LXR ligands induce apoptosis of EGFR-TKI-resistant human lung cancer cells \textit{in vitro} by inhibiting Akt-NF-κB activation

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\textbf{Abstract.} Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are efficient in treating patients with non-small cell lung cancer (NSCLC) harboring EGFR activating mutations. Unfortunately, nearly all patients ultimately acquire resistance to EGFR-TKI treatment. Liver X receptors (LXRs) can regulate tumor growth in various cancer cell lines. The present study indicated that LXR agonist combined with gefitinib weakened Akt-nuclear factor (NF)-κB activation and inhibited the expression levels of apoptosis-related proteins \textit{in vitro}. By contrast, LXR ligands alone exhibited no significant effect on gefitinib-resistant lung cells. In conclusion, the study provided evidence for the combination treatment of acquired TKI resistance in NSCLC.

\textbf{Introduction}

Lung cancer is the leading cause of cancer-related mortality worldwide (1), and non-small cell lung cancer (NSCLC) is the most common clinicopathological type. Although early diagnosis of lung cancer has developed in recent years, most patients are initially diagnosed with an advanced stage (2).

The epidermal growth factor receptor (EGFR) is a well characterized mutated oncogene in NSCLC with 10-20% cases in Western countries and is predominantly associated with adenocarcinoma histology. EGFR-mutated tumors are dependent on EGFR signaling for proliferation and survival (3,4). EGFR tyrosine kinase inhibitors (EGFR-TKIs) have shown dramatic therapeutic effects in patients with NSCLC harboring EGFR-activating mutations. Based on the positive results of several phase III clinical trials, National Comprehensive Cancer Network (NCCN) has recommended EGFR-TKI as the standard first-line therapy in NSCLC patients with sensitive EGFR mutations (5,6). However, nearly all patients eventually developed drug resistance after a median period of ~10 months (7). Thus, innovative treatment strategies are urgently needed for increasing sensibility to EGFR-TKI and improving the survival of patients with NSCLC.

Recently, a number of nuclear receptor superfamily, including liver X receptors (LXRs), have been shown to mediate tumor proliferation and enhance chemotherapeutic efficacy (8-11). LXRs (LXRα/NR1H3 and LXRβ/NR1H2) can be activated by natural ligands, including oxysterols and synthetic agonists GW3965 (12). High level of LXRα expression can be found in liver, kidney, intestine, fat tissue and macrophages, whereas LXRβ expression is ubiquitous (13). Some studies have demonstrated that LXR ligands exhibited anti-cancer activities in a variety of cancer cell lines. For example, LXR ligands can suppress the proliferation of breast, ovarian, prostate, colon and leukemia cancer cells \textit{in vitro} (14). Recently, a study has found that LXR ligands combined with gefitinib could suppress cell cycle progression by inhibiting cyclinD1 and cyclinB expression in NSCLC cells (15).

Based on these previous reports, the present study aimed to investigate the effects of synthetic LXR ligands (GW3965) on increasing sensibility of gefitinib-resistant NSCLC cell to EGFR-TKI and explored their potential mechanisms.

\textbf{Materials and methods}

\textbf{Cell lines and reagents.} Human NSCLC H827 cell line harboring the EGFR exon 19 deletion (Del E746-A750) was obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Cell Resource Center, and maintained in RPMI-1640 supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO\textsubscript{2} and at humidified atmosphere. Gefitinib (Iressa) was purchased from AstraZeneca, and GW3965 was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Cells were treated with gefitinib and GW3965 in RPMI-1640 supplemented with 5% FBS. Primary antibodies against MET,
PTEN, AKT, p-Akt (Ser473), NF-κB, p-NF-κB, Bax, Bcl-2 and β-actin were obtained from cell signaling technology.

*Generation of gefitinib-resistant H827 cells in vitro.* To generate a resistant cell line, we exposed H827 cells to increasing concentrations of gefitinib according to previously described methods (16). The resistant H827 cells were passed 25 times in the absence of gefitinib and were found to maintain their resistance as confirmed by Cell Counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assays. Six individual clones were isolated and all were confirmed independently to be resistant to gefitinib by CCK-8 assay. No significant change was observed in the sensitivity to gefitinib in parental cells during the period.

*Cell proliferation assay.* Cancer cells were seeded in 96-well plates and exposed to different doses of gefitinib alone, GW3965 alone, and both drugs for 96 h. Each combination of cell line and drug concentration was set up in 5 replicate wells and repeated at least thrice. Cell proliferation was measured by the CCK-8 assay. IC₅₀ values were determined by interpolation from the dose-response curves. Among the six gefitinib-resistant clones, we selected the most sensitive to LXR ligands for further study.

*Sequencing of the EGFR gene.* To determine the EGFR sequence of cells, DNA was extracted from each cell line using a QIAamp DNA mini kit (Qiagen, Tokyo, Japan), and the exons encoding the intracellular domain (exons 18–21) were amplified by PCR. Primer sequences are shown in Table I (17). Sequencing was conducted using an ABI 3500 sequencer (ABI).

*Colony formation assay.* Clonogenic survival assays were prepared according to literature (18). Briefly, H827-7-2 and H827-7-4 cells were seeded at a density of 600 cells/well in flat-bottomed 6-well plates. According to pre-experimental results, cells were treated with 2 ml of gefitinib (1 µM) alone, LXR ligands (GW3965, 1 µM) alone, and both drugs diluted with the medium to appropriate concentrations after 24 h of incubation. Then, cells were cultured for an additional 15 days, and subsequently stained with Giemsa. Experiments were performed forthrice.

*Cell apoptosis analysis.* H827-7-2 and H827-7-4 cells were seeded in 6-well plates (2x10⁴ cells/well) for 12 h and treated with gefitinib (5 µM) alone, LXR ligands (GW3965, 5 µM) alone, and both drugs for another 96 h, and then cells were harvested and washed twice with ice-cold PBS. Annexin V-FITC/PI staining was used to detect apoptotic cells. Three individual experiments were conducted.

*Quantitative PCR (qPCR) analysis.* Total RNA was isolated from H827-7-2 and H827-7-4 cells treated with GW3965 at different concentrations using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and cDNA was synthesized with PrimeScript RT Master Mix and premix EX Taq™ Probe qPCR Mix (Takara Bio, Dalian, China) on a 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Quantitative PCR was performed using SYBR Green PCR Mix (Roche, Mannheim, Germany). β-actin was used as an internal control to normalize the amount of total RNA in each sample. Primer sequences were summarized in Table I. The relative levels of gene expression were determined using the ΔΔCt method relative to internal control gene β-actin. All reactions were repeated thrice for each sample.

Moreover, qPCR was performed using premix EX Taq™ Probe qPCR Mix on a 7300 real-time PCR system. The copy number ratio of MET to GAPDH, a housekeeping gene, was calculated using a genomic DNA sample. The sequences of the Taqman probe and primers for MET and GAPDH were previously described (19). Quantification was based on standard curves from a serial dilution of normal human genomic DNA. All specimens were analyzed triplicates.

*Western blot analysis.* Cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China). Proteins were separated on 10% Bis-Tris Mini gels and transferred to a PVDF membrane. The primary antibodies used were AKT, p-Akt (Ser473), NF-κB, p-NF-κB, Bax, Bcl-2, MET, PTEN (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), and β-actin (1:1,000; Sigma-Aldrich). Proteins were detected with secondary antibodies (1:5,000; Immunology Consultanta Laboratory, Portland, OR, USA) and enhanced chemiluminescence solution (ECL; Beyotime Institute of Biotechnology).

*Statistical analysis.* Data were expressed as means ± SD. Statistical analysis was conducted using one-way ANOVA and Student's t-test with Graphpad 5.0 and SPSS 13.0. P<0.05 was considered statistically significant.

**Results**

*Effect of combined treatment of GW3965 and gefitinib.* After establishing six monoclonal gefitinib-resistant H827 cell lines, we characterized their drug resistance indices through
As illustrated in Table II, a ~300-fold increase was observed in IC_{50} for each monoclonal cell line compared with those of parental H827 cells (Fig. 1A). We found that 5 \mu M GW3965 had no inhibitory effects on the growth of TKI-resistant lung cancer cell lines and its monoclonal cell lines (Fig. 1B). Therefore, we continued using this concentration for further analysis. The amount of gefitinib (5 \mu M), which was less than 50% of the inhibitory concentration (IC_{50}) had no significant effect on all monoclonal cells (Fig. 1A). Then, we explored the combinational therapeutic potential of GW3965 and gefitinib, in monoclonal cell lines. Combined treatment showed significant growth inhibitory proliferation compared with each drug alone in H827-7-2 and H827-7-4 cells (Fig. 1C). These data suggested that GW3965 may increase the sensitivity of NSCLC to EGFR-TKI.

To exclude the mechanisms of acquired resistance, we conducted the DNA sequencing of EGFR at exons 18-21 and examined genetic alterations, including the levels of MET (20) and PTEN (21) and the well-known T790M mutation in these two monoclonal cells (Table I). T790M mutation at exon 20 was not observed in the H827-7-2 and H827-7-4 cells (Fig. 2A). The expression of PTEN was similar in both monoclonal cells, whereas MET was higher in H827-7-2 and H827-7-4 cells than in parental H827 cells (Fig. 2B and C).

**LXR ligands increase gefitinib-induced apoptosis in H827-7-2 and H827-7-4 cells.** We then analyzed the induction of apoptosis in the two monoclonal cells treated with GW3965 alone, gefitinib alone, or in combination. As shown in Fig. 3, flow cytometric analysis revealed that the percentage of apoptosis induced by gefitinib in H827-7-2 cells was 6.802%. When GW3965 was added together with gefitinib, the percentage of apoptosis dramatically increased to 20.69%. Similarly, the percentage of apoptosis induced by gefitinib increased from 8.88 to 22.06% after treatment with GW3965 in H827-7-4 cells (Fig. 3). Colony-forming assays revealed that GW3965 alone did not enhance the apoptosis of H827-7-2 and H827-7-4 cells, but the combination therapy significantly augmented apoptosis (Fig. 4A).

Furthermore, we examined the effect of GW3965 on apoptotic proteins. Cells were treated with gefitinib (5 \mu M) alone, LXR ligand (GW3965, 5 \mu M) alone, or both drugs for 96 h. Western blot analysis showed that gefitinib enhanced Bcl-2 expression and decreased Bax expression compared with that of the control. GW3965 treatment with gefitinib weakened the effect of cell apoptotic proteins compared with gefitinib alone (Fig. 4B). Overall, these in vitro data suggest that gefitinib resistant cells have a stress response to gefitinib, but GW3965 can reduce this stress response to gefitinib in the H827-7-2 and H827-7-4 cells. The combination of GW3965 with gefitinib resensitized the resistant cells to TKIs. Reduced stress response may be one of the important mechanisms underlying the synergistic effects of LXR ligands on gefitinib.

**Effect of GW3965 on the transcriptional level of nuclear receptor LXR.** As GW3965 activates LXR receptors, we then applied Quantitative PCR assay to confirm whether GW3965 affects the level of LXR receptors (LXR\alpha and LXR\beta) in...
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the cell nucleus. As shown in Fig. 5A, with the treatment of GW3965 in H827-7-2 and H827-7-4 cells, the expression level of LXRα did not exhibit any differences. However, GW3965 increased the expression level of LXRβ distinctly at a higher dose (5 and 10 µM), whereas it had no effect at low dose (1 µM).

GW3965 treatment with gefitinib influences the activation of AKT-NF-κB. To further investigate the underlying mechanism of apoptosis introduced by GW3965 combined with gefitinib in H827-7-2 and H827-7-4 cells, these two cells were first treated with GW3965 (5 µM) separately at various periods (0-96 h). The phosphorylation of AKT and NF-κB was decreased after treatment with T0901317 for 96 h (Fig. 5B). Therefore, we analyzed the expression of EGFR and its downstream genes in two cells treated with gefitinib (5 µM) alone, LXR ligands (GW3965, 5 µM) alone or both drugs for 96 h. As shown in Fig. 5C, the phosphorylation levels of AKT and NF-κB when combined with GW3965 were downregulated compared with that of gefitinib alone.

Discussion

LXR is a ligand-dependent nuclear receptor. Previous study demonstrated that LXR had antiproliferative effects on cancer cells (22). Our results demonstrated that GW3965 exhibited no cytotoxicity in H827-7-2 and H827-7-4 cells. However, the proliferation rates of the two cells being treated with GW3965 combined with gefitinib were obviously inhibited when compared with that of gefitinib alone.

We found that GW3965 sensitized gefitinib in the two cells by inducing apoptosis and inhibiting colony formation.
To further investigate whether GW3965 could affect cells apoptotic proteins, we detected the protein expression of Bcl-2 and Bax. Western blot analysis showed that gefitinib increased the expression level of Bcl-2 and decreased the expression level of Bax compared with that in the control. These results further illustrated that lung cancer cells had been resistant to gefitinib. As expected, GW3965 alone did not change the expression level of cell apoptotic proteins compared with that in the control. Meanwhile, when GW3965 was combined with gefitinib, the expression levels of Bcl-2 decreased while Bax increased compared with that in gefitinib alone.

In addition, synthetic agonists including TO-901317 and GW3965, were biphasic activators of LXRα and LXRβ. Compared with endogenous ligands, synthetic agonists could activate LXR more effectively and generate stronger effect on the transcription of downstream target genes. Colon cancer cell apoptosis could be induced by the LXRβ-dependent pathway. However, LXR could be synergistic in human carcinomas owning to signaling interactions mediated through LXRα. In this study, the expression level of LXRβ increased with higher dose of GW3965, prompting that LXRβ may be the primary subtype expressed in our experimental models.

Human NSCLCs with activating EGFR mutations showed an excellent response to treatment with EGFR-TKIs, including gefitinib and erlotinib. However, most patients with prolonged exposure to the drug developed relapse of cancer with drug resistance. Two principal mechanisms accounting for ~50% of acquired resistance were secondary mutations of threonine-to-methionine substitution at amino acid position 790 (T790M) of EGFR and amplification of the N-methyl-1-N0-nitro-N-nitroso-guanidine (MNNG) HOS-transforming gene (MET) oncogene. In this study, T790M mutation at exon 20 was not observed in the cells, but we found that the expression level of MET was higher in H827-7-2 and H827-7-4 cells than that in parental H827 cells, whereas PTEN had similar expression between two cell lines.

Various molecular mechanisms of acquired resistance to EGFR-TKIs in lung cancer were reactivation of EGFR...
downstream signaling pathways, especially Akt-NF-κB signal pathway (33). MET amplification could lead to gefitinib resistance in lung cancer by activating ERBB3-phosphoinositide 3-kinase (PI3K)/Akt (34). NF-κB was known to have a role in survival signaling and could promote the transcription of anti-apoptotic genes. Previous study found that inhibition of NF-κB enhanced apoptosis in EGFR-mutant lung cancer models (35). Furthermore, the inhibition of NF-κB could enhance EGFR TKI-induced apoptosis (36). Previous studies demonstrated that the LXR agonist could inhibit activation of the EGFR-AKT-NF-κB pathway and also had a potential synergistic effect with EGFR-TKI treatment (37,38). The phosphorylation of Akt and NF-κB reduced time dependently with GW3965 treatment in the two cells in our study. Thus, we hypothesized that the Akt-NF-κB signaling pathway could be suppressed by the LXR agonist, so that it could sensitize gefitinib-resistant non-small cell lung cancer cells to gefitinib treatment. Our results proved this hypothesis and confirmed the inhibitory effect of the LXR agonist on the Akt-NF-κB signaling pathway. The combination of gefitinib with GW3965 suppressed Akt-NF-κB signaling pathway, which promoted cell apoptosis and inhibited cell proliferation.

In conclusion, our results demonstrated that the LXR agonist GW3965 could effectively enhance gefitinib-resistant cell sensitivity to gefitinib. In addition, the results suggested that the resensitization of H827-7-2 and H827-7-4 was achieved by the inhibited activation of Akt-NF-κB pathway, which mediated by the LXRβ expression. Overall, these findings provided evidence for the combination treatment of acquired TKI resistance NSCLC.

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Conflict of interest

The authors declare that they have no conflict of interest.
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