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Original Article

The antitumor activity study of ginsenosides and metabolites in lung cancer cell

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Abstract: Ginseng and its components exert various biological effects, including antioxidant, anti-carcinogenic, anti-mutagenic, and antitumor activity. Ginsenosides are the main biological components of ginseng. Protopanaxadiol (PPD) and protopanaxatriol (PPT) are two metabolites of ginsenosides. However, the difference between these compounds in anti-lung cancer is unclear. The present study aimed to evaluate the antitumor activity of PPD, PPT, Ginsenosides-Rg3 (G-Rg3) and Ginsenosides-Rh2 (G-Rh2) in lung cancer cell. After treatment with cisplatin, PPD, PPT, G-Rg3 or G-Rh2, the viability, apoptosis level and invasiveness of lung cell lines (A549 cell, a lung adenocarcinoma cell line and SK-MES-1 cell, a lung squamous cell line) *in vitro* were analyzed by Cell Counting Kit-8 (CCK8), Annexin V/PI apoptosis and Matrigel invasion assays, respectively. Here we found that all these compounds led to significant decreases of viability and invasiveness and an obvious increase of apoptosis of A549 and SK-MES-1 cells. Among these, the viability of SK-MES-1 cell treated with PPT was decreased to 66.8%, and this effect was closest to Cisplatin. G-Rg3 had the highest stimulatory effect on apoptosis, and PTT had the highest inhibitory effect on cell invasiveness in A549 and SK-MES-1 cells. These results indicate that both ginsenosides and two metabolites have antitumor activity on lung cancer cell *in vitro*. However, PPT is more powerful for inhibiting the viability and invasiveness of lung cancer cell, especially lung squamous cell. G-Rg3 has the best pro-apoptosis effects. This study provides a scientific basis for potential therapeutic strategies targeted to lung cancer by further structure modification.

Keywords: Ginsenosides, PPD, PPT, antitumor activity, lung cancer cell

Introduction

Lung cancer is one of the most common cancers and its prognosis is poor. Of all people with lung cancer, about 15% survive for five years after diagnosis [1]. Treatment of lung cancer depends on the cancer's histological type, staging and the person's performance status. Common treatments include surgery, radiation and chemotherapy, either alone or in combination. In these therapies, chemotherapy improves survival and is used as first-line treatment [2, 3]. Among these, cisplatin is most commonly used [4]. However, chemotherapeutic agents are not only harmful to tumor cells, but also to normal cells, limiting their therapeutic use in the clinic. Thus, new natural anticancer compounds are urgently needed, especially those compounds derived from long-term used traditional Chinese medicinal herbs [5], such as ginseng.

Ginseng is a medicinal herb widely used in Asian countries and the North American. Ginsenosides are main components extracted from ginseng, and ginsenoside Rg3 is one of the most important parts. Ginsenosides have various pharmaceutical activities, such as antitumor, antioxidant, immunomodulatory and anti-inflammatory etc [6]. Most ginsenosides are composed by a dammarane skeleton (30 carbons in a four-ring structure) and sugar moieties which attached to the C-3 and C-20 positions [7]. Differences in saponin structure can

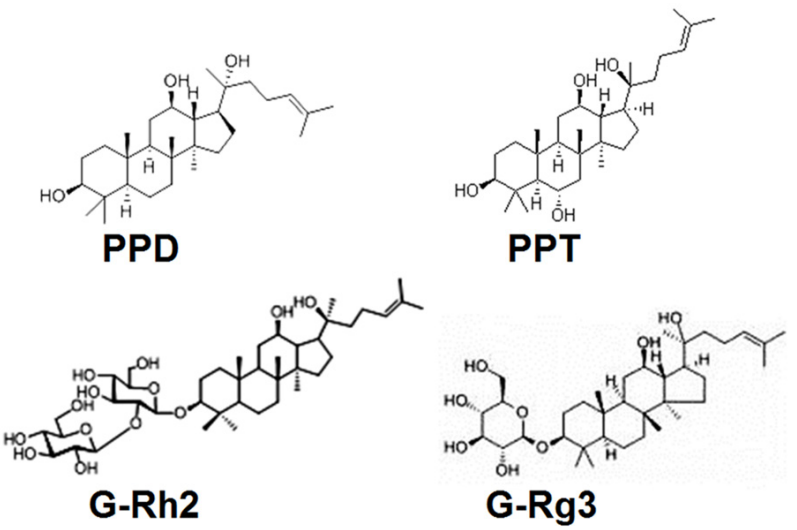


Figure 1. The Chemical structures of PPD, PPT, G-Rg3 and G-Rh2.

influence bioactivity and bioavailability, especially for the antitumor activity. The antitumor activities of ginsenosides are related to the number of sugar molecules, number and position of hydroxyl groups and stereoselectivity [8].

Researches indicate that ginsenoside-Rg3 (G-Rg3) exhibits anticancer activity *in vitro* and *in vivo* models as a relatively safe medicine in several kinds of tumors [9-15]. Ginsenoside Rh2 (G-Rh2) is also one of the bioactive components extracted from ginseng [16]. Several health benefits of Rh2 have been reported due to its anti-inflammatory, anti-osteoclastogenic, anti-hyperglycemic and antitumor effects [17-21]. As two metabolites of ginsenoside, protopanaxadiol (PPD) and protopanaxatriol (PPT), also exhibit activity against a variety of cancer cells [22, 23]. The chemical structures of PPD, PPT, G-Rg3 and G-Rh2 were shown in **Figure 1**. However, so far it still has not reported about a comparative study of antitumor activities of these four compounds.

Therefore, the present study is undertaken to evaluate the antitumor activities of PPD, PPT, G-Rg3 and G-Rh2 on lung cancer cells *in vitro*, with cisplatin as a positive control. This project will provide a new direction for research on antitumor effects of ginsenosides. In addition, this study is expected for us to find a more effective antitumor compounds. On this basis, it will provide targeted compounds used to further chemical structural modification for finding new strategies to treat lung cancer.

Materials and methods

Reagents

All these compounds (PPD, PPT, G-Rg3, and G-Rh2) were bought from Sigma-Aldrich Co. LLC., (USA).

Cell culture

The human lung cancer cells (Human lung adenocarcinoma cell line, A549 cell, and human lung squamous cell line, SK-MES-1 cell) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). These cells were grown were grown in RPMI-1640 medium (Gibco, USA) supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), and were incubated in a humidified incubator at 37°C and 5% CO_2.

The cell-counting kit-8 (CCK-8) assay

A549 cells or SK-MES-1 cells were seeded in 96-well plates (3×10^3 cells/well), and then treated with different concentration of PPD, PPT, G-Rg3 or G-Rh2 (0, 0.4, 4 or 40 uM) for 48 h, with cisplatin (2 ug/ml) as a positive control and 0.1% DMSO as a blank control. Then these cells were collected and detected the viability by the cell-counting Kit-8 (CCK-8) assay (Dojindo, Japan). According to the manufacturer's protocol, the CCK8 reagent was added to each well and cells were incubated at 37°C for 1-4 h. The absorbance (optical density) at 450 nm was measured and used to represent the viability of cells. In addition, A549 cells (3×10^4 cells/well) in 12-well flat-bottom microplates after treatment were counted at a magnification of 100x with microscope (Olympus 1X71, Olympus, Tokyo, Japan). Each experiment was performed in six parallel wells, and repeated three times.

Annexin V/PI apoptosis assay

A549 or SK-MES-1 cells were seeded at a density of 2×10^5 cells/well in 12-well flat-bottom microplates, and then treated with cisplatin (2

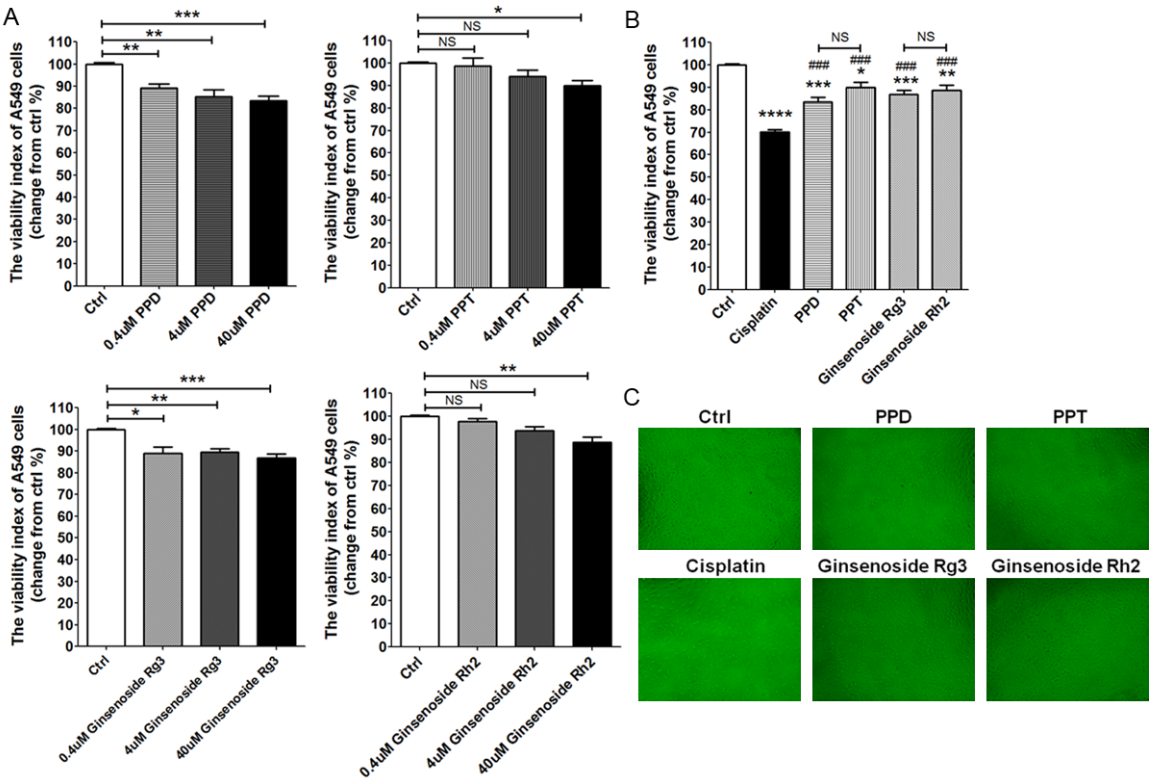


Figure 2. PPD, PPT, G-Rg3 and G-Rh2 suppresse the viability of A549 cell. (A) A549 cells were treated with different concentrations of PPD, PPT, G-Rg3 or G-Rh2 (0.4 uM, 4 uM, 40 uM) for 48 h, the media was added to some wells as a negative control, and then we performed CCK-8 assay to detect the viability of A549 cells. Meanwhile, we compared the antitumor effects between cisplatin (2 ug/ml), PPD (40 uM), PPT (40 uM), G-Rg3 (40 uM) and G-Rh2 (40 uM) (B, C). The data are expressed as the mean ± SEM. Original magnification: X100. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ or $^{***}P<0.0001$ to the vechile control (one-way ANOVA); $^{\#\#\#}P<0.001$ compared to cisplatin (one-way ANOVA). NS: no statistically difference.

ug/ml), PPD (40 uM), PPT (40 uM), G-Rg3 (40 uM) or G-Rh2 (40 uM) for 48 h, with 0.1% DMSO as the control. Subsequently, these A549 or SK-MES-1 cells were digested by 0.25% trypsin without EDTA, and then centrifuged 1000 g for 5 min, resuspended with PBS, labeled the cells by Annexin V and PI according to the instruction manual, and detected the percentage of early apoptosis cells by flow cytometry. The apoptosis detection kit was obtained from Invitrogen Company (Invitrogen, USA). The experiments were carried out in triplicate, and repeated three times.

Matrigel invasion assay

The invasiveness of the A549 or SK-MES-1 cells across matrigel was evaluated objectively in an invasion chamber, based on our previous procedure [24]. Briefly, the cell inserts (8-um pore size, 6.5-mm diameter, Corning, USA) coated with 20 uL of matrigel (BD, USA) were placed in a 24-well plate. The A549 or SK-MES-1 cells of 2 $\times 10^4$ were plated in the upper chamber (the media contained 1% charcoal stripped FCS). Cisplatin (2 ug/ml), PPD (40 uM), PPT (40 uM), G-Rg3 (40 uM), G-Rh2 (40 uM) or vehicle (0.1% DMSO) was added, respectively. The lower chamber (the media was contained 5% charcoal stripped FCS) was filled with 800 ul of medium. The cells were then incubated at 37°C for 48 hours. The inserts were removed, washed in PBS, and the non-invading cells together with the matrigel were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in methanol for 10 minutes at room temperature and stained with hematoxylin. The result was observed under Olympus BX51+DP70 microscope (Olympus, Tokyo, Japan). The cells migrating to the lower surfaces were counted at a magnification of X200. The cells migrating to the lower surfaces were counted in five predetermined fields. The invasion index of each group was

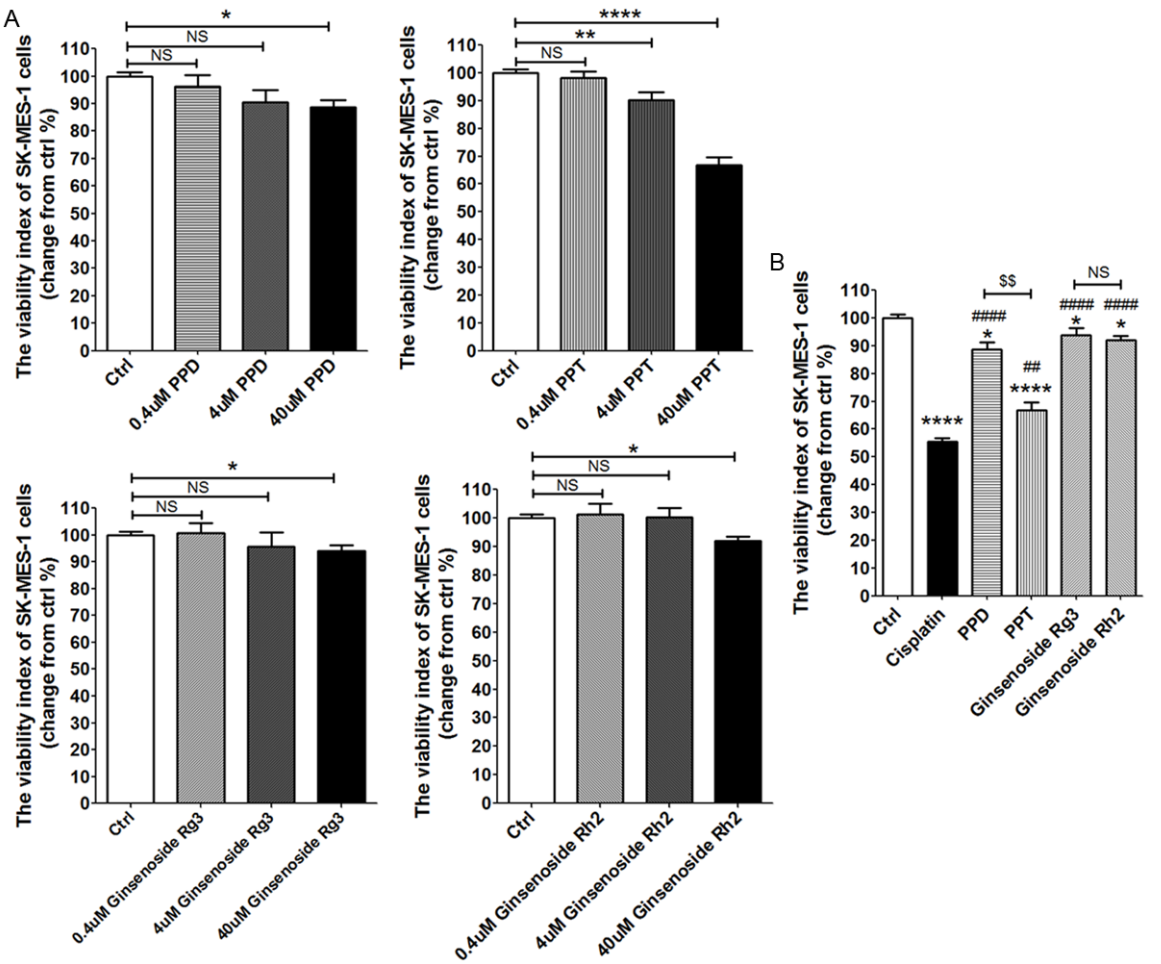


Figure 3. SK-MES-1 cell is more sensitive to PPT. (A, B) CKK-8 assay analysis of SK-MES-1 cells viability, which were treated as described in 2A and 2B. The data are expressed as the mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ and $^{****}P<0.0001$ to the vechile control (one-way ANOVA); $^{\#\#}P<0.01$ and $^{\#\#\#\#}P<0.0001$ compared to cisplatin (one-way ANOVA). $^{\$\$}P<0.01$ compared to PPD. NS: no statistically difference.

calculated as the ratio of the cells number migrated to the lower surfaces to the vehicle control. Each experiment was carried out in triplicate, and repeated three times.

Statistics

All values are shown as the mean ± SEM. The data were analyzed with GraphPad Prism version 5 by one-way ANOVA with Bonferroni post hoc t tests. Differences were considered statistically significant at $P<0.05$.

Results

PPD, PPT, G-Rg3 and G-Rh2 suppress the viability of A549 cell

To evaluate whether PPD, PPT, G-Rg3 and G-Rh2 regulate viability of lung cancer cells, the CCK-8 assay was performed to analysis the effect of these four compounds on the viability of A549 or SK-MES-1 cells. For A549 cells, as shown in **Figure 2**, all these compounds (PPD, PPT, G-Rg3 and G-Rh2) could notably inhibit the viability in a dosage-dependent manner ($P<0.05$, $P<0.01$ or $P<0.001$) (**Figure 2A**), especially at the concentration of 40 uM. But these effect was weaker than that of cisplatin ($P<0.001$) (**Figure 2B** and **2C**).

SK-MES-1 cell is more sensitive to PPT

To analysis the effect of these four compounds on lung squamous cell carcinoma, we treated SK-MES-1 cells with different concentration of PPD, PPT, G-Rg3 and G-Rh2 for 48 h. As shown, all these compounds also led to a marked decrease of viability of SK-MES-1 cells ($P<0.05$,

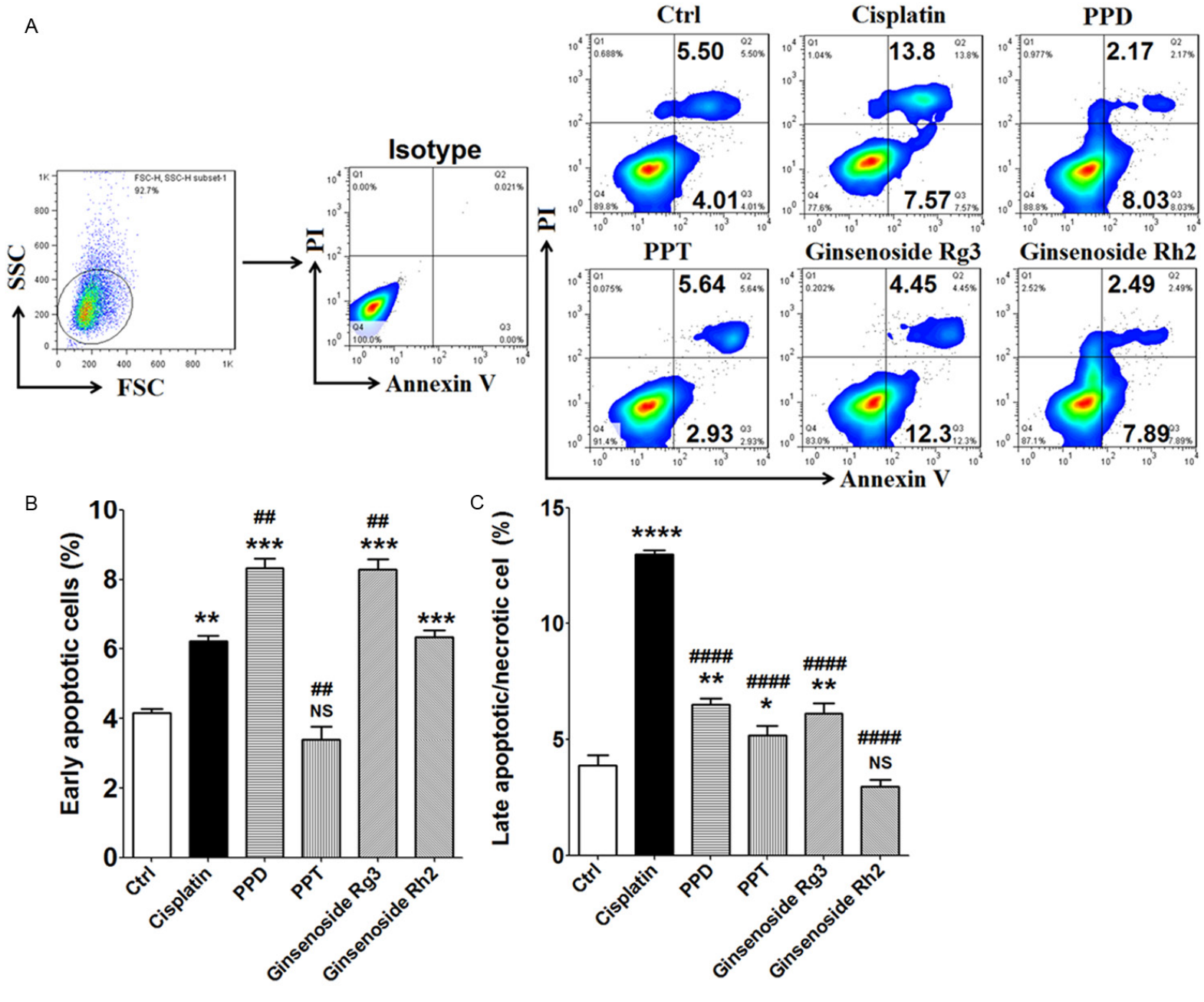

A
Ctrl
Cisplatin
PPD
PPT
Ginsenoside Rg3
Ginsenoside Rh2
Isotype
SSC
FSC
PI
Annexin V
5.50
4.01
13.8
7.57
2.17
8.03
5.64
2.93
4.45
12.3
2.49
7.89
B
Early apoptotic cells (%)
C
Late apoptotic/necrotic cel (%)
Ctrl
Cisplatin
PPD
PPT
Ginsenoside Rg3
Ginsenoside Rh2

Figure 4. PPD and G-Rg3 significantly promote early apoptosis of A549 cell. (A-C) A549 cells (2×10^5 cells/well) were treated with cisplatin (2 ug/ml), PPD (40 uM), PPT (40 uM), G-Rg3 (40 uM) or G-Rh2 (40 uM) for 48 h. Subsequently, Annexin V-FITC apoptosis assay was used to analyze the apoptosis of A549 cells. Results were highly reproducible in three independent experiments. The early apoptotic cells were Annexin V^+PI^- cells; the late apoptotic/necrotic cells were Annexin V^+PI^+ cells. The data are expressed as the mean ± SEM. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ compared to the vechile control (one-way ANOVA); $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to cisplatin (one-way ANOVA); NS: no statistically difference.

P<0.01 or *P*<0.0001) (**Figure 3A**). Among these, PPT was more powerful (*P*<0.01) (**Figure 3A** and **3B**). After treatment with PPT, the viability of SK-MES-1 cell was decreased to 66.8%, and this effect was closest to cisplatin (*P*<0.01) (**Figure 3B**). The antitumor effect on squamous cell carcinoma (SK-MES-1 cell) induced by PPT was more powerful than it on adenocarcinoma (A549 cell). These results suggest that lung squamous cell carcinoma may be more sensitive to PPT.

G-Rg3 significantly promote the apoptosis in A549 and SK-MES-1 cells

In order to evaluate whether these compounds modulate the apoptosis of lung cancer cells, A549 and SK-MES-1 cells were analyzed for the apoptosis level after treatment with cisplatin (2 ug/ml), PPD (40 uM), PPT(40 uM), G-Rg3 (40 uM) or G-Rh2 (40 uM) for 48 h, respectively. As shown, PPD and G-Rg3 and G-Rh2 not PPT could promote early apoptosis in A549 cells (*P*<0.01 and *P*<0.001) (**Figure 4A** and **4B**), especially PPD and G-Rg3. The stimulatory effect on the early apoptosis of A549 cell mediated by PPD and G-Rg3 was more powerful compared to cisplatin (*P*<0.01) (**Figure 4A** and **4B**). However, compared to PPD, G-Rg3 and G-Rh2, treatment with cisplatin led to an obvious increase of late apoptotic or necrotic A549 cells (*P*<0.0001) (**Figure 4A** and **4C**).

Meanwhile, PPD and G-Rg3 also had the stimulatory effect on early apoptosis of SK-MES-1 cells (*P*<0.01, *P*<0.001 or *P*<0.0001) (**Figure 5A** and **5B**). Treatment with PPT or G-Rh2 did not change apoptosis level of SK-MES-1 cells (*P*>0.05) (**Figure 5A** and **5B**). Similar with A549 cells, treatment with G-Rg3 led to higher level of early apoptotic SK-MES-1 cell compared to cisplatin (*P*<0.01) (**Figure 5A** and **5B**). However, there was no significant difference between PPD and cisplatin (*P*>0.05) (**Figure 5A** and **5B**). Further analysis showed that cisplatin resulted in the highest elevation of late apoptotic or necrotic SK-MES-1 cells (*P*<0.001) (**Figure 5A** and **5C**), which echoed the results of A549 cells. Our current results suggest that G-Rg3 significantly stimulate the apoptosis of lung cancer cells (both adenocarcinoma and squamous cell carcinoma).

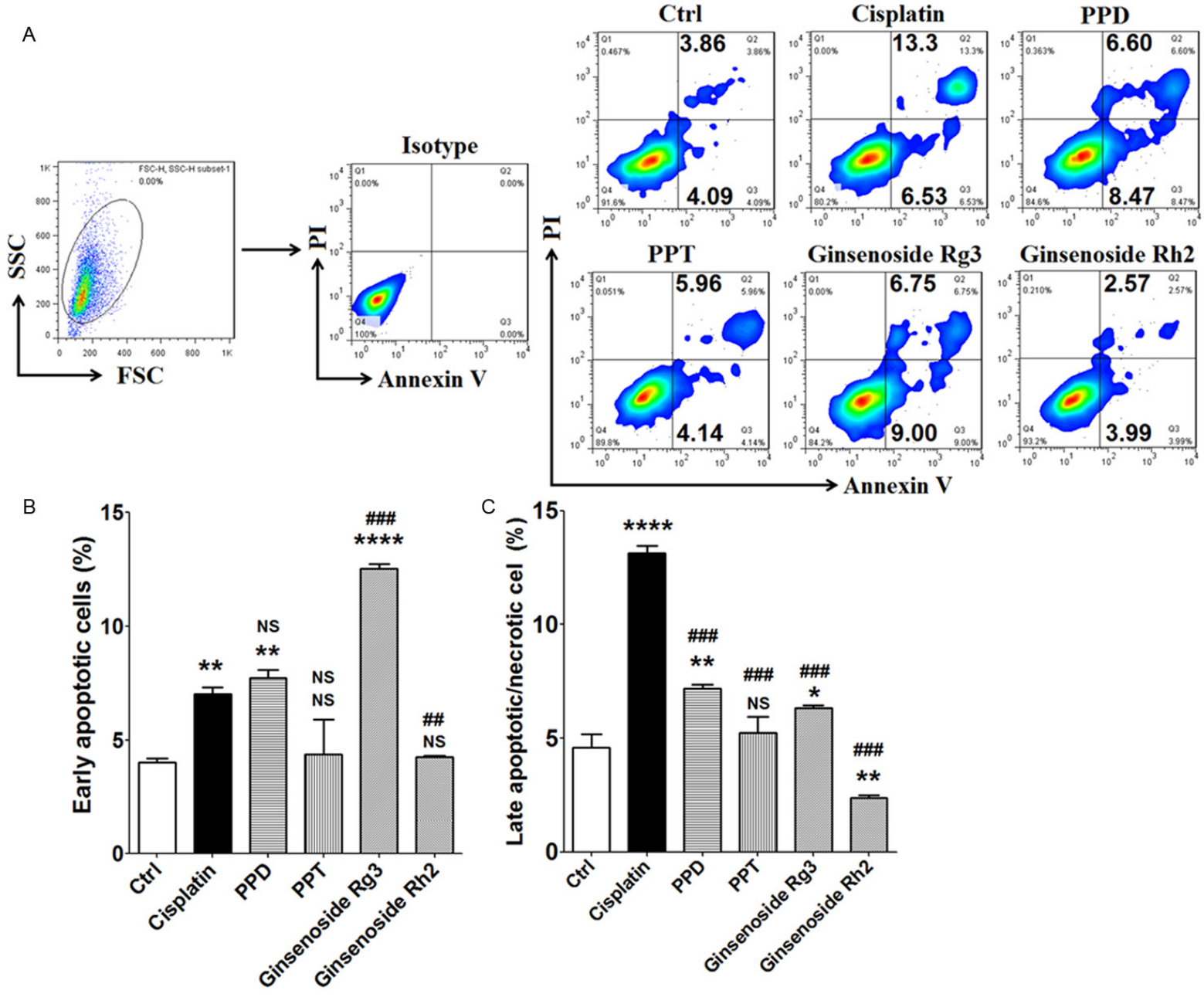
PPT obviously inhibits the invasiveness of A549 and SK-MES-1 cells

We next investigated whether PPD, PPT, G-Rg3 and G-Rh2 could regulate the invasion of lung cancer cell. The results of Matrigel invasion assays showed that four compounds could restrict the invasion of A549 (*P*<0.01 and *P*<0.0001) (**Figure 6A** and **6B**) and SK-MES-1 cells (*P*<0.01, *P*<0.001 and *P*<0.0001) (**Figure 6C** and **6D**). The invasiveness regulation of A549 induced by PPT was similar with cisplatin (*P*>0.05) (**Figure 6A** and **6B**). For SK-MES-1 cells, the inhibitory effects on cell invasion mediated by PPD, PPT, G-Rg3 and cisplatin had no obviously differences (*P*>0.05) (**Figure 6C** and **6D**). These data suggest that both ginsenosides and metabolites have a powerful role in suppressing the invasion and metastasis of lung cancer cell, especially PPT.

Discussion

Lung cancer is the leading cause of cancer-related deaths among both men and women [25], which accounts for about 27% of all cancer deaths [26]. Non-small cell lung cancer (NSCLC), is approximately 80% of all lung cancers. Surgery is to be considered the curative treatment of NSCLC. Overall, surgically-treated patient survival is only around 40% at 5 years. The majority of NSCLC patients present with advanced disease at diagnosis and a large part of those diagnosed with early stage disease eventually recur, experiencing metastatic disease. The advanced disease palliation and the patients' quality of life are still the primary goals of therapy, although total cure is remaining elusive.

With overall disappointing results, the therapy of non-small cell lung cancer (NSCLC) has reached a plateau in improving patient survival.


A
SSC
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Isotype
PI
Annexin V
Ctrl
Cisplatin
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PPT
Ginsenoside Rg3
Ginsenoside Rh2
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13.3
6.53
6.60
8.47
5.96
4.14
6.75
9.00
2.57
3.99
B
Early apoptotic cells (%)
C
Late apoptotic/necrotic cel (%)

Figure 5. G-Rg3 stimulates early apoptosis of SK-MES-1 cell. (A-C) Annexin V-FITC apoptosis detection assay were used to analyze the apoptosis of SK-MES-1 cells, which were treated as described in 4A and 4B. The data are expressed as the mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ compared to control; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to cisplatin; NS: no statistically difference.

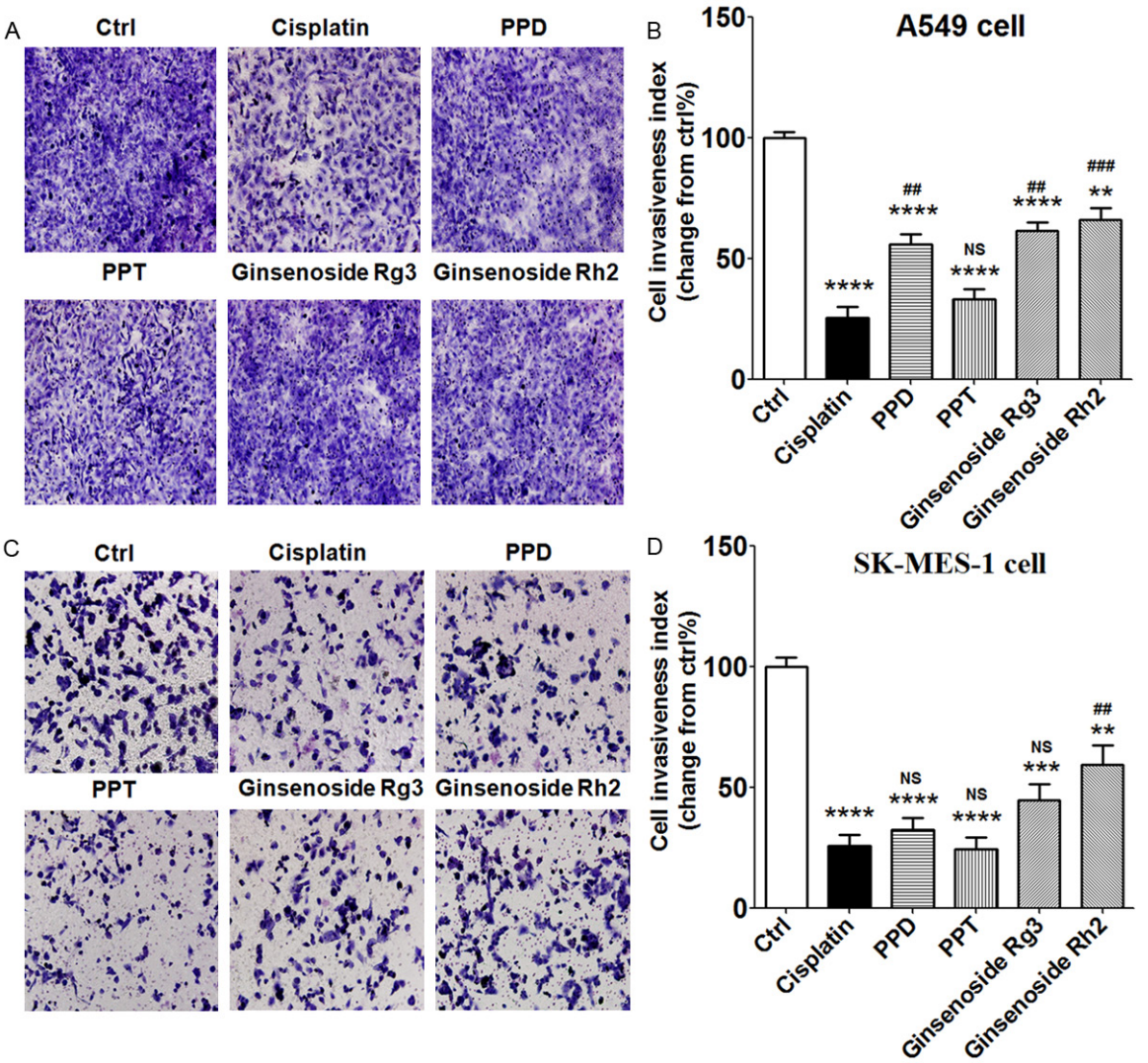

Figure 6. PPT obviously inhibits the invasiveness of A549 and SK-MES-1 cells. A549 or SK-MES-1 cells were treated with cisplatin (2 ug/ml), PPD (40 uM), PPT (40 uM), G-Rg3 (40 uM) or G-Rh2 (40 uM) for 48 h, with vehicle as controls. Then the Matrigel invasion assay was used to detect the invasiveness of A549 cells (A, B) and SK-MES-1 cells (C, D). Results were highly reproducible in three independent experiments. Original magnification: X200. The data are expressed as the mean ± SEM. $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ compared to control; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to cisplatin; NS: no statistically difference.

Therefore, clinical research for looking for new treatment strategies is warranted. The main clinical data currently available on targeted agents in the treatment of NSCLC, focusing on epidermal growth factor receptor family inhibitors, signal transduction inhibitors, angiogenesis inhibitors, eicosanoid pathway inhibitors, vaccines and gene therapy.

Natural products are of increasing interest and importance to cancer patients. Ginseng has been used in Asia for millennia, and is believed to have effective antitumor activity. As the major active chemical components of ginseng, the ginsenosides mainly consist of dammarane-type saponin derivatives. Ginsenosides are a special group of triterpenoid saponins

that can be classified into two groups by the skeleton of their aglycones, namely dammarane- and oleanane-type. Ginsenosides are found nearly exclusively in Panax species (ginseng). Up to now more than 150 naturally occurring ginsenosides have been isolated from roots, fruits, leaves/stems, and/or flower heads of ginseng [27]. Ginsenosides have been the target of a lot of research, and they are believed to be the main active principles behind the claims of ginsengs efficacy. The potential health effects of ginsenosides include anticarcinogenic, immunomodulatory, anti-inflammatory, antiallergic, antiatherosclerotic, antihypertensive, and antidiabetic effects as well as antistress activity, regulation of blood pressure and metabolism [28-30]. Ginsensoides can be metabolized in the stomach (acid hydrolysis) and in the gastrointestinal tract (bacterial hydrolysis) or transformed to other ginsenosides by drying and steaming of ginseng to more bioavailable and bioactive ginsenosides. Ginsenosides within the dammarane-type consist mainly of three types classified according to their genuine aglycone moieties: PPD, PPT and ocotillol.

In our current study, we observed that these compounds (PPD, PPT, G-Rg3 and G-Rh2) could repress the viability and invasiveness, and promote the apoptosis in A549 and SK-MES-1 cells. Among these, PPT induced more powerfully antitumor activity (anti-viability and anti-invasiveness), especially for squamous cell carcinoma (SK-MES-1 cell). This effect may be dependent on the down-signaling pathways, such as mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways and downstream transcription factors such as NF-κB and AP-1 [31, 32]. But the exact molecular mechanism need to research. Compared to cisplatin, treatment with G-Rg3 led to a higher level of early apoptotic lung cancer cells. These data above indicate that different compounds have different antitumor activities of lung cancer cell.

Most patients with lung cancer are diagnosed at the advanced stages of disease and approximately the half of lung cancer patients is inoperable due to metastasized diseases. Lung cancer frequently metastasizes to the brain, liver, bones, and adrenals et al [33-35]. Here we found that these four compounds could inhibit the invasiveness of A549 and SK-MES-1 cells. In addition, the invasiveness regulation of lung cancer cells induced by PPT was similar with cisplatin. Previous reports have paid more attention on the antitumor activities of Ginsensoides, such as G-Rg3, G-Rh2 and G-Rg1 [36-39]. However, our current study in the first time evaluates the antitumor activity difference between PPD, PPT, G-Rg3 and G-Rh2 in lung cancer cells, suggesting that PPT may be a potential compound for targeting lung cancer, especially metastasized lung cancer. Further research should be focused on whether the combination of PPT and cisplatin has a synergistic effect, and whether PPT also plays an effective anti-lung cancer effect *in vivo*, especially during distant metastasis.

A major barrier to restrict the clinical use of ginseng or ginsenosides now is their low oral bioavailability, usually <5% in rodent model [40, 41]. The low oral bioavailability of ginsenosides was attributed previously to their poor oral absorption, which is caused by a large molecular weight and bulky sugar moieties [42]. Differences in saponin structure can fundamentally influence biological responses, especially for the antitumor activity, which is also estimated in the current study. Therefore, chemical synthesis and structure modification plays an important role in the acquirement of various ginsenosides, and finding out the better ideal drugs for treating cancer.

Therefore, based on our findings, it can be concluded that PPT is the best effective compound for anti-lung cancer, especially for lung squamous cell carcinoma or the advanced stages of metastasized lung cancer. Further study should be focused on the structure modification of PPT, which will be helpful for finding out the better ideal drugs for treating lung cancer.

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Disclosure of conflict of interest

None.

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