TPCA-1 Is a Direct Dual Inhibitor of STAT3 and NF-κB and Regresses Mutant EGFR-Associated Human Non–Small Cell Lung Cancers

Jing Nan1, Yuping Du1, Xing Chen1, Qifeng Bai2, Yuxin Wang1, Xinxin Zhang1, Ning Zhu1, Jing Zhang1, Jianwen Hou1, Qin Wang1, and Jinbo Yang1,3

Abstract

Epidermal growth factor receptor (EGFR) is a clinical therapeutic target to treat a subset of non–small cell lung cancer (NSCLC) harboring EGFR mutants. However, some patients with a similar kind of EGFR mutation show intrinsic resistance to tyrosine kinase inhibitors (TKI). It indicates that other key molecules are involved in the survival of these cancer cells. We showed here that 2-[aminocarbonyl]amino)-5-(4-fluorophenyl)-3-thiophene-carboxamide (TPCA-1), a previously reported inhibitor of IkB kinases (IKK), blocked STAT3 recruitment to upstream kinases by docking into SH2 domain of STAT3 and attenuated STAT3 activity induced by cytokines and cytoplasmic tyrosine kinases. TPCA-1 is an effective inhibitor of STAT3 phosphorylation, DNA binding, and transactivation in vivo. It selectively repressed proliferation of NSCLC cells with constitutive STAT3 activation. In addition, using pharmacologic and genetic approaches, we found that both NF-κB and STAT3 could regulate the transcripts of interleukin (IL)-6 and COX-2 in NSCLC harboring EGFR mutations. Moreover, gefitinib treatment only did not efficiently suppress NF-κB and STAT3 activity. In contrast, we found that treatment with TKIs increased phosho-STAT3 level in target cells. Inhibiting EGFR, STAT3, and NF-κB by combination of TKIs with TPCA-1 showed increased sensitivity and enhanced apoptosis induced by gefitinib. Collectively, in this work, we identified TPCA-1 as a direct dual inhibitor for both IKKs and STAT3, whereas treatment targeting EGFR only could not sufficiently repress NF-κB and STAT3 pathways for lung cancers harboring mutant EGFR. Therefore, synergistic treatment of TPCA-1 with TKIs has potential to be a more effective strategy for cancers. Mol Cancer Ther; 13(3); 617–29. ©2014 AACR.

Introduction

NF-κB and STAT3 signaling pathways are found to play pivotal roles in various aspects of tumorigenic process in a number of malignancies. Most often, NF-κB and STAT3 are constitutively activated in neoplastic cells (1). However, mere disruption of either NF-κB or STAT3 signaling does not lead to cell death. Previously, we have found STAT3 blockage by small chemical inhibitor often increases NF-κB activity. Therefore, a dual inhibitor that is able to simultaneously block STAT3 and NF-κB signaling may be a novel strategy for cancer therapy.

Lung cancer is the leading cause of cancer-related deaths in both men and women in the United States and worldwide. Approximately 85% to 90% of all cases of lung cancer are non–small cell lung cancer (NSCLC; ref. 2). Some adenocarcinoma contains constitutive epidermal growth factor receptor (EGFR) activity with mutant EGFR (3, 4). Gefitinib is the first-generation tyrosine kinase inhibitor (TKI) targeting on EGFR. Nearly all gefitinib responsive lung cancers have somatic EGFR mutation with kinase domain. Exon 19 of EGFR deletion and EGFR L858R missense substitutions are found in more than 80% of patients with NSCLC who respond to gefitinib treatment (5, 6). Although EGFR-TKI treatment therapy shows good responsive and survival rates in patients with NSCLC with EGFR mutation as mentioned above (6, 7), about 30% of patients with NSCLC with activated EGFR mutations are not respond to those TKIs. In addition, TKIs responsive patients also showed different sensitivity to the treatment. These findings indicated that other causes might also contribute to the intrinsic resistance (8–10).

Therefore, completely understanding of the causes for responsiveness to EGFR TKIs is worth pursuing to improve the clinical benefits of targeted therapies.

STAT3 and NF-κB are key pathways downstream of EGFR. STAT3 is frequently associated with deregulated cell growth and neoplasia (11). The activation of STAT3 often involves a ligand–receptor interaction. STAT3 can...
be activated by many various cytokines, including interferons, EGF, G-CSF, and interleukin (IL-6) family cytokines. Binding of cytokines to their cognate receptors leads to JAKs phosphorylation, STAT3 dimerization, nuclear translocation, DNA binding, and gene activation (12, 13). In addition, STAT3 phosphorylation can also be induced by cytoplasmic tyrosine kinase, such as Src family kinase (14). It had been reported that elevated EGFR activity and STAT3 activation is positive correlated in many primary tumor specimens and tumor-derived cell lines, including NSCLC, breast cancer, and head and neck carcinomas (15, 16).

Increased STAT3 activity was observed in lung adenocarcinomas and cell lines expressing mutant EGFRs (17). STAT3 is required by mutant EGFRs and is necessary for its downstream phenotypic effects. Inhibiting STAT3 function in fibroblasts abrogates transformation by mutant EGFR (18). However, TKIs cannot completely abrogate STAT3 activity in NSCLC cell lines. Previous study suggests mutant EGFR induces activation of gp130/JAK/STAT3 pathway by means of IL-6 upregulation (19). Tumor expression of IL-6 and IL-6 receptor components gp80 and gp130 had been found in NSCLC specimens (20). Increased levels of pro-inflammatory cytokines such as IL-6 and IL-8 are also associated with NSCLC tumorigenesis and prognosis. These indicate that IL-6 and its downstream pathway are potential to be the target for patient treatment (21). Therefore, we hypothesized that NF-κB and STAT3 signaling were regulating IL-6 autocrine in lung cancer.

NF-κB is a dimeric complex formed by RelA, RelB, and c-Rel. Activation of the NF-κB is initiated by the signal-induced degradation of IκB protein (22, 23). Known inducers of NF-κB activity include TNF-α, IL-1β, and EGF (24, 25), etc. NF-κB p65 nuclear expression is an early and frequent phenomenon in the pathogenesis of lung cancers. NF-κB subunit p65/RelA is determined to be required for K-Ras-induced lung tumorigenesis (26). Furthermore, NSCLC containing EGFR mutation shows elevated NF-κB activity (25). Recently, an increase of the IκBα level predicts improved progression-free and overall survival in patients with EGFR mutant NSCLC treated with erlotinib (27). However, underlying mechanisms involved in NF-κB promoting EGFR mutant NSCLC cancer cell proliferation is remain unclear.

In this work, we found that, TPCA-1, a previously used IκB kinase (IKK) antagonist, not only inhibited NF-κB signaling but also blockage STAT3 signaling pathway via binding to STAT3 SH2 domain directly. Thus, TPCA-1 is a dual inhibitor of IκKβ and STAT3 that represses IL-6 autocrine and COX-2 transcription in EGFR mutant cells. Moreover, the functional significance of TPCA-1-induced STAT3 and NF-κB inhibition was determined by examining its effect on the sensitivity to gefitinib.

Materials and Methods

DNA construct and stable cell line establishment

plV-c-src plasmid was generated by subcloning a human c-Src cDNA into plV-puro plasmid and the lentiviral plasmid was infected into HEK-293T cells. Human STAT3WT and STAT3Y705F cDNA fragments were inserted into plV-puro vector, respectively. Constructed plasmids were introduced into HCC827 cells by lentiviral infection and selected by puromycin (2.5 μg/mL) for 3 days. IL-6 and scramble short hairpin RNAs (shRNA) were prepared according to the introduction of pLKO.1. These constructs were introduced into lung cancer cell lines by lentiviral infection and selected with puromycin (2.5 μg/mL) for 3 days.

Cell culture, inhibitors, and cytokines

NCI-H1650, A549, MDA-MB231, and HEK-293T cell lines were obtained from American Type Culture Collection (ATCC). PC9 were gifted by George R. Stark (Lerner Research Institute). HCC827, Sk-br-3, and NCI-H1975 cell lines were purchased from Shanghai cell bank. HCC827, PC9, and NCI-H1975 were authenticated by short tandem repeat (STR) analysis in Jianlian Gene. Results of STR matched the data of ATCC, DSMZ, and JCRB cell banks. HEK-293T cells were maintained in Dulbecco’s Modified Eagle Medium with 10% FBS. All other cells were maintained in RPMI 1640 supplemented with 10% FBS. IKK antagonists TPCA-1 and BAY11-7082 and cytokine TNF-α were purchased from Sigma. Gefitinib was purchased from LC Laboratories. IL-6 was purchased from PeproTech.

Cell viability assay

Cell were seeded into a 96-well plate at density of 5 $\times$ 10^3 cells/well and incubated for 24 hours. After cells were exposed to drugs for 72 hours, 0.5 mg/mL of MTT reagent was added to the medium in the well. After incubation for 4 hours at 37°C, formazan crystals in viable cells were solubilized with 100 μL dimethyl sulfoxide (DMSO). The absorbance at 490 nm was determined using a plate reader.

Luciferase reporter assays

To assess STAT3 transcriptional activity, HEK-293T cells were stably transfection with the pGL4.20-SIE-luc luciferase reporter, which contains 9 copies of the STAT3 binding site plus with TATA box. This cell line was named as 293T-SRL. The cells were harvested with lysis buffer after 24 hours of treatment. Luciferase activity was presented relative to pGL4.20-SIE-luc-transfected samples treated with DMSO, arbitrarily set at 1. The results of the luciferase assay represent the averages from 3 independent experiments.

Cell apoptosis analysis by flow cytometry

For apoptosis assay, cells were collected by trypsinization and washed with cold PBS, 1 $\times$ 10^6 cells were resuspended in 100 μL PBS containing 4 μL propidium (PI) and 4 μL Annexin V-FITC. These cells were incubated in the dark at room temperature. After 15 minutes of incubation, 400 μL...
binding buffer were added. The percent of apoptotic (Annexin V-positive) cells was determined by the flow cytometry.

RNA extraction and quantitative real-time-PCR

Total RNA was collected from cells following the manufacturer’s instructions using RNA Prep Pure Cell kit (TIANGEN). Total RNA (2 μg) was subjected to a reverse transcriptase reaction using the M-MLV Retro-Transcription Kit (Invitrogen). Real-time PCR was performed using SYBR GREEN on a BIO-RAD CFX96 Real Time system machine. Expression data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. Data are presented in arbitrary units and were calculated as 2ΔΔCt (ΔCt GAPDH—gene of interest). Primer sequences of tested genes are listed as follows: IL-6 forward primer, 5′-GAGAAAGGAGACATGTAACAAGAGT-3′; reverse primer, 5′-GCCGAGAATGAGATGAGTTGT-3′; COX-2 forward primer, 5′-CCCTTGCTTCTCAAAAGTAA-3′; reverse primer, 5′-AACGTGATCCGTGAAGTGTC-3′; SOCS3 forward primer, 5′-CCATGGTGTTGGTAAGACGCC-3′; reverse primer, 5′-CCCTGCCAGCCCAATACCTGA-3′. GAPDH forward primer, 5′-TGCCAAATTCCTCATGGC-3′; reverse forward, 5′-CCATGGTGTTGGTAAGACGCC-3′. The results of the real-time PCR represent the averages from 3 independent experiments.

Immunocytochemical analysis

Cells were plated on coverslips. After incubation, cells were fixed with 4% formaldehyde and absolute methanol. Then and incubate for 10 minutes in blocking buffer at room temperature. After the coverslips were washed with PBS, anti-STAT3 antibody (Cell Signaling Technology) was diluted in blocking buffer at a concentration of 1:200. The slides were incubated at 4 °C overnight and washed 3 times with PBS. Cells were then incubated with secondary antibody for 2 hours at room temperature. Removed antibody and added 4',6-diamidino-2-phenylindole at a final concentration of 0.4 μg/mL for 5 minutes and wash the cells 5 times with PBS. Cells were viewed under a fluorescent microscope.

Western blot analysis and molecular modeling

Cells were lysed in radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 5 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na3VO4, 20 mmol/L NaF, 50 mmol/L, Tris-Cl, pH 7.5) with proteinase inhibitors. Equal amounts of protein were fractionated by 8% to 10% SDS-PAGE and then transfer them to polyvinylidene difluoride (PVDF). Antibodies used were as follows: anti–phospho-STAT3(Tyr705), phospho-JAK2(Tyr1007/1008), JAK2, phospho-p65(Ser536), p65, cyclin D3, cyclin D1, BCL-XL, p-IkBα (Ser32), IkBα, survivin, PARP (Cell Signaling Technology); anti-β-actin (Santa Cruz Biotechnology); and anti-GAPDH, HRP-conjunction secondary antibodies (Zhong Shan Jin Jiao). Protocol about molecular modeling is given in Supplementary Methods.

Xenograft studies

BALB/c female nude mice were purchased from Vital Rival. All experiments were performed in the Animal Center of Gansu University of Traditional Chinese Medicine. Six-week-old mice were injected subcutaneously with HCC827 cells (5 × 106). HCC827 cells were suspended in serum-free RPMI 1640. When tumor volumes were reached approximately 80 mm3, mice were randomized to groups of 6 animals to receive either vehicle control, TPCA-1 alone, gefitinib alone, or TPCA-1 and gefitinib together. Gefitinib was suspended in 0.5% (w/v) methylcellulose and administered once daily by oral gavage (2 mg/kg). TPCA-1 was dissolved in PBS and administered by intraperitoneally at a daily dosage of 10 mg/kg. Mice in the untreated group were given the same volumes of PBS by injection and 0.5% (w/v) methylcellulose by oral gavage. Tumor size was measured every 2 days using calipers. The average tumor volume was calculated according to the equation: tumor volume = 0.5 × (largest diameter) × (small diameter)2. Tumor weight was measured at the endpoints of this study.

Results

TPCA-1 inhibits STAT3 Y705 phosphorylation induced by cytokines and c-Src

TPCA-1 and BAY11-7082 are reported to be selective inhibitors of IKK (Fig. 1A; refs. 28 and 29). Both of them inhibited TNF-α-induced p65 activation (Supplementary Fig. S1A). However, as shown in Fig. 1B, TPCA-1 abrogated STAT3 activation induced by IL-6, IFN-α, and IFN-γ. By contrast, TPCA-1 had no effect on phospho-AKT. To determine whether the STAT3 activity suppression by TPCA-1 is depend on its IKK inhibition, we examined the total IkB and phospho-p65 levels and found no obvious changes (Fig. 1B). Further evidence showed that another IKK inhibitor BAY11-7082 had little influence on STAT3 phosphorylation (Supplementary Fig. S1B). In addition, we found that TPCA-1 also can repress STAT3 phosphorylation induced by c-src overexpression (Fig. 1C). Same results were observed in HeLa cells (Supplementary Fig. SIC). SOCS3 is a major end product of STAT3 signaling and is broadly used as a marker for STAT3 activation. As shown in Fig. 1D, SOCS3 transcription induced by IL-6 was absolutely diminished by TPCA-1 but not BAY11-7082.

We further found that TPCA-1 severely blocked phospho-STAT3 induced by IL-6 or IFN-α at 500 or 100 nmol/L (Fig. 2A and Supplementary Fig. S2A). Constitutive activated STAT3 were inhibited fiercely at 250 nmol/L (Supplementary Fig. S2B). NSCLC having mutant EGFR with high STAT3 activity were treated with TPCA-1 at different time points. As seen in Fig. 2B, phospho-STAT3 was almost abrogated as early as 15 minutes and phospho-p65 was just slightly inhibited. These results further indicated that inhibition of TPCA-1 on constitutively activated STAT3 is more efficient than its impact on NF-κb pathway. Furthermore, STAT3-dependent luciferase activity...
was inhibited at 100 nmol/L (Fig. 2C). All together, these data suggest that TPCA-1 can inhibit STAT3 phosphorylation and transactivation induced by cytokines and non-receptor tyrosine kinase in dose- and time-dependent manner. And this inhibition is not because cross-talking between STAT3 and NF-κB pathway.

TPCA-1 binds to SH2 domain of STAT3

J. Saez-Rodriguez reported that TPCA-1 inhibits JAK2 kinase to suppress STAT3 phosphorylation induced by IL-6 (30). But JAK2 is not required for IFN-α and c-Src–induced STAT3 activation (31). Our finding showed that TPCA-1 can inhibit STAT3 phosphorylation induced by both IFN-α and c-Src. We tested whether TPCA-1 affects JAKs phosphorylation. Results showed that TPCA-1 had no effect on JAK2 induced by IFN-γ (Fig. 3A). So, we supposed there may be the other mechanism about inhibition of TPCA-1 on STAT3 pathway. Next, we want to examine whether TPCA-1 abrogates STAT3 anchoring to upstream receptors. In the cytoplasm, via the 2 docking autophosphorylated tyrosines (Y1068 and Y1086), cell surface EGFR physically interacts with STAT3 SH2 domain (32). STAT3 activation is partly dependent on EGFR in MDA-MB-231 cells (Supplementary Fig. S3). As the Fig. 3B shown, we found that TPCA-1 blocked interaction of EGFR with STAT3 (Fig. 3B). Based on these findings, we presumed that TPCA-1 directly target on STAT3 SH2 domain. To establish our assumption, we did molecular docking experiment subsequently.

The possible binding cavity domains have been reported (33–35). Both BAY11-7082 and TPCA-1 are docked into SH2 domain of STAT3 using AutoDock Vina program, automatically. The TPCA-1 is placed into the STAT3 pocket suitably as show in Fig. 3C. The STAT3–TPCA-1 has about 1.0 kcal/mol lower affinity energy than STAT3–BAY11-7082. In addition, there is no hydrogen bond...
between the STAT3 and BAY11-7082. However, one hydrogen bond is detected between STAT3 and TPCA-1 using the hydrogen bond module of AutoDockTools1.5.4 (36). The carboxyl oxygen atom of Glu594 forms the hydrogen bond with the amidogen hydrogen atom of TPCA-1 (Fig. 3D). This hydrogen bond contributes to more affinity energy between the TPCA-1 and STAT3. Whether the affinity energy and the hydrogen bonding are compared between STAT3–BAY11-7082 and STAT3–TPCA-1, TPCA-1 shows stronger affinity on the SH2 domain of STAT3.

TPCA-1 selectively suppresses growth of EGFR mutant NSCLC harboring constitutively active STAT3

We estimated growth repressive effect of TPCA-1 on EGFR mutant NSCLC harboring constitutively activated STAT3 to other NSCLC with lower STAT3 activity. TPCA-1 suppressed proliferation of HCC827 and H1975 cells but had little effect on A549 (Fig. 4A). Comparing with EGFR mutant NSCLC, A549 cell has lower STAT3 activity (Fig. 4B), which is consistent with Song’s observation (37). TPCA-1 completely inhibits STAT3 phosphorylation without changing total STAT3 levels. STAT3 downstream proteins, including c-Myc, cyclin D, and survivin, were severely decreased (Fig. 4B). SOCS3 mRNA levels in HCC827 and H1975 cells were also eliminated upon TPCA-1 treatment (Supplementary Fig. S4A). Previous studies suggested that STAT3 activation in EGFR mutant NSCLC was driven by autocrine IL-6 (19, 37). Exogenous IL-6 did not compensate the reduction of phospho-STAT3 by TPCA-1 (Supplementary Fig. 5B). In addition, STAT3 nucleocytoplasmic shuttling was also blocked by TPCA-1 but not BAY11-7082 (Fig. 4C). TPCA-1 also led to a G2–M cell-cycle arrest in HCC827 but not A549 (Supplementary Fig. S5). We observed that STAT3 activity restoration partly rescued growth inhibition caused by TPCA-1 (Fig. 4D). Taken together, these results suggest that TPCA-1 as a direct STAT3 inhibitor selectively suppresses NSCLC with EGFR mutant.

TPCA-1 represses IL-6 autocrine and COX-2 expression in EGFR mutant NSCLC

Although STAT3 activity restoration rescued cell growth suppression in present lower dose of TPCA-1, this compensation was partial when TPCA-1 was applied in higher concentration. This might be because of the inhibition of IKKβ by TPCA-1. Overexpression of NF-κB, STAT3, and IL-6 might lead to the progression of lung cancer (26, 38). To determine the role of NF-κB signaling in lung cancer with mutant EGFR, we knocked down RelA in HCC827 and H1975. The growth of cells with RelA knockdown was inhibited (Fig. 5A). We next sought to elucidate how NF-κB contributed to NSCLC survival. Some pro-proliferative and antiapoptotic downstream genes of NF-κB were tested. Results showed there was no difference in the levels of cyclin D1 and BCL-XL except for IxBα (Fig. 5B). Intracellular IL-6 is required to control of cell proliferation in a subset of human lung cancer cells. COX-2 is reported to be regulated by NF-κB and is frequently expressed in lung adenocarcinoma (39). Compared with less malignancy NSCLC cells, HCC827 (EGFR mutant) contained more abundance of IL-6 and COX-2 transcripts (Supplementary Fig. S6). We found that mRNA levels of IL-6 and COX-2 were sharply repressed when RelA was knocked down (Fig. 5C). These results showed that NF-κB signaling was requisite for IL-6 autocrine and COX-2 expression in lung cancer.

Based on these finding, we further examined the role of NF-κB pathway in mediating IL-6 and COX-2 expressions. Fig. 5D showed that TPCA-1 inhibited IL-6 transcripts severely but not BAY11-7082. Because TPCA-1 abrogated STAT3 activity directly as above mentioned, so we supposed IL-6 autocrine and COX-2 expression in EGFR mutant NSCLC are also mediated by STAT3. STAT3 dominant-negative expressing decreased about 50% of IL-6 and COX-2 mRNA levels. Furthermore, STAT3 overexpression increased IL-6 and COX-2 transcripts (Fig. 5E).
These findings suggested that NF-κB was prerequisite to IL-6 and COX-2 transcription, and STAT3 signaling was also involved in regulating IL-6 autocrine and COX-2 expression in HCC827 cells. Therefore, TPCA-1 as a novel inhibitor of STAT3 can block IL-6 autocrine and COX-2 transcription in EGFR mutant NSCLC cells.

Rationale of combined STAT3, NF-κB, and EGFR inhibition in EGFR mutant lung cancer

Effects of TKI on STAT3 and NF-κB are not fully understood. We next investigated whether gefitinib represses activation of STAT3 and p65. Gefitinib inhibited AKT activity and STAT3 ser727 phosphorylation but no effect on STAT3 tyrosine phosphorylation (Fig. 6A). Surprisingly, although there is lower STAT3 activity in PC9, TKI treatment obviously increased phospho-STAT3, and TPCA-1 blocked this increase. Combination of TKI with TPCA-1 abrogated both phospho-STAT3 Y705 and S727 phosphorylation (Fig. 6A). Although gefitinib and TPCA-1 both slightly reduced the levels of phospho-p65 in HCC827 cells, gefitinib had no impact on the level of phospho-IκBα S32. However, TPCA-1 could diminish IκBα S32 phosphorylation. Furthermore, we then discovered that cotreatment with 2 antagonists inhibited p65 activation potently (Fig. 6B). These findings indicate that there exist 2 redundant mechanisms of NF-κB activation in EGFR mutant NSCLCs. The one is IKKs dependent, and the other is not rely on IKKs activity but depends on EGFR activity.

We subsequently tested whether gefitinib affect mRNA levels of IL-6 and COX-2. Twenty-four hours after gefitinib treatment, IL-6 mRNA level was significantly elevated by 5.4-fold compared with control (DMSO) and the change of COX2 level was just slight. TKI neither diminished IL-6 nor COX-2 transcript in sensitive NSCLCs, but combination of TPCA-1 with gefitinib repressed their transcription potently (Fig. 6C).

To further examined the effect of inhibition of STAT3, NF-κB, and EGFR pathways in lung cancer cells containing EGFR mutant. We found TPCA-1 increased sensitivity to gefitinib in both TKI sensitive cells and insensitive cells (Fig. 6D and Supplementary Fig. S7). To understand whether the combination effect was additive or synergistic, a Bliss independent criterion analysis was performed in HCC827 and PC9 cells. As shown in Fig. 6D, the inhibition of drugs used in combination (E\textsubscript{observed}) was greater than the theoretical inhibition (E\textsubscript{bliss}). The effect of these 2 drugs is considered synergistic. Taken together, these findings indicated that blocking STAT3, NF-κB, and EGFR pathways by combination of TPCA-1 with gefitinib may be served as a new strategy to EGFR mutant lung cancer.

Dual inhibition of STAT3 and NF-κB pathway enhances TKI-induced apoptosis via extrinsic pathway

To examine whether inhibition of STAT3 and NF-κB pathways enhanced apoptosis induced by gefitinib in lung cancer cells containing EGFR mutant, we checked the effects of gefitinib and combination of gefitinib with TPCA-1 on HCC827 cells. HCC827 cells showed a pronounced increase in the percentage of apoptotic cells when compared with control, gefitinib alone (P < 0.05), or TPCA-1 alone (P < 0.01; Fig. 7A). Furthermore, we found that dual inhibition of STAT3 and NF-κB pathway via TPCA-1 increased the level of cleaved PARP induced by gefitinib.
TPCA-1 enhanced apoptosis through elevating cleaved caspase-8 and then caspase-3 cleavage, but not caspase-9 (Fig. 7C).

TPCA-1 inhibits growth of NSCLC with EGFR mutation and potentiates antitumor effect of gefitinib in xenograft models

To further examine whether TPCA-1 as a dual antagonist of STAT3 and IKKs has impact on the tumor growth and TKI sensitivity of EGFR mutant NSCLC in vivo, we constructed HCC827 xenograft model and tested the effect of TPCA-1, gefitinib, and their combination on tumor growth. As shown in Fig. 8A and B, we found that blockade of p65 and STAT3 activity by TPCA-1 showed inhibition of tumor growth ($P < 0.001$). TPCA-1 combined with gefitinib also showed strong repression compared with TPCA-1 or gefitinib alone. Bliss-independent criterion analysis was also

Figure 4. TPCA-1 blocks STAT3 phosphorylation and inhibits proliferation of NSCLC cells harboring EGFR mutant. A, cells were incubated with TPCA-1, and then cell viabilities were estimated with MTT assays. B, cells were treated with TPCA-1. Proteins were analyzed by Western blot analysis. C, STAT3 intracellular distribution in HCC827 cells treated with drugs was analyzed by immunocytochemical analysis. D, STAT3 was overexpressed in HCC827 and H1975 cells. Viabilities of cells treated with TPCA-1 were estimated by MTT assay.
performed in xenograft studies. The tumor weight inhibition rate of TPCA-1, gefitinib, and their combination are 0.419\( (E_{\text{TPCA-1}}) \), 0.680\( (E_{\text{gefitinib}}) \), and 0.837\( (E_{\text{observed}}) \), respectively. The expected combinational inhibition rate is 0.814\( (E_{\text{bliss}}) \). \( E_{\text{observed}} \) is greater than theoretical inhibition \( (E_{\text{bliss}}) \). Therefore, we considered the antitumor effect of combination TPCA-1 and gefitinib was synergistic. Figure 8C is a model depicting the role of NF-κB and STAT3 and the effect of combination TPCA-1 with TKI in EGFR mutant lung cancer cells.

**Discussion**

STAT3 and NF-κB are ubiquitously expressed and control numerous physiologic processes including development, immunity, and cancer. Activated STAT3 and NF-κB cooperatively control the expression of antiapoptotic, pro-proliferative, immune responsive genes (40). TPCA-1 has been reported to be an ATP-competitive and selective inhibitor of IKK2. Our results differed from previous. In our studies, we first found that TPCA-1 diminished STAT3 Y705 phosphorylation induced by IL-6, IFN-α, IFN-γ, and...
c-src in lower concentration. However, p-JAK2 was not suppressed by TPCA-1. Actually, we found that TCPA-1 binds into SH2 domain and blocks TPCA-1 recruitment with upstream tyrosine kinases. TPCA-1 also diminished constitutive phospho-STAT3 in HCC827 and H1975 cells and selectively repressed their growth. In addition, it was reported that STAT3 activation in EGFR mutant lung cancer cells is rely on JAK1 activity but not JAK2 (37). It further indicated that STAT3 inhibition via TPCA-1 is not correlated with JAK2 activity. Despite, TPCA-1 was initially found to be IKK\(\beta\) antagonist, in our study we found its inhibition on p-p65 in cancer cells was not as potent as...
on p-STAT3. It may because that NF-κB activation in these cell lines is a more complex process, not only dependent on IKKs activity but may be the other factor involving in. TPCA-1 as a new dual inhibitor of STAT3 and NF-κB may show superiority in next cancer therapy. Subsequently, we explored the potential of TPCA-1 on lung cancer treatment.

EGFR is characterized as a "driver oncogene" in EGFR mutant NSCLC. High STAT3 activity is commonly correlated with lung cancer EGFR mutant. Our results uncovered that gefitinib did not decrease phospho-STAT3 Y705. It is consistent with previous observation (18). However, we first found STAT3 activity was obviously upraised by TKI treatment in PC9 cells. Notably, the elevation of phospho-STAT3 in HCC827 was slight; this may because of the higher background of p-STAT3 in HCC827 cells. Whatever, this elevation may impair the effect of TKIs. The mechanism under this phenomenon is obscure. However, we found that gefitinib treatment elevated IL-6 transcription and autocrine. It may be the cause leads to STAT3 activation upon TKI treatment. Recently, a study had been reported that lung cancer cell lines with secondary EGFR mutation showed de novo resistance to irreversible EGFR inhibitors through induction of IL-6R/JAK1/STAT3 upon afatinib treatment (41).

Many studies reported that NF-κB is constitutively activated by ErbB family in breast, ovarian, prostate, and colorectal cancers (42, 43). We first demonstrated that NF-κB activation through 2 pathways in HCC827 and PC9...
cells. The one is IKK2 kinase dependent and the other is independent of IKK2 but requires EGFR activity. In addition, the former pathway led to phosphorylation of IκB S32. By contrast, that mutant EGFR induced NF-κB activation occurred without phosphorylation of IκB at Ser32. This finding is consistent with previous report by G. Sethi, who showed that EGF-induced NF-κB activation is require of phosphorylation of IκB at tyrosine 42 but not serine 32/36 (25). These 2 pathways are redundant in EGFR mutant lung cancer cell lines. Mere blockade one pathway had slightly impact on NF-κB activation. Simultaneously treatment with TKIs and IKK inhibitor made more potent repression of NF-κB. Nonetheless, the upstream factor of IKKs/NF-κB activation in NSCLC with EGFR mutation remains to be identified.

In addition, we further discovered that NF-κB and STAT3 co-operated the expression of IL-6 and COX-2. Downregulating STAT3 activity decreased IL-6 and COX-2 transcription. IL-6 is reported to be one of the genes downstream of COX-2 in oropharyngeal carcinoma, and COX-2 activates STAT3 by inducing IL-6 expression in lung cancer (44, 45). Our findings inferred there were maybe 2 positive feedback loops for NF-κB–IL-6–STAT3–IL-6 axis and NF-κB–COX-2–IL-6–STAT3–COX-2 axis in EGFR mutant lung cancer cells. In addition, we found TKI treatment failed to decrease the mRNA levels of IL-6 and COX-2. In contrast, TPCA-1, as a novel dual IKK2 and STAT3 inhibitor, sharply inhibited COX-2 and IL-6 autocrine in HCC827 cells. Overall, these findings suggest that combination of EGFR and STAT3 as well

Figure 8. TPCA-1 inhibits growth of NSCLC with EGFR mutation and enhances antitumor effect of gefitinib in vivo. A, HCC827 cells were injected into the flanks of nude mice. When tumor volumes reached approximately 80 mm³, mice were treated daily with TPCA-1 alone, gefitinib alone, or TPCA-1 with gefitinib at indicated doses. B, tumor weight was determined after 28 days drug treatment. *, P < 0.05; **, P < 0.001. C, model depicting the role of EGFR, NF-κB, and STAT3 and the effect of combination TPCA-1 with TKI in EGFR mutant lung cancer cells. TKI treatment is not efficient. Turn off NF-κB and STAT3 can abrogate transcription of IL-6 and COX-2 and intensify apoptosis of HCC827 and PC9 cells.

TPCA-1 Inhibits STAT3 and NF-κB Activation in Lung Cancer

www.aacrjournals.org Mol Cancer Ther; 13(3) March 2014
627

Published OnlineFirst January 8, 2014; DOI: 10.1158/1535-7163.MCT-13-0464
NF-xB inhibition may be a more effective therapeutic strategy. In conclusion, we showed that treatment with only TKIs could not suppress activity of STAT3 and NF-xB but inversely upregulate phosphorylation of STAT3. Dual inhibition of STAT3 and NF-xB enhanced apoptosis induced by Gefitinib. In addition, we found that TPCA-1 is an efficient dual inhibitor of STAT3 and NF-xB. This inhibitor may represent a unique strategy for cancer therapy. Currently, treatments with a combination of drugs are commonly used to improve the efficacy of NSCLC treatment. Therapies for EGFR-addicted NSCLC drugs are commonly used to improve the efficacy of therapy. Currently, treatments with a combination of inhibition of STAT3 and NF-kB could not suppress activity of STAT3 and NF-kB but EGF receptor-kinase dependent tyrosine 42 phosphorylation of IGF1R could not suppress activity of IGF1R. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

References

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc): J. Nan, Y. Du, J. Yang
Analysis and interpretation of data (e.g., statistical analysis, biosatistics, computational analysis): J. Nan, Y. Du, X. Chen, Q. Bai, J. Hou, Q. Wang, J. Yang
Writing, review, and/or revision of the manuscript: J. Nan, Y. Du, Q. Bai, X. Zhang, J. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Nan, Y. Du, J. Zhang, J. Yang
Study supervision: J. Nan, J. Yang

Acknowledgments
The authors thank Dr. G.R. Stark, Cleveland Clinic Foundation, for critical reading of this manuscript

Grant Support
This work was supported by grants 2009DF30900 from the Ministry of Science and Technology of the People’s Republic of China, 0708WCGA149 from the Gansu Provincial Science and Technology, and 2009AA01A130 from National Natural Science Foundation of China to J.B. Yang.

Published OnlineFirst January 8, 2014; DOI: 10.1158/1535-7163.MCT-13-0464

Downloaded from mct.aacrjournals.org on April 3, 2017. © 2014 American Association for Cancer Research.
TPCA-1 Inhibits STAT3 and NF-κB Activation in Lung Cancer


