TPCA-1 is a direct dual inhibitor of STAT3 and NFκB 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 & Jinbo Yang¹,³*

¹School of Life Sciences, ²College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou, Gansu 730000, China
³Department of Molecular Genetics, Lerner Research Institute, The Cleveland Clinic, Cleveland, OH 44195, USA

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*Correspondence address:
Jinbo Yang

School of Life Science, Lanzhou University, Lanzhou, Gansu 730000, P. R. China

Tel.: (+86-931)-8915350; Fax: (+86-931)-8915350

E-mail: yangjb@lzu.edu.cn
Abstract

Epidermal growth factor receptor (EGFR) is a clinical therapeutic target to treat a subset of NSCLC harboring EGFR mutants. However, some patients with similar kind of EGFR mutation show intrinsic resistance to TKIs. It indicates that other key molecules are involved in the survival of these cancer cells. We showed here that TPCA-1, a previously reported inhibitor of IKKs, 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 \textit{in vivo}. It selectively repressed proliferation of NSCLC cells with constitutive STAT3 activation. In addition, using pharmacological and genetic approaches, we found that both NFκB and STAT3 could regulate the transcripts of IL6 and COX2 in NSCLC harboring EGFR mutations. Moreover, gefitinib treatment only did not efficiently suppress NFκB and STAT3 activity. In contrast, we found that treatment with TKIs increased phosho-STAT3 level in target cells. Inhibiting EGFR, STAT3 and NFκB 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, while treatment targeting EGFR only could not sufficient to repress NFκB and STAT3 pathways for lung cancers harboring mutant EGFR. Therefore, synergistic treatment of TPCA-1 with TKIs is potential to be a more effective strategy for cancers.
Introduction

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

Lung cancer is the leading cause of cancer deaths in both men and women in the U.S and worldwide. Approximately 85-90% of all cases of lung cancer are non-small-cell lung cancer (NSCLC) (2). Some adenocarcinoma contains constitutive EGFR activity with mutant EGFR (3, 4). Gefitinib is the first-generation tyrosine kinase inhibitor 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 non-small cell lung cancer patients that respond to gefitinib treatment (5, 6). Although EGFR-TKI treatment therapy shows good responsive and survival rates in NSCLC patients with EGFR mutation as mentioned above (6, 7), about 30% of NSCLC patients 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 NFκB are key pathways down-stream 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 IL6 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 can’t 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 up-regulation (19). Tumor expression of IL6 and IL6 receptor components gp80 and gp130 had been found in NSCLC specimens (20). Increased levels of pro-inflammation cytokines such as IL6 and IL8 are also associated with non-small cell lung cancer tumorigenesis and prognosis. These indicate that IL6 and its downstream pathway are potential to be the target for patient with NSCLC harboring EGFR mutation. However, the mechanism about IL6 induction by oncogenic EGFR mutations in NSCLC is remaining unclear. IL6 had been reported to induce an autocrine IL6 loop in breast cancer (21). Therefore, we hypothesized that NFκB and STAT3 signaling were regulating IL6 autocrine in lung cancer.

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

In this work, we found that, TPCA-1, a previously used IKKs antagonist, not only inhibited NFκB signaling but also blockage STAT3 signaling pathway via binding to STAT3 SH2 domain directly. Thus, TPCA-1 is a dual inhibitor of IKKβ and STAT3 that
represses IL-6 autocrine and COX2 transcription in EGFR mutant cells. Moreover, the functional significance of TPCA-1-induced STAT3 and NFκB 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 three days. IL6 and scramble shRNAs 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 three days.

Cell culture, inhibitors, and cytokines
NCI-H1650, A549, MDA-MB231 and HEK-293T cell lines were obtained from 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, PC-9 and NCI-H1975 were authenticated by STR analysis in Jianlian Gene, Beijing. Results of STR matched the data of ATCC, DSMZ and JCRB cell banks. HEK-293T cells were maintained in Dulbecco’s Modified Eagle’s Medium with 10% fetal bovine serum (FBS). All other cells were maintained in RPMI 1640 supplemented with 10% FBS. IKK antagonists TPCA-1 and BAY11-7082 and cytokine TNFα were purchased from SIGMA. Gefitinib was purchased from LC Laboratories. IL6 was purchased from PeproTech.

Cell viability assay
Cell were seeded into a 96-well plat at density of 5 × 10³ cells/well and incubated for 24 h. After cells were exposed to drugs for 72 h, 0.5 mg/ml of MTT reagent was added to the medium in the well. After incubation for 4 h at 37 °C, formazan crystals in viable cells were solubilized with 100 μl 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 transfected with the pGL4.20-SIE-luc luciferase reporter, which contains nine 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 h of treatment. Luciferase activity was presented relative to a pGL4.20-SIE-luc transfected samples treated with DMSO, arbitrarily set at 1. The results of the luciferase assay represent the averages from three 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 \(\mu\)l PBS containing 4 \(\mu\)l PI and 4 \(\mu\)l Annexin V-FITC. These cells were incubated in the dark at room temperature. After 15 min incubation, 400 \(\mu\)l binding buffer were added. The percent of apoptotic (Annexin V- positive) cells was determined by the flow cytometry.

RNA extraction and quantitative RT-PCR

Total RNA was collected from cells following the manufacturer’s instructions using RNA Prep Pure Cell kit (TIANGEN). Total RNA (2 \(\mu\)g) 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 GAPDH mRNA expression. Data are presented in arbitrary unites and were calculated as \(2^{-\Delta\Delta Ct\ _{\text{gapdh-gene of interest}}}}\). Primer sequences of tested genes are listed as follows: IL6 forward primer, 5’-GAGAAAGGAGACATGTAACAAAGGT-3’; reverse primer, 5’-GCCACAATGAGATGAGTTGT-3’. COX2 forward primer, 5’-CCCTTGGGTGTCAAAGGTAA-3’; reverse primer, 5’-AACTGATGCGTGAAGTGCTG-3’. SOCS3 forward primer, 5’-CCATGGTGGTGAAAGACGC-3’; reverse primer, 5’-CCTGTCCAGCCCAATACCTGA-3’. GAPDH forward primer, 5’-TGGCAAATTCATGGCAC-3’; reverse forward, 5’-CCATGGTGGTGAGACGC-3’. The results of the real time PCR represent the
averages from three independent experiments.

Immunocytochemical analysis (ICC)

Cells were plated on coverslips. After incubation, cells were fixed with 4% formaldehyde and absolute methanol. And then incubate for 10 min in blocking buffer at room temperature. After the coverslips were washed with PBS, anti-STAT3 antibody (CST) 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 h at room temperature. Removed antibody and added DAPI at a final concentration of 0.4 \( \mu \text{g/ml} \) for 5 min and wash the cells 5 times with PBS. Cells were viewed under a fluorescent microscope.

Western blot and Molecular modeling

Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 5 mM EDTA, 1 mM EGTA, 1 mM Na3V3O4, 20 mM NaF, 50 mM Tris-HCl, PH 7.5) with proteinase inhibitors. Equal amounts of protein were fractionated by 8%-10% SDS-PAGE and then transfer them onto PVDF. Antibodies used were as follows: anti-phospho-STAT3(Tyr705), phospho-STAT3(Ser727), STAT3, phospho-AKT (Ser473), AKT, phospho-JAK1(Tyr1022/1023), phospho- JAK2(Tyr1007/1008 ), JAK2, phospho-p65(Ser536), p65, cyclinD3, cyclinD1, BCL-XL, p-IκBα(Ser32), IκBα, 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 in supplementary method.

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 nude mice were injected subcutaneously with HCC827 cells \((5 \times 10^6)\). HCC827 cells were suspended in serum-free RPMI-1640. When tumor volumes were reached approximately 80 mm\(^3\), mice were randomized to groups of six 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 i.p. 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 × (large 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) (28, 29). Both of them inhibited TNFα induced p65 activation (Supplementary Fig. S1A). However, as shown in Fig. 1B, TPCA-1 abrogated STAT3 activation induced by IL6, IFNα and IFNγ. 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 IκBα 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 over-expression (Fig. 1C). Same results were observed in Hela cells (Supplementary Fig. S1C). 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 IL6 was absolutely diminished by TPCA-1 but not BAY11-7082.

We further found that TPCA-1 severely blocked phospho-STAT3 induced by IL6 or IFNα at 500 nM or 100 nM (Fig. 2A and Supplementary Fig. S2A). Constitutive activated STAT3 were inhibited fiercely at 250 nM (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 min and phosho-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 NFκB pathway. Furthermore, STAT3-dependent luciferase activity was inhibited at 100 nM (Fig. 2C). These data suggest 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 due to cross-talking between STAT3 and NFκB pathway.
TPCA-1 binds to SH2 domain of STAT3

Julio Saez-Rodriguez reported that TPCA-1 inhibits JAK2 kinase to suppress STAT3 phosphorylation induced by IL6 (30). But JAK2 is not required for IFNα and c-Src induced STAT3 activation (31). Our finding showed that TPCA-1 can inhibit STAT3 activation induced by both IFNα and c-Src. We tested whether TPCA-1 affects JAKs phosphorylation. Results showed that TPCA-1 had no effect on JAK2 induced by IFNγ (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 two 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. While, 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 or not, 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 NSLCC 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 down-stream genes including c-myc, cyclinD 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 IL6 (19, 37). Exogenous IL6 didn’t compensate the reduction of phospho-STAT3 by TPCA-1 (Supplementary Fig. S4B). 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 IL6 autocrine and COX2 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 due to the inhibition of IKKβ by TPCA-1. Over-expression of NFκB, STAT3 and IL6 might lead to the progression of lung cancer (26, 38). To determine the role of NFκB 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 NFκB contributed to NSCLC survival. Some pro-proliferative and anti-apoptotic downstream genes of NFκB were tested. Results showed there was no difference between scramble shRNA and RelA knockdown cells in the levels of cyclinD1 and BCL-XL except for IκBα (Fig. 5B). Intracellular IL6 is required to control of cell proliferation in a subset of human lung cancer cells. COX2 is reported to be regulated by NFκB and is frequently expressed in
lung adenocarcinoma (39). Compared with less malignancy NSCLC cells, HCC827 (EGFR mutant) contained more abundance of IL6 and COX2 transcripts (Supplementary Fig. S6). We found that mRNA levels of IL6 and COX2 were sharply repressed while RleA knocked down in HCC827 and H1975 cell (Fig. 5C). These results showed that NFκB signaling was requisite for IL6 autocrine and COX2 expression in lung cancer.

Based on these finding, we further examined the role of NFκB pathway in mediating IL6 and COX2 expressions. Fig. 5D showed that TPCA-1 inhibited IL6 transcripts severely but not BAY11-7082. Because TPCA-1 abrogated STAT3 activity directly as above mentioned, so, we supposed IL6 autocrine and COX2 expression in EGFR mutant NSCLC are also mediated by STAT3. STAT3 dominant-negative expressing decreased about 50% of IL6 and COX2 mRNA levels. Furthermore, STAT3 over-expression increased IL6 and COX2 transcripts (Fig. 5E). These finding suggested that NFκB was prerequisite to transcript of IL6 and COX2, and STAT3 signaling pathways was also involved in regulating IL6 autocrine and COX2 expression in HCC827 cells. Therefore, TPCA-1 as a novel inhibitor of STAT3 can block IL6 autocrine and COX2 transcription in EGFR mutant NSCLC cells.

Rationale of combined STAT3, NFκB and EGFR inhibition in EGFR mutant lung cancer

Effects of TKI on STAT3 and NFκB are not fully understood. We next investigated whether gefitinib represses activation of STAT3 and p65. Gefitinib inhibited AKT activity and STAT3 serie727 phosphorylation but no effect on STAT3 tyrosine phosphorylation (Fig. 6A). Surprisingly, although there is lower STAT3 activity in PC9 cell line, TKI treatment obviously increased phospho-STAT3, and TPCA-1 blocked this rise. Combination of TKI with TPCA-1 abrogated both phospho-STAT3 Y705 and S727 phosphorylation (Fig. 6A). Although gfitinib and TPCA-1 both slightly reduced the levels of phospho-p65 in HCC827 cells, gfitinib had no impact on the level of phospho-IκBα S32. However, TPCA-1 could diminish IκBα S32 phosphorylation. Furthermore, we then discovered that co-treatment with two antagonists inhibited p65 activation potently (Fig. 6B). These findings indicate that there exist two redundant mechanisms of NFκB
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 IL6 and COX2. Twenty four hours after gefitinib treatment, $IL6$ mRNA level was significantly elevated by 5.4-fold compared to control (DMSO) and the change of $COX2$ level was just slight. TKI neither diminished IL6 autocrine nor COX2 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$\kappa$B and EGFR pathways in lung cancer cells containing EGFR mutant. We found the combination of TPCA-1 treatment increased sensitivity to gefitinib in both TKI sensitive cells and insensitive cells (Fig. 6D and Fig. S7). To understand whether the combination of the drugs 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 observed) was greater than the theoretical inhibition (E bliss). The effect of these two drugs is considered synergistic. Taken together, these findings indicated that blocking STAT3, NF$\kappa$B 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$\kappa$B pathway enhances TKI induced apoptosis via extrinsic pathway**

To examine whether inhibition of STAT3 and NF$\kappa$B 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 to 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$\kappa$B pathway via TPCA-1 increased the level of cleaved PARP induced by Gefitinib (Fig. 7B). 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 anti-tumor**
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 *in vivo*. As shown in Fig 8A, 8B, 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 performed in xenograft studies. The tumor weight inhibition rate of TPCA-1, gefitinib and their combination are 0.419(*E*$_{TPCA-1}$), 0.680(*E*$_{gefitinib}$) and 0.837(*E*$_{observed}$) respectively. The expected combinational inhibition rate is 0.814(*E*$_{bliss}$). *E*$_{observed}$ is greater than theoretical inhibition (*E*$_{bliss}$). Therefore, we considered the antitumor effect of combination TPCA-1 and gefitinib was synergistic. Fig. 8C is a model depicting the role of NFκB and STAT3 and the effect of combination TPCA-1 with TKI in EGFR mutant lung cancer cells.
Discussion

STAT3 and NFκB are ubiquitously expressed and control numerous physiological processes including development, immunity and cancer. Activated STAT3 and NFκB cooperatively control the expression of anti-apoptotic, 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 firstly found that TPCA-1 diminished STAT3 Y705 phosphorylation induced by IL6, IFNα, IFNγ 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. Additionally, 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 IKKβ 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 NFκB 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 NFκB 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 didn’t decrease phospho-STAT3 Y705. It is consistent with previous observation (18). However, we firstly 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 IL6 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 NFκB is constitutively activated by ErbB family in breast, ovarian, prostate and colorectal cancers (42, 43). We firstly demonstrated that NFκB activation through two 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 leaded to phosphorylation of IκB S32. By the contrast, that mutant EGFR induced NFκB activation occurred without phosphorylation of IκB at Ser32. This finding is consistent with previous report by G Sethi, who showed that EGF-induced NFκB activation is require of phosphorylation of IκB at tyrosine 42 but not serine 32/36 (25). These two pathways are redundant in EGFR mutant lung cancer cell lines. Mere blockade one pathway had slightly impact on NFκB activation. Simultaneously treatment with TKIs and IKK inhibitor made more potent repression of NFκB. Nonetheless, the upstream factor of IKKs/ NFκB activation in NSCLC with EGFR mutation remains to be identified.

In addition, we further discovered that NFκB and STAT3 co-operated the expression of IL6 and COX2. Down regulating STAT3 activity decreased IL6 and COX2 transcripption. IL6 is reported to be one of the genes downstream of COX2 in oropharyngeal carcinoma, and COX2 activates STAT3 by inducing IL6 expression in lung cancer (44, 45). Our findings inferred there were maybe two positive feedback loops for NFκB-IL6-STAT3–IL6 axis and NFκB-COX2-IL6-STAT3-COX2 axis in EGFR mutant lung cancer cells. Additionally, we found TKI treatment failed to decrease the mRNA levels of IL6 and COX2. In contrast, TPCA-1, as a novel dual IKK2 and STAT3 inhibitor, sharply inhibited COX2 and IL6 autocrine in HCC827 cells. Overall, these findings suggest that combination of EGFR and STAT3 as well NFκB inhibition may be a more effective therapeutic strategy.

In conclusion, we showed that treatment with only TKIs could not suppress activity of STAT3 and NFκB but inversely up-regulate phosphorylation of STAT3; dual inhibition of STAT3 and NFκB enhanced apoptosis induced by Gefitinib. In addition, we found that TPCA-1 is an efficient dual inhibitor of STAT3 and NFκB. This inhibitor may represent a
unique strategy for cancer therapy. Currently, treatments with a combination of drugs are commonly employed to improve the efficacy of NSCLC treatment. Therapies for EGFR addicted NSCLC are under clinical trials including using TKIs together with inhibitors of MET, PI3 kinase pathway and COX2 (46, 47). Our data suggest an intriguing therapeutic opportunity combined TPCA-1 with TKIs for EGFR addicted NSCLC.
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FIGURE LEGENDS

**Figure 1.** TPCA-1 inhibits STAT3 Y705 phosphorylation induced by cytokines and c-Src independent NFκB pathway.

A, Chemical structures of TPCA-1 and BAY11-7082. B, 293T cells were pretreated with DMSO or TPCA-1 (2 μM) for 2 h, then stimulated the cells with 50 μg/ml IL-6 for 2 h or 2,500 U/ml IFNα for 30 min or IFNγ for 1h. Cell extracts were analyzed by Western blot. C, c-Src over-expressed 293T cells were treated with DMSO and TPCA-1 for 2 h, extracts from these cells were analyzed. D, 293T cells were incubated with IL6, or IL 6 and BAY11-7082, or IL6 and TPCA-1 for 4 h. *soсs3* mRNA levels were determined by real-time PCR.

**Figure 2.** TPCA-1 inhibits STAT3 activation in a time and dose dependent manner. A, Proteins from 293T cells, pre-incubated with different dose of TPCA-1 before stimulation with IL6, were analyzed. B, HCC827 were treated with TPCA-1 for 0 - 2 h, cells were harvested for Western blot analysis. C, 293T-SRL cells were incubated with 0 - 2 μM TPCA-1 and IL6 or DMSO and IL6. The cell lysates were collected and assayed for luciferase activity. Data are the averaged results from 3 independent experiments, and error bars mark SDs. (*, P < 0.05, **, P < 0.01, ***, P < 0.001).

**Figure 3.** TPCA-1 disrupts STAT3 SH2 domain-phosphotyrosine interactions.

A, Equal proteins prepared from 293T stimulated by IFNγ following DMSO or TPCA-1 incubation were analyzed for JAK2 phosphorylation. B, Immunoblotting analysis of STAT3 immune complex (upper panel) or whole cell lysates (lower panel) from TPCA-1 treated MDA-MB-231 cells. C, TPCA-1 binds to the cavity of STAT3. D, The hydrogen bond between Glu594 and TPCA-1.

**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. C, STAT3 intracellular distribution in HCC827 cells treated with drugs was analyzed by ICC. D, STAT3 was over-expressed in HCC827 and H1975 cells. Viabilities of cells treated with TPCA-1 were estimated by MTT assay.

**Figure 5.** IL6 and COX2 are regulated by NFκB and STAT3 in NSCLC with EGFR mutant.

A, Proliferation of RelA knockdown cells was determined daily by MTT assays. B, RelA knockdown cells were harvested for Western blot analysis. C, IL6 and COX2 mRNA levels from parent cells or RelA knocked-down cells were determined by Real-time PCR. D, IL-6 mRNA levels from HCC827 cells upon treatment with DMSO, TPCA-1 or BAY11-7082 were determined. E, Empty vector, pLV–STAT3WT and pLV–STAT3Y705F lentivirus were introduced into HCC827 cells. Three days after selection, IL-6 and COX2 mRNA levels were estimated. (*, P < 0.05, **, P < 0.01, ***, P < 0.001).

**Figure 6.** Combined TPCA-1 and gefitinib represses NSCLC with EGFR mutant *in vitro.*

A-B, Extracts from cells treated with DMSO, TPCA-1(1 μM), gefitinib(1 μM), or TPCA-1 combined with gefitinib for 3 h were analyzed by Western blot. C, IL6 and COX2 mRNA levels in HCC827 cells treated with DMSO, gefitinib, or TPCA-1 and gefitinib combination were determined. D, Cells were incubated in gefitinib alone or combined with TPCA-1. Cell viabilities were measured by MTT assay. Bliss independent criterion analysis was tested on both cell lines. Data are the averaged results from 3 independent experiments, and error bars mark SDs. (*, P < 0.05, **, P < 0.01, ***, P < 0.001).

**Figure 7.** TPCA-1 enhances apoptosis induced by gefitinib in NSCLC cells.

A, HCC827 cells were incubated with indicated treatments for 16 h. Apoptosis was assayed using PI and Annexin-V staining. B, Cleavage PARP levels in cells treated with gefitinib or TPCA-1 or gefitinib combined with TPCA-1 for indicated time points were measured by Western blot. C, Levels of caspase 8, caspase 9 and caspase 3 in cells treated
as above for 12 h were examined.

Figure 8. TPCA-1 inhibits growth of NSCLC with EGFR mutation and enhances anti-tumor 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. C, Model depicting the role of EGFR, NFκB and STAT3 and the effect of combination TPCA-1 with TKI in EGFR mutant lung cancer cells. TKI treatment is not efficient. Turn off NFκB and STAT3 can abrogate transcription of IL6 and COX2 and intensify apoptosis of HCC827 and PC9 cells.
Figure 5

A

- HCC827 scramble shRNA
- HCC827 RelA shRNA
- H1975 scramble shRNA
- H1975 RelA shRNA

Cell viability

Day

0 1 2 3 4 5 6 7

B

ctrl scramble shRNA RelA shRNA

p65
IkBa
cyclinD1
Bcl-xl
β-actin

C

HCC827

IL6
COX2

Fold change

scramble shRNA RelA shRNA

0.2 0.4 0.6 0.8 1.0 1.2

H1975

IL6
COX2

Fold change

scramble shRNA RelA shRNA

0.2 0.4 0.6 0.8 1.0 1.2

D

E

IL6

Fold change

ctrl TPCA-1 BAY11-7082

0.0 0.2 0.4 0.6 0.8 1.0

Fold induction

empty vector STAT3 Y705F STAT3 wt

0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0

** ** ***
Figure 7

A

Ctrl  TPC-1  Gefitinib  T+G

B

HCC827

Gefitinib (100 nM)  TPCA-1 (2 μM)

Time (hours)
0  3  6  12  24  3  6  12  24

116 KDa  89 KDa

PARP  β-actin

C

HCC827

Ctrl  T  G  T+G

PARP  Cleaved caspase 8  Cleaved caspase 9  Cleaved-caspase 9  Cleaved-caspase 3  GAPDH
Molecular Cancer Therapeutics

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