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Anti-lung cancer effects of novel ginsenoside 25-OCH₃-PPD

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Summary

20(S)-25-methoxyl-dammarane-3 β , 12 β , 20-triol (25-OCH₃-PPD), a newly identified natural product from *Panax notoginseng*, exhibits activity against a variety of cancer cells. Herein, we report the effects of this compound on human A549, H358, and H838 lung cancer cells, and compare these effects with a control lung epithelial cell line, BEAS-2B. 25-OCH₃-PPD decreased survival, inhibited proliferation, and induced apoptosis and G1 cell cycle arrest in the lung cancer cell lines. The *Panax notoginseng* compound also decreased the levels of proteins associated with cell proliferation and cell survival. Moreover, 25-OCH₃-PPD inhibited the growth of A549 lung cancer xenograft tumors. 25-OCH₃-PPD demonstrated low toxicity to non-cancer cells, and no observable toxicity was seen when the compound was administered to animals. In conclusion, our preclinical data indicate that 25-OCH₃-PPD is a potential therapeutic agent *in vitro* and *in vivo*, and further preclinical and clinical development of this agent for lung cancer is warranted.

Keywords

Panax notoginseng; 25-OCH₃-PPD; ginsenoside; natural products; lung cancer

1. Introduction

Lung cancer is the leading cause of cancer death in the United States. It is estimated that in 2008, more than 215,020 new cases will be diagnosed, and more than 161,840 people will succumb to the disease [1]. The prognosis for lung cancer is grim, with only approximately 40% of patients surviving one year after diagnosis [2]. This is largely due to the late stages at which lung cancer is generally diagnosed, and to the lack of effective therapeutic approaches for late-stage disease. Despite the variety of treatment options, ranging from surgery to small molecule inhibitors, lung cancer remains a major cause of cancer mortality. New therapeutic agents, especially those that work by novel mechanisms of action, are urgently needed.

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Natural products are of increasing interest and importance to cancer patients. Ginseng has been used in Asia for millennia, and is believed to have anti-cancer activity. The ginsenosides are the major active chemical components of ginseng, and mainly consist of dammarane-type saponin derivatives. To date, more than 60 ginsenosides have been discovered, and many of these have been shown to possess anti-angiogenic and anti-proliferative effects [3-5]. Ginsenosides can also be derived from the closely related *P. notoginseng*, which is cultured extensively in China.

We have recently isolated novel ginseng and notoginseng-derived compounds, and have found that a notoginseng compound, 20(S)-25-methoxyl-dammarane-3 β ,12 β ,20-triol (25-OCH₃-PPD), was the most effective against a variety of human cancer cells among the more than 60 ginsenosides identified so far. We have demonstrated that the compound is active against breast and prostate cancer [6,7], but hypothesize that it will be effective against a broader spectrum of cancers. Given the lack of effective therapies for lung cancer, and the high incidence of the disease, we wanted to determine whether 25-OCH₃-PPD could represent a novel therapeutic agent for lung cancer. To test our hypothesis, we evaluated the anti-lung cancer effects of the compound *in vitro* and *in vivo*, and accomplished preliminary studies elucidating its mechanisms of action.

2. Materials and Methods

2.1. Reagents

The identity and purity of 25-OCH₃-PPD and Rg3 were established previously [6,8]. All chemicals and solvents were of the highest analytical grade available. Cell culture media, fetal bovine serum (FBS); phosphate-buffered saline (PBS), HEPES buffer, sodium pyruvate, penicillin-streptomycin and other cell culture supplies were obtained from the Comprehensive Cancer Center Media Preparation Shared Facility (University of Alabama at Birmingham). The anti-human MDM2 (SMP14), p21 (C-19), Bcl-2 (100), Bax (N-20), E2F1 (KH95), p27 (C-19), CDK2 (M2), CDK4 (H-22), CDK6 (C-21), Cyclin D1 (DCS-6), and PARP (H-250) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-human p53 (Ab-6) antibody was from EMD Chemicals, Inc (Gibbstown, NJ).

2.2. Cell Culture

Human cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The lung cancer cell lines used were: A549, H358 and H838. A549 cells were grown in Ham's F12K medium supplemented with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate. H358 and H838 cells were grown in RPMI 1640 supplemented with 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES buffer, 1 mM sodium pyruvate and 2 mM L-glutamine. Normal lung epithelial cells (BEAS-2B) were also obtained from the ATCC, and were cultured according to the ATCC's instructions. All media contained 10% FBS and 1% penicillin/streptomycin.

2.3. Cell Survival Assay

The effects of the test compound on human lung cancer cell growth, expressed as the percentage of cell survival, were determined using the MTT assay. Rg3, a compound approved for clinical use as an anti-cancer agent in China, was used as a reference. The cells were grown in 96-well plates at $4-5 \times 10^3$ cells per well and exposed to various concentrations of 25-OCH₃-PPD or Rg3 (0, 1, 10, 25, 50, 100 μ M) for 72 hr. The absorbance at 570 nm was recorded using an OPTImax microplate reader (Molecular Devices; Sunnyvale, CA). The cell survival percentages were calculated by dividing the mean OD of compound-containing wells by that of control wells.

2.4. Cell Proliferation

The effects of 25-OCH₃-PPD on cell proliferation were determined by the BrdUrd incorporation assay (Oncogene, La Jolla, CA), following the manufacturer's protocol. Cells were seeded in 96-well plates (8×10^3 to 1.2×10^4 cells per well) and incubated with various concentrations of the compound (0-100 μ M) for 24 hr. BrdUrd was added to the medium 10 hr before termination of the experiment. The BrdUrd incorporated into cells was determined by anti-BrdUrd antibody, and absorbance was measured at dual wavelengths of 450/540 nm with an OPTImax microplate reader (Molecular Devices; Sunnyvale, CA).

2.5. Detection of Apoptosis

Following a similar protocol as above, cells in early and late stages of apoptosis were detected using an Annexin V-FITC apoptosis detection kit from BioVision (Mountain View, CA). In brief, $2-3 \times 10^5$ cells were exposed to the test compound (0, 1, 5, 10, 25, or 50 μ M) and incubated for 48 hr prior to analysis. Cells that were positive for Annexin V-FITC alone (early apoptosis) and Annexin V-FITC and PI (late apoptosis) were counted.

2.6. Cell Cycle Measurements

To determine the effects of 25-OCH₃-PPD on the cell cycle, $2-3 \times 10^5$ cells were exposed to the compound (0, 1, 10, or 25 μ M) and incubated for 24 hr prior to analysis. Cells were trypsinized, washed with PBS, and fixed in 1.5 mL of 95% ethanol at 4°C overnight, followed by incubation with RNase and staining with propidium iodide (Sigma). The DNA content was determined by flow cytometry.

2.7. Western Blot Analysis

The expression levels of various proteins after 24 hr exposure to different concentrations of 25-OCH₃-PPD (0-25 μ M) were assessed using methods described previously [9].

2.8. Animals

Pathogen-free male athymic nude mice (nu/nu, 4-6 weeks) were purchased from Frederick Cancer Research and Development Center (Frederick, MD). Animals were fed a commercial diet and provided water *ad libitum*. All animal studies were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

2.9. Xenograft model and treatment protocol

The A549 xenograft model was established by subcutaneous implantation of A549 cells into athymic nude mice. The mice were injected subcutaneously with cultured cells suspended in serum-free medium:Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, MA) at a ratio of 3:1 into the left inguinal area. Tumor-bearing mice were randomly divided into multiple treatment and control groups ($n = 5$ mice per group). 25-OCH₃-PPD was dissolved in PEG400: ethanol: saline (57.1%: 14.3%: 28.6%, v/v/v), and given by i.p. injection at doses of 1 mg/kg/d and 10 mg/kg/d, 5 days/wk for 6 weeks. The control group received the vehicle only. All animals were monitored for activity, physical condition, body weight, and tumor growth. Tumor size was determined by caliper measurement of two perpendicular diameters of the implant every other day. Tumor weight (in g) was estimated by the formula $1/2a \times b^2$, where “a” is the long diameter and “b” is the short diameter (in cm).

3. Results

3.1. 25-OCH₃-PPD decreases the survival of malignant lung epithelial cells

We evaluated the effects of 25-OCH₃-PPD (Fig. 1A) and Rg3 (Fig. 1B) on the survival of several human non-small cell lung cancer cell lines, including A549, H358, and H838, as well as normal (non-malignant) BEAS-2B lung epithelial cells (Fig. 1C and D). 25-OCH₃-PPD exerted potent effects against the various cell lines, leading to significant decreases in cell viability, especially in the malignant cells. As seen in Table 1, the compound demonstrated IC₅₀ (the concentration that inhibits the survival of cells by 50%) values of less than 20 μ M (4.88-19.12 μ M), and IC₈₀ values between 35 μ M and 82 μ M in the lung cancer cells. BEAS-2B cells were also affected by the compound, although they demonstrated a somewhat higher IC₅₀ value (24.61 μ M), and a much higher IC₈₀ value (>100 μ M) than the cancer cell lines. This suggests that 25-OCH₃-PPD may affect malignant cells more readily than normal cells.

3.2. The novel ginsenoside inhibits cell proliferation

Next, we examined whether 25-OCH₃-PPD could inhibit cell proliferation (Fig. 2A). The A549 lung cancer cells were the most sensitive, with the 50 μ M concentration inhibiting their proliferation by more than 90% ($p < 0.01$). The proliferation of the other cancer cell lines was also inhibited by more than 70% at this dose. Surprisingly, although the BEAS-2B cells demonstrated almost 50% inhibition of proliferation at the 25 μ M dose, the higher dose did not decrease their proliferation much further.

3.3. 25-OCH₃-PPD induces apoptosis

In addition to inhibiting proliferation, 25-OCH₃-PPD also induced apoptosis in lung cancer cells. As seen in Figure 2B, all three of the examined lung cancer cell lines exhibited a significant increase ($p < 0.01$) in apoptosis following exposure to the 25 μ M concentration of the compound. The “normal” BEAS-2B cells also exhibited increased apoptosis following exposure to 25-OCH₃-PPD; however, they did not exhibit a significant increase in apoptosis until they were exposed to the 50 μ M concentration ($p < 0.01$).

3.4. The compound causes cell cycle arrest

We also observed that 25-OCH₃-PPD inhibited cell cycle progression, leading to arrest in the G1 phase of the cell cycle. Minimal effects could be seen at the lower doses, but a significant increase in the number of cells in the G1 phase occurred for all of the cell lines ($p < 0.01$). The “normal” BEAS-2B cells also exhibited cell cycle arrest (Fig. 3). The lower cytotoxicity of the compound in these cells may stem from differences in their ability to recover from cell cycle arrest.

3.5. 25-OCH₃-PPD down-regulates lung cancer-associated proteins

Given the range of effects of 25-OCH₃-PPD (growth inhibition, apoptosis and cell cycle arrest) it was of interest to determine the possible mechanism(s) of action for the compound. We screened a panel of proteins in A549 cells (which were most sensitive to the compound) to determine which proteins were altered. We found that the expression of numerous proliferation-associated and cell cycle regulatory proteins were affected by the compound. 25-OCH₃-PPD reduced the expression of MDM2, E2F1, Cyclin D1, Cyclin E, cdc25c and cdk4. Conversely, p21 expression increased (Fig. 4). Given the involvement of MDM2 with many of these proteins and its importance for both cell cycle regulation and oncogenesis [10-13], inhibition of MDM2 may be at least partially responsible for the effects of the compound.

3.6. The ginsenoside inhibits the growth of lung cancer xenograft tumors

Since it exerted potent effects *in vitro*, we wanted to determine whether 25-OCH₃-PPD could also exert anti-tumor effects *in vivo*. Nude mice bearing A549 xenograft tumors were treated with either 1 or 10 mg/kg of the compound. The 10 mg/kg dose inhibited tumor growth by more than 35% ($p < 0.01$) after six weeks of treatment (Fig. 5A) compared to mice administered the vehicle only. Additionally, there were no significant differences in body weights from treatment with 25-OCH₃-PPD, nor any gross organ abnormalities at necropsy (Fig 5B). This data suggest that the ginsenoside can be safely given as a novel therapeutic agent. Optimization of the dose and dosing schedule will likely yield even better anti-tumor efficacy.

4. Discussion

The mortality rate of lung cancer is high both in the United States and world-wide, and existing therapies are insufficient for eradicating the disease. Moreover, many of the conventional treatments (e.g. surgery, chemotherapy) pose high risks for the patient and decrease quality of life. The discovery of a new agent that effectively destroys lung cancer cells while preserving quality of life would be beneficial to patients. We have demonstrated that the newly identified natural product, 25-OCH₃-PPD, can exert potent anti-lung cancer effects *in vitro* and *in vivo*.

The ginsenoside decreased proliferation, increased apoptosis, arrested cells in the G1 phase of the cell cycle, inhibited the expression of various pro-proliferation proteins, and decreased tumor growth in animals. While the present study is only a preliminary examination of the effects of the compound against lung cancer, it appears that it is effective against lung cancer cells with different genetic backgrounds.

Implicated in the development and progression of lung cancers are multiple pathways, including Rb/p16/cyclin D1, wnt/APC, EGFR/Ras, Pin1 and p53/MDM2/p19^{Arf} [14-16]. The presence of oncogenes such as c-myc and mutated K-ras, as well as overexpression of EGFR, cyclin D1, Bcl-2, and MDM2 are associated with the disease and with disease prognosis. By inhibiting the expression of these molecules, including MDM2, 25-OCH₃-PPD could decrease and/or prevent the growth of primary, and perhaps metastatic, lung cancer.

5. Conclusion

Further investigation is needed to fully examine the anti-cancer activity of 25-OCH₃-PPD and to determine its primary mechanism of action. However, we believe that the data reported in this manuscript demonstrate that the compound may represent an effective agent for lung cancer therapy. The present study highlights at least four novel discoveries involving 25-OCH₃-PPD in human lung cancer. First, the ginsenoside is cytotoxic to non-small cell lung cancer cells, with IC₅₀ values in the low micromolar range. Second, the ginsenoside decreases proliferation, induces apoptosis, and causes cell cycle arrest in lung cancer cells. Third, the compound exerts a lesser effect on normal bronchial epithelial cells compared to cancer cells. Finally, the compound decreases the growth of mouse xenograft tumors without any apparent toxicity. These findings provide the basis for further evaluation of the compound for lung cancer therapy.

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The abbreviations used are**25-OCH₃-PPD**20(S)-25-methoxyl-dammarane-3 β ,12 β ,20-triol**Rg3**

ginsenoside Rg3

PBS

phosphate-buffered saline

MTT

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

BrdUrd

bromodeoxyuridine

25-OCH₃-PPD**Rg3****A** **B** **C** **D** **Figure 1.**

Chemical structures of (A) 25-OCH₃-PPD and (B) Rg3. Cytotoxicity of 25-OCH₃-PPD to lung cancer cells. Growth-inhibitory effects of 25-OCH₃-PPD (C) and Rg3 (D) on A549, H358, and H838 cells, in comparison with the control lung epithelial cell line BEAS-2B. Effects of cell growth and viability were assessed using the MTT assay following a 72 h exposure to various concentrations of the compound (0-100 μM). All assays were performed in triplicate.

A.

B.

Figure 2.

(A) Anti-proliferative effects of 25-OCH₃-PPD on lung cancer cells, A549, H358, and H838, in comparison with control cell line BEAS-2B. These cells were exposed to various concentrations of the ginsenosides for 24 h followed by the BrdU incorporation assay. (B) Induction of apoptosis by 25-OCH₃-PPD in A549, H358, and H838 lung cancer cells, in comparison with the control cell line BEAS-2B. Cells were exposed to various concentrations of the ginsenosides for 48 h followed by the flow cytometry-based apoptosis assay. All assays were performed in triplicate.

Figure 3.

Effects of the ginsenosides on the cell cycle distribution of A549, H358, and H838 lung cancer cells, in comparison with the control cell line BEAS-2B. Cells were exposed to various concentrations of the compounds for 24 h, followed by flow cytometry analysis.

Figure 4. Effects of the ginsenosides on protein expression in A549 lung cancer cells. Cells were exposed to various concentrations of the compounds for 24 h, followed by Western blot analysis.

Figure 5.

Antitumour activity and effects on body weight of 25-OCH₃-PPD administered to nude mice bearing A549 xenograft tumors. (A) 25-OCH₃-PPD was given by intraperitoneal injection at doses of 1 mg/kg/d, or 10 mg/kg/d, 5 d/wk for 6 wk. (B) No body weight loss was observed during the treatments.

Table 1

Cytotoxicity of 25-OCH₃-PPD and Rg3

Cell Line	IC ₂₀ (μM) 25-OCH ₃ -PPD	IC ₅₀ (μM) 25-OCH ₃ -PPD	IC ₈₀ (μM) 25-OCH ₃ -PPD	Rg3	Rg3	Rg3
A549	0.66	4.88	35.86	>100	>100	>100
H358	4.48	19.12	81.7	>100	>100	>100
H838	3.37	13.39	53.29	>100	>100	>100
BEAS-2B	4.46	24.61	>100	>100	>100	>100