling pathway could overcome EGFR-TKI resistance induced by HGF in EGFR mutant lung cancer. We found that transient exposure of class I PI3K inhibitor plus gefitinib was sufficient to overcome HGF-mediated resistance by inducing apoptosis of EGFR mutant lung cancer cells.

Materials and Methods

Cell cultures and reagents

The EGFR mutant human lung adenocarcinoma cell lines, with exon 19 deletion in EGFR PC-9 (del E746_A750) and HCC827 (del E746_A750), were purchased from Immuno-Biological Laboratories Co. and American Type Culture Collection, respectively (19). The H1975 human lung adenocarcinoma cell line with EGFR-L858R/T790M double mutation (20) was kindly provided by Dr. J.D. Minna (University of Texas Southwestern Medical Center, Dallas, TX) and Dr. Y. Sekido (Aichi Cancer Center Research Institute, Nagoya, Japan). Human lung embryonic fibroblasts, MRC-5, were obtained from RIKEN Cell Bank. The PC-9, HCC827, and H1975 cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics. The MRC-5 (p30–p35) was cultured in 10% FBS DMEM (Dulbecco’s modified Eagle’s medium). All cells were passage for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for mycoplasma with the use of a MycoAlert Mycoplasma Detection Kit (Lonza). Gefitinib, erlotinib, PI-103 (PI3K α inhibitor 1), and GDC-0941 were obtained from AstraZeneca, Chugai Pharmaceutical Co., Calbiochem, and Selleck Chemicals, respectively. Human recombinant HGF was prepared as described previously (21).

Cell proliferation assay

Cell proliferation was measured by the MTT dye reduction method (22). Tumor cells (2 × 10^3/100 μL per well) were plated into each well of 96-well plates in RPMI 1640 with 10% FBS. After 24 hours incubation, several concentrations of gefitinib, erlotinib, PI-103, and/or HGF were added to each well, and incubation was continued for a further 72 or 48 hours. For short exposure to gefitinib and/or PI-103, tumor cells (8 × 10^5/800 μL per well) were incubated in 24-well plates. After 24 hours incubation, several concentrations of PI-103 and gefitinib were added for 1 hour, then washed 2 times with PBS, and then replated with fresh medium. Viability was assessed at 48 hours after initial exposure. Cell proliferation was determined with MTT solution (2 mg/ml; Sigma) as described previously (7). Each experiment was done at least 3 times, each with triplicate samples.

Determination of drug synergy

Cells were seeded at a density of 2 × 10^3 per well of a 96-well plate. Concentration ranges were chosen to span the complete dose-response range of both drugs. All treatments were done in quadruplicate. Cell proliferation/viability was determined after 3 days by using MTT assay. Multiple drug effect analysis was done by using Calcusyn Software (Biosoft), which quantitatively describes the interaction...
between 2 or more drugs (23). This method assigns combination index (CI) values to each drug combination and defines drug synergy when a CI value is less than 1 or drug antagonism when a CI value is greater than 1.

**Coculture of lung cancer cells with fibroblasts**

Cells were cocultured in transwell chambers separated by 8 μm pore filters. Tumor cells (8 × 10^4 cells/700 μL) with gefitinib and or PI-103 different doses were placed in the bottom chamber, and fibroblasts (10^4 cells/300 μL) were placed in the top chamber. After 72 hours, the top chamber was removed and cell proliferation was measured by MTT assay. For short exposure to PI-103 and/or gefitinib, the proliferation was assessed after 48 hours and drugs were administered for only 1 hour in different concentration, and then each well was washed twice with PBS and new fresh media were added. Each experiment was done at least 3 times, each with triplicate samples.

**Assay for RNA interference**

Duplexed Stealth RNAi (Invitrogen) against Akt1-1 (5'-AUACCCGCAAGAACAGUUGCUAGCA-3'), Akt1-2 (5'-AACCCUCUUCCAAUAUGGCACGCUC-3'), and Akt1-3 (5'-UACCGUGGCGCGAGUCIUIUAUGU-3') was used for RNA interference assay. One day before transfection, aliquots of 2 × 10^5 tumor cells in 400 μL of antibiotic-free medium were plated on 24-well plates. After incubation for 24 hours, the cells were transfected with siRNA (50 pmol) with Lipofectamine 2000 (1 μL) in accordance with the manufacturer’s instructions. After 24 hours incubation, the cells were washed with PBS and incubated with or without gefitinib (0.1 μmol/L) and/or rhHGF (recombinant human HGF, 20 ng/mL) for an additional 72 hours in antibiotic-containing medium. Cell proliferation was measured by a Cell Counting Kit-8 (Dojin) in accordance with the manufacturer’s instructions. Each experiment was done at least in triplicate and 3 times independently.

**Xenograft studies in SCID mice**

Suspensions of PC-9 cells (5 × 10^6) with MRC-5 (5 × 10^6) were injected subcutaneously into the backs of 5-week-old female severe combined immunodeficient mice (SCID). Mice (n = 6 per group) were randomized to: (a) control group, (b) gefitinib only (25 mg/kg/d) orally, (c) PI-103 only prepared in 20% 4-hydroxypropyl β-cyclodextrin (5 mg/kg/d) intraperitoneally, or (d) gefitinib (25 mg/kg/d) and PI-103 (5 mg/kg/d). After 4 days (tumors diameter >4 mm), the treatment was started. The tumor volume was calculated (mm^3 = width^2 × length/2). All animal experiments complied with the guidelines for the Institute for Experimental Animals, Advanced Science Research Center, Kanazawa University, Kanazawa, Japan (approval no. AP-081088).

**Antibodies and Western blotting**

For Western blotting analysis, 40 μg of total protein were resolved by SDS polyacrylamide gel (Bio-Rad) electrophoresis and the proteins were then transferred onto polyvinylidene difluoride membranes (Bio-Rad). After washing 4 times, membranes were incubated with Blocking One (Nacalai Tesque, Inc.) for 1 hour at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-MET (25H12), anti-phospho-MET (anti-p-MET, Y1234/Y1235; 3D7), anti-p-EGFR (Y1068), anti-ErbB3 (1B2), anti-p-ErbB3 (Tyr1289; 21D3), anti-Akt or p-Akt (Ser473), anti-cleaved caspase-9 (Asp315), anti-cleaved caspase-3 (Asp175), anti-cleaved PARP (Asp214) antibodies (1:1,000 dilution, Cell Signaling Technology), anti-human EGFR (1 μg/mL), anti-human/mouse/rat ERK-1/ERK-2 (0.2 μg/mL), or anti-p-ERK-1/ERK-2 (T202/ Y204; 0.1 μg/mL) antibodies (R&D Systems). After washing thrice, membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate, an enhanced chemiluminescent substrate (Pierce Biotechnology). Each experiment was done at least thrice independently.

**TUNEL assay**

Terminal deoxynucleotidyl transferase–mediated nick end labeling staining was performed by the Apoptosis Detection System (Promega). Briefly, the frozen tissue sections (9 μm thick) were fixed with PBS containing 4% formalin. The slides were washed with PBS and permeabilized with 0.2% Triton X-100. The samples were then equilibrated, and DNA strand breaks were labeled with fluorescein-12-dUTP (fluorescein-12-deoxy-uridine-5-triphosphate) by adding nucleotide mixture and terminal deoxynucleotidyl transferase enzyme. The reaction was stopped with saline sodium citrate, and the localized green fluorescence of apoptotic cells was detected by fluorescence microscopy (×200).

**Reverse transcriptase-PCR analysis**

Total RNA was isolated from MRC-5 cells treated with various concentration of PI-103 for 24 hours, with ISOGEN RNA extraction. Total RNAs were reversely transcribed by an Omniscript RT Kit (Qiagen) according to the manufacturer’s protocols. The primers for HGF and β-actin were as follow: HGF forward, 5'-CAGTGTTGAGGTAATGC-3', reverse, 5'-GTGTCATTCATAGTATTGTGAG-3'; and β-actin forward, 5'-AAGAGGGCATCCCTCACCT-3', reverse, 5'-TACATGGCTGGGGTGTTGAA-3'. Polymerase chain reaction was done by Ex Taq Hot Start Version (Takara). Cycles for HGF and β-actin were 28 and 26, respectively. The bands were visualized by ethidium bromide staining.

**Cell apoptosis assay**

Cell apoptosis induced by gefitinib was detected with an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen) in accordance with the manufacturer’s protocols as described previously (7). The analysis was done...
on a FACS Calibur flow cytometer with CellQuest Software (Becton Dickinson).

**Statistical analysis**

Two-tailed Student’s t test was performed, when noted, by GraphPad Software. Differences of \( P < 0.001 \) were considered statistically different (24).

**Results**

**Continuous exposure of PI-103 effectively suppresses the in vitro proliferation of EGFR mutant lung cancer cells in the presence of HGF**

Both PC-9 and HCC827 cells were highly sensitive to continuous exposure (72 hours) to gefitinib and erlotinib. HGF alone did not affect proliferation of PC-9 cells, but it slightly stimulated the proliferation of HCC827 cells. Under these experimental conditions, HGF dose dependently induced resistance to gefitinib and erlotinib of PC-9 and HCC827 cells (Fig. 1), as reported previously (7). Under the same experimental conditions, continuous exposure (72 hours) of PI-103 inhibited the proliferation of PC-9 and HCC827 cells though the IC\(_{50}\) (half maximal inhibitory concentration) to PI-103 was higher (0.3 \( \mu \)mol/L for HCC827; 0.8 \( \mu \)mol/L for PC-9 cells) than gefitinib (0.01 \( \mu \)mol/L for HCC827; 0.03 \( \mu \)mol/L for PC-9 cells) and erlotinib (0.01 \( \mu \)mol/L for HCC827; 0.02 \( \mu \)mol/L for PC-9 cells). Importantly, HGF did not decrease the sensitivity of PC-9 and HCC827 cells to PI-103, suggesting the potential of PI-103 to overcome HGF-induced resistance to gefitinib and erlotinib in vitro.

We recently reported that HGF induces resistance in lung cancer cells (H1975) with EGFR T790M second mutation to irreversible EGFR-TKI that is expected to overcome T790M second-mutation–mediated resistance to gefitinib or erlotinib (25). Interestingly, continuous exposure (72 hours) of PI-103 inhibited proliferation of H1975 cells in a dose-dependent manner (Supplementary Fig. S1). HGF slightly stimulated proliferation of H1975 cells and induced the resistance to irreversible EGFR-TKI, CL-387,785 (N-[4-[[3-bromophenyl]amino]-6-quinazolinyl]-2-butynamide). However, HGF did not affect the sensitivity of H1975 cells to PI-103. These results suggest that PI-103 has potential to overcome HGF-induced resistance to not only irreversible EGFR-TKIs but also irreversible EGFR TKIs. Moreover, combined use of PI-103 with CL-387,785 further inhibited proliferation of H1975 cells, irrespective of the presence of HGF.

We next examined whether PI-103 sensitized EGFR mutant lung cancer cells when combined with gefitinib in the presence or absence of HGF. PI-103 inhibited the proliferation of PC-9 and HCC827 cells in a dose-dependent manner. Gefitinib markedly suppressed the proliferation, and HGF induced the resistance to gefitinib. Surprisingly, PI-103 combined with gefitinib further inhibited the proliferation of PC-9 and HCC827 cells not only in the absence of HGF but also in the presence of HGF (Fig. 2A). Proliferation data were analyzed by the established method of Chou and Talalay (23) by using CalcuSyn software. The resulting CI values were less than 1 over most dose levels of PI-103 with improved pharmacokinetic and pharmacodynamic properties that are active against all isoforms of class
PI3Ks and now used in clinical trials in patients with solid tumors (26; Supplementary Fig. 2). This synergy was also observed in a coculture system where HGF high producing fibroblasts, MRC-5, induced gefitinib resistance. While PI-103 did not inhibit HGF expression in MRC-5 cells (Supplementary Fig. S3), PI-103 synergistically inhibited the proliferation of PC-9 and HCC827 cells, irrespective of the presence of MRC-5 cells, when combined with gefitinib (Fig. 2B). These results suggest that PI3K inhibitor combined with gefitinib may be more beneficial than either monotherapy.

**PI-103 with or without gefitinib suppresses PI3K/Akt pathway even in the presence of HGF**

To explore the molecular mechanism by which PI-103 combined with gefitinib showed greater antiproliferative effect, we examined the phosphorylation of MET, EGFR, ErbB3, and their downstream pathways (PI3K/Akt and ERK1/2) by Western blotting (Fig. 3A). PC-9 and HCC827 cells expressed EGFR, ErbB3, and MET proteins, and these molecules were phosphorylated at various levels. These receptors and downstream molecules, such as Akt and ERK1/2, were also phosphorylated. While HGF alone did not affect phosphorylation of EGFR or ErbB3, it stimulated phosphorylation of MET and thereby activated Akt and ERK1/2. Although gefitinib inhibited phosphorylation of Akt and ERK1/2 in the absence of HGF, it failed to inhibited Akt and ERK1/2 phosphorylation in the presence of HGF. Importantly, PI-103 did not affect the phosphorylation of ERK1/2 or upstream molecules such as MET, EGFR, and ErbB3, but did inhibit the phosphorylation of Akt, regardless of presence of HGF. In addition, PI-103 combined with gefitinib inhibited phosphorylation of both Akt and ERK1/2 in the absence of HGF. The combination of PI-103 and gefitinib inhibited Akt phosphorylation, even in the presence of HGF, but failed to inhibit ERK1/2 phosphorylation. These results confirm our previous observations (7) and further suggest that PI-103 overcomes this resistance by inhibiting phosphorylation of downstream PI3K/Akt. The importance of PI3K/Akt as a target of PI-103 was further supported the evidence obtained in experiments with siRNA for Akt. Treatment with siAkt1 alone knocked down the Akt expression in PC-9 cells (Fig. 3B) and resulted in inhibition of cell proliferation by 20% (Fig. 3C). Notably, the treatment with siAkt1 reversed HGF-induced gefitinib resistance in combination with gefitinib (Fig. 3C).

**PI-103 combined with gefitinib overcomes HGF-induced gefitinib resistance in vivo**

We recently established an in vivo model by inoculating PC-9 cells premixed with HGF high producing MRC-5 cells and showed that gefitinib resistance that was abrogated by HGF-MET inhibition (9). By using this model, we next evaluated whether PI-103 overcomes HGF-induced resistance to gefitinib in vivo. Consistent with previous observations, we found that treatment with gefitinib alone prevented the enlargement of tumors produced by the mixture of PC-9 cells and MRC-5 cells, but did not cause tumor regression. Since gefitinib induces tumor shrinkage of PC-9 tumors (9), our results suggest that MRC-5 cells induced gefitinib resistance in vivo. Under these experimental conditions, treatment with PI-103 alone did not inhibit tumor growth, whereas combined treatment with PI-103 and gefitinib dramati-
cally regressed the tumors (Fig. 4A and B). Similar results were reproduced by GDC-0941 that is now used in clinical trials in patients with solid tumors (Supplementary Fig. S4). These combined treatments did not cause obvious side effects, such as weight loss of the mice.

We further examined apoptotic cells in the tumors treated with gefitinib and/or PI-103. Although there were few apoptotic cells in control-treated (Fig. 4C) or PI-103–treated tumors (Fig. 4D), discernible numbers of apoptotic cells were detected in gefitinib-treated tumors (Fig. 4E). However, more apoptotic cells were found in tumors treated with both PI-103 and gefitinib (Fig. 4F; Fig. 4A and B). Immunoblots with these tumors revealed that treatment with PI-103 with or without gefitinib did not affect the phosphorylation of ERK1/2. On the other hand, PI-103 alone or in combination with gefitinib inhibited Akt phosphorylation in the tumor. Most notably, PI-103 combined with gefitinib induced cleaved caspase-3, the effector caspase that mediates death signaling (Fig. 4G). These results strongly suggested the importance of PI3K/Akt as a target of this combined therapy.

Short exposure of PI-103 combined with gefitinib further inhibits Akt mediating signal and proliferation of EGFR mutant lung cancer cells

Our finding that PI-103 monotherapy did not inhibit tumor growth, whereas PI-103 overcame synergistically HGF (MRC-5)-induced gefitinib resistance when combined with gefitinib, was unexpected. Although PI-103 has been reported to have very rapid tissue distribution and tissue clearance in vivo (half-life of PI-103 in the major organs is 0.7–1.3 hours; ref. 12), we hypothesized that rapid tissue clearance of PI-103 might be responsible for its insufficient therapeutic effect in vivo. To mimic pharmacodynamics of PI-103 in vivo, we exposed PC-9 and HCC827 cells to PI-103 transiently for 1 hour, washed the cells, and incubated the resultant cultures in fresh medium for 48 hours (Fig. 5A). Transient exposure to PI-103 resulted only in approximately 15% inhibition of the proliferation of PC-9 or HCC827 cells, whereas transient exposure to gefitinib resulted in higher inhibition of proliferation (>30%). Importantly, transient exposure to both PI-103 and gefitinib inhibited the proliferation of these 2 cell lines, reaching IC50 (Fig. 5B). Analysis using CalcuSyn software (23) indicates that the effect was synergistic. These phenomena were also observed when EGFR mutant cancer cells were cocultured with MRC-5 cells (Fig. 5C) to induce gefitinib resistance, representing the therapeutic efficacy seen in vivo model. In contrast, transient exposure to PI-103 and/or gefitinib did not inhibit proliferation of MRC-5 cells (Fig. 5D).

We further evaluated the kinetics of PI3K/Akt phosphorylation after transient exposure of PC-9 cells to PI-103 and gefitinib (Fig. 6A). As shown earlier (Fig. 3), 1 hour treatment with either PI-103 or gefitinib completely inhibited Akt phosphorylation in the absence of HGF. We found out that in the absence of HGF, gefitinib, alone or combined with PI-103, inhibited Akt phosphorylation for up to
1 hour; subsequently, however, Akt phosphorylation in PC-9 cells treated with PI-103 alone started to recover. In the presence of HGF, gefitinib did not inhibit Akt phosphorylation, whereas Akt phosphorylation in PC-9 cells treated with PI-103 alone recovered by 1 hour after washing. However, cells treated with PI-103 plus gefitinib showed inhibition of Akt phosphorylation 1 hour after washing (Fig. 6A). More importantly, transient exposure to PI-103 plus gefitinib, but not either alone, resulted in the induction of cleaved caspase-9 and caspase-3, the initiator and effector caspsases that mediate death signaling and cleaved PARP (Fig. 6A). Flow cytometry analyses with Annexin V further confirmed that transient exposure with PI-103 plus gefitinib induced apoptosis of HGF-treated PC-9 cells (Fig. 6B). These findings indicate that transient exposure to PI-103 combined with gefitinib is sufficient for inducing death signaling even in the presence of HGF, supporting the results observed in vivo experiments.

Discussion

Accumulating evidence indicate that HGF-MET axis is considerable therapeutic target for several solid tumors. HGF can act as autocrine growth factors for glioblastoma, thyroid cancer, and gastric cancer. Moreover, HGF stimulates the invasion and dissemination of various types of cancers (27), and inducing EGFR-TKI resistance in EGFR mutant lung cancer. In contrast to MET amplification–induced resistance, restoring the PI3K/Akt pathway and mediated by ErbB3 as an adaptor, HGF activates normal MET receptor and induces the resistance that restores PI3K/Akt pathway mediated by Gab1/2 as an adaptor (7–8). HGF also accelerates the expansion of preexisting clones with MET gene amplification and facilitates the induction of EGFR-TKI resistance in a population of EGFR mutant lung cancer (8). In addition, HGF frequently detected in chronic myelogenous leukemia (CML; ref. 28). Our results illustrate the possibility that transient double blockade of EGFR and PI3K may be useful for controlling HGF-induced resistance to EGFR-TKIs in EGFR mutant lung cancer.

We have shown here that the combination of gefitinib and class I PI3K inhibitors, PI-103 and GDC-0941, overcame HGF-mediated gefitinib resistance in EGFR mutant lung cancer cells. Although HGF restored ERK1/2 and PI3K/Akt phosphorylation via MET activation even in the presence of gefitinib, transient exposure of PI-103 plus gefitinib efficiently inhibited PI3K/Akt phosphorylation, induced death signaling, and caused apoptosis of PC-9 cells. The combined treatment with PI-103 and gefitinib did not inhibit phosphorylation of ERK1/2 (Fig. 3) or STAT3 (data not shown). Recently, it was shown that transient potent inhibition of BCR-ABL kinase activity is associated with maximal clinical benefit in patients with chronic myelogenous leukemia (CML; ref. 28). Our results illustrate the possibility that transient double blockade of EGFR and PI3K may be useful for controlling HGF-induced resistance to EGFR-TKIs in EGFR mutant lung cancer.

A large number of PI3K inhibitors are developed and are being evaluated in the preclinical and clinical trials (18,
PI3K inhibitors have been found to induce G0/G1 cell arrest rather than apoptosis, and primarily causing stasis of tumor growth in vivo without substantial tumor shrinkage. For example, intraperitoneal administration of high doses of PI-103 (30–70 mg/kg) resulted in growth inhibition rather than regression in a range of human tumor xenografts (12, 30). Moreover, in EGFR mutant or k-ras mutant lung cancer models, tumor regression associated with apoptosis was observed only when PI3K/Akt pathway and MEK/MAPK pathway were simultaneously blocked (24, 31). Since these 2 pathways collaborate with each other to maintain cell survival, simultaneous blockade of both pathways is necessary to induce apoptosis. Surprisingly, our in vitro experiments revealed that though transient exposure of PI-103 plus gefitinib failed to inhibit ERK1/2 phosphorylation, it caused sustained PI3K/Akt inhibition, induced proapoptotic molecules, such as cleaved caspase-3 and caspase-9 and PARP, and thereby induced PC-9 cell apoptosis even in the presence of HGF. The mechanism by which combined use of PI-103 and gefitinib induces apoptosis of PC-9 cells, even in the presence of HGF, is not fully understood at present. One possible explanation is that double blockade of PI3K/Akt signaling pathway at upstream (EGFR level) and downstream (PI3K/Akt level) is efficient for inducing the apoptosis. The other possibility is that combined use of PI-103 and gefitinib inhibited the unknown pathway(s) that are responsible for survival of EGFR mutant lung cancer cells. While the combined therapy did not inhibit phosphorylation of STAT3 (data not shown) in PC-9 cells, we cannot rule out the involvement of other unknown pathway(s). Further experiments are warranted to clarify the mechanisms in future.

PI-103 is a class I PI3K inhibitor that reported favorable antitumor activity without any obvious side effects in preclinical animal models (12, 32). Pharmacokinetically, PI-103 is metabolized to form glucuronide and is cleared rapidly from plasma, with metabolism of more than 70% of PI-103 after 30 minutes of incubation with human and mouse microsomes (12). This is consistent with our results, showing that Akt phosphorylation in PC-9 cells treated with PI-103 alone was recovered by 1 hour (Fig. 6). PI-103 is not in clinical trials and work is now in progress to optimize its pharmacokinetics properties by structural modification (13). While continuous exposure for 72 hours of PI-103 inhibited the proliferation of PC-9 and HCC827 cells, transient exposure for 1 hour of PI-103 failed to do so in vitro condition. In vivo treatment with PI-103 (5 mg/kg intraperitoneally, once a day) also failed to inhibit the growth of PC-9 cells mixed with HGF high producing fibroblasts (MRC-5). Collectively, the insufficient effect of PI-103 monotherapy may be, at least in part, due to short half-life and rapid metabolism of this drug in vivo. GDC-0941 (5 mg/kg pharmacokinetics of oral, once a day) also failed to inhibit the growth of PC-9 cells mixed with MRC-5 cells. Though GDC-0941 is a derivative of PI103 with improved pharmacokinetic and pharmacodynamic properties, intratumoral concentration of GDC-0941 might not be enough to inhibit tumor growth in our in vivo experimental conditions. However, GDC-0941,
like PI-103, successfully reversed the resistance when combined with gefitinib (Supplementary Fig. S4). Since we did not use maximum tolerated dose of these PI3K inhibitors (70–150 mg/kg; refs. 12, 26), our findings suggest that suboptimal dose of PI3K inhibitors may overcome HGF-induced resistance if combined with EGFR-TKIs.

In this study, we showed the possibility that double blockade of PI3K/Akt signaling pathway at upstream (EGFR level) and downstream (PI3K/Akt level) may be useful for overcoming HGF-induced resistance to EGFR-TKIs in EGFR mutant lung cancer cells. This concept can also be applicable to circumvent HGF-induced resistance to irreversible EGFR-TKIs (25). Moreover, cancer cell populations were recently shown to exhibit reversible tolerance to EGFR-TKIs by maintaining a phenotypical distinct subpopulation of cells that can protect the overall population from eradication by EGFR-TKIs (33). This reversible tolerance is mediated by activation of insulin like growth factor (IGF)-1 receptor (IGF-1R), but can be overcome by IGF-1R inhibitor combined with gefitinib ((35). Since PI3K/Akt is involved in IGF-1R–mediating signaling, the combined use of PI3K inhibitor may also protect against the emergence of reversibly tolerant subpopulations and may potently eradicate EGFR mutant lung cancer. Further investigations in PI3K/Akt signaling pathway are warranted for developing more successful compounds with better activity and safety for EGFR mutant lung cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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