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Original Research

TW37 enhances the pro-apoptosis and anti-migration ability of gefitinib in Non-Small Cell Lung Cancer

Xiaofan Chen[1], Guangxian Mao[2], Hua Chen[2], Suyue Liu[2], Sichao Wang[2], Xiaoqiang Li[2], Yiwang Ye[2], Hao Wu[2], Jixian Liu[2*]

[1] Biomedical Research Institute, Shenzhen Peking University-the Hong Kong University of Science and Technology Medical Center, Shenzhen, Guangdong, China
[2] Department of Thoracic Surgery, Peking University Shenzhen Hospital, Shenzhen, Guangdong, China

Correspondence to: liujixiancmb@163.com

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Abstract: B cell leukemia-2 (Bcl-2) plays important roles in the development of tumor and drug resistance. The growth of tumor cells can be inhibited by downregulating the abnormal expression of Bcl-2 protein. TW37, an effective inhibitor of Bcl-2 protein, has now been widely studied in many tumors. In our study, it was found that TW37 exerted a significant effect on the proliferation, apoptosis and migration of Non-Small Cell Lung Cancer cells. Bcl-2 is also a key downstream factor of many signaling pathways such as Epidermal Growth Factor Receptor (EGFR). TW37 enhanced the inhibition of tumorigenesis by gefitinib, an EGFR-Tyrosine Kinase Inhibitor drug. Moreover, TW37 can promote apoptosis ability by inhibiting the phosphorylation level of EGFR protein in H1975 cells. Overall, TW37 enhances the pro-apoptosis and anti-migration ability of gefitinib in Non-Small Cell Lung Cancer.

Key words: TW37; Gefitinib; Apoptosis; Migration; Non-Small Cell Lung Cancer.

Introduction

Non-Small Cell Lung Cancer (NSCLC) is one of worldwide malignant tumors with the highest mortality rate. The overall 5 year survival rate of NSCLC is less than 15%(1). Since twenty-first century, the lung cancer treatment with Epidermal Growth Factor Receptor - Tyrosine Kinase Inhibitor (EGFR-TKI) has helped the patients whom traditional chemotherapy cannot cure. EGFR-TKI drugs, such as gefitinib and Erlotinib, have been approved by many countries and are widely used in patients with advanced or refractory NSCLC. Above 10% of patients with NSCLC benefited from the treatment of EGFR-TKI drugs, whose symptoms and survival time could be improved after failure of chemotherapy. In recent years, a large number of clinical data have shown that EGFR-TKI can significantly prolong the progression-free survival of sensitive patients with non-small cell lung cancer, which can reach improvements of 80% in efficiency. However, drug resistance emerged inevitably about ten months later (2-4), because T790M mutation in the twentieth exon of EGFR can prevent the competitive binding of Mg-ATP sites in the catalytic domain of TKI and EGFR-TK (5-7).

Apoptosis was also known as programmed cell death. The oncogene B cell leukemia -2 (Bcl-2) can inhibit apoptosis, which play an important role in the development of NSCLC (8). Recent studies also reported that the high expression level of Bcl-2 protein was associated with the resistance to chemotherapy and radiation in tumor cells (9). Bcl-2 is a key downstream factor in EGFR resistance mechanisms, such as T790M mutation and insµlin-like growth factor 1 receptor (IGF-1R) overexpression. Since 2009, a number of studies have suggested that high expression of Bcl-2 was closely related to secondary resistance of EGFR-TKI in lung cancer cell lines (10).

TW37, a gossypol derivative, is considered as an effective inhibitor of Bcl-2 protein. Gossypol was firstly studied as a kind of male contraceptive pills in China. Then it was found that Gossypol has an anticancer effect by inducing apoptosis and inhibiting the growth of a broad range of tumor cells (11). Gossypol combined with a variety of chemotherapy drugs can produce synergistic effect, which improves the efficacy and reduces the side effects of chemotherapy drugs (12). Compared with gossypol, the higher affinity and selectivity of TW37 for Bcl-2 showed significant antiproliferative effect on diffuse large cell lymphoma cell lines obtained from patients with lymphoma, but not on normal peripheral blood lymphocytes (13). A recent study found that TW37 can inhibit the growth of breast cancer cells, pancreatic cancer cells and prostate cancer cell, through inhibition of Bcl-2 protein and regulation of several important genes which arrest cells in the S phase (14,15).

In our study, TW-37 can potently enhance the pro-apoptosis and anti-migration ability of gefitinib in NSCLC by inhibiting the phosphorylation level of EGFR protein. The combinatory of TW-37 and gefitinib in NSCLC exhibit high efficiency, which may solve the bottleneck of EGFR-TKI treatment in NSCLC.

Materials and Methods

Cell culture and reagents

H1975 cells were grown in RPMI-1640 Medium (Hyclone, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA), and 1% Penicillin/Streptomycin. Cells were cultured in an incubator with an atmosphere of 5% CO2 at 37°C. H1975 cells were cultured in 10-cm dishes and 6-well plates with 70%-80% confluence. Then they were treated with different concentrations of TW37 (Selleck Chemicals, USA) and gefitinib (Selleck Chemicals, USA) in fresh RPMI-1640 containing 10% FBS.

Cell proliferation test

Proliferation of H1975 cells was measured by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma Aldrich, St. Louis, Missouri, USA). Cells were seeded into 96-well culture plate for 24 hours prior to drug treatment. The blank control was set up with medium only. After 24 hours, different concentrations of TW37 and gefitinib diluted by RPMI-1640 containing 10% FBS were added and cells were incubated. Then 20 µl MTT was added and cells were incubated in the incubator at 37 °C. Then, 150 µl dimethylsulphoxide (DMSO, Sigma, Shanghai, China) was added into each well and the plates were thoroughly mixed for 10 min. The optical density (OD) was measured by an ELISA microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm (with 630 nm as the reference wave length). Percent of cell proliferation was calculated by considering 100% growth in the control group (no drug added). The combination index (CI) values of TW37 and gefitinib was calculated by Chou Talalay Formula(16). The CI values resided in the "nearly additive" range ($0.9 < CI < 1.1$). Experiments were repeated at least three times.

Cell wound-healing experiment

For wound-healing experiments, cells were seeded in 6-well plates (Corning Life Sciences) and grown to approximately 100% confluence. After scratching with a sterile 200 µl pipette tip, cells were washed by serum-deprived RPMI-1640 for three times, and then different conditioned mediums were added and incubated at 37 °C. Images of cells migration were captured at time points of 0 and 24 hours by digital camera system. The software program MIAS-2000 was used to determine the migration distance. Experiments were run in three independent repeats and analyzed in a double-blind fashion by at least two observers.

Apoptosis analysis

The percentage of apoptotic cells was calculated from the data originating from flow cytometry. PE Annexin V Apoptosis Detection Kit I (BD pharmingen, USA) was used to identify apoptosis following the manufacturer's instructions. H1975 cells were seeded on 6-well plates and incubated with different concentrations of TW37 and gefitinib (Selleck Chemicals, USA). Then they were resuspended in binding buffer, mixed with Annexin V-PE and 7-AAD, and incubated in room temperature for 10 min. flow cytometry (FCM) was applied to detect apoptotic cells. The experiments were repeated at least three times.

Western blotting analysis

H1975 cells were washed with ice-cold PBS and lysed in each well of a 6-well plate with 60 µl RIPA Solution containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and 1 mM PMSF. Lysate was incubated 30 min on ice and centrifuged to remove insoluble materials (12,000 g for 10 min at 4 °C). The supernatant was collected for the following experiments. 15 µl lysate were separated by SDS-PAGE and electro blotted onto nitrocellulose membrane. The membrane was blocked by 5% non-fat milk in Triton X-100 (Sigma, USA) / Tris-buffered saline (TTBS) for two hours at room temperature, then incubated with primary antibody (1:1000 diluted) at 4°C overnight. The primary antibodies including anti-Phospho-EGF Receptor (Tyr1068) and anti-β-Actin were purchased from Cell Signaling Technology. Then peroxidase-conjugated secondary antibody (1:10000 diluted, MBL) was added on the membrane at room temperature for another 1 hour. The expressions of related proteins were assessed with ECL reagents (Pierce Chemical, Rockford, IL) and measured by X-ray film (Fuji Safelight Glass, Japan).

Statistical analyses

All experiments were carried out in triplicate, and all data were represented as mean ± standard deviation (SD). Statistical significances between groups were determined by Student's t-test. P values less than 0.05 were considered statistically significant. One-way ANOVA was used to analyze multiple comparisons. The IC50 values of TW37 and gefitinib for inhibition of H1975 cells viability were obtained by linear regression analysis (SPSS version 17.0 software was used).

Results

TW37 cytotoxicity in H1975 cells is dose dependent

It was reported that proliferating endothelial cells are susceptible to TW37 and suggested that the cytotoxic effect of TW37 is cell type specific (17). The viability of H1975 cells treated with TW37 and gefitinib were examined by MTT assay. As shown in Figure 1, the

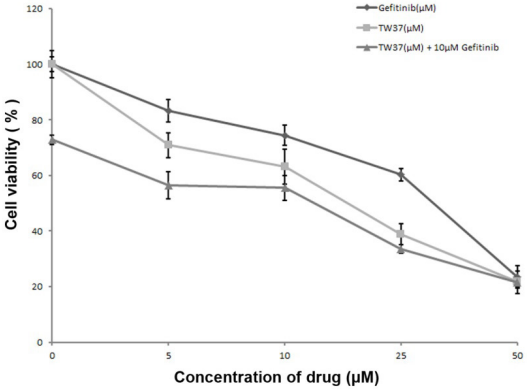

Figure 1. The inhibitory effect of TW-37 and gefitinib on the proliferation ability of NSCLC cell lines. The cell growth curves of different dose of TW-37 or/and gefitinib was shown in the H1975 cell lines. All data are presented as means ± SD for three different experiments performed in duplicate.

Table S1. The combination index of different dose TW-37 with 10μM gefitinib.

Gefitinib Dose (μM)	TW-37 Dose (μM)	combination index
10.0	5.0	0.97120
10.0	10.0	1.36902
10.0	25.0	1.08011
10.0	50.0	1.03358

proliferation of H1975 cells treated with either TW37 or gefitinib was inhibited in a dose-dependent manner. IC50 value is the efficient concentration at which the viability of H1975 cells was inhibited by 50%, and was gained by interpolation from linear regression analysis. The IC50s were approximately 25.781 μM, 28.682 μM for TW37 and gefitinib, respectively. When 10 μM gefitinib and TW37 were both added, the IC50 of H1975 cells decreased prominently to 23.099 μM for TW37. Moreover, TW37 may have additive effects with gefitinib and play an important role in the proliferation ability of the H1975 cells (Table S1).

TW37 enhances the apoptosis in H1975 cells

Bcl-2 is a key survival checkpoint factor in the apoptosis signaling pathway (18), while small-molecule inhibitors of Bcl-2 have been found to induce apoptosis in tumor cells (19)**.** Therefore, overall growth inhibition induced by an inhibitor of Bcl-2 may be expected to involve apoptosis. FCM analysis is a sensitive method to distinguish between the normal cells and the apoptotic cells, so it was used to elucidate the effect of TW37 on the apoptosis of H1975 cells in this study. It was observed that increasing concentrations of TW37 were correlated with significantly increased apoptosis of H1975 cells. Similar results were found in the combined treatment with TW37 and gefitinib, which suggested that TW37 enhance the apoptosis of H1975 cells induced by gefitinib (Figure 2).

TW37 attenuates migration in H1975 cells

Because the migration of lung cancer cells is necessary in the tumorigenesis process, the effect of TW37 on H1975 cells migration was investigated by the wound healing assay. It revealed that TW37 dose-dependently prevented the migration ability of H1975 cells. What's more, the results of wound healing assays showed that the migration ability of H1975 cells was significantly decreased in the drug combination group (Figure 3).

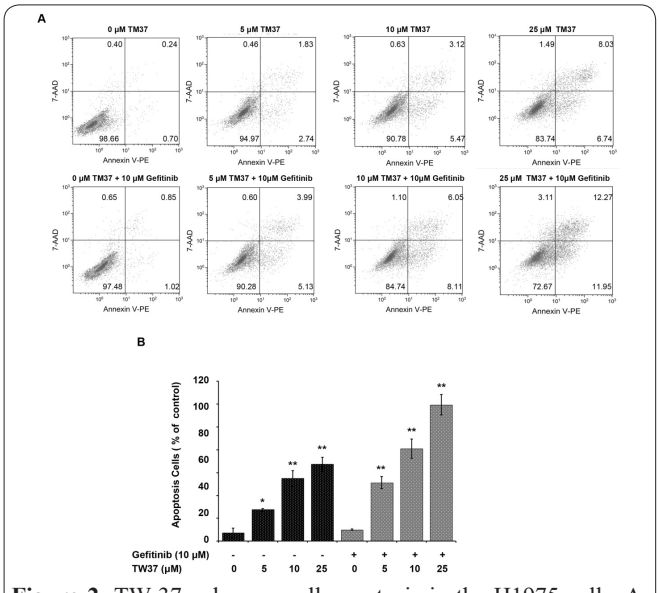

Figure 2. TW-37 enhances cell apoptosis in the H1975 cells. A, In situ apoptosis was assessed by Annexi-V/7-AAD staining. B, The statistical analysis of the data from FCM analysis. Data was presented as mean ± SD. n=3, *, P<0.05. **, P<0.01.

High dose of TW37 attenuates apoptosis and migration of H1975 cells by inactivating the EGFR pathway

Epidermal growth factor receptor (EGFR) is a therapeutic target of EGFR-TKI. The EGFR protein can be phosphorylated and thereby activated, which participates in tumor growth, infiltration and metastasis. After treated with different concentrations of TW37 and gefitinib, the phosphorylation level of EGFR proteins in the H1975 cells were tested. The results showed that high dose of TW37 can inhibit the phosphorylation level of EGFR proteins (Figure 4), which promoted the apoptosis and prevented the migration ability in H1975 cells.

Discussion

Gossypol was initially used as a male contraceptive but was subsequently investigated as a novel antitumor

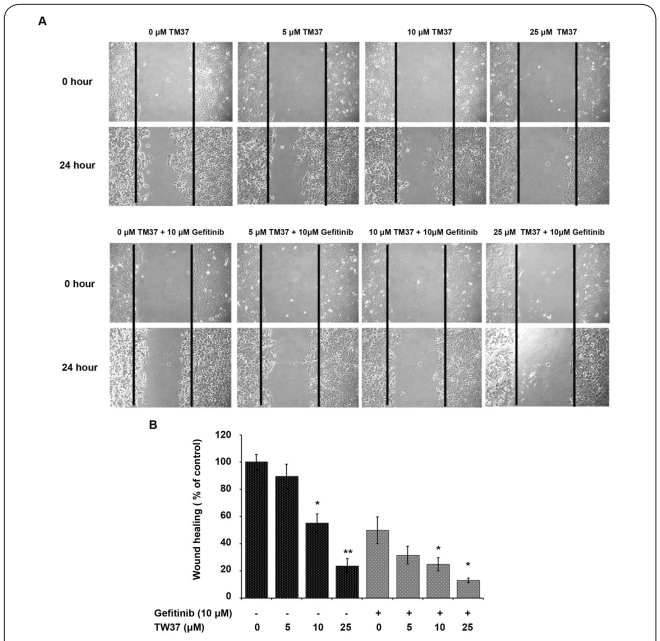

Figure 3. TW-37 and gefitinib on the migration ability of the H1975 cells. A, H1975 cells were divided into several groups: Control group, TW-37 single groups, gefitinib with TW-37 groups. In these groups gefitinib was used as 10μM and TW-37 was used as 0μM, 5μM, 10μM, 25μM. B, all data are presented as means ± SD from three different experiments performed in duplicate. *, P<0.05; **, P<0.01 versus the control group.

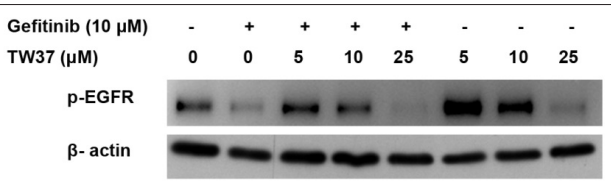

Figure 4. TW-37 induces decreased phosphorylation of EGFR. All experiments were carried out in triplicates and a representative blot was shown.

agent. The isopropyl moiety of the gossypol molecule, like the aldehyde group, is essential for its mechanism of antifertility(20). Gossypol and its derivatives exert anti-tumor effects on different cancer types and demonstrate synergistic effects with other chemo- and radio- therapeutic treatments (21). Previous studies indicated that the expression of Bcl-2, an antiapoptotic protein upregulated in several tumor types, have strong asosiation to development of resistance to therapy and poor prognosis (22-26). TW-37 is a gossypol derivative, which was originally designed to target the BH3-binding groove in the Bcl-2 family protein by researchers at University of Michigan(17). Like other gossypol derivatives lacking the essential isopropyl moiety, TW-37 may avoid the antifertility effect of gossypol and exhibit powerful anti-tumor effect in many kinds of cancers, however the underlying mechanisms are still obscure (17,27,28).

According to our results, TW37 can promote its apoptosis and prevent its migration of the H1975 cells. It was found that TW-37 significantly inhibited the viability of H1975 cells in a dose dependent manner. Further investigations indicated that TW-37 at these concentrations could also prevent the migration and apoptosis of the H1975 cells. After H1975 cells were treated with high concentrations of TW37, the downregulated phosphorylation of EGFR was detected. The phosphorylated EGFR protein is important for tumor growth, infiltration and metastasis. When H1975 cells were treated with low-dose TW37, increased phosphorylated EGFR might be due to the negative feedback loop that contributed to intracellular homeostasis. When H1975 cells were treated with higher dose of TW37, however, the balance was broken, and phosphorylated EGFR was decreased dramatically. Thus, more efforts are required to investigate possible signaling pathways which are related with TW37 and tumorigenesis.

The pro-apoptosis ability of gefitinib through its inhibition of EGFR autophosphorylation has been confirmed in many tumors. However, T790M mutation of EGFR caused resistance to EGFR-TKI, because T790M mutation can prevent the competitive binding of Mg-ATP sites in the catalytic domain (5-7). Bcl-2 is one of key factors in the life-death balance. Recently, Bcl-2 proteins have been considered as drug targets in the design of new anti-tumor therapies. It was observed that Bcl-2 inhibitors could induce cell death in vitro and to exhibits potent anti-tumor activity in animal models of myeloma and leukemia (29). It was shown that gossypol and its derivatives trigger apoptosis through the canonical intrinsic mitochondrial pathway (30-32). Recently, it was reported that gossypol also cause autophagy through Atg5 and Beclin-1 in apoptosis-resistant glioma cells (33). Similarly, apogossypolone led to autophagy in hepatocellular and nasopharyngeal carcinoma (34,35). In NSCLC, gefitinib with different dose of TW-37 was added to enhance apoptosis ability. It was indicated that high dose of TW-37 can inhibit the phosphorylation level of EGFR proteins.

In conclusion, TW-37 can potently enhance the pro-apoptosis and anti-migration ability of gefitinib in NSCLC by inhibiting the phosphorylation level of EGFR proteins. The combinatory use of TW-37 and gefitinib in NSCLC can exhibit high efficiency, which may provide a new therapeutic regimen for NSCLC and a new method against the resistance of EGFR-TKI in the treatment of lung cancer.

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Conflict of interest

No potential conflicts of interest are disclosed.

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