Polyphyllin I (PPI), a small molecular monomer extracted from Rhizoma of Paris polyphyllin, shows strong anticancer effects in previous study. Human lung adenocarcinoma A549 cells, human lung squamous cell carcinoma SK-MES-1 cells, and human lung large cell carcinoma H460 cells were cultured and then treated with PPI. Cell proliferation and apoptosis were measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, flow cytometry, western blot analysis, and DNA ladder. Athymic nude mice bearing tumors were injected with PPI, and tumor growth was recorded. Our results showed that PPI significantly inhibited the proliferation of three non-small cell lung cancer (NSCLC) cell lines, with the inhibitory concentrations (IC50) of 1.24, 2.40, and 2.33 μg/ml for A549, H460, and SK-MES-1 cells, respectively. After being treated with 2.5 μg/ml of PPI for 24 h, the apoptotic rate of A549 cells was 39.68%, which was remarkably higher than that of the control. Tumor growth was significantly inhibited in the PPI-treated group compared with the group treated with cisplatin (DDP) or PBS in the nude mice. PPI exhibits antitumor ability in NSCLC cells in vitro and in vivo, which might be related to the apoptosis induced by PPI.

**Keywords** polyphyllin I; non-small cell lung cancer; apoptosis; xenograft

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**Introduction**

Lung cancer accounts for 32% of cancer deaths in men and 24% in women, and represents the leading cause of cancer death worldwide. Approximately 75—80% of lung cancer is non-small cell lung cancer (NSCLC), and its overall 5-year survival rate is only 8—14% when diagnosed and 40% after complete surgical resection [1,2]. Meta-analysis reveals that chemotherapy is the standard therapy for NSCLC, however, the efficacy of this treatment is limited, because the 5-year overall survival benefit in favor of cisplatin-based chemotherapy was 5.3% [3]. Therefore, novel strategies for treatment of lung cancer are urgently needed.

Polyphyllin I (PPI), a small molecular monomer extracted from Rhizoma of Paris polyphyllin, is a steroidal saponin (Fig. 1). The systematic name of PPI is diosgenyl α-L-rhamnopyranosyl-(1–2)-β-L-arabinofuranosyl-(1–4)-β-D-glucopyranoside with a molecular weight of 855.02 Da, and the molecular structure is C44H70O16 [4]. In China, the rhizome of P. polyphylla, known as Chong-Lou, is reported to have effects on many tumors and xenograft, including the pancreas, urinary bladder, breast cancer and liver tumor, showing strong anticancer effects in previous study [5–7]. Siu et al. [8] reported an induction of endoplasmic reticulum stress and mitochondria-mediated apoptotic pathways by polyphyllin D (the molecular structure is the same as the PPI) in the NSCLC cell line H460 cells, but the effects of PPI on all three cell lines of NSCLC and their mechanisms are still unclear. In this study, we investigated the anticancer effects and mechanisms of PPI on NSCLS cells in vivo and in vitro.

**Materials and Methods**

**Materials**

Human lung adenocarcinoma A549 cells were a gift from Dr. XY Lu (School of Medicine, Zhejiang University, Hangzhou, China); human lung squamous cell carcinoma SK-MES-1 cells and human lung large cell carcinoma H460 cells were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). Procaspase-3 PcAb (sc-1223) and Bcl-2 McAb (sc-7382) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). PPI was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). DNA ladder kit was purchased from Axygen Biosciences (Union City, USA). Specific pathogen-free male BALB/C...
cAnNCrj-nu mice (five weeks old) were purchased from the Shanghai Cancer Institute (Shanghai, China). The mice were cared for and used according to Zhejiang University’s guidelines.

Cell culture and treatment
The lung cancer cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO2. For treatment, cells were seeded in plates at the concentration of 3 × 10⁵ cells/ml for 16 h. Cells were treated with PPI at the concentrations of 0, 0.625, 1.25, 2.5, 5, and 10 μg/ml, respectively and cultured for 6, 12, 24, 48, and 72 h.

Effects of PPI on the cell proliferation measured by MTT assay
Cancer cells (4 × 10⁵ cells/well) were seeded in a 96-well plate for 16 h, and PPI was added to every well at the concentrations of 0, 0.625, 1.25, 2.5, 5, and 10 μg/ml, respectively, with PBS as a negative control. Cell viability was measured at 24, 48, and 72 h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The OD values were measured at a wavelength of 570-nm after PPI or PBS treatment. The suppression rate was calculated using the formula: suppression rate = (1 − ODtreatment group/ODcontrol group) × 100%. The suppression rate curve at different concentrations was made to calculate the IC50 using curve regression. All the experiments were performed in triplicate.

Apoptotic DNA ladder detection
Cancer cells were seeded into 6-well plates for 16 h, then treated with 2.5 μg/ml of PPI for another 48 h. Total DNA was separated by 2% (w/v) agarose gel electrophoresis in order to analyze the internucleosomal DNA cleavage.

Western blot analysis of caspase-3 and Bcl-2 proteins
Cancer cells were seeded into 6-well plates for 16 h, and then treated with 2.5 μg/ml of PPI for another 48 h. Total proteins were extracted from each well. Total cellular proteins (30 μg) were separated on 10% SDS-polyacrylamide gels, and then transferred to Hybond-P polyvinylidene difluoride membranes (Amersham, Piscataway, USA). After the non-specific binding sites were blocked by incubating the membranes in Tris-buffered saline and 0.1% Tween-20 (TBST) with 5% skimmed milk for 0.5 h at room temperature, the membranes were probed with primary antibodies at the proper concentrations [9] (1:400 dilutions for pro-caspase-3, caspase-3 p20 and Bcl-2; 1:1000 dilution for β-actin) at room temperature for 3 h, and then washed three times with TBST. The membranes were then incubated with HRP-conjugated goat antimouse (or goat antirabbit) IgG (1:2500 dilution) for 1 h at room temperature, and washed three times with TBST. The membranes were developed by chemiluminescence kit (Roche, Indianapolis, USA), and analyzed using Scion Image software (Scion Corporation, Frederick, USA).

Effects of PPI on cell apoptosis measured by flow cytometry
A549 cells were seeded into 6-well plates for 16 h and then treated with PPI at the concentration of 2.5 μg/ml for 6, 12, and 24 h. PBS was used as a negative control. Cells were removed by trypsinization, washed in PBS, and resuspended in Annexin V-FITC/PI (propidium iodide; Sigma,
Effects of PPI on tumorigenicity in vivo
To assess the effect of PPI on tumorigenicity, 33 male BALB/c nude mice (5 weeks old) were subcutaneously inoculated with 100 µl of exponential growing A549 cells at the concentration of 5 × 10^7 cells/ml for formatting NSCLC xenograft, as described in our previous work [10]. The mice were randomly divided into three groups: PPI group (n = 11), cisplatin (DDP) group (n = 11) and PBS group (n = 11). The animals were treated twice daily by intraperitoneal (i.p.) injection from day 2 to day 11. The PPI group mice at 1.5 mg/kg body weight and the DDP group mice at 1 mg/kg body weight were administrated to study the growth inhibition effect of PPI on NSCLC cells in vivo. Tumor size was measured every 5 d and calculated by the formula: volume (mm^3) = 1/2(width^2 × length). On the 30th day, the mice were euthanized. The tumors were removed, fixed by 4% polyformaldehyde, paraffin embedded, and sectioned.

Statistical analysis
All the quantitative data were presented as the mean ± SD. The statistical significance of the differences was determined using Student’s two-tailed t-test for two groups and one-way ANOVA for multiple groups. P-value less than 0.05 was considered statistically significant. All the data were analyzed with the SPSS 13.0 software (SPSS, Chicago, USA).

Results

The inhibitory effects of PPI on the proliferation of three NSCLC cell lines
The inhibiting effect was detected by MTT assay in A549, H460, and SK-MES-1 cells at 24, 48, and 72 h after being treated with PPI at different concentrations (0, 0.625, 1.25, 2.5, 5, and 10 µg/ml). The obviously inhibitory effects were found in the three NSCLC cell lines at 24, 48, and 72 h after being treated with 2.5 µg/ml of PPI. The inhibitory rates of cells are shown in Table 1. When being treated with 10 µg/ml of PPI, the inhibiting rates reached about 90%. PPI significantly inhibited the proliferation of three NSCLC cell lines, with the IC50 of 1.24, 2.40, and 2.33 µg/ml for A549, H460, and SK-MES-1 cells at 48 h, respectively. The results show that cells treated with PPI exhibited a markedly reduced proliferation capacity in vitro and in a concentration-dependent manner (Fig. 2).

Cell apoptosis induced by PPI in vitro
The DNA fragmentation analysis yielded the evidence of apoptotic induction. As shown in Fig. 3, cells treated with 2.5 µg/ml of PPI for 48 h showed an enhanced DNA ladder on agarose gel electrophoresis, compared with control groups in three cell lines, which is a typical hallmark of apoptosis. In addition, an immunoblot analysis was carried out to detect Bcl-2, pro-caspase-3, and caspase-3 p20 (a cleaved fragment protein of caspase, in order to confirm the apoptosis induced by PPI via the activation of caspase-3). Anti-apoptosis protein Bcl-2 showed a reduction in NSCLC cells after being treated with PPI. As expected, the cleaved fragment of caspase-3 p20 was up-regulated in the cells treated with PPI compared with the control, which suggested that the caspase-3 was activated after being treated with PPI (Fig. 4).

Table 1 The inhibitory effects of PPI on the proliferation of three NSCLC cell lines

<table>
<thead>
<tr>
<th>PPI (µg/ml)</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>0.625</td>
<td>10.0 ± 8.7*</td>
<td>27.2 ± 5.6*</td>
<td>27.9 ± 11.9*</td>
<td>27.9 ± 10.1*</td>
<td>16.1 ± 7.3*</td>
<td>12.9 ± 8.4*</td>
<td>14.5 ± 8.9*</td>
<td>8.6 ± 4.3*</td>
<td>19.3 ± 5.0*</td>
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<td>1.25</td>
<td>16.9 ± 3.2*</td>
<td>60.4 ± 5.9*</td>
<td>66.6 ± 6.6*</td>
<td>39.6 ± 3.6*</td>
<td>22.8 ± 9.0*</td>
<td>24.5 ± 7.0*</td>
<td>31.9 ± 8.9*</td>
<td>25.3 ± 5.9*</td>
<td>39.7 ± 8.4*</td>
</tr>
<tr>
<td>2.5</td>
<td>51.9 ± 7.1*</td>
<td>76.9 ± 2.8*</td>
<td>84.7 ± 4.8*</td>
<td>60.3 ± 10.6*</td>
<td>49.1 ± 7.5*</td>
<td>52.1 ± 2.2*</td>
<td>67.8 ± 8.9*</td>
<td>60.2 ± 2.7*</td>
<td>71.9 ± 2.9*</td>
</tr>
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<td>5</td>
<td>68.7 ± 3.2*</td>
<td>87 ± 1.5*</td>
<td>93.9 ± 0.5*</td>
<td>81.2 ± 11.8*</td>
<td>79.3 ± 3.4*</td>
<td>87.5 ± 1.5*</td>
<td>83.1 ± 3.3*</td>
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</tr>
<tr>
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<td>87.8 ± 1.2*</td>
<td>93.7 ± 0.7*</td>
<td>93.4 ± 0.6*</td>
<td>88.8 ± 2.8*</td>
<td>94.8 ± 0.4*</td>
<td>80.3 ± 4.5*</td>
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<td>90.8 ± 0.8*</td>
</tr>
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</table>

Human lung adenocarcinoma A549 cells, human lung squamous cell carcinoma SK-MES-1 cells, and human lung large cell carcinoma H460 cells were cultured. The growth inhibition effects of different concentrations (0 – 10 µg/ml) of PPI on NSCLC cells were detected by MTT assay.

*P < 0.05 vs. control. **P < 0.05 vs. 24 h group.
To determine the apoptosis-inducing potential of PPI in A549 cells, the flow cytometric analysis was performed to detect the Annexin V-FITC/PI double-stained cells. Apoptotic cells presented Annexin V positive and PI negative, death cells were double positive and live cells were double negative. After being treated with PPI for 24 h, the percentage of the apoptosis cell (Annexin V<sup>+</sup> PI<sup>−</sup>) was much higher (39.68%) than that of the control group (3.40%) (Fig. 5).

Finally, we determined whether the PPI could inhibit the tumor growth in the nude mice. The incidence of subcutaneous tumors derived from A549 cells were 100%. The time for tumorigenicity was 20 d for the groups treated with PPI or DDP, and 15 d for the PBS-treated group. All mice survived for 30 d after inoculation, and the body weight did not have difference in the three groups. The tumor growth rate in the PPI group was much lower than that in any other group \((P < 0.05)\). On the 30th day, the tumor size and weight in the PPI group were \(18.23 \pm 5.36 \text{ mm}^3\) and \(0.03 \pm 0.009 \text{ g}\), respectively, which was markedly lower than the DDP group \((P < 0.05)\) with the size \((69.60 \pm 7.93 \text{ mm}^3)\) and weight \((0.23 \pm 0.04 \text{ g})\) and the PBS group with the tumor size \((333.89 \pm 44.95 \text{ mm}^3)\) and weight \((0.37 \pm 0.05 \text{ g})\) (Fig. 6). The tumors in the groups treated with PPI and DDP were oval and had a smooth surface whereas the tumors in the PBS group were irregular, nodular, and rich in vessels (Fig. 7). There was no invasion in any of the three groups. These results suggested that the PPI could significantly inhibit the tumor growth of A549 cells in the nude mice.

**Discussion**

Lung cancer is the leading cause of cancer-related mortality worldwide. Even after adequate resection and adjuvant systemic chemotherapy, more than half of patients died of recurrent disease, which might be due to the multiple drug resistance of NSCLC cells to chemical agents [11]. Moreover, the failure of treatment with drugs was usually found in late-stage NSCLC [12,13]. So the development of a better drug for NSCLC is crucial.

In our study, the results demonstrated for the first time that PPI was an effective anticancer agent for the lung cancer. We have shown that PPI was able to kill all three NSCLC cell lines: human lung adenocarcinoma A549...
cells, human lung squamous cell carcinoma SK-MES-1 cells and human lung large cell carcinoma H460 cells. The concentration of PPI that reduced cell survival by 50% (IC50) for each cell line was analyzed by the MTT assay and determined from cell survival curves with the values from 1.2 to 2.4 μg/ml.

Many anticancer agents function by inducing apoptosis in tumor cells [14]. To test whether PPI induced cytotoxic effects on NSCLC cells via apoptosis, we tried to detect DNA fragmentation by agarose gel electrophoresis. As shown in Fig. 3, ladder-like DNA fragmentation was observed in the three cell lines after being treated with PPI for 24 h. To confirm these results, cells treated with PPI were analyzed for the presence of early apoptotic events such as phosphatidylserine (PS) externalization and no loss of membrane integrity with Annexin-V and PI staining. In our results, a very low percentage of Annexin-V positive and PI negative cells (3.4%) was observed in the untreated population. However, 2.5 μg/ml of PPI induced apoptosis in A549 cells with nearly 40% of apoptotic cells detected after PPI treatment.

PPI was found in the present studies to exert its effect by inducing apoptosis in the three NSCLC cell lines, as proved by inducing DNA fragmentation, formation of hypodiploid peak (data not shown) and externalization of PS. Furthermore, we evaluated the effect of PPI on the activation of caspase-3, one of the important effector caspases in the downstream execution apoptotic pathway [15], and the expression level of Bcl-2, an important apoptosis-associated protein [16]. According to the results from western blot...
analysis, incubation of NSCLC cells with PPI cleaved the zymogen pro-caspase-3, and diminished the anti-apoptosis effect of Bcl-2 in A549 lung cancer cells (Fig. 4).

For drug development, it is extremely important to evaluate the toxicity and the potency of the drug in vivo [17]. To address this important issue, we evaluated the anticancer efficacy of PPI in vivo in the nude mice xenografted with A549 cells. We also evaluated its probable toxicity to the host, compared with the DDP group, a basic control [18]. Our results are promising because PPI can effectively reduce the burden of A549 xenograft in the nude mice via the tumor volume and tumor weight with potency even greater than that of the DDP in vivo (Figs. 6 and 7). Furthermore, injection curative dose of PPI did not have obvious side-effects to the host within a short period of time based on the body weight of mice and the morphology of liver and kidney. These findings further strengthened the potential of PPI to be a possible antilung cancer agent for further clinical trial.

In conclusion, developing novel and effective anticancer agents for NSCLC has been a goal of cancer drug discovery research. One of the mechanisms by which cancer cells survive in the presence of chemotherapeutic drugs is to increase anti-apoptotic activities [19]. PPI is a potential antilung cancer drug. It induces apoptosis in the three NSCLC cell lines, A549 cells, SK-MES-1 cells and H460 cells. Though its mechanism of inhibiting the proliferation of NSCLC cells is not yet completely known, the in vivo efficacy and low toxicity of PPI make it have potential implications as an anticancer agent.

### Funding

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### References


