Parthenolide Augments the Chemosensitivity of Non-small-Cell Lung Cancer to Cisplatin via the PI3K/AKT Signaling Pathway

Li-Mei Wu1†, Xiao-Zhong Liao2†, Yan Zhang1†, Zi-Rui He3, Shi-Qing Nie1, Bin Ke4, Lin Shi4, Jian-Fu Zhao1* and Wen-Hui Chen1*

1 Department of Oncology, The First Affiliated Hospital of Jinan University, Guangzhou, China, 2 Department of Oncology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, China, 3 Department of Traditional Chinese Medicine, Cancer Center of Sun Yat-sen University, Guangzhou, China, 4 Department of Traditional Chinese Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou, China

The mortality rate of non-small-cell lung cancer (NSCLC) remains high worldwide. Although cisplatin-based chemotherapy may greatly enhance patient prognosis, chemotherapy resistance remains an obstacle to curing patients with NSCLC. Therefore, overcoming drug resistance is the main route to successful treatment, and combinatorial strategies may have considerable clinical value in this effort. In this study, we observed that both parthenolide (PTL) and cisplatin (DDP) inhibited the growth of NSCLC cells in a dose- and time-dependent manner. The combination of PTL and DDP presented a synergistic inhibitory effect on NSCLC at a ratio of 50:1. The combination of PTL and DDP synergistically inhibited cell migration and invasion, inhibited cell cycle progression, and induced apoptosis of A549 and PC9 cells. Bioinformatics and network pharmacology analysis indicated that PTL may primarily affect the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway. After treatment with PTL and DDP either alone or in combination, Western blot analysis revealed that the proteins levels of Bax and cleaved Caspase-3 were upregulated, while p-PI3K, p-Akt, Caspase-3, and Bcl-2 proteins were downregulated. Among these alterations, the combination of PTL and DDP was found to exhibit the most significant effects. PTL might therefore be considered as a new option for combination therapy of NSCLC.

Keywords: PTL, DDP, combination, synergistic effect, NSCLC, PI3K/Akt pathway

INTRODUCTION

Lung cancer is the most common and most aggressive malignancy, as well as the principal cause of cancer-related deaths in both men and women all over the world (Siegel et al., 2018) and in China (Chen et al., 2016). About 80–85% patients with lung cancer are diagnosed with non-small cell lung cancer (NSCLC) (Chen et al., 2016; Siegel et al., 2018). In recent decades, important progress has been made in the diagnosis and treatment strategies of NSCLC, although there has been no significant improvement in its prognosis, and the 5-year overall survival rate is still <15% (Chen et al., 2014). Platinum-based chemotherapy is still the standard-of-care for most patients who suffer from advanced NSCLC (Rossi and Di Maio, 2016). Nevertheless, toxicity, drug resistance,
and high risk of death are seen clinically, underlining that the medication strategies require to be optimization. There are two types of drug resistance: primary resistance which appertains to chemoresistance prior to chemotherapy, and acquired resistance which emerges following chemotherapy (Kelland, 2007). The common mechanisms of drug resistance mainly include increased drug efflux from cancer cells, reduced uptake of drugs, modification of oncogenes, and inhibition of drug-induced apoptosis (Hamilton and Rath, 2014). Ultimately, the resistance leads to chemotherapy failure and therefore a poor prognosis.

DDP, also known as cisplatinum or cis-diamminedichloroplatinum (II), is a chemotherapeutic drug (Dasari and Tchounwou, 2014). It has been used to treat a variety of solid malignancies, including testicular, ovarian, head and neck, colorectal, bladder, and lung cancers (Gridelli et al., 2015). Cisplatin exerts anticancer effects through multiple mechanisms, but its utmost (and best understood) mode of action involves the generation of DNA lesions followed by the activation of DNA damage response and the induction of mitochondrial apoptosis (Dasari and Tchounwou, 2014). Cisplatin treatment often induces the development of chemoresistance, leading to therapeutic failure and plentiful unfavorable side effects such as serious kidney problems, allergic reactions, declining immunity to infections, gastrointestinal diseases, hemorrhage, as well as hearing loss found particularly in young patients (Dasari and Tchounwou, 2014). Because cisplatin is the main therapeutic option in some clinical settings, the development of chemosensitization strategies has become a clinically significant goal. In addition, combination therapies with cisplatin and other drugs have been taken into high consideration to mount drug-resistance and lessen toxicity.

Fortunately, natural products with various chemical structures and pharmacological effects can serve as effective drug-resistant substances (Thomford et al., 2018). PTL, originally isolated from Tanacetum parthenium L., is a prominent and naturally occurring germacranolide, which has shown cytotoxicity in multifarious human cancer cells but not in normal cells (Ghanous et al., 2013). PTL has been found to have anti-inflammatory (Wang et al., 2016), antioxidant (Farzadfar et al., 2016), and antitumor activity in a variety of cancers, including breast (Araujo et al., 2019), acute myeloid leukemia (Darwish et al., 2019), and non-small cell lung cancer (Zhang et al., 2009).

Despite the anticancer effect of PTL reported previously in several cancer cell lines, the effect of co-treatment with PTL and DDP for synergistic inhibition of NSCLC cells has not been well-explored. The aim of this study was to investigate the potential synergistic effects of the combination of PTL and DDP on NSCLC as well as the related mechanism.

Abbreviations: 2D, two dimensional; CCK8, Cell Counting Kit-8; CI, combination index; CID, compound ID; cisplatin, DDP; ECL, electrochemiluminescence system; Fa, fraction affected; FCM, flow cytometry; HRP, horseradish peroxidase; NSCLC, non-small-cell lung cancer; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PTL, parthenolide; PVDF, polyvinylidenedifluoride; TCM, Traditional Chinese medicine.
allowed to heal the wounds for 48 h. At the same place where cells were scratched, pictures (magnification, 10 ×) were taken at 0 and 24 h. Ultimately the Adobe Photoshop CS6 software was used to determine the migration length of cells according to the change of wound size.

Transwell Invasion Assay
A549 and PC9 cells were incubated in serum-free RPMI1640 for 24 h. Subsequently, cells (6 × 10⁴) in 600 µL serum-free medium containing various drugs were plated on the top compartment of transwell filters, which were covered by thin layers of matrigel basement membrane matrix, with 700 µL medium containing 10% FBS in the bottom compartment. The transwell filters were cultured at 37°C with 5% CO₂ for 48 h. After that, the cells adhering to the bottom membrane were fixated in 4% paraformaldehyde for 30 min, and subsequently dyed with 0.5% CV solution for 15 min at room temperature. Ultimately, the transwell filters were inverted and observed under a microscope (magnification, 100 ×) for photographic recording and the number of cells on the bottom surface was counted. Five random fields were counted per filter in all groups.

Cell Colony Formation Assay
Cells were trypsinized single cells were obtained and seeded in 6-well plates at a density of 500 cells/well. After 10 days of culture, colonies were fixed with methyl alcohol and stained with crystal violet, and the colony formation ratio was calculated.

Cell Cycle Distribution Analysis
A Cell Cycle Detection Kit obtained from 4A Biotech Co., Ltd. (Beijing, China) was employed to detect cell cycle distribution. Briefly, A549 and PC9 cells were incubated in six-well plates (1 × 10⁶ cells/well) and cultured overnight, and then cells were incubated with PTL or DDP alone or in combination for 48 h. After that, cells were rinsed with cold phosphate-buffered saline (PBS) and in mobilized with 70% ethyl alcohol overnight. After washes with PBS, cells were stained with propidium iodide (10 µg/mL) in the presence of calcium (1 g/L), 1 g/L sodium citrate, and 0.5% Triton X 100 (v/v) in the dark for 30 min. Then cells were collected for cell cycle distribution analysis using an AEC NovoCyte flow Cytometer equipped with Novoexpress (Becton Dickinson, San Jose, CA, USA).

Cell Apoptosis Analysis
An Annexin V-FITC apoptosis detection kit obtained from 4A Biotech Co., Ltd. (Beijing, China) was used to detect cell apoptosis. A549 and PC9 cells were seeded in six-well plates and cultured overnight. After exposure to PTL or DDP alone or in combination for 48 h, the cells were harvested, resuspended in 500 µL of incubation buffer containing Annexin V-FITC and PI, and incubated for 30 min in the dark. The cells were then washed and subjected to apoptosis analysis using an AEC NovoCyte flow cytometer furnished with Novoexpress.

Xenograft Tumor Assay in Nude Mice
Nude female BALB/c-nu/nu mice (4–6 weeks) were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences, Beijing, China, and placed in a specific pathogen-free (SPF) environment. A549 cells (3 × 10⁶) in 0.2 mL of PBS were inoculated into the flanks of the mice. When tumors became palpable, mice were subdivided into four groups of six. Figure 7A shows the in vivo treatment regimen with a variety of concentrations of PTL or DDP. PTL and vehicle control were administrated daily via intraperitoneal (I. P.) injection, while DDP was administrated every 5 days via I. P. injection. The tumor volumes were measured at the beginning of the treatment and every 4 days during treatment by measuring the length (L) and width (W) of the tumors. The tumor volume was calculated by the following formula: v = length × (width)²/2. Tumors were excised and weighed on the second day after the last injection. All experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University and performed in accordance with national ethical guidelines.

Potential Target Identification Based on Pharmmapper
PharmMapper (http://lilab.ecust.edu.cn/pharmmapper/index.php) consists of a huge internal repertoire of a pharmacophore database, which is pulled out from all the targets in TargetBank, DrugBank, BindingDB, and PDTD. PharmMapper stores and accesses over 20,000 receptor-based pharmacophore models (information about 1,627 drug targets can be found, and 459 of which are human protein targets). First, the SDF format of PTL was downloaded from PubChem Compound (https://www.ncbi.nlm.nih.gov/pcpdcompound/) and then uploaded to PharmMapper after exactly setting the parameters, target recognition was performed, and the information relating to the first 300 potential protein targets was acquired.

Bioinformatics and Network Pharmacology Analysis
Based on the DAVID database (https://david.ncifcrf.gov/), we imported the top 300 potential targets, selected Homo sapiens, and then carried out Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The information associated with the first 100 potential pathways was acquired. A parameter enrichment gene count > 2 and hypergeometric analysis were used for testing significance threshold with a P < 0.05. For a KDR value < 0.05 (Q), we selected the Top 20 pathway, and mapped senior bubbles by the OmicShare website (http://www.omicshare.com/).

Western Blot Analysis
Cells were harvested and processed in RIPA lysis buffer supplemented with 1% phenylmethylsulfonyl fluoride and 1% phosphatase inhibitor. The soluble protein fractions were extracted after centrifugation at 1.35 × 10⁴ g for 10 min. The protein concentrations were tested using a BCA kit. Around 30 mg of proteins were separated by 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes, and incubated with different primary antibodies overnight at 4°C. Membranes were then washed and incubated with proper secondary antibodies. Signals were detected using an ECL chemiluminescence detection kit.
Statistical Analysis
SPSS 24.0 software (IBM, NY) was employed to performed statistical analysis. Statistical comparisons were performed through independent samples t-test or one-way analysis of variance (ANOVA). Data measurements are indicated as mean values ± standard deviation. The value of $p < 0.01$ was considered statistically significant.
RESULTS

Co-treatment With PTL and DDP Concurrently Inhibited the Proliferation of NSCLC Cells

It was observed that both PTL and DDP inhibited the proliferation of NSCLC cells in a dose-dependent manner. After 48 h treatment with PTL, the IC₅₀ values were 29.423, 23.21, 47.70, and 119.67 µM for A549, PC9, H1299, and BEAS-2B cell lines, respectively. Similarly, the IC₅₀ values obtained after DDP treatment were 0.89, 0.73, 0.93, and 0.68 µM for A549, PC9, H1299, and BEAS-2B cell lines, respectively (Figure 2). We also treated the cell lines with a combination of PTL and DDP (the PTL:DDP molar ratio of 50:1) for 48 h. The results showed that, when compared with single drug therapy, the combination drug therapy had a stronger inhibitory effect on cell proliferation. The synergistic effects of drug combination therapy were observed in the A549, PC9, and H1299 cell lines with different Fa values, but not in the BEAS-2B cell line. Table 1 lists the summary of the CI and the concentrations of the individual drugs used in combination at 50% Fa.

Table 1

| Drug combination | Fa = 0.5 |
|------------------|----------|
|                  | A549     | PC9     | H1299   | BEAS-2B |
| DDP + PTL       |          |         |         |
| CI              | 0.69832  | 0.71184 | 0.74962 | 1.71719 |
| DDP (µM)       | 0.38906  | 0.35321 | 0.48497 | 0.44289 |
| PLT (µM)        | 9.72652  | 8.83035 | 12.1244 | 11.0722 |

Cooperation of PTL and DDP Synergistically Suppressed the Migration and Invasion of NSCLC Cells

We used wound healing assay and transwell assay to evaluate the effects of individual PTL and DDP or combination of these two drugs on the migration and invasion of A549 and PC9 cells. Figure 3 shows that both the migration distances and invaded cell numbers were reduced markedly after 48 h treatment with either individual drugs or a combination of PTL and DDP. Furthermore, the combination treatment resulted in the smallest values for both migration distance and aggressive cell number, suggesting the combination of PTL and DDP exerted a more significant inhibition on cell migration and invasion than each of them alone.

Co-treatment With PTL and DDP Coordinately Inhibited the Cell Colony Formation of NSCLC Cells

As showed in Figure 4, compared with untreated controls, PTL, DDP, and the combination of them all significantly inhibited...
cell colony formation of A549 and PC9 cells. Similarly, the combination of PTL and DDP exerted a more significant inhibition on cell colony formation than each of them alone.

**Co-treatment With PTL and DDP Synergistically Arrested the Cell Cycle of NSCLC Cells**

After verifying the anti-proliferative effect of PTL and DDP, flow cytometry (FCM) was used to analyze the cell cycle of the treated NSCLC cells. As illustrated in Figure 5, PTL and DDP both arrested A549 and PC9 cells at S and G2 phases, while the combination of PTL and DDP showed a more significant effect in arresting PC9 cells at S and G2 phases.

**Co-treatment With PTL and DDP Synergistically Induced Apoptosis in NSCLC Cells**

As shown in Figure 6, both the PTL and DDP individual drug treatments and the drug combination enhanced the ratio of early and late apoptosis in A549 and PC9 cells. Additionally, the combination of PTL and DDP was more efficient at inducing apoptosis compared to the single treatment (PTL + DDP vs. PTL, \( p = 0.0004 \); PTL + DDP vs. DDP, \( p = 0.0006 \)).

**Co-treatment With PTL and DDP Synergistically Suppressed A549 Cell Xenograft Tumor Growth**

Figure 7 shows the experimental setup of the in vivo animal experiment, including A549 cell seeding and drug treatment. Our next step was to study the effects of PTL and DDP on the growth of xenograft NSCLC tumors. It was found that the xenograft tumors in the control group grew faster than those in the group treated with the tested drugs. In addition, compared with PTL and DDP monotherapy, the combination of PTL and DDP had a more significant inhibitory effect on tumor growth (PTL + DDP vs. PTL, \( p = 0.004 \); PTL + DDP vs. DDP, \( p = 0.005 \)). These results indicated that the anti-tumor effect of DDP in vivo could be effectively enhanced by PTL.

**Potential Target Proteins of PTL and the Bioinformatics and Network Pharmacology Analysis**

The relevant information of the first 300 potential protein targets of PTL was obtained utilizing PharmMapper (Table 2). We conducted gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis according to the DAVID database (https://david.ncifcrf.gov/). The obtained bioinformatics analysis was depicted in Figure 8, suggesting that PTL might play a role, mainly through affecting the PI3K/Akt signaling pathway.

**The Combination of PTL and DDP Synergistically Inhibited the Activity of the PI3K/Akt Signaling Pathway in NSCLC Cells**

Western blotting analysis demonstrated that both drug monotherapy and combination therapy increased the expression levels of Bax and cleaved Caspase-3, but decreased the expression levels of p-PI3K, p-Akt, Caspase-3, and Bcl-2, with total expression levels of Akt, PI3K, and glyceraldehyde 3-phosphate dehydrogenase remaining unchanged. Importantly, the efficacy of drug combination treatment was more significant than the single drug treatments (PTL + DDP vs. PTL, \( p < 0.01 \); PTL + DDP vs. DDP, \( p < 0.01 \)). Moreover, the inhibitory effect of
PTL + DDP combined treatment on the signaling pathway was partially restored by a PI3K activator (740 Y-P), as shown in Figure 9 (p < 0.01 for p-PI3K, p-Akt, Caspase-3, Bcl-2, Bax, and cleaved Caspase-3 between 740 Y-P + PTL + DDP and PTL + DDP groups).

**DISCUSSION**

In the process of tumor chemotherapy, one of the toughest problems is that cancer cells develop resistance to chemotherapy drugs. Despite the fact that cisplatin-based chemotherapy is the first-line therapy for NSCLC, the occurrence of acquired resistance to cisplatin still presents a great challenge (Dasari and Tchounwou, 2014; Rossi and Di Maio, 2016). The development of cisplatin resistance is a key issue in the failure of NSCLC therapy, and can lead to cancer palindromia and metastasis.

Artémisinin (qinghaosu) and arsenic oxide (III) (As$_2$O$_3$) have achieved remarkable success in clinical practice, and have attracted the attention of many researchers to natural extracts. Considering their safety, long-term use, and ability to target various pathways, there has been great interest in re-understanding the molecular mechanisms of their activities. In the clinical practice, many traditional Chinese medicines have shown synergistic effects in chemotherapy.
In recent years, PTL has shown the ability to comprehensively prevent tumor progression, such as the prevention of NSCLC through the induction of apoptosis. Although PTL has been reported to have anti-tumor ability, the mechanism by which it inhibits tumorigenesis remains unclear. Therefore, to fully explain its biological activity on different types of cancer, including NSCLC, will require further research. According to modern pharmacological research, the combined application of two drugs could inhibit the growth, proliferation, migration, and invasion of a variety of tumor cells, as well as induce tumor cell apoptosis and inhibit the effect of tumor-promoting substances on potential tumor cells. In this study, we found that the combined use of PTL and DDP had a synergistic effect on NSCLC. This can be considered as a new adjuvant treatment strategy for NSCLC.

Some recent studies have also provided evidence that PTL could act against many varieties of cancers, such as NSCLC (Talib and Al Kury, 2018) and breast cancer (Araujo et al., 2019; Berdan et al., 2019). Nevertheless, there is a lack of evidence at both cellular level and in animal models to show the effect of PTL and DDP combination on the development of NSCLC. This study revealed that PTL combined with DDP had an inhibitory effect on the growth and metastasis of NSCLC and the PI3K/Akt pathway, providing a potential basis for the promising strategy of PTL and DDP combination for the treatment of NSCLC.

According to the IC_{50} values analyzed using CCK8 assay, we proved that PTL and DDP could inhibit the proliferation of A549,
## TABLE 2 | Potential targets of parthenolide by PharmMapper.

| Uniplot | Uniprot | Go | p | q | p-value |
|---------|---------|----|---|---|---------|
| 1reu    | BMP2    | 3  | 2.975 0.9917 1.7192 | | |
| 2p3g    | MAPK2   | 3  | 2.966 0.9888 1.75996 | | |
| 196     | AK1C2   | 3  | 2.954 0.9848 1.89686 | | |
| 1w8l    | PP1A    | 3  | 2.935 0.9783 1.64874 | | |
| 2oji    | MK01    | 3  | 2.916 0.9719 1.42276 | | |
| 3dej    | CASP3   | 3  | 2.723 0.9076 0.89396 | | |
| 3gam    | NCO2    | 3  | 2.606 0.8866 0.64813 | | |
| 1xdd    | ITAL    | 3  | 2.543 0.8478 0.90326 | | |
| 2xwu    | EPHB4   | 3  | 2.465 0.8218 1.1296 | | |
| 1okl    | CAH2    | 3  | 2.382 0.794 –0.3892 | | |
| 1n7i    | PNMT    | 4  | 2.99 0.7474 1.73609 | | |
| 1mkp    | DUS6    | 4  | 2.975 0.7437 1.5315 | | |
| 1x7f    | ALDR    | 3  | 2.202 0.7341 –0.4667 | | |
| 1du    | HS017B1 | 4  | 2.928 0.7319 1.38111 | | |
| 2h8h    | SRC1    | 4  | 2.927 0.7317 1.5454 | | |
| 2zas    | ERR3    | 4  | 2.922 0.7304 1.27297 | | |
| 1m17    | EGFR    | 4  | 2.891 0.7228 1.33827 | | |
| 2o65    | PIM1    | 3  | 2.158 0.7194 –1.4526 | | |
| 1dc    | CF4D    | 4  | 2.87 0.7176 1.3316 | | |
| 1vly    | TGF1    | 4  | 2.848 0.7112 1.1212 | | |
| 1sqn    | PRQR    | 4  | 2.827 0.7068 1.05616 | | |
| 1s7f    | VTDB    | 4  | 2.789 0.6972 0.87214 | | |
| 2w1    | HS090AA1| 5  | 2.78 0.6951 0.96209 | | |
| 1dg    | C1TC    | 4  | 2.761 0.6903 1.07111 | | |
| 1soj    | PDE3B   | 4  | 2.758 0.6895 1.00205 | | |
| 1kp    | PDE5A   | 4  | 2.737 0.6842 0.91313 | | |
| 2ao6    | ANDR    | 4  | 2.734 0.6855 0.95089 | | |
| 1e7a    | ALBU    | 3  | 2.047 0.6639 1.0033 | | |
| 2b1v    | ESR1    | 4  | 2.729 0.6822 0.9393 | | |
| 3fl2    | GSK3B   | 5  | 2.666 0.6772 0.98083 | | |
| 35p    | IGF1R   | 4  | 2.677 0.6691 0.5812 | | |
| 1mkd    | PDE4D   | 4  | 2.647 0.641 1.43777 | | |
| 1mx1    | EST1    | 4  | 2.633 0.6582 0.2642 | | |
| 2wyw    | CHK1    | 4  | 2.604 0.6511 0.43158 | | |
| 2pe0    | PDK1    | 4  | 2.558 0.637 0.93929 | | |
| 1hak    | ANXA5   | 4  | 2.482 0.6204 –0.8744 | | |
| 1b6a    | AMPM2   | 4  | 2.481 0.6201 0.15918 | | |
| 1c5b    | CATB    | 4  | 2.443 0.6107 –0.2588 | | |
| 1shl    | CASP7   | 4  | 2.416 0.6041 –0.3164 | | |
| 1p0p    | CHLE    | 4  | 2.403 0.6008 –0.501 | | |
| 1d39    | PYR1D   | 4  | 2.403 0.6007 –0.5732 | | |
| 1s1p    | AK1C3   | 5  | 2.982 0.5964 1.44379 | | |
| 2abi    | MCR1    | 5  | 2.976 0.5953 1.42687 | | |
| 3zrr    | DH11    | 5  | 2.97 0.5939 0.80992 | | |
| 1tzw    | PLGF    | 5  | 2.958 0.5915 1.4615 | | |
| 1ov4    | ST2A1   | 5  | 2.90 0.5859 0.36594 | | |
| 2zaz    | MAPK14  | 5  | 2.915 0.5829 0.92383 | | |
| 1oxi    | TTPA    | 5  | 2.912 0.5824 0.87911 | | |
| 1x70    | DPP4    | 5  | 2.908 0.5817 1.12942 | | |
| 2c6i    | CDK2    | 5  | 2.904 0.5808 0.91187 | | |

(Continued)
### TABLE 2 | Continued

| Pharma model | Uniplot | Num feature | Fit  | Norm fit | z-score |
|--------------|---------|-------------|------|----------|---------|
| 1w6j         | ERG7    | 5           | 2.12 | 0.424    | −1.749  |
| 1hmt         | FABP4   | 7           | 2.96 | 0.4228   | 0.2111  |
| 1fcy         | RARG    | 8           | 3.38 | 0.4225   | 1.70098 |
| 2bk3         | AOFB    | 7           | 2.953| 0.4219   | −0.046  |
| 1sa4         | FNTA    | 7           | 2.952| 0.4217   | 0.68966 |
| 1otv         | FABP6   | 7           | 2.94 | 0.42     | 0.258   |
| 1mnz         | RXRA    | 7           | 2.939| 0.4199   | 0.66477 |
| 1dkf         | RARA    | 8           | 3.348| 0.4185   | 1.5054  |
| 1svh         | PRKACA  | 7           | 2.928| 0.4183   | 0.10117 |
| 1p62         | DCK     | 7           | 2.916| 0.4166   | 0.87197 |
| 2w1g         | AURKA   | 5           | 2.08 | 0.418   | −1.6433 |
| 1nd5         | PPAP    | 6           | 2.496| 0.4159   | −0.9438 |
| 2b7a         | JAK2    | 7           | 2.888| 0.4126   | 0.33259 |
| 1uwj         | B RAF1  | 7           | 2.883| 0.4119   | 0.34593 |
| 1tou         | FABP4   | 6           | 2.471| 0.4119   | −0.3267 |
| 1y6b         | VGF R2  | 7           | 2.874| 0.4106   | −0.48752|
| 2ko          | REN1    | 7           | 2.867| 0.4095   | 0.45273 |
| 1upw         | NR1H2   | 6           | 2.444| 0.4073   | −1.0423 |
| 2tq9         | CATS    | 5           | 2.035| 0.4069   | −1.1074 |
| 1nhz         | GCR     | 8           | 3.238| 0.4048   | 0.71596 |
| 1f63         | FABP7   | 7           | 2.804| 0.406    | −0.2902 |
| 1nav         | P10B27  | 7           | 2.803| 0.4044   | −0.0991 |
| 1egc         | ACDM    | 7           | 2.793| 0.3988   | 0.90037 |
| 2ph6         | BACE1   | 7           | 2.782| 0.3974   | 0.42086 |
| 1bn8         | TGM3    | 8           | 3.112| 0.3889   | 0.43289 |
| 1q4n         | AMY1    | 6           | 2.309| 0.3849   | −0.8531 |
| 1sj          | BST1    | 7           | 2.648| 0.3762   | −0.135  |
| 1xap         | RARB    | 9           | 3.388| 0.3763   | 0.55752 |
| 2gpq         | E SF4E  | 8           | 2.995| 0.3699   | 1.48143 |
| 1t7b         | DCAM    | 8           | 2.407| 0.3684   | 1.48162 |
| 1qje         | H NMT   | 8           | 2.946| 0.3683   | 0.07142 |
| 1gzu         | NMMAT1  | 7           | 2.568| 0.3691   | −0.5435 |
| 2aeb         | ARG1I   | 7           | 2.651| 0.3688   | −0.382  |
| 1hrk         | FECH    | 8           | 2.925| 0.3666   | 0.3439  |
| 2gqg         | ABL1    | 7           | 2.926| 0.3654   | 0.68279 |
| 11v2         | HNF4G   | 7           | 2.555| 0.365    | −0.8227 |
| 1qcf         | HCK     | 5           | 2.542| 0.3631   | −0.8023 |
| 1kms         | D YR    | 7           | 2.542| 0.3631   | −0.6051 |
| 1kuq         | PLA2G2A | 8           | 2.894| 0.3617   | 0.02781 |
| 2dli         | BIRC7   | 8           | 2.883| 0.3603   | 0.71932 |
| 1xv5         | FPS     | 8           | 2.872| 0.359    | 0.01297 |
| 1nmr         | KTHY    | 8           | 2.864| 0.358    | 0.32451 |
| 1mlw         | TPH1    | 7           | 2.505| 0.3578   | −0.832  |
| 1xbt         | TK1     | 7           | 2.499| 0.3571   | −0.0525 |
| 1n46         | THR8    | 7           | 2.496| 0.3568   | −0.5149 |
| 3dcu         | NR1H4   | 8           | 2.852| 0.3565   | −0.5149 |
| 1ogn         | OAT     | 8           | 2.832| 0.354    | 0.19086 |
| 1sxx         | NR1I2   | 10          | 3.519| 0.3519   | 1.03581 |
| 1tqvn        | IL2     | 8           | 2.797| 0.3496   | 0.02755 |

(Continued)
PC9, and H1299 cells in a dose-dependent and time-dependent manner. In comparison with PTL, DDP had a stronger inhibitory effect on the tested cell line when treated as a single agent. The combination of drugs exerted a synergistic inhibitory effect on A549, PC9, and H1299 cells. Based on the above results, we proposed the hypothesis that PTL may increase the sensitivity of NSCLC to DDP.

Furthermore, PTL can not only inhibit hypoxia-inducible factor-1α signaling transduction in colorectal cancer, but could also inhibit hypoxia induced epithelial-mesenchymal transition (Kim et al., 2017). As suggested by our findings, the combination of PTL and DDP could synergistically suppress the migration and invasion ability of A549 and PC9 cells.

In addition, this study analyzed the mechanism of the synergistic effect of PTL and DDP on cell cycle distribution in A549 and PC9 cells using FCM. The results indicated that PTL, DDP, and combined treatment caused S and G2 phase arrest in A549 and PC9 cells. Tang found that PTL treatment inhibited survivin, arrested cancer cells at G2/M phases, and triggered cell death in human malignant glioblastoma cells (Tang et al., 2015). However, there is still a controversy in terms of the effect of PTL on cell cycle distribution. One study using human uveal melanoma cells discovered diverse outcomes that PTL exerted growth-inhibiting and apoptosis-inducing effects in UM cells by blocking G1 phase and regulating the mitochondrial pathway (Che et al., 2019). These findings showed that PTL may be conducive to cell cycle arrest at G1 phase in human uveal melanoma cells, while PTL might lead to G2/M phase cell cycle arrest in human malignant glioblastoma cells, both of which necessitate further discussion.

Numerous studies have showed that PTL suppresses tumor-promoting effects of nicotine in lung cancer through inducing p53-dependent apoptosis (Talib and Al-Kanani, 2016), autophagy-induced mitochondrial autophagy induced by oxidative stress, and plays an inhibitory role in the apoptosis of C2C12 myoblasts (Ren et al., 2019). Our study also revealed that, in A549 and PC9 cells, the same results were achieved with both PTL and DDP, and that apoptosis rates could be improved remarkably with their combined application rather than with monotherapy. Overall, these results indicated that apoptosis could be synergistically promoted by a combination treatment of PTL and DDP.

Moreover, PTL was discovered to induce intrinsic apoptosis in thyroid carcinoma cells both in vivo and in vitro by Li C’s team (Yang et al., 2019). Our research showed that both PTL and DDP could suppress the growth of NSCLC xenograft tumors, and that more significant effects were observed with their combination in vivo. As a result, our results showed that the anti-tumor effect of DDP could be effectively enhanced by PTL in vitro. Because of lack of funds, the number of animals we were able to experiment on was limited. Therefore, we did not have different (i.e., less frequent) Schedules of Administration.

Next, the relevant information of the first 300 potential protein targets of PTL was acquired utilizing PharmMapper (Wang et al., 2017). Due to the KEGG pathway analysis, we realized that PTL, in line with recent studies, may chiefly affect the PI3K/Akt signaling pathway (Jeyamohan et al., 2016; Yang et al., 2019). Likewise, according to numerous current investigations, the chemosensitivity of NSCLC cells to DDP could be increased through the inactivation of the PI3K/Akt pathway (Chen et al., 2017; Shi et al., 2017; Gong et al., 2018; Liu et al., 2018; Xia et al., 2018; Zhao et al., 2018). We therefore speculated that PTL may improve the anti-tumor ability of DDP in NSCLC by obstructing the PI3K/Akt pathway.

There are various cellular processes such as survival, proliferation, growth, metabolism, angiogenesis, and metastasis that can be regulated by the hyperactivated or altered PI3K/Akt/mammalian target of the rapamycin (mTOR) signaling pathway in many cancer types (Katso et al., 2001; Engelmann et al., 2006; Martini et al., 2014). The development of cancer resistance to anticancer therapies is closely related to the activation of the PI3K/AKT/mTOR pathway in several tumor types (Martini et al., 2014). PI3Ks are a group of lipid kinases related to the plasma membrane and are composed of three subunits: the p85 regulatory subunit, the p55 regulatory subunit, and the p110 catalytic subunit (Donahue et al., 2002). Based on their diverse structures and particular subunits, PI3Ks are divided into three classes: I, II, and III (Hennessy et al., 2007; Engelmann et al., 2006; Martini et al., 2014; Aziz et al., 2009). Over the past few decades, the PI3K pathway has been thought to be deregulated in multiple human cancers, including NSCLC (Dillon et al., 2007). The PI3K pathway is inactivated by various mechanisms, including the tumor suppressor PTEN, variation or expansion of PI3K, and activation of the upstream tyrosine kinase growth factor receptor or oncogene of PI3K (Stemke-Hale et al., 2008). It was found that overactivity of the PI3K signaling pathway was significantly correlated with the progression of human tumors, increased tumor microvascular density, chemotaxis, and enhanced invasion of cancer cells. Therefore, the PI3K signaling pathway has been considered one of the main targets for cancer therapy (Hennessy et al., 2005; Dillon et al., 2007). Great efforts have been made to develop drugs targeting the PI3K signaling pathway, with many presently being evaluated in clinical tests (Aziz et al., 2009). The suppression of PI3K signaling is a promising and valid means for the treatment of NSCLC.

Through Western blotting, we found that drug treatment resulted in a significant increase in the expression levels of cleaved Caspase-3 and Bax, but the expression levels of Bcl-2, Caspase-3, p-Akt and p-PI3K proteins were lower, and total Akt and PI3K protein expression remained unchanged. In comparison with single-agent therapy, the combination treatment exhibited a greater effect. Therefore, we proposed a hypothesis that the drug-induced pro-apoptotic process may be related to the downregulation of the PI3K/Akt signaling pathway. In keeping with numerous other research studies, our findings suggested that based on the upregulation of Bax and cleaved Caspase-3, the mitochondrial apoptotic pathway was related to PTL-induced A549 cell death. Unfortunately, we have not validated studies from other perspectives, such as whether AKT inhibition reproduces the effect of PTL on DDP sensitivity, and there remains a lack of further study of the upstream mechanisms of AKT.

The above studies showed that inhibiting the PI3K/Akt signaling cascade could be considered an effective strategy for...
NSCLC therapy. Drugs targeting the apoptosis pathway (such as PTL) may also serve as an effective strategy for NSCLC therapy, and might play vital roles in minimizing adverse reactions, maximizing clinical efficacy, and helping to increase the quality of life for patients.

According to the results of our research, PTL combined with DDP could synergistically suppress NSCLC cells through the downregulation of the PI3K/Akt signaling pathway. In combination therapy, PTL was able to increase the sensitivity of NSCLC cells to DDP, allowing for its reduced dose, thereby potentially decreasing its side effects. This discovery provides the basis for PTL as a new option for combination therapy in the treatment of NSCLC.

In summary, our study showed that PTL could strengthen the pro-apoptotic effect of DDP on NSCLC cells through arresting cells at S and G2/M phases, thus functioning as
FIGURE 9 | Suppressive effect of PTL, DDP, and the combination on the PI3K/AKT signaling pathway in A549 cells. Protein expression levels of p-PI3K, PI3K, p-Akt, Akt, Caspase-3, cleaved Caspase-3, Bcl-2, Bax, and GAPDH in A549 cells treated with 30 μM PTL, 0.75 μM DDP alone, and 0.2 μM DDP and 10 μM PTL in combination with or without 30 μM 740 Y-P for 48 h (A). Histograms depicting the relative gray value of the related proteins measured using ImageJ (B–E). All data are shown as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01 or ***P < 0.001 vs. the control group.

an inducer of apoptosis. In the xenograft models, PTL and DDP combination demonstrated distinct anti-cancer activity and reduced tumor volumes and weights. Therefore, PTL has potential as a synergistic drug in combination with DDP to prevent NSCLC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Sun Yat-sen University.

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