

The Anticancer Mechanism of an Approved Disease Modifying Herb Medicine: Total Glucosides of Paeony Target both EGFR and HER-2 in Lung Cancer Cell Lines

Yang Tang¹, Yidi Cui¹, Lingling Xu¹, Siwen Wang¹, Meng Zhang¹, Yue Sun¹, Hang Xiao², Huashi Guan¹, Mingzhuang Zhu³, Peiju Qiu^{1*} and Lijuan Zhang^{1*}

¹School of Medicine and Pharmacy, Ocean University of China, Qingdao, 266003, P.R. China

²Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA

³Marine Biodiversity and Evolutionary Institute, Ocean University of China, Qingdao 266003, P.R. China

*Corresponding authors: Peiju Qiu, Ocean University of China, 5 Yushan Road, Qingdao, P.R. China, Tel: 086-532-82031615; Fax: 086-532-82031615; E-mail: grtqiupaiju@hotmail.com

Lijuan Zhang, Ocean University of China, 5 Yushan Road, Qingdao, P.R. China, Tel: 086-532-82031615; Fax: 086-532-82031615; E-mail: lijuanzhang@ouc.edu.cn

Rec date: Apr 29, 2014; Acc date: May 30, 2014; Pub date: June 03, 2014

Copyright: © 2014 Tang Y, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

The high cost and slow pace of the pharmaceutical industry in bringing novel anti-cancer drugs for waiting patients indicates that the current business model for drug development needs innovation. We assumed that studying anticancer activities from approved disease modifying medicines might provide a possible way to lower the cost and speed up anti-cancer drug development. To test the idea, we studied the molecular mechanisms of anti-cancer effect of Total Glucosides of Paeony (TGP), a Chinese State Food and Drug Administration approved drug, in non-small cell lung cancer (NSCLC) cell-based systems. NSCLC is the leading cause of death from all cancers. Epidermal growth factor receptor (EGFR) is a major and effective molecular target of anti-NSCLC therapy because EGFR is over-expressed in 50-80 percent of NSCLC. We discovered that TGP inhibited phosphorylation of two most prominent members of EGFR family, EGFR and HER-2, in addition to suppressing EGFR and HER-2 expression in lung cancer cell lines tested. Subsequently, TGP showed expected anti-cellular proliferation, pro-apoptosis, and cell cycle arrest properties of EGFR inhibition in the cancer cell lines tested. Therefore, searching anti-EGFR and HER-2 activities among approved medicines might be a reasonable approach for novel anti-NSCLC drug discovery.

Keywords: Total glucosides of paeony; Lung cancer; Cell cycle arrest; Apoptosis; EGFR; HER-2

Introduction

Despite exponentially increased investment, the total numbers of approved drugs by US FDA have been about the same each year during the past 50 years [1]. Market capitalization of 9 top-tier pharmaceutical companies from 2001 to 2009 had accumulative loss of \$626 billion [1].

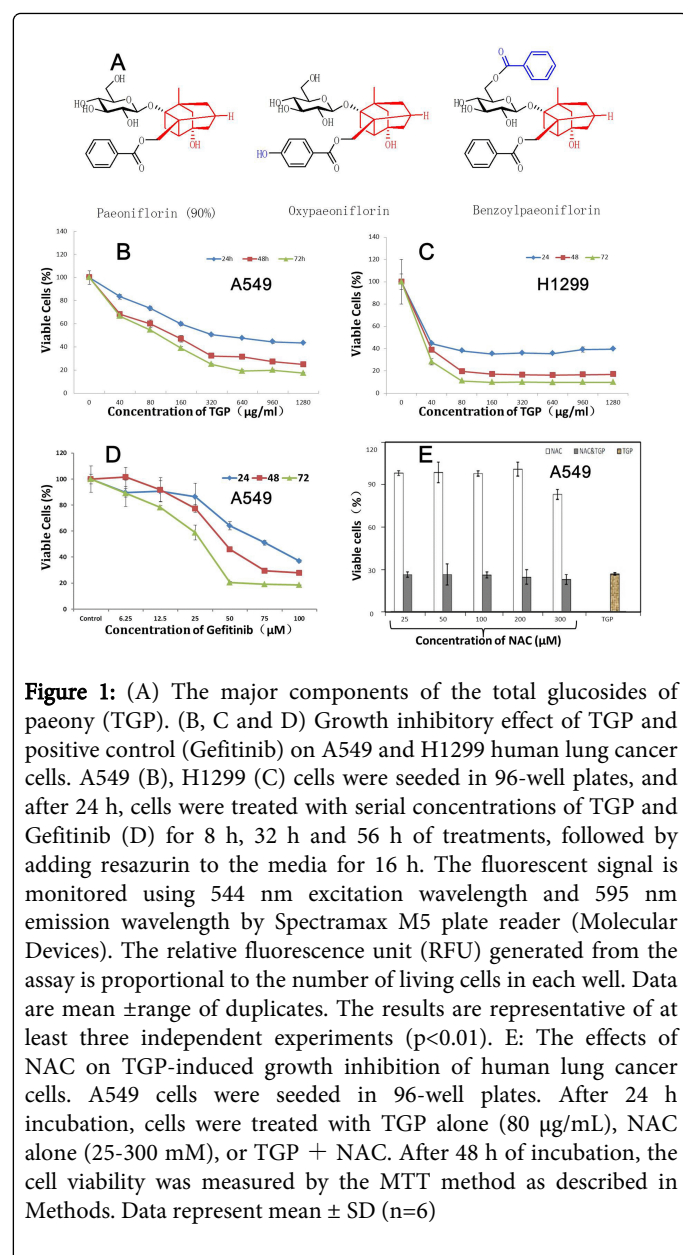
Therefore, identifying and successfully developing new drug candidates while trying to contain the costs might be the key to make pharmaceutical industry profitable and novel drugs available for waiting patients. New ideas, new sources, and new data might help the pharmaceutical industry to speed up the process of identifying new drug candidates. Based on this idea, we studied the molecular mechanisms of anti-cancer effect of Total Glucosides of Paeony (TGP), a Chinese State Food and Drug Administration approved rheumatoid arthritis modifying drug, in a non-small cell lung cancer (NSCLC) cell-based system.

Non-small-cell lung cancer (NSCLC) is the leading cause of death from all cancers [2,3]. Epidermal growth factor receptor (EGFR) is a major and effective molecular target of anti-NSCLC therapy because EGFR is over-expressed in 50–80 percent of NSCLC [4].

Autophosphorylation of tyrosine residue of EGFR by its tyrosine kinase domain initiates activation of the downstream signaling

cascades PI3K–Akt, which has anti-apoptotic and pro-survival effects that promote cancer cell growth [5]. Tyrosine kinase inhibitors (TKIs) directed against EGFR, such as gefitinib or erlotinib, are among the first molecular-targeted agents to be approved in the US and other countries for the treatment of NSCLC, breast, head and neck cancers [6], esophagus cancer [7], glioblastoma [8], and epithelial ovarian cancer [9]. However, cancer patients who have benefited from costly EGFR TKI therapy quickly generate resistance to the drug with 70% known [10-12] and 30% unknown reasons [4]. Hence there is a great need to develop cheaper and more effective TKIs.

Paeonia radix, the dried root of Paeonia lactiflora Pall, has been prescribed in various formulations of Chinese medicines for over 1500 years [13]. Modern research indicates that the total glucosides of paeony (TGP), an active fraction extracted from Paeonia radix, is responsible for many of its therapeutic properties. The major components (Figure 1A) of TGP include paeoniflorin (~90%), oxypaeoniflorin, and benzoylpaeoniflorin, which all have the same florin core structure [14-16].



TGP has shown to be anti inflammatory [17], anticoagulative [18], cognition-enhancing [19], antihyperglycemic [20,21], neuroprotective [22], analgesic [23], antioxidative [24], immune regulatory [25,26], neuromuscular blocking [27], myelosuppression [13], and hepatic injury protective [28]. In 1998, the use of TGP as a rheumatoid arthritis (RA) modification drug was approved by the Chinese State Food and Drug Administration (SFDA). Similar to cancer growth, hyperplastic and invasive RA synovium expresses EGFR and its ligands [29]. Activation of EGFR signaling is responsible for synovial fibroblast proliferation in RA [29]. Furthermore, EGFR and its ligands can induce cytokine production of synovial fibroblasts during the pathogenesis of RA [29]. Agents that target EGFR have yielded promising results in animal experiments involving RA [29]. Since two most prominent members of EGFR family, EGFR and HER-2 are responsible for initiating multiple biological processes in vivo, the multiple biological functions of TGP intrigues us to hypothesize that

both EGFR and HER-2 might be a target of TGP, and that TGP might work as a TKI in Lung Cancer Cell Lines.

Indeed, the cytotoxic effect of TGP seems to be the consequence of affecting several cellular processes. It has been reported that the albiflorin, a component of TGP, inhibits bladder cancer growth via phosphorylation of Chk2 [30]. Paeoniflorin, another component of TGP, induces cancer cell apoptosis via the Fas/Fas ligand (FasL) pathway [31]. Recently, it has also been demonstrated that paeoniflorin inhibits cell growth through cell cycle arrest specifically in G1 phase and causing apoptosis in human colon cancer HT29 cells [32].

We discovered in our studies that TGP inhibited both EGFR and HER-2 tyrosine phosphorylation in addition to suppressing EGFR and HER-2 expression in two lung cancer cell lines tested. At the same time, TGP showed anti-proliferation, anti-apoptosis, and cell cycle arrest activities. Based on current understanding of the signal transduction network in cancer cells [33], all the desirable anti-lung cancer cell properties of TGP might be mediated through its ability to deactivate EGFR signaling by acting as a TKI and an EGFR expression suppressor. Therefore, TGP might be an interesting drug candidate for EGFR and HER-2 inhibition.

Materials and methods

Cell culture and TGP

Human lung cancer cell lines A549 and H1299 were obtained from the Chinese Academy of Sciences (Shanghai, China). They were maintained in RPMI 1640 media supplemented with 5% FBS, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin at 37°C with 5% CO₂. TGP was purchased in Liwah Pharmaceutical. TBS was used as the vehicle to deliver TGP [34].

Cell proliferation assay

A549 or H1299 (2000 cells/well) cells were seeded in 96-well plates. After 24 h incubation, the cells were treated with 200 μ L complete media containing serial TGP. After 8, 32, and 56 h treatments, each well was added 20 μ L of resazurin (2 mg/mL). The fluorescent signal was monitored using 544 nm excitation and 595 nm emission wavelengths by Spectramax M5 plate reader (Molecular Devices) [35]. The assay was performed in triplicate for each TGP treatment along with control. Same experiment was repeated twice.

NAC assay for cellular viability

A549 (2000 cells/well) cells were seeded in 96-well plates. After 24h, the cells were treated with TGP, N-acetylcysteine (NAC), or TGP plus NAC in 200 μ L of complete media and then cells were subject to MTT assay [34]. The absorbance was monitored at 570 nm using a Spectramax M5 plate reader (Molecular Devices).

Cell cycle analysis

A549 and H1299 cells (6 \times 10⁴ cells/well, 2 mL/well) were seeded in 6-well plates. After 24 h, cells were treated with different concentrations of TGP in 2 mL of complete media. After another 24 h, media were collected and combined with adherent cells that were detached by brief trypsinization. Cell pellets were resuspended in 70% ethanol at 4°C overnight. After centrifugation, the supernatant was removed and cells were incubated with 0.5 mL PI/RNase Staining

Buffer (BD Biosciences) for 15 min at room temperature. Cell cycle was analyzed using the Beckman cell analyzer FC500-mpl [36].

Detection of apoptosis

Apoptotic cells were quantified by Annexin V/propidium iodide (PI) double staining assay kit (BD Biosciences, San Diego CA, USA). Briefly, A549 and H1299 cells were treated with TGP the same as described for the cell cycle analysis. After washing with binding buffer, cells were suspended in 100 μ L binding buffer containing Annexin V and PI, and incubated for 15 min at room temperature. The apoptotic cells were identified using the Beckman cell analyzer FC500-mpl.

Immunoblot analysis

Proteins from TGP treated A549 and H1299 cell lysates were quantified by BCA Protein Assay Kit [37]. Equal amount of proteins (50 μ g) was resolved over 12% or 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked and then incubated with appropriate primary antibody overnight at 4°C. After incubation with appropriate secondary antibodies, the membranes were visualized using Western Lightning (PerkinElmer, USA).

Antibodies for Rb, CDK-2, CDK-4, CyclinD1, phospho-EGFR (Tyr1068), phospho-Akt (Ser473), HER-2, Phospho-HER2/ErbB2 (Tyr1248), Bax, Bcl-2, Caspase 3, PARP, EGFR, CyclinE1 and β -Actin were from Cell Signaling Technology (USA).

Results

TGP inhibits the growth of human lung cancer cells

The effects of TGP and Gefitinib were studied on the growth of A549 and H1299 human lung cancer cells. In both A549 (Figure 1B) and H1299 (Figure 1C) cells, proliferation of the cells was significantly suppressed in the presence of TGP in a concentration- and time-dependent manner compared with the positive control Gefitinib (Figure 1D). In A549 cells, TGP had IC₅₀ values of 464.57, 124.78, 91.49 μ g/mL in 24 h, 48 h, 72 h. Similar trend and even stronger effect was also observed in H1299 cells.

We used antioxidant NAC to eliminate possible influence of reactive oxygen species (ROS) for ROS has growth inhibition ability [38]. We optimized the concentrations of NAC that acts as a cellular ROS scavenger. Based on cell viability assay, the NAC concentration range used in A549 cells was 25-300 μ M. At these concentrations, the lung cancer cell viability was not significantly affected by NAC. We used TGP at 80 μ g/mL as positive controls in A549 cells to cause considerable growth inhibition (about 30%). However, the addition of NAC (from low to high concentrations) to TGP-treated cells did not affect the growth inhibition caused by TGP in A549 cells (Figure 1E). These results indicated that ROS was not associated with growth inhibition observed in Figure 1B.

TGP inhibits EGFR expression and phosphorylation

Data in Figure 1 showed that TGP inhibited the growth of two human lung cancer cells. Decades of research have shown that EGFR forms part of a complex signal transduction network that is essential for many important cellular processes, including cell proliferation, migration, survival and adhesion [39], we therefore tested if the

observed growth inhibition was related to suppressed EGFR expression and/or phosphorylation of EGFR by TGP.

We examined the effect of TGP on the expression and activation of EGFR and the AKT. AKT is an important downstream effector of EGFR signaling. The phosphorylation of AKT (P-AKT) is conducted by phosphorylated EGFR (P-EGFR), followed by a survival signaling cascade.

As shown in Figure 2, TGP decreased the levels of EGFR, P-EGFR, P-AKT in a concentration-dependent manner in the two lung cancer cell lines. At 40 μ g/mL, TGP decreased EGFR expression to 81% and phosphorylated EGFR to 58% compared to the treatment-free control in A549 cells (Figure 2A). Consistently, at 40 μ g/mL, TGP decreased EGFR expression to 85% and phosphorylated EGFR to 46% compared to the treatment-free control in H1299 cells (Figure 2B). In both lung cancer cells, TGP reduced EGFR phosphorylation more than EGFR expression at all the concentrations tested, which indicated that TGP might act as a TKI.

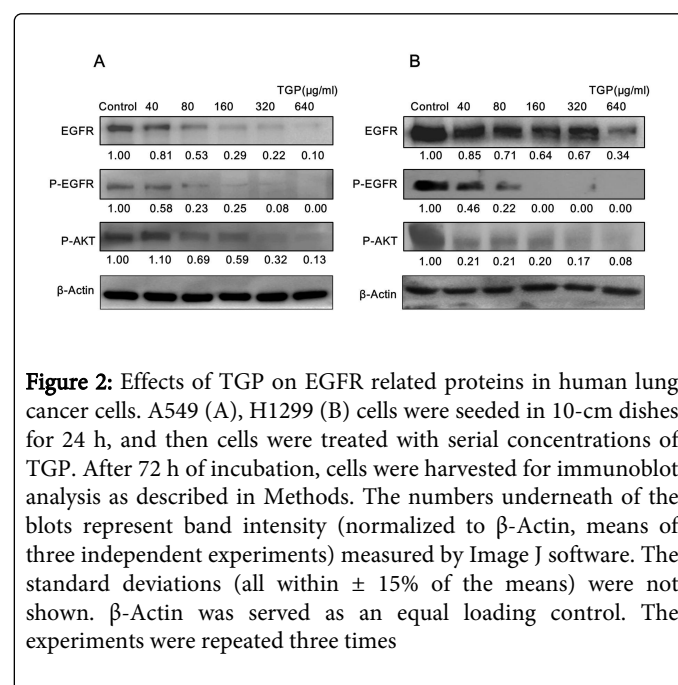


Figure 2: Effects of TGP on EGFR related proteins in human lung cancer cells. A549 (A), H1299 (B) cells were seeded in 10-cm dishes for 24 h, and then cells were treated with serial concentrations of TGP. After 72 h of incubation, cells were harvested for immunoblot analysis as described in Methods. The numbers underneath the blots represent band intensity (normalized to β -Actin, means of three independent experiments) measured by Image J software. The standard deviations (all within $\pm 15\%$ of the means) were not shown. β -Actin was served as an equal loading control. The experiments were repeated three times

TGP induces apoptosis in human lung cancer cells

Lapatinib, a clinically approved TKI, has been studied for its anti-cellular proliferation, pro-apoptosis, and cell cycle arrest properties in the same lung cancer cell line A549 [40] as used in our studies. Therefore, we decided to perform similar experiments with TGP but in both A549 and H1299 lung cancer cells.

The published report indicates that lapatinib induces apoptosis in A549 cells through analysis of key proteins in apoptotic pathway induced by lapatinib [40]. We determined the extent of apoptosis contributing to TGP treatment by flow cytometry analysis. To this end, A549 and H1299 cells were stained with Annexin V/propidium iodide and analyzed by flow cytometry. Viable cells (FITC-negative) and early apoptotic cells (FITC-positive) were PI-negative, whereas late apoptotic and necrotic cells were PI-positive and FITC-positive. In both A549 and H1299 cells, control cells showed only viable cells (K3) whereas treatment with TGP increased the percentage of early and late

apoptotic cells in a TGP concentration-dependent manner (Figure 3A and B). For example, treatments with 640 $\mu\text{g/mL}$ TGP increased early apoptotic cell population 27.7-fold compared to that of control cells in A549 cells (Figure 3A), while similar effects were observed in H1299 cells (Figure 3B).

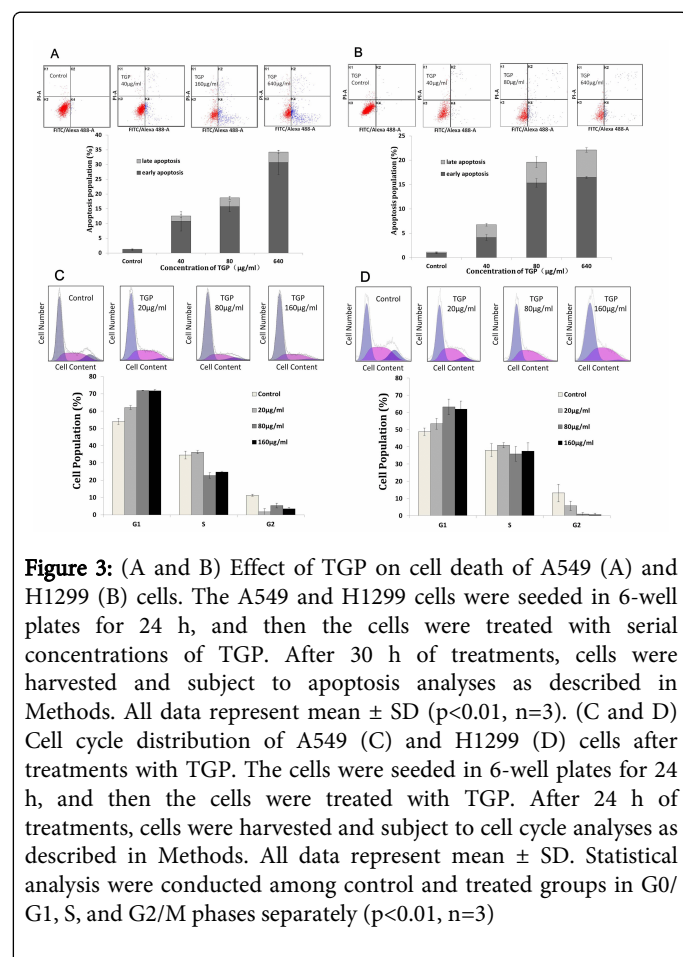


Figure 3: (A and B) Effect of TGP on cell death of A549 (A) and H1299 (B) cells. The A549 and H1299 cells were seeded in 6-well plates for 24 h, and then the cells were treated with serial concentrations of TGP. After 30 h of treatments, cells were harvested and subject to apoptosis analyses as described in Methods. All data represent mean \pm SD ($p < 0.01$, $n = 3$). (C and D) Cell cycle distribution of A549 (C) and H1299 (D) cells after treatments with TGP. The cells were seeded in 6-well plates for 24 h, and then the cells were treated with TGP. After 24 h of treatments, cells were harvested and subject to cell cycle analyses as described in Methods. All data represent mean \pm SD. Statistical analysis were conducted among control and treated groups in G0/G1, S, and G2/M phases separately ($p < 0.01$, $n = 3$).

Effect of TGP on cell cycle distribution of human lung cancer cells

We then studied TGP's effect on cell cycle of both lung cancer cell lines. In A549 cells, treatment with TGP for 24 h increased cell population in G1/G0 phases, and decreased cell population in S phase in a concentration-dependent manner (Figure 3C). At the concentrations of 80 $\mu\text{g/mL}$, TGP increased G1/G0 cell population to 71.8% of total cells, which was 1.3-fold of that of the control cells. We also observed a G2 arrest effect of TGP treatment at higher concentration (data not shows), which was parallel to the effect observed in previous report of *Paeonia lactiflora* Pall [30]. Similar results were observed in lung cancer H1299 cells after treatments with TGP (Figure 3D). These results were the same as the effect of lapatinib on the cell cycle in A549 cells in the published report [40].

TGP profoundly modified cell cycle and apoptosis-related signaling proteins

Finally, we examined the effects of TGP on key proteins in cell cycle progression and apoptotic pathway in lung cancer cells. Since TGP

had the similar effects on both A549 and H1299 as data shown in Figures 1-3, we decided to use A549 cells for the following experiments. Western blotting was performed on the whole-cell lysates after the treatments with TGP. In the G1 checkpoint of the cell cycle, CyclinD1-CDK4/6 complex and CyclinE1-CDK2 complex are required to allow cell cycle progression through G1 phase. Our results showed that after 72h treatment, TGP decreased the levels of CDK-2, CDK-4, CyclinD1 and CyclinE1 in a concentration-dependent manner. Furthermore, Treatments with TGP increased the levels of total Rb (Figure 4A). Rb stands for retinoblastoma protein, which is a tumor suppressor protein that is dysfunctional in several major cancers. One function of Rb is to prevent excessive cell growth by inhibiting cell cycle progression.

Activation of HER-2 plays an important role in the process of apoptosis, since it has been reported that switching off HER-2 could lead to apoptosis [41]. In addition, the reported lapatinib [40] acts as a dual TKI for both EGFR and HER-2 in A549 cells. Thus we tested how TGP affects HER-2 expression and phosphorylation. Data in Figure 4B shows that TGP treatment decreased the expression of HER-2 and also the phosphorylation of HER-2 in a concentration-dependent manner. At 80 $\mu\text{g/mL}$, TGP decreased HER-2 expression to 64% and phosphorylated HER-2 to 21% compared to the treatment-free control in A549 cells. The data showed that TGP reduced HER-2 phosphorylation more than HER-2 expression at all the concentrations tested, which indicated that TGP might resemble lapatinib in that it acted as a dual TKI for both EGFR and HER-2.

Bcl-2, and Bax are members of the expanding Bcl-2 family that play key roles in the regulation of apoptosis [42]. Bcl-2 is an anti-apoptotic protein whereas Bax is a pro-apoptotic protein. Decreased ratio of Bcl-2/Bax allows Bax to act on mitochondrial membrane by releasing cytochrome c from mitochondria, which leads to cells apoptosis. It could be seen from Figure 4B that treatment of TGP decreased the Bcl-2 level in a concentration-dependent manner, coincided with no noticeable change in Bax. Consistent with decreased ratio of Bcl-2/Bax that induces apoptosis, caspase 3, a crucial player in the process of apoptosis, was significantly activated by the treatments with TGP in a concentration-dependent manner. PARP is a substrate of caspase 3. PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [43]. Our data showed a concentration-dependent increase of the cleaved PARP as well (Figure 4B).

Discussion

EGF receptor family with their most prominent members EGFR and HER-2 represents validated targets for anti-cancer therapy. Anti-EGFR and HER-2 MoAbs (cetuximab, panitumumab, and trastuzumab) and TKIs (gefitinib, erlotinib, and lapatinib) have now been approved for the treatment of advanced colorectal cancer, squamous cell carcinoma of the head and neck, advanced NSCLC, as well as pancreatic and breast cancer. However, the benefits of costly TKIs and Anti-EGFRs MoAbs to most cancer patients are very limited due to complications of drug-resistant and side effects. The median survival rates increase only for a few months compared to patients treated with conventional therapies.

Our data showed that TGP inhibited both EGFR and HER-2 tyrosine phosphorylation in addition to suppressed EGFR and HER-2 expression in two lung cancer cell lines tested. Subsequently, TGP showed expected anti-proliferation, anti-apoptosis, and cell cycle arresting properties of EGFR/HER-2 TKI in the cancer cell lines tested.

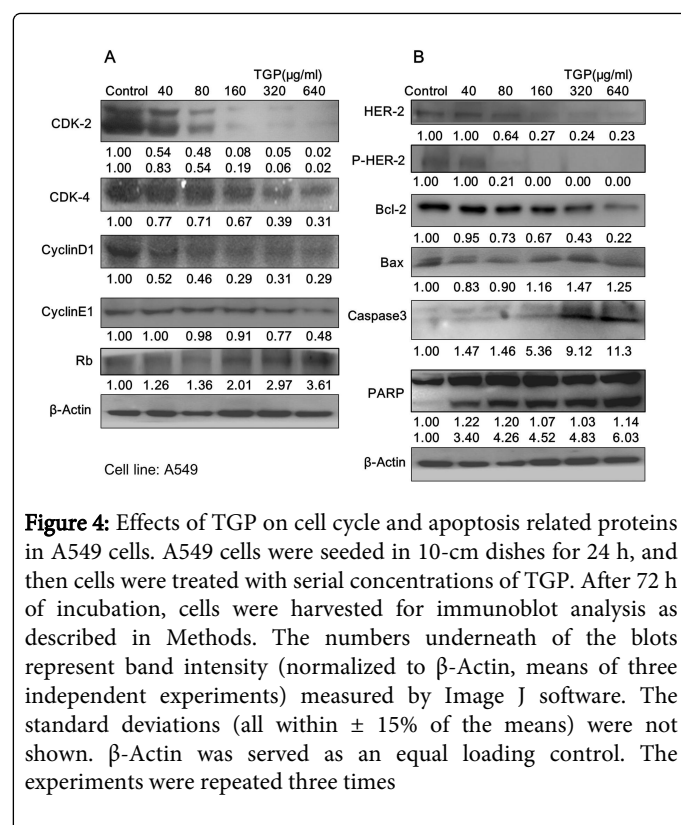


Figure 4: Effects of TGP on cell cycle and apoptosis related proteins in A549 cells. A549 cells were seeded in 10-cm dishes for 24 h, and then cells were treated with serial concentrations of TGP. After 72 h of incubation, cells were harvested for immunoblot analysis as described in Methods. The numbers underneath of the blots represent band intensity (normalized to β -Actin, means of three independent experiments) measured by Image J software. The standard deviations (all within $\pm 15\%$ of the means) were not shown. β -Actin was served as an equal loading control. The experiments were repeated three times

Therefore, TGP is promising in treating EGFR/HER-2 over-activated cancers, including NSCLC, breast, squamous cell carcinoma of the head and neck and others. Based on current understanding of the signal transduction net work in cancer cells [33], the desirable anti-lung cancer cell properties of TGP might be mediated through its ability to deactivate both EGFR and HER-2 signaling pathways by acting as a TKI and an EGFR and HER-2 expression suppressors. Hence our studies of TGP might provide with useful information towards developing inexpensive and more effective anti-EGFR drugs for cancer patients.

There is considerable interest in finding anticancer drugs among Chinese medicinal herbs. Currently, the cost of developing an approvable drug is frequently cited as about \$1 billion across 15 years in the US, although recent estimates have ranged as high as \$4 billion to \$12 billion per drug, depending upon how many failures are included in the estimate. Therefore, it will be a daunting job to develop novel and cheaper TKIs. In a recently published study, it was discovered that among 656 US FDA approved drugs tested, each drug hits more than 7 targets in the 73 total targets tested [44]. Many drugs are less effective to the previously known targets compared to off-targets based on the affinity or biological data. This published report provides valuable guidance in efforts to repurpose existing drugs, especially thousands of established herb-based medicines, for new diseases and conditions. Our studies of TGP are a good proof of this principle by discovering TGP worked as a potential TKI. We presume that further clinical studies of TGP in cancer treatment might be a cost-effective way in developing novel cancer modifying drugs.

Acknowledgements

This work was supported by Natural Science Foundation of China (Grant No. 91129706), Program for Science and Technology Development of Shandong Province (Grant No. 2012GSF11912).

References

- Kaitin KI (2010) Deconstructing the drug development process: the new face of innovation. *Clin Pharmacol Ther* 87: 356-361.
- Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics, 2014. *CA Cancer J Clin* 64: 9-29.
- Maione P, Rossi A, Sacco PC, Bareschino MA, Schettino C, et al. (2010) Advances in chemotherapy in advanced non-small-cell lung cancer. *Expert Opin Pharmacother* 11: 2997-3007.
- Wu K, House L, Liu W, Cho WC (2012) Personalized targeted therapy for lung cancer. *Int J Mol Sci* 13: 11471-11496.
- Giaccone G (2005) HER1/EGFR-targeted agents: predicting the future for patients with unpredictable outcomes to therapy. *Ann Oncol* 16: 538-548.
- Box C, Zimmermann M, Eccles S (2013) Molecular markers of response and resistance to EGFR inhibitors in head and neck cancers. *Front Biosci (Landmark Ed)* 18: 520-542.
- Xu Y, Sheng L, Mao W (2013) Role of epidermal growth factor receptor tyrosine kinase inhibitors in the treatment of esophageal carcinoma and the suggested mechanisms of action. *Oncol Lett* 5: 19-24.
- Hegi ME, Rajakannu P, Weller M (2012) Epidermal growth factor receptor: a re-emerging target in glioblastoma. *Curr Opin Neurol* 25: 774-779.
- Gui T, Shen K (2012) The epidermal growth factor receptor as a therapeutic target in epithelial ovarian cancer. *Cancer Epidemiol* 36: 490-496.
- Cheng L, Alexander RE, MacLennan GT, Cummings OW, Montironi R, et al. (2012) Molecular pathology of lung cancer: key to personalized medicine. *Mod Pathol* 25: 347-369.
- Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, et al. (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2: e73.
- Ma C, Wei S, Song Y (2011) T790M and acquired resistance of EGFR TKI: a literature review of clinical reports. *J Thorac Dis* 3: 10-18.
- Xu W, Zhou L, Ma X, Chen Y, Qin B, et al. (2011) Therapeutic effects of combination of paeoniflorin and albiflorin from *Paeonia radix* on radiation and chemotherapy-induced myelosuppression in mice and rabbits. *Asian Pac J Cancer Prev* 12: 2031-2037.
- Xie P, Chen S, Liang YZ, Wang X, Tian R, et al. (2006) Chromatographic fingerprint analysis--a rational approach for quality assessment of traditional Chinese herbal medicine. *J Chromatogr A* 1112: 171-180.
- Kaneda M, Iitaka Y, Shibata S (1972) Chemical studies on the oriental plant drugs—XXXIII: The absolute structures of paeoniflorin, albiflorin, oxypaeoniflorin and benzoylpaeoniflorin isolated from chinese paeony root. *Tetrahedron* 28: 4309-4317.
- Zhao X, Sun Y (2003) Analysis of *Paeoniae Radix* by high-performance liquid chromatography-electrospray ionization-mass spectrometry. *Anal Sci* 19: 1313-1315.
- Yamahara J, Yamada T, Kimura H, Sawada T, Fujimura H (1982) Biologically active principles of crude drugs. II. Anti-allergic principles in "Shoseiryu-To" anti-inflammatory properties of paeoniflorin and its derivatives. *J Pharmacobiodyn* 5: 921-929.
- Ishida H, Takamatsu M, Tsuji K, Kosuge T (1987) Studies on active substances in herbs used for oketsu ("stagnant blood") in Chinese medicine. V. On the anticoagulative principle in moutan cortex. *Chem Pharm Bull (Tokyo)* 35: 846-848.
- Ohta H, Matsumoto K, Watanabe H, Shimizu M (1993) Involvement of beta-adrenergic systems in the antagonizing effect of paeoniflorin on the

- scopolamine-induced deficit in radial maze performance in rats. *Jpn J Pharmacol* 62: 345-349.
20. Hsu FL, Lai CW, Cheng JT (1997) Antihyperglycemic effects of paeoniflorin and 8-debenzoylpaeoniflorin, glucosides from the root of *Paeonia lactiflora*. *Planta Med* 63: 323-325.
21. Wu Y, Ren K, Liang C, Yuan L, Qi X, et al. (2009) Renoprotective effect of total glucosides of paeony (TGP) and its mechanism in experimental diabetes. *J Pharmacol Sci* 109: 78-87.
22. Liu DZ, Xie KQ, Ji XQ, Ye Y, Jiang CL, et al. (2005) Neuroprotective effect of paeoniflorin on cerebral ischemic rat by activating adenosine A1 receptor in a manner different from its classical agonists. *Br J Pharmacol* 146: 604-611.
23. Kobayashi M, Ueda C, Aoki S, Tajima K, Tanaka N, et al. (1990) [Anticholinergic action of Paeony root and its active constituents]. *Yakugaku Zasshi* 110: 964-968.
24. Okubo T, Nagai F, Seto T, Satoh K, Ushiyama K, et al. (2000) The inhibition of phenylhydroquinone-induced oxidative DNA cleavage by constituents of Moutan Cortex and Paeoniae Radix. *Biol Pharm Bull* 23: 199-203.
25. Wu H, Wei W, Song L, Zhang L, Chen Y, et al. (2007) Paeoniflorin induced immune tolerance of mesenteric lymph node lymphocytes via enhancing beta 2-adrenergic receptor desensitization in rats with adjuvant arthritis. *Int Immunopharmacol* 7: 662-673.
26. Xu HM, Wei W, Jia XY, Chang Y, Zhang L (2007) Effects and mechanisms of total glucosides of paeony on adjuvant arthritis in rats. *J Ethnopharmacol* 109: 442-448.
27. Kimura M, Kimura I, Nojima H (1985) Depolarizing neuromuscular blocking action induced by electropharmacological coupling in the combined effect of paeoniflorin and glycyrrhizin. *Jpn J Pharmacol* 37: 395-399.
28. Liu D, Wei W, Song L (2006) Protective effect of Paeoniflorin on immunological liver injury induced by bacillus calmette-guerin plus lipopolysaccharide: modulation of tumor necrosis factor-alpha and interleukin-6 mRNA. *Clin Exp Pharmacol Physiol* 33: 332-339.
29. Swanson CD, Akama-Garren EH, Stein EA, Petralia JD, Ruiz PJ, et al. (2012) Inhibition of epidermal growth factor receptor tyrosine kinase ameliorates collagen-induced arthritis. *J Immunol* 188: 3513-3521.
30. Ou TT, Wu CH, Hsu JD, Chyau CC, Lee HJ, et al. (2011) *Paeonia lactiflora* Pall inhibits bladder cancer growth involving phosphorylation of Chk2 in vitro and in vivo. *J Ethnopharmacol* 135: 162-172.
31. Hung J, Yang C, Tsai Y, Huang H, Huang M (2007) Antiproliferative activity of Paeoniflorin is through cell cycle arrest and the FAS/FAS ligand-mediated apoptotic pathway in human non-small cell lung cancer A549 cells. *Clin Exp Pharmacol Physiol* 35: 141-147.
32. Wang H, Zhou H, Wang CX, Li YS, Xie HY, et al. (2012) Paeoniflorin inhibits growth of human colorectal carcinoma HT 29 cells in vitro and in vivo. *Food Chem Toxicol* 50: 1560-1567.
33. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144: 646-674.
34. Qiu P, Guan H, Dong P, Guo S, Zheng J, et al. (2011) The inhibitory effects of 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone on human colon cancer cells. *Mol Nutr Food Res* 55: 1523-1532.
35. Pan J, Qian Y, Zhou X, Lu H, Ramacciotti E, et al. (2010) Chemically Oversulfated Glycosaminoglycans Are Potent Modulators of Contact System Activation and Different Cell Signaling Pathways. *J Biol Chem* 285:22966-22975.
36. Qiu P, Dong P, Guan H, Li S, Ho CT, et al. (2010) Inhibitory effects of 5-hydroxy polymethoxyflavones on colon cancer cells. *Mol Nutr Food Res* 54 Suppl 2: S244-252.
37. Xiao H, Yang CS, Li S, Jin H, Ho CT, et al. (2009) Monodemethylated polymethoxyflavones from sweet orange (*Citrus sinensis*) peel inhibit growth of human lung cancer cells by apoptosis. *Mol Nutr Food Res* 53: 398-406.
38. Pan M, Lai Y, Lai C, Wang Y, Li S, et al. (2007) 5-Hydroxy-3,6,7,8,3',4'-hexamethoxyflavone Induces Apoptosis through Reactive Oxygen Species Production, Growth Arrest and DNA Damage-Inducible Gene 153 Expression, and Caspase Activation in Human Leukemia Cells. *J Agr Food Chem* 55: 5081-509.
39. Yarden Y (2001) The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. *Eur J Cancer* 37 Suppl 4: S3-8.
40. Diaz R, Nguewa PA, Parrondo R, Perez-Stable C, Manrique I, et al. (2010) Antitumor and antiangiogenic effect of the dual EGFR and HER-2 tyrosine kinase inhibitor lapatinib in a lung cancer model. *BMC Cancer* 10: 188.
41. Schiffer IB, Gebhard S, Heimerdinger CK, Heling A, Hast J, et al. (2003) Switching off HER-2/neu in a tetracycline-controlled mouse tumor model leads to apoptosis and tumor-size-dependent remission. *Cancer Res* 63: 7221-7231.
42. Hsu YT, Wolter KG, Youle RJ (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci U S A* 94: 3668-3672.
43. Oliver FJ, de la Rubia G, Rolli V, Ruiz-Ruiz MC, de Murcia G, et al. (1998) Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. *J Biol Chem* 273: 33533-33539.
44. Lounkine E, Keiser MJ, Whitebread S, Mikhailov D, Hamon J, et al. (2012) Large-scale prediction and testing of drug activity on side-effect targets. *Nature* 486: 361-367.