Original Paper

Pinostilbene Hydrate Suppresses Human Oral Cancer Cell Metastasis by Downregulation of Matrix Metalloproteinase-2 Through the Mitogen-Activated Protein Kinase Signaling Pathway

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Key Words

Oral squamous cell carcinoma • Pinostilbene hydrate • MMP-2 • migration • MAPK

Abstract

Background/Aims: Cancer is the most common cause of death worldwide with approximately one third of people being diagnosed with cancer in their lifetime. Pinostilbene hydrate (PSH) A methylated derivative of resveratrol Has been reported to possess antioxidative Cardioprotective and anticancer properties. However the antimetastatic effect of pinostilbene in oral squamous cell carcinoma (OSCC) remains unknown. **Methods:** In this study We investigated the effect of PSH on antimetastatic activity and the relevant signaling pathways underlying mechanisms of SCC-9 SAS and HSC-3 oral cancer cell lines by MTT assay Wound healing Transwell assay Zymography and western blot analysis. **Results:** Our findings indicated that PSH inhibits migration and invasion ability by reducing the protein activity and expression of matrix metalloproteinases-2 (MMP-2) in all three cell lines. Moreover • The phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (p38) had significant inhibitory effects in the presence of PSH in the SCC9 and SAS cell lines. A combination of ERK1/2 and p38 inhibitors with PSH also reduced the migration and activity of MMP-2 in the SCC9 and SAS cell lines. **Conclusion:** This study demonstrated that PSH suppresses MMP-2 enzymatic activity by downregulating the p38/ERK1/2 pathway and that it might be a promising agent for preventing OSCC cell metastasis.

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Introduction

Oral squamous cell carcinoma (OSCC), the most common primary malignancy occurring in the head and neck, has approximately 500,000 new cases reported each year [1] and is the fourth most common male cancer and the fourth leading cause of male cancer death in Taiwan [2]. The use of tobacco and alcohol has been associated with oral cancer [3, 4]. Primary treatment for OSCC is surgical operation, but for patients in the intermediate or advanced stage of the disease, radiotherapy and chemotherapy are also standard treatments [5]. After treatment, the 5-year survival rate of patients with OSCC is below 50%, owing to cancer cells that are highly metastatic and prone to relapse [6, 7].

Metastasis of cancer is a complex molecular mechanism that is often associated with cancer deaths [8]. During metastasis, degradation of the extracellular matrix (ECM) by extracellular proteinases contributes to the progression of tumor cell migration and invasion [9, 10]. Tumor invasion of the ECM initiates a metastatic cascade, and furthermore, it is an active process that can be divided into several subprocesses: (1) changes in tumor cell-cell interaction; (2) degradation of the ECM; (3) attachment to novel ECM components; and (4) migration of tumor cells [11]. ECM degradation involves various proteases such as matrix metalloproteinases (MMPs), which are calcium-dependent zinc-containing endopeptidases. MMP-2 and MMP-9 are type-IV collagenases of MMPs, and they have been correlated with lymph node metastasis and advanced tumor stage groups [12-14]. Numerous studies have indicated that MMP-2 and MMP-9 expression can be regulated through growth factors, cytokines, and mitogen-activated protein kinase (MAPK) signaling pathways or chemicals [15-17]. Furthermore, endogenous inhibitors, such as tissue inhibitor of metalloproteinases (TIMPs), play a crucial role in cancer cell metastasis and invasion [18].

Natural medications such as resveratrol have been demonstrated to have a beneficial effect on human health. Resveratrol exists in berries, grapes, peanuts, and red wine [19]. Resveratrol has antioxidative, neuroprotective, and cardioprotective properties [20]; however, resveratrol is unstable in environments with strong pH conditions or upon exposure to light. Resveratrol derivatives are able to effectively stabilize upon methylation of their hydroxyl groups [21]. Naturally occurring methylated derivatives of resveratrol exist, including pterostilbene, pinostilbene, and desoxyrhapotigenin [22]. One of these, pinostilbene hydrate, has been demonstrated to have a stronger neuroprotective effect against 6-hydroxydopamine-induced neurotoxicity than resveratrol in SH-SY5Y cells [21]. Moreover, one study reported pinostilbene hydrate, as a major colonic metabolite of pterostilbene, can induce apoptosis in human colon cancer cells [23]. However, the antimetastatic effect of pinostilbene hydrate in OSCC cells remains unknown.

In the present study, we investigated the antimetastatic properties of pinostilbene hydrate on OSCC cells and revealed the involved inhibitory mechanism. Our results demonstrated that pinostilbene hydrate can suppress cell migration and invasion ability by downregulating MMP-2 expression through activation of the ERK1/2 and p38 signaling pathways. These findings suggest the potential of pinostilbene hydrate in preventing oral cancer cell metastasis, as well as indicate potential future directions for medical treatment.

Materials and Methods

Reagents

Pinostilbene hydrate (3, 4'-Dihydroxy-5-methoxy-trans-stilbene hydrate, PSH) ($\geq 95\%$ purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock was dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO for all treatments was consistently less than 0.1%, and stored at -20°C . Other chemicals were obtained from the following companies: Antibodies against MMP-2, p-ERK1/2, ERK1/2, p-JNK1/2, JNK1/2, p-p38, p38, were purchased from cell Signaling Technology (Danvers, MA) and

β -actin from Novus Biologicals (Littleton, CO), all antibodies were stored at -20°C . 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO) and stored at 4°C . Specific inhibitors for ERK1/2 (U0126), p38 (SB203580) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and stored at -20°C .

Cell culture

SCC-9 and HSC-3, the human tongue squamous carcinoma cell lines, was obtained from ATCC (Manassas, VA). The other cell lines SAS, were purchased from and validated by the Japanese Collection of Research Bioresources Cell Bank (JCRB, Shinjuku, Japan). SCC-9 cells were cultured in Dulbecco's Modified Eagle Medium (DEME; Life Technologies, Grand Island, NY) supplemented with Ham's F12 Nutrient Mixture (Life Technologies, Grand Island, NY), 10% fetal bovine serum (FBS), 1.2 g/L sodium bicarbonate, 15 mM HEPES, 1% penicillin/streptomycin, hydrocortisone (400 ng/ml), 1% nonessential amino acids. SAS and HSC-3 cells were cultured in Dulbecco's Modified Eagle Medium (DEME; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 1.2 g/L sodium bicarbonate, 15 mM HEPES, 1% penicillin/streptomycin. All cell cultures were maintained in 5% CO_2 at 37°C .

Cell viability (MTT assay)

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to determine cell viability of PSH. SCC-9 (8×10^4 cells/well), SAS (10^5 cells/well) and HSC-3 (1.2×10^5 cells/well) were seeded in 24-well plates and treated with various concentrations of PSH (0, 20, 40 and 80 μM) for 24 h. Next, the medium was removed and the cells were incubated with MTT reagent 800 μl (0.5 mg/mL) at 37°C in 5% CO_2 for 4 h. The amount of MTT formazan crystals was dissolved in isopropanol, and using an enzyme-linked immunosorbent assay plate reader at a wavelength of 595 nm to measure the absorbance.

Assessment of MMP-2 activity by gelatin zymography

SCC-9, SAS and HSC-3 cells were plated at an appropriate cell numbers (8×10^4 , 10^5 , 1.2×10^5 cells/well) in 24-well plates and then treated with PSH (0, 20, 40 and 80 μM) for 24 h. The cultured medium was collected after treatment, mixed with a loading buffer and subjected to 0.1% gelatin-8% SDS-PAGE electrophoresis. After electrophoresis were completed, the gels were washed 30 minutes twice in 2.5% Triton X-100 then incubated in reaction buffer (40 mM Tris-HCl, pH 8.0, 0.02% NaN_3 , 10 mM CaCl_2) at 37°C for 20 h. Finally, the gel was stained with Coomassie brilliant blue R-250 for visualization as previously described [24]. Bands corresponding to proteolytic activity of MMP-2 were evaluated by ImageJ software.

Cell migration and invasion assays

Cell migration and invasion was assayed according to the methods described previously [25, 26]. After treatment with PSH for 24 h, the surviving SCC-9, SAS and HSC-3 cells were harvested and seeded to upper Transwell (Greiner Bio-One, North Carolina, USA), and DMEM-F12 medium supplemented with 10% FBS (600 μl) was added in advance into Transwell lower chambers in a 24-well plate, then incubated for 24 h at 37°C . The invasion assay was carried out as described above used matrigel (25 mg/50 mL, 60 μl ; BD Biosciences, MA) coating on the upper Transwell. Finally, the migrating or invading cells were fixed with methanol and stained with Giemsa for 2 hs, and used a light microscope to count the cells.

Wound healing assay

As previously described [27], SCC-9 cells (3×10^5 cells/well) were seeded into 6-well plate, after 24 h we created wounded by scratching with a pipette tip and treat PSH (0, 20, 40 and 80 μM) for 24 h. SAS and HSC-3 cells (6×10^5 cells/well) were seeded into culture inserts 2 well for self-insertion (ibidi, Martinsried, Germany). After wounding, cell migration was observed and photographed at 0, 2, 4, 6, 24 h after wounding and compared with the 0 h images.

Western blot analysis

The cell lysates preparation using the methods as previously described [27, 28]. The cell extracts were separated by 10% polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in TBS-T buffer (20 mM Tris, 137 mM NaCl, pH 7.4) with 5% nonfat milk for 1 h, and the blots were probed with the indicated primary antibodies for 24 h at 4°C. After washing steps with TBS-T (10 min each) for 3 times, the blots were incubated with horseradish peroxidase anti-mouse IgG or goat anti-rabbit for 1 h at room temperature. Last, the blots were visualized by ECL reagent (Millipore), and the protein expression was detected by chemiluminescence. The relative photographic density was quantified by ImageQuant LAS 4000 mini Biomolecular Imager (GE Healthcare Bio-Sciences AB, Björkgatan 30 751 84 Uppsala, Sweden).

Statistical analysis

Results were expressed as the means $\pm$ standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using SigmaStat 2.0 (Jandel Scientific, San Rafael, CA). Data comparisons were used Student's t test (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA) between control and treated groups, when P values < 0.05 was considered statistically significant.

Results

Effects of pinostilbene hydrate on the viability of human oral cancer cells

The chemical structure of pinostilbene hydrate (PSH) is presented in Fig. 1A. The cytotoxic effects of PSH at various concentrations (0, 20, 40, and 80 μ M) on SCC-9, SAS, and HSC-3 cells were determined using MTT assay. The results demonstrated no significant cytotoxic effects on the viability of the SCC-9, SAS, and HSC-3 cell lines (Fig. 1B–D). On the basis of the MTT assays, a concentration range of 0–80 μ M of PSH was chosen for subsequent experiments.

Effects of PSH on motility, migration, and invasion of SCC-9, SAS, and HSC-3 cells

To identify the metastasis ability in the SCC-9, SAS, and HSC-3 cells, we used

wound-healing and transwell assays to detect cell migration and invasion. The results suggested that SCC9 cells had inhibited motility after treatment with various concentrations of PSH (0, 20, 40, and 80 μ M) at 24 hs compared with a control group (Fig. 2A). In the SAS and HSC-3 cell lines, the same result was recorded at 6 hs (Fig. 2B and C). Using a transwell assay, we demonstrated that PSH was able to reduce the migration and invasion ability of all three cell lines significantly after 24 hs in a dose-dependent manner (Fig. 2D and E). These results demonstrate that PSH had an inhibitory effect on cell motility, migration, and invasion.

Fig. 1. Effects of Pinostilbene hydrate (PSH) on the Viability of human oral cancer cells. (A) Chemical structure of Pinostilbene hydrate (PSH). (B) SCC-9, (C) SAS and (D) HSC-3 cell lines treat with different concentrations (0, 20, 40 and 80 μ M) of PSH for 24 h, and the cell viability was determined by MTT assay. The values represented the means $\pm$ SD of at least three independent experiments.

Fig. 2. Effects of PSH on motility, migration, and invasion of SCC-9, SAS and HSC-3 cells. The motility of SCC-9 (A), SAS (B) and HSC-3(C) cells were assessed by wound healing assay with different concentration of PSH (0, 20, 40, and 80 μM) at different time points. The cell migration (D) and invasion (E) were measured using transwell for 24 h in three cell lines. The migration and invasion abilities was measured with underside of polycarbonate filters, respectively. The values represented the means ± SD of at least three independent experiments. *P<0.05, compared with the control group.

Fig. 3. PSH inhibits MMP-2 enzyme activities and protein expression of SCC-9, SAS and HSC-3 cells. SCC-9 (A), SAS (B), and HSC-3 (C) cell lines were treated with PSH in serum free medium for 24 h and analyzed the activity of MMP-2 of gelatin zymography assay. The MMP-2 protein expression was detected by Western blot assay after treatment with PSH for 24 h in SCC-9 (D), SAS (E), HSC-3 (F) cells. Quantitative results of MMP-2 protein expression after being adjusted with β -actin. The values represented the means $\pm$ SD of at least three independent experiments. * $P < 0.05$, compared with the control group.

PSH inhibits MMP-2 enzyme activities and protein expression of SCC-9, SAS, and HSC-3 cells

To identify the effect of PSH on MMP-2 activities and protein expression in human oral cancer cells, we treated SCC-9, SAS, and HSC-3 cells with various concentrations of PSH (0, 20, 40, and 80 μ M) for 24 h, and collected the conditioned media to detect the activities of MMP-2 using a gelatin zymography assay. MMP-2 enzyme activities were decreased with increasing PSH concentrations in SCC9, SAS, and HSC-3 cells (Fig. 3A–C). In addition, Western blot was performed to analyze MMP-2 protein expression in the three cell lines. The same result is illustrated in Fig. 3D–F, which indicates that PSH can inhibit MMP-2 activity and the protein expression of SCC-9, SAS, and HSC-3 cells.

Effect of PSH on the MAPK pathway in SCC-9 and SAS cell lines

Studies have reported the MAPK signaling pathway as being involved in the regulation of MMP-2 expression in oral cancer [1, 8]; thus, we examined the expression of three MAPK pathway-related proteins—ERK1/2, JNK1/2, and p38—using Western blot analysis. The results revealed that PSH reduced the phosphorylation level of ERK2 and p38 to 29% and 33%, respectively, in SCC-9 cells at 40 μ M (Fig. 4A and B). In addition, PSH reduced the phosphorylation level of ERK2 and p38 in the SAS cell line by 26% and 32%, respectively, at 40 μ M (Fig. 4D and E). Notably, after treatment with PSH, the expression levels of p-JNK 1/2 were increased in both the SCC9 and SAS cell lines (Fig. 4C and F).

Fig. 4. Effect of PSH on MAPK pathway in SCC-9 and SAS cell lines. SCC-9 and SAS cell lines were treated with different concentrations of PSH (0, 20, 40, and 80 μM) and the cell lysates were subjected to SDS-PAGE followed by Western blots with (A), (D) anti-ERK1/2, (B), (E) anti-p38, (C), (F) anti-JNK antibodies in SCC-9 and SAS cell lines as described in Materials and Methods. Quantitative results of each protein expression after being adjusted with β-actin. The values represented the means ± SD of at least three independent experiments. *P<0.05, compared with the control group.

Effect of ERK1/2 and p38 inhibitors on cell migration and MMP-2 expression in SCC-9 and SAS cell lines

Following analysis of the data, we used ERK and p38 inhibitors—U0126 (20 μM) and SB203580 (20 μM)—combined with PSH treatments (40 μM) to confirm the effects of specific inhibitors on the ERK 1/2 pathway (U0126) and p38 pathway (SB203580) in SCC-9 and SAS cells. The results indicated that a combined treatment of U0126 and SB203580 with PSH could further decrease the MMP-2 activity in both cell lines (Fig. 5A and B). Furthermore, Western blot analysis demonstrated similar MMP-2 protein expression (Fig. 5C and D). In addition, wound-healing and transwell migration assays were performed for further confirmation. The results of these assays demonstrated that, compared with the PSH treatment (40 μM) only, ERK and p38 inhibitors combined with PSH treatments (40 μM) had significantly greater inhibiting effects on both motility and migration in the SCC9 (Fig. 6A and C) and SAS (Fig. 6B and D) cell lines. These data indicate that PSH inhibited SCC-9 and SAS cell migration through inactivation of the ERK1/2 and p38 signaling pathways.

Fig. 5. Effect of ERK1/2 and p38 inhibitor on MMP-2 enzyme activities and protein expression in SCC-9 and SAS cell lines. SCC-9 and SAS cells were pretreated with U0126 or SB203580 combined with or without 40 μM PSH for 1 h and then incubated in the presence or absence of PSH for 24 h. Analysis of (A), (B) of MMP-2 activity with gelatin zymography assay in different cell lines. (C), (D) showed the western blot of MMP-2 expression as described in Methods section. Quantitative results of each protein expression after being adjusted with β-actin. The values represented the means ± SD of at least three independent experiments. *P<0.05, compared with the control group.

Fig. 6. Effect of ERK1/2 and p38 inhibitor on cell motility and migration in SCC-9 and SAS cell lines. SCC-9 and SAS cells were pretreated with U0126 or SB203580 for 1 h, and then incubated in the presence or absence of 40 μ M PSH for 24 h. Two cells were then used for (A), (B) wound healing assay and (C), (D) migration assay, as described in Methods section. The values represented the means $\pm$ SD of at least three independent experiments. * $P < 0.05$, compared with the control group.

Discussion

Oral cancer is a common type of head and neck cancer, with squamous cell carcinoma being the most common single entity [29]. However, surgical or chemotherapy treatment for oral cancer often fails because of metastasis [30]. The SAS, SCC-9 and HSC-3 cell lines belong to tongue squamous cell carcinoma with different degrees of migration/invasion ability. The expression levels of MMP-2 and MMP-9 are different. In this study, the effect of PSH on three different malignant squamous cell carcinoma cells was examined to evaluate/confirm the applicability of PSH anti-metastatic ability to different oral cancer cells. Confirm the applicability of PSH anti-metastatic ability to different oral cancer cells. Studies have

demonstrated that high expression of MMP-2 in the early stages of tongue squamous cell carcinoma is associated with distant metastasis [31]. In the present study, using zymography assay we determined that the activity of MMP-2 was gradually suppressed by increased concentrations of PSH. In addition, the gene expression of MMP-2 was not affected (data not show). The effect of PSH inhibited MMP-2 expression and activity may through zymogen post-transcriptional activation regulation and protein translation regulation. The expression of MMP-9 was no significant difference (data no show). The correlation between high expression of MMP-2 and metastasis has not only been confirmed in oral cancer but also in other malignant tumors. Yang et al. noted that andrographolide can inhibit MMP-2 expression in glioblastoma multiform [32]. Hsin et al. confirmed that *Rubus idaeus* has an inhibitory effect on the migration of human nasopharyngeal carcinoma cells through suppressing the expression and activity of MMP-2 by downregulating the ERK1/2 signaling pathway [33]. All of these studies demonstrate that MMPs can be used as biomarkers for metastasis.

Numerous studies have indicated that MAPK pathways are vital for controlling cell growth, survival, migration, differentiation, and apoptosis through phosphorylation or dephosphorylation [34-36]. Higher p38 MAPK and ERK expression has been noted with oral cancers [37, 38]. The present study determined that PSH suppresses ERK1/2 and p38 protein phosphorylation in both SCC-9 and SAS cells; however, the phosphorylation of JNK1/2 was increased. Liang et al. established that IL-8 produced through the activation of the p38 MAPK/ERK-NF- κ B pathway and reduction of the JNK level promotes inflammatory mediators in head and neck squamous cell carcinoma progression and migration [39]. Furthermore, their experimental results revealed that IL-8 increased MMP2 and MMP9 expression [39]. Therefore, evidence suggests that the phosphorylation of JNK differs from ERK and p38, which may have implications for the mechanisms involved in the regulation of MMPs and migration.

In addition, we used U0126 and SB203580 as ERK and p38 inhibitors, respectively, for further molecular confirmation. Our findings revealed that combining these inhibitors with PSH had greater inhibitory effects (as shown through zymography and Western blot assay) than PSH alone in both the SCC-9 and SAS cells. In addition, the results revealed that U0126 and SB203580 produced improved cell motility and migration-inhibitory effects when combined with PSH in both the SCC-9 and SAS cell lines. Notably, the results demonstrated that SB203580 used alone could strongly suppress MMP-2 enzyme activity and expression in both the SCC9 and SAS cell lines, and that it may also have the potential to inhibit cell migration in both cell lines. This implies that p38 inhibitor may be a cancer suppressant.

Conclusion

The present study is the first to report that PSH can suppress migration and invasion of SCC-9 and SAS by inhibiting MMP-2 activity through the ERK and p38 pathways. Hence, these finding serve as strong initial evidence for the role of PSH as a preventive and therapeutic agent in oral cancer metastasis.

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M.J.H. and M.K.C. study conception and design; C.C.L., Y.S.L., Y.T.H., Y.C.C., and M.C.C. acquisition, analysis and/or interpretation of data; M.C.C and M.J.H. drafting/revision of the work for intellectual content and context; M.J.H. final approval and overall responsibility for the published work. All authors read and approved the final manuscript.

Disclosure Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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