The herbal agent plantamajoside, exerts a potential inhibitory effect on the development of hepatocellular carcinoma

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Abstract. Plantamajoside (PMS), a major component of Plantago asiatica L, has several pharmacological properties, including anti-proliferative, anti-inflammatory and anti-tumor effects. However, the effects of PMS on hepatocellular carcinoma (HCC) have yet to be determined. The aim of the present study was to investigate the effects of PMS on HCC and elucidate the underlying mechanism. All assays were conducted using 5 groups, namely control, sorafenib, and PMS 100, 50, and 25 µg/ml groups. Cell proliferation was determined by the MTT assay. Cell migration was evaluated with the wound healing and Transwell assays, respectively. Cell apoptosis and cell cycle distribution were evaluated via flow cytometry. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis and western blotting were used to further investigate the mechanism of action of PMS. Sorafenib and PMS both significantly attenuated the proliferation and migration of HCC cells, and markedly promoted cell apoptosis. PMS induced cell cycle arrest in the G0/G1 phase. The efficacy of PMS increased in a dose-dependent manner. Further study evaluated the expression of peroxisome proliferator-activated receptor (PPARγ), nuclear factor (NF)-κB and cyclooxygenase (Cox-2) using RT-qPCR analysis and western blotting. The results demonstrated that PMS promoted the expression of PPARγ and suppressed the expression of NF-κB and Cox-2. In conclusion, PMS was shown to affect cell proliferation, migration, apoptosis and cell cycle distribution. Furthermore, PMS promoted the expression of PPARγ and inhibited the expression of NF-κB and Cox-2, which may be the mechanism underlying its biological effects. Based on the results of the present study, PMS appears to be a promising agent for HCC therapy.

Key words: plantamajoside, hepatocellular carcinoma, herbal medicine, peroxisome proliferator-activated receptor γ

Introduction

Hepatocellular carcinoma (HCC) is an aggressive cancer and is the third leading cause of cancer related mortality worldwide (1). A number of patients with HCC are not considered candidates for surgery due to the late stage diagnosis (2) and HCC is highly resistant to chemo therapy (3). Therefore, novel drugs and treatment targets are urgently needed to develop therapies for HCC.

Peroxisome proliferator-activated receptor γ (PPARγ) is a class of ligand activated nuclear transcription factor. Upon activation by its ligand, PPARγ can inhibit tumor cell proliferation and metastasis, as well as promote apoptosis (4,5). For example, activated PPARγ promotes the expression of the pro-metastatic genes MMP9, MMP13 to regulate cell metastasis; overexpression of PPARγ also promotes expression of caspase-3, caspase-7 and other caspases (6,7). Previous studies have also indicated that PPARγ transcriptionally inhibits NF-κB signaling in HCC (8,9). Cyclooxygenase-2 (Cox-2), a prostaglandin synthetase, is a rate-limiting enzyme that is highly expressed in various types of cancer, including HCC, and exerts anticancer effects (10). The promoter region of Cox-2 contains several known sequences, including a binding site for NF-κB (11).

Plantamajoside (PMS) is a major component of Plantago asiatica L, which has several pharmacological properties, including anti-proliferative, anti-inflammatory and anti-tumor effects (12,13). Previous studies have reported that PMS suppresses the growth and metastasis of breast cancer and squamous cell carcinoma (14,15). Furthermore, it has been reported that the biological effects of PMS are mediated through the regulation of MMP9 and 2; NF-κB; PI3K/Akt; and MAPK signaling (13,15-17).

To the best of our knowledge, the present study is the first study that has demonstrated that PMS inhibits the biological functions of HCC cells and as such, may be employed as a novel therapeutic agent for human HCC.

Materials and methods

Reagents. PMS was purchased from Shifeng Biological Technology Co., Ltd., and dissolved in a solution of ethanol.
and double distilled water at a ratio of 1:1. Sorafenib (10 mM) was purchased from Selleck Chemicals to be used as positive control and was digested in a solution containing DMSO (63 mg/ml, warmed to 25°C). T0070907 was purchased from Selleck Chemicals. It has been previously reported that T0070907 is a selective ligand for PPARγ, functioning as an antagonist (18).

**Cell culture and treatment.** Huh7 cells, PLC/PRF 5 and THLE-2 cells (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an atmosphere containing 5% CO₂. Cells were treated with PMS at a concentration of 25, 50 or 100 µg/ml according to a previous study (14), or were treated with sorafenib at 5 or 20 µM. T0070907 was used as a pre-treatment to inhibit PPARγ.

**Cell viability assay.** To investigate the effects of PMS on cell growth, MTT assays were used. Huh7 cells were seeded in 96-well plates at a density of 1x10⁴ cells/well for 24 h. After cell attachment, the cells were treated with sorafenib or PMS at 25, 50 or 100 µg/ml, or were not treated at 37°C. The Optical density (OD) value at 490 nm was detected at 12, 24, 36 and 48 h.

**Wound healing assay.** Cells (1x10⁶ cells/well) were seeded in 6-well plates and incubated for 48 h until ~100% confluent. Cells were washed with serum-free medium and pre-treated with mitomycin C (10 µg/ml at 37°C for 30 min). Subsequently, the incubation medium was replaced with serum-free DMEM. A scratch was created in the cell layer using a 200 µl pipette tip, followed by incubation with medium (non-treated control), sorafenib (positive control) or PMS at 25, 50 or 100 µg/ml for 48 h. Cell migration was examined under a light microscope (magnification, x100; Olympus Corporation). The wound healing distance was calculated using the following equation: (Initial width at 0 h-final width at 48 h)/0 h width. The relative wound healing distance was obtained by normalizing to the control group.

**Transwell assay.** Cells were seeded at a density of 1x10⁵ cells/well in the upper chamber of a transwell plate (8 µm pore-size filter; Merck KGaA) and received no treatment (control), were treated with sorafenib (positive control) or were treated with PMS at 25, 50 or 100 µg/ml at 37°C for 48 h. Following addition of 100 µl FBS free medium and treatment with mitomycin C at 37°C for 30 min, the upper chambers were placed in a 24-well plate. The lower chambers were filled with 500 µl medium supplemented with 10% FBS for 24 h. The cells that had migrated to the lower surface of the filter were stained with 0.1% crystal violet solution. Images were captured using a light microscope (magnification, x100; Olympus Corporation) and processed by IPP 6.0 software (Media Cybernetics, Inc.).

**Cell apoptosis and Cell cycle.** Cells (4x10⁴ cells/well) were cultured with sorafenib and PMS at 25, 50 and 100 µg/ml for 48 h prior to analysis. The cells were subsequently fixed with 70% pre-cooled ethanol for 12-14 h, washed with PBS and resuspended in PBS containing RNase and PI/Triton X-100 (20 µg/0.1% Triton X-100) for 15 min at 37°C, then the cell cycle was analyzed (1x10⁴ cells/sample) using the BD AccuriC6 flow cytometer (BD Biosciences).

Cells were cultured with sorafenib and PMS at 25, 50 and 100 µg/ml for 48 h prior to analysis, stained with Annexin V-FITC Apoptosis Detection kit (Beckman Coulter, Inc.) and double distilled water at a ratio of 1:1. Sorafenib (10 mM) was purchased from Selleck Chemicals to be used as positive control and was digested in a solution containing DMSO (63 mg/ml, warmed to 25°C). T0070907 was purchased from Selleck Chemicals. It has been previously reported that T0070907 is a selective ligand for PPARγ, functioning as an antagonist (18).

**Reverse transcription-quantitative PCR (RT-qPCR) analysis.** Total RNA was extracted from Huh7 cells using TRizol® (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RNA concentrations were evaluated using a spectrophotometer (Beckman Coulter, Inc.). cDNA was synthesized using a Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions manual. The temperature protocol for RT was as follows: 37°C for 15 min, followed by 85°C for 5 sec and holding at 4°C. cDNA was amplified by PCR using SYBR (Invitrogen; Thermo Fisher Scientific, Inc.), and the specific primers used were as follows: GAPDH (reference gene) forward, 5'-GAACGGAAGACTCAGTTG-3' and reverse, 5'-GCTGCTTCACCATCTTCT-3'; NF-κB forward, 5'-AAGCAC GTAAGACAGAGCC-3' and reverse, 5'-TTGGCAGATTAGCTCTTTT-3'; Cox-2 forward, 5'-GTGCAACTTGTAGGCT-3' and reverse, 5'-CTTCTGTGACCGTTTGG-3'; and PPAR-γ forward, 5'-GCAGGACGAGGCAAAGAAG-3' and reverse, 5'-GAGGAGTAACTTGGTCCTCT-3'. The thermocycling conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 20 sec and 65°C for 40 sec. Expression levels of target genes were normalized to the endogenous control GAPDH using the 2^(-ΔΔCt) method (19).

**Western blotting.** Cells (1x10⁶ cells/well) were seeded into 6-well plates and received no treatment (control) or were treated with sorafenib (positive control) and PMS at 25, 50 or 100 µg/ml for 48 h. Cells were lysed using lysis buffer (Shanghai Yanjin Biological Technology Co., Ltd.) and the protein concentration was detected using spectrophotometry. Equal masses of protein samples (30 µg extract loaded per lane) were subjected to 12% SDS-PAGE transferred to PVDF membranes (Invitrogen; Thermo Fisher Scientific, Inc.). After blocking with 5% non-fat milk at room temperature for 2 h, samples were incubated with the indicated primary antibody, namely: NF-κB (p65; cat. no. MW 65; 1:1,000; Cell Signaling Technology, Inc.), Cox-2 (cat. no. MW 74; 1:1,000; Cell Signaling Technology, Inc.), PPAR-γ (cat. no. MW 55; 1:1,000; Cell Signaling Technologies, Inc.), MMP2 (cat. no. MW 72; 1:1,000; Cell Signaling Technology, Inc.), MMP9 (cat. no. MW 92; 1:1,000; Cell Signaling Technology, Inc.), cyclin D1 (cat. no. MW 36; 1:1,000; Cell Signaling Technology, Inc.), cleaved-caspase-3 (cat. no. MW 35; 1:1,000; Cell Signaling Technology, Inc.), caspase-3 (cat. no. MW 17; 1:1,000; Cell Signaling Technology, Inc.), cytochrome C (Cyt-C; cat. no. MW 14; 1:1,000; Cell Signaling
HCC cell migration is inhibited by PMS. As shown in Fig. 4, wound healing assays showed a significant decrease in the cell migration in the sorafenib and PMS 100 µg/ml groups compared with the untreated cells, with no significant difference being found between these two treatments. PMS 50 µg/ml caused a significant reduction in the cell migration in both cells. The low dose group (PMS 25 µg/ml) did not exhibit a notable change compared with the untreated control group. To further investigate whether PPARγ participated in the effects of PMS, transwell assays were performed and it was demonstrated that 50 and 100 µg/ml PMS markedly inhibited cell migration compared with the untreated control group, and that treatment with the PPARγ inhibitor, T0070907, reversed the effects of PMS on cell migration compared with 100 µg/ml PMS (Fig. 5).

**Results**

**PMS inhibits the viability of HCC cells.** To investigate the anti-HCC effect, Huh7 and PLC/PRF5 cells were treated with PMS at various doses and MTT assays were performed to determine cell viability. Sorafenib, as a positive control, significantly reduced the viability of Huh7 and PLC/PRF5 cells at all measured time-points. PMS, at 100 µg/ml, also exhibited a similar efficacy to sorafenib in Huh7 and PLC/PRF5 cells compared with the untreated control, but the treatment in PLC/PRF5 did not show the same level of significance as that in Huh7 cells. In addition, PMS at 25 µg/ml did not exhibit a significant impact compared with the untreated control in both HCC cells, only having a significant impact on the PCL/PRF5 cells at 48 h (Fig. 1). PMS at 100 µg/ml promoted the expression of mRNA and proteins of PPARγ in the Huh7 and PLC/PRF5 cell line compared with the control group (Fig. 2). The cell viability of Huh-7 and PLC/PRF5 cells, which was reduced by Sorafenib and 100 µg/ml PMS, was reversed by the PPARγ inhibitor, T0070907 when compared with PMS 100 µg/ml (Fig. 3). PPARγ may be a potential mechanism by which PMS exerted its anti-tumor effects.

**Statistical analysis.** Graphpad PRISM 6.0 (GraphPad Software, Inc.) was used to analyze the data. Numerical results are presented as the mean ± SD from three independent experiments. Differences between groups were compared using one-way ANOVA and two-way ANOVA, followed by Tukey's post-hoc multiple comparisons tests. A P-value <0.05 was considered to indicate statistically significant differences.

**PMS induces cell cycle arrest at the G0/G1 phase and promotes apoptosis in HCC cells.** To further investigate the effects of PMS on cell proliferation, the cell cycle was analyzed in Huh-7 and PLC/PRF5 cells. It was found that there were significantly more cells in the G0/G1 phase in the sorafenib, PMS 100 µg/ml and PMS 50 µg/ml treated groups compared with the untreated control group. The effect of PMS on the cell cycle appeared to be directly associated with the dose administered. Cell cycle arrest at the G0/G1 phase was reversed by treatment with T0070907 (Fig. 6).

In addition, cell apoptosis in Huh-7 and PLC/PRF5 cells was significantly enhanced by PMS and sorafenib treatment. PMS at a high dose (100 µg/ml) exerted effects comparable to those of sorafenib and the efficacy of PMS was directly associated with the dose administered, that is, the higher the dose of PMS, the stronger the promotive effect on apoptosis. The raised apoptosis observed following PMS 100 µg/ml treatment to both cell lines was significantly reversed by treatment with a PPARγ inhibitor (T0070907) in both cell lines, although this did not reach levels observed in the untreated cells (Fig. 7).

**Further mechanisms of PMS on cell migration, proliferation, the cell cycle and cell apoptosis.** To further confirm that the effects of PMS on cell migration, proliferation, the cell cycle and cell apoptosis are PPARγ-dependent, the expression levels of biomarkers involved in the above processes including MMP2, MMP9, Cyclin D1, cleaved caspase-3/caspase-3 and Cyt-C were detected (Fig. 8). MMPs play a role in the degradation of the extracellular matrix, leading to metastasis (20).
Consistently, the expression levels of MMP2 and MMP9 were significantly decreased in the sorafenib and PMS 100 µg/ml treated groups, compared with the untreated control. The PPARγ inhibitor, T0070907 reversed the inhibitory effect of PMS. Cyclin D1, a major cell cycle regulators of G1 phase progression (21), was expressed at significantly lower levels in the sorafenib, PMS 100 µg/ml and PMS 50 µg/ml treated groups, than in the control. Again, the effects of PMS on the expression levels of cyclin D1 were significantly reversed by T0070907. In regards to apoptosis related proteins, caspase-3 and Cyt-C are involved in apoptosis activation (22). The expression levels of cleaved-caspase-3, a compound caused by the activation of caspase-3 activation (22), were significantly increased in the sorafenib and PMS 100 and 50 µg/ml
Figure 4. Cell migration is inhibited by PMS treatment. Wound healing assays were performed to detect the cell migration at 48 h in (A) Huh-7 and (B) PLC/PRF5 cells. Data are presented as the mean ± SD. **P<0.01, ***P<0.001 vs. the control group; #P<0.05, ##P<0.01, ###P<0.001 vs. sorafenib. PMS, plantamajoside.

Figure 5. PMS inhibits cell migration through interactions with PPARγ. Transwell assays was performed to investigate the effects of PMS on cell migration in (A) Huh-7 and (B) PLC/PRF5 cells. PPARγ inhibitor was used to reverse the effects of PMS. Sorafenib was used as a positive control and non-treated cells were used as a control. Data are presented as the mean ± SD. **P<0.01, ***P<0.001 vs. the control group; #P<0.05, ##P<0.01, ###P<0.001 vs. sorafenib; ∆P<0.05 vs. PMS 100 µg/ml. PMS, plantamajoside; T0070907, PPARγ inhibitor.
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Figure 6. Cells are arrested in the G0/G1 phase after treatment with PMS and mediated by PPARγ. PMS affects cell cycle progression through upregulating PPARγ, as shown by flow cytometry in (A) Huh7 cells and (B) PLC/PRF5 cells. Sorafenib was used as a positive control and non-treated cells were used as a control. Data are presented as the mean ± SD. *P<0.01, **P<0.001 vs. the control group; *P<0.05, ***P<0.001 vs. sorafenib; #P<0.05, ###P<0.001 vs. PMS 100 µg/ml. T0070907 was used for PPARγ inhibition. PMS, plantamajoside; PPARγ, peroxisome proliferator-activated receptor γ.

PMS exerts its biological effects through the PPARγ/NF-κB/ Cox-2 signaling pathway. Compared with the normal liver cell line THLE-2, the expression levels of PPARγ were significantly decreased, whereas that of Cox-2 and NF-κB was significantly increased in both untreated Huh7 and PLC/PRF5 cells. Western blotting analysis results revealed that the expression levels of NF-κB and Cox-2 were significantly decreased following treatment with sorafenib and high dose PMS (100 µg/ml), with no significant difference between the two groups. The intermediate (50 µg/ml) and low (25 µg/ml) PMS dose groups also exhibited a decrease in the expression of these proteins, but to a lesser degree compared with the high dose group, compared with the control group. PPARγ expression was increased in the sorafenib and PMS groups, with the effects of PMS appearing to be concentration-dependent. Inhibition of PPARγ significantly upregulated the expression of Cox-2 and NF-κB, compared with the PMS 100 µg/ml group (Fig. 9).

treated cells compared with the control. The expression levels of Cyt-C were also increased in the sorafenib treated group, as well as in the PMS 100 and 50 µg/ml treated groups, compared with the control. Furthermore, the expression levels of both cleaved-caspase-3 and Cyt-C were significantly reversed by T0070907 treatment compared with the 100 µg/ml treated group, although this did not reach the levels of the control group.
Discussion

Herbal medicine is becoming increasingly attractive as a potential cancer therapy (23). PMS is extracted from Plantago major L, which has been reported to exert inhibitory effects on certain types of cancer, such as breast cancer and esophageal squamous cell carcinoma (13,14). However, the effect of PMS on HCC and the underlying mechanism of action remain unclear. It was hypothesized that PMS may be beneficial for the treatment of HCC.

To investigate this hypothesis, the biological effects of PMS on HCC cells were investigated. A high dose of PMS was found to significantly inhibit cell proliferation and migration, as well as to promote apoptosis. Sorafenib is used as a positive control and non-treated cells were used as a control. Data are presented as the mean ± SD. ***P<0.001 vs. control; ****P<0.001 vs. sorafenib; ΔΔΔP<0.001 vs. PMS 100 µg/ml. T0070907 was used for PPARγ inhibition. PMS, plantamajoside; PPARγ, peroxisome proliferator-activated receptor γ.
sorafenib was used as a control due to its proven efficacy (23). The present study herein demonstrated that a high dose of PMS exerted similar effects with the sorafenib positive control when inhibiting HCC, indicating the potential role that PMS played on HCC cells in vitro.

It has previously been shown that PMS regulates cell migration, proliferation and apoptosis, and promotes the expression of PPARγ (13,14). To examine whether PPARγ was involved in these biological effects in HCC cells, HCC cells were pretreated with the PPARγ inhibitor, T0070907, before PMS treatment. PPARγ has been reported to inhibit MMP2 and MMP9, to exert an anti-tumor metastasis effect in mice with HCC (26,27). Activation of PPARγ by its ligands are attributed to the suppression of proliferation and induction of apoptosis (28). The present results revealed that PPARγ mediated the effects of PMS on cell migration, proliferation

Figure 8. The role of PMS on cell proliferation, migration and apoptosis. The expression levels of proteins involved in migration (MMP2 and MMP9), apoptosis (caspase-3 and Cyt-C) and cell cycle (cyclin D1) were detected using (A) western blotting and (B) gray scan analysis from the Huh-7 and PLC/PRF5 cells following treatment with sorafenib, PMS and T0070907 + PMS. Data are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. the control group; #P<0.05, ##P<0.01, ###P<0.001 vs. sorafenib; ΔP<0.05, ΔΔP<0.01 vs. PMS 100 µg/ml. T0070907 was used for PPARγ inhibition. Cyt-C, cytochrome C; MMP, matrix metalloproteinase; PMS, plantamajoside; PPARγ, peroxisome proliferator-activated receptor γ.
and apoptosis. Moreover, inhibition of PPARγ also blocked the expression levels of caspase-3 and Cyt-C, which were promoted by PMS. Cyt-C is a marker of the mitochondrial respiration chain (29), therefore, PMS may promote mitochondrial apoptosis in a PPARγ-mediated manner. Cyclin D1 is a regulator of the progression from G1 to S, as reflected by the accumulation in G1 (30). The present study showed that PMS inhibits cell proliferation and this effect is linked with the downregulation of cyclin D1 expression. In addition, T0070907 reversed the effects of PMS on cell proliferation.

In the present study, it was also observed that Cox-2 expression was significantly decreased following treatment with PMS, while PPARγ expression was significantly increased, and the two may have mutual constraints. PPARγ is a member of the ligand regulated nuclear receptor superfamily, which exerts diverse biological effects, such as promoting tumor cell growth, angiogenesis and invasion (31-33). PPARγ ligands can interact with Cox-2 and affect shared pathways. Previous studies have suggested that Cox-2 expression can be inhibited with PPARγ activators in human cervical cancer (34,35). The dysregulation of the NF-κB signaling pathway has recently been confirmed to be involved in the biological response to various types of cancer (36). NF-κB not only promotes tumor cell survival and protects cells against apoptotic stimuli, but may also promote proliferation and metastasis of tumor cells (37). The human Cox-2 promoter includes several transcription factor-binding sites, such as for NF-κB and NF-IL6 (34). It has been previously demonstrated that increased PPARγ expression inhibits the expression of Cox-2 and its promoter activity (38). In the present study, the expression of PPARγ was significantly promoted by PMS, whereas the expression of Cox-2 and NF-κB was downregulated in Huh7 and PLC/PRF5 cells. Taken together, these findings indicated that PMS may have acted as an activator of the PPARγ/NF-κB/Cox-2 signaling pathway in HCC cells.

In conclusion, the present study evaluated the effects of PMS on HCC cell metastasis, apoptosis, cell cycle distribution and proliferation. PMS was found to inhibit the proliferation and migration and promote the apoptosis of Huh7 and PLC/PRF5 cells. PMS also triggered G0/G1 phase arrest in Huh7 and PLC/PRF cells. The PPARγ/NF-κB/Cox-2 axis may be the mechanism underlying its regulatory biological effects. These results indicated that PMS may be a promising agent for the treatment of HCC. However, more HCC cell lines as well as in vivo investigations should be explored in further research to confirm the findings of the present study.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors' contributions

YL and SL conceived and designed the current study. SL, XJ, GY and YL acquired the data. ZL, LM, JW and HW analyzed the data. YL and SL drafted the manuscript and revised it for critically important intellectual content. All authors read and approved the final manuscript. YL and SL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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