Dehydroandrographolide inhibits oral cancer cell migration and invasion through NF-κB-, AP-1-, and SP-1-modulated matrix metalloproteinase-2 inhibition

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Abstract

Background and purpose: Oral cancer is a type of head and neck cancer that is characterized by cancerous tissue growth in the oral cavity. Andrographolide and dehydroandrographolide (DA) are the two principal components of Andrographis paniculata (Burm.f.) Nees and are the main contributors to its therapeutic properties. However, the pharmacological activities of DA remain unclear.

Experimental approach: In this study, we used wound closure assay and Boyden chamber assay to determine the effects of DA on oral cancer cell migration and invasion.

Key results: DA treatment significantly inhibited the migration and invasion abilities of SCC9 cells in vitro. Gelatin zymography and Western blotting results revealed that DA inhibited MMP-2 activity and reduced its protein levels. DA inhibited the phosphorylation of ERK1/2, p38, and JNK 1/2 in SCC9 cells. According to the mRNA levels detected using real-time PCR, DA inhibited MMP-2 expression in SCC9 cells. This inhibitory effect was associated with the upregulation of the TIMP-2 and downregulation of NF-κB, AP-1, and SP-1 expression. In addition, DA suppressed carcinoma-associated epithelial–mesenchymal transition in SCC9 cells. Finally, DA administration effectively suppressed MMP-2 expression and tumor metastases in the oral carcinoma xenograft mouse model in vivo.

Conclusions & implications: DA inhibits the invasion of human oral cancer cells and is a potential chemopreventive agent against oral cancer metastasis.

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1. Introduction

OSCC is the most common head and neck cancer and has a poor prognosis and low survival rate. The conventional treatment of OSCC includes surgery, radiotherapy, and chemotherapy [1]. Many vegetables, fruits, and grains provide substantial protection against various cancers, and natural phytochemicals can be used for advanced-stage cancer treatment [2–4]. Furthermore, by using these phytochemicals as chemopreventive and chemotherapeutic
agents, cell death can be induced in various diseases, including OSCC [5,6].

Andrographis paniculata (Burm.f.) Nees (family: Acanthaceae), is grown widely in many Asian countries and has various pharmacological properties, such as anticancer, anti-HIV, anti-influenza virus, and cardioprotective properties [7–9]. The primary active ingredients of A. paniculata include several diterpene lactones, flavonoids, and polyphenols [10,11]. Two principal components of A. paniculata, namely andrographolide and dehydroandrographolide (DA), are considered the main contributors of its therapeutic properties. DA inhibits LPS-induced oxidative stress by inactivating iNOS [12] as well as inhibiting viral DNA replication [13], confirming, respectively, that DA is an iNOS inhibitor and antiinflammatory agent [14] as well as an antiviral agent. However, the effects of DA on OSCC invasion and metastasis and the mechanism underlying its antitumor effects have yet to be evaluated.

Cancer cell metastasis involves multiple processes and various cytophysiological changes, including invasion of surrounding tissue, alteration of the adhesive ability of the ECM, penetration into blood or lymphatic vessels, and formation of new tumors [15]. Thus, enzymes causing proteolytic degradation and dysfunctional intracellular interaction, such as MMPs, cathepsins, and PA, are critical in tumor invasion and metastasis [16,17]. Among these, MMP-2 and MMP-9 are associated with cancer invasion and metastasis [18,19]. The endogenous TIMP-2 is a specific inhibitor of MMP-2. Imbalance between MMP and TIMP levels may contribute to the degradation or deposition of the ECM [20,21]. Therefore, the MMP- or PA-mediated inhibition of migration or invasion can be a putative preventive measure against cancer metastasis. This study determined the effects of DA on human oral cancer cells and elucidated the molecular mechanism underlying DA-induced antitumor effects, affecting cancer cell invasion and migration.

2. Materials and methods

2.1. Cell lines

The SCC9 and HSC-3 human oral cancer cell lines were purchased from ATCC (ATCC: American Type Culture Collection, Manassas, VA, USA). SCC9 cells were cultured in Dulbecco’s modified Eagle’s medium-F12 (Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 1% NEAA (Gibco, Karlsruhe, Germany), 1% penicillin streptomycin [20] (Gibco, Karlsruhe, Germany), 1.5 g L⁻¹ sodium bicarbonate (Sigma, St. Louis, Mo, USA), 25 mM HEPES (pH 7.4) (Sigma, St. Louis, Mo, USA), hydrocortisone (0.4 mg L⁻¹) (Sigma, St. Louis, Mo, USA) and 1 mM sodium pyruvate (Sigma, St. Louis, Mo, USA). HSC-3 cells were cultured in Dulbecco’s modified Eagle’s medium-F12 supplemented with 10% FBS, 1% penicillin streptomycin [20], 1.5 g L⁻¹ sodium bicarbonate, 25 mM HEPES (pH 7.4) and 1 mM sodium pyruvate. The cells culture was maintained at 37 °C in a humidified atmosphere of 5% CO₂.

2.2. Antibodies and other reagents

Dehydroandrographolide (DA) of >98% (HPLC) purity was purchased from ChemFaces (Wuhan, PRC). Stock solution of DA was made at 10, 20 and 40 mM concentration in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, Mo, USA) and stored at −20 °C. The final concentration of DMSO for all treatments was less than 0.1%. Other chemicals were obtained from USB Chemical Co. The ERK1/2 inhibitor U0126, p38 inhibitor SB203580 and JNK inhibitor SP600125 were purchased from LC Laboratories. Antibodies for MMP-2, TIMP-2, βcatenin, NF-κB, GAPDH, IKKα, p-IKKα, IKKβ, p-IKKβ, p-IKIkα/pβ, Akt, p-Akt, ERK1/2, p-ERK1/2, p38, p-p38, JNK1/2 and p-JNK1/2 were obtained from Cell Signaling; antibodies for Epithelial–mesenchymal transition (EMT) were obtained from Cell Signaling EMT antibody sampler kit #9782; antibodies for β-actin was obtained from Millipore Corporation, Milford, MA, USA; IKK16 and antibodies for c-Fos, c-Jun, SP1, C23 was obtained from Santa Cruz Biotechnology (California, USA).

2.3. Cell cytotoxicity

As previously described [22]. Briefly, cells (2 × 10⁴ cells well⁻¹) were cultured in 96-well plates and stimulated with different concentrations of DA. After 24, 48 or 72 h, MTt was added to each well and incubated for further 4 h. The viable cell number was directly proportional to the production of formazan, reflected by the color intensity measured at 595 nm, following the solubilization with DMSO.

2.4. Colony formation assays

As previously described [23]. SCC-9 cell line was seeded at a concentration of 5 × 10³ cells well⁻¹ in 6-well cell culture plates in appropriate media. After 24 h of incubation, media were replaced with fresh media containing DA at 10, 20, 40 μM. Both treated and untreated cells were incubated with compound being changed every 3 days. Colonies were allowed to form for 2 weeks and then stained with 0.3% crystal violet solution.

2.5. In vitro wound closure

Cells (2 × 10⁵ cells well⁻¹) were plated in 6-well plates for 24 h, wounded by scratching with a pipette tip, then incubated with DMEM-F12 medium containing 0.5% FBS and treated with or without DA (0, 10, 20, 40 μM). Cells were photographed at 0, 12 and 24 h using a phase-contrast microscope (Olympus, Tokyo, Japan). The wounded areas were quantified as wound width by ImageJ software (National Institutes of Health) using 10× objectives.

2.6. Invasion and migration assays

According to the methods described by Chien et al. [23]. For invasion assay, 10 μl Matrigel (25 mg 5 BD Biosciences, MA, USA) was applied to 8 l Matrigel (25 mg 5 BD Biosciences, MA, USA) under the bottom chamber contained DMEM-F12 medium containing 0.5% FBS and treated with or without DA (0, 10, 20, 40 μM). Cells were subjected to 0.2% gelatin and 8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and incubated in reaction buffer (40 mM Tris–HCl, pH 8.0; 10 mM CaCl₂ and 0.01% NaN₃) for 24 h at 37 °C. The gel was then, stained with Coomassie Brilliant Blue R-250.

2.7. Gelatin zymography

The gelatinolytic activities of MMP-2 in conditional medium were measured using gelatin zymography protease assays, as described previously [24]. Briefly, collected media of an appropriate volume (300 μl well⁻¹) (adjusted by vital cell number) were subjected to 0.2% gelatin and 8% SDS-PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and incubated in reaction buffer (40 mM Tris–HCl, pH 8.0; 10 mM CaCl₂ and 0.01% NaN₃) for 24 h at 37 °C. The gel was then, stained with Coomassie Brilliant Blue R-250.
2.8. Western blot analysis

Cell lysates were separated in a polyacrylamide gel and transferred onto a PVDF membrane. The blot was subsequently incubated with 5% non-fat milk or 0.1% BSA in TBST for 1 h and probed with a corresponding antibody against a specific protein for 37°C at 2 h or overnight at 4°C, and then with an appropriate peroxidase conjugated secondary antibody for 1 h. Signal was developed by ECL detection system and relative photographic density was quantitated by a gel documentation and analysis system.

2.9. Preparation of cell nuclear and cytosolic extracts

Nuclear extracts and cytosolic extracts were prepared essentially as described and used to prepare extracts [23]. Briefly, 1 × 10⁶ cells well⁻¹ were cultured in 10 cm dish and stimulated with different concentrations of DA for 24 h. Cells were rinsed with cold PBS twice and then harvested with HyQTase™ cell detachment solution (HyClone). Removal supernatant, cold reagents were added to the cell pellet with a volume ratio of CER I:CER II:NER at 200:11:100. All of the reagents contain protease inhibitors cocktail. Add cold CER I and vortex for 15 s, the reactions were incubated on ice for 10 min and then cold CER II were added. After a 5 min centrifugation at the 15,000 rpm, resultant supernatant (cytoplasmic extract) was stored at −80°C. Meanwhile, the pellet fraction was resuspended in cold NER. This vortex procedure was repeated for four times with at 10 min incubation on ice between each vortex. After centrifugation, the resultant supernatant (nuclear extract fraction) was stored at −80°C.

2.10. In situ immunofluorescence assay

Cells were seeded into 6-well dish at a density of 4 × 10⁵ cells per dish. After DA incubation, cells were fixed with 4% paraformaldehyde for 20 min. PBS washing was conducted between each reaction to remove any residual solvent. Afterwards, fixed cells were incubated with blocking buffer at room temperature for 2 h and then with the appropriate primary antibodies in antibody dilution buffer at 4°C for overnight. After overnight incubation, cells were washed and then incubated with Alexa Fluor 488-conjugated or Dylight 594-conjugated affinity goat anti-rabbit IgG secondary antibody (Jackson Immuno Research, West Grove, PA, USA) with light protection for 1 h. At the end of incubation, cells were observed under fluorescence microscopy equipped with filters for UV, Blue 488 nm and Green 594 nm.

2.11. In vivo anti-tumor growth and anti-metastasis effects on xenograft transplantation

As previously described [25]. For experimental xenograft growth inhibition study, 5–6 week male BALB/c nude mice (18–22 g) (National Taiwan University Animal Center, Taiwan) were used. HSC-3 cells (2 × 10⁶ per mouse) were resuspended in 200 μl of sterile PBS and injected s.c. into the right flank of the mouse. Mice were randomized into two groups (5 mice/group). All animals were housed with a regular 12-h light/12-h dark cycle and water and ad libitum access to standard rodent chow diet (LaboratoryRodent Diet 5001, LabDiet, St. Louis, MO), and kept in a pathogen-free environment at the Laboratory Animal Unit (temperature 22°C, humidity 30–70%, 5 mice/cage). Seven days after tumor cell injection, the mice were orally fed DA (20 and 40 mg kg⁻¹) or vehicle three times per week. The control group received an equal volume of 0.5% carboxymethyl cellulose vehicle. Tumor volumes were determined from caliper measurements obtained every six days. At the end of the experiment, mice were sacrificed and primary tumors were removed for further analysis.

The primary tumors were separated from the surrounding muscles and dermis, and then weighed. The tumor volume was calculated by the following formula: 0.5 × length × width². The number of metastatic tumor nodules in the lung surface was counted under a dissecting microscope. Mean weight of mice at initiation of study and termination of study did not differ significantly between the groups. All of the procedures involving animals were conducted in accordance with the institutional animal welfare guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Laboratory Animal Center, Changhua Christian Hospital.

2.12. Tumor immunohistochemistry (IHC)

As previously described [25]. Paraffin embedded squamous cell carcinoma and paired non-cancerous tissue sections (4 μm) on poly-l-lysine-coated slides were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was blocked with 3% H₂O₂. The antigen was retrieved by heating at 100°C for 20 min in 10 mM citrate buffer (pH 6.0). After antigen retrieval, slides were washed with PBS and incubated with anti-Ki67, anti-MMP-2, and anti-mouse or anti-rabbit immunoglobulin G (IgG) antibodies for 2 h at room temperature. After washing in PBS, slides were incubated with a horseradish peroxidase (HRP)/Fab polymer conjugate for another 30 min. The sites of peroxidase activity were visualized using 3,3′-diaminobenzidine tetrahydrochloride as a substrate. Gill Hematoxylin Solution II (MERCK, Darmstadt, Germany) was utilized as the counterstain. All specimens were deparaffinized and stained with hematoxylin and eosin (H&E) which was used as a light counterstain.

2.13. Statistical analysis

Statistically significant differences were calculated using Student’s t-test (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA). A p value < 0.05 was considered to be statistically significant. Values represent the mean ± standard deviation and the experiments were repeated three times (n = 3).

3. Results

3.1. Cytotoxic effects of DA in a human oral cancer cell line

The chemical structure of DA is shown in Fig. 1A. To assess the effects of DA on cell viability, a human oral cancer cell line, SCC9, was treated with DA at various concentrations (0–40 μM) for 24, 48, and 72 h and then analyzed using MTT assay. DA did not reduce cell viability even after 72 h of treatment compared with untreated cells (Fig. 1B). In addition, even 40 μM DA did not affect the growth rate of SCC9 cells with regard to long-term colony formation (Fig. 1C).

3.2. DA inhibits wound closure, invasion, and migration of SCC9 cells

To investigate the pharmacological potential of DA against OSCC, we next studied the effect of DA on the wound closure, migration, and invasion abilities of SCC9 cells by performing wound closure assays after 12 and 24 h of DA treatment. The number of cells migrating into the wound decreased in a DA concentration-dependent manner (Fig. 2A and B). The results indicated that DA reduced the number of migrating SCC9 cells by 33% at 24 h. Boyden chamber assays were also used to observe the effects of 16 h (cell migration) and 24 h (cell invasion) of DA treatment on SCC9 cells. At 40 μM, DA reduced cell invasion and migration by 40% (Fig. 2C) and 45% (Fig. 2D), respectively. Therefore, DA
kinases, and IKK

Fig. 3B. Treatment with DA (0–40 

considerably reduced the invasion and migration abilities of SCC9 

cells in a dose-dependent manner.

3.3. Effects of DA on MMP-2 and TIMP-2 expression in SCC9 cells

The metastatic potential of tumor cells is correlated with the activity of MMP-2, also known as gelatinase [26]. In this study, DA (0–40 

µM) did not alter the viability of SCC9 cells in a serum-free condition for 24 h (Fig. 3A). Therefore, we next investigated the reasons that DA inhibits SCC9 cell invasion and migration; we measured and analyzed MMP-2 expression and activity. As shown in Fig. 3B, treatment with DA (0–40 

µM) for 24 h significantly inhibited MMP-2 activity (Fig. 3B and C) and expression (Fig. 3D and E) in SCC9 cells. TIMP-2 expression also increased in these DA-treated SCC9 cells. These findings indicated that DA inhibited MMP-2 expression and activity and induced TIMP-2 expression.

3.4. DA suppresses MMP-2 expression by inhibiting IκB kinase expression to regulate nuclear factor kappa B and AP-1 expression

To evaluate whether transcription factors are involved in the DA-mediated regulation of MMP-2 expression, we analyzed the effects of DA on nuclear translocation by using nuclear and cytosolic extracts. MMP2 activation is mediated by the NF-κB in different cancers [27,28], Western blotting results (Fig. 4A and B) demonstrated that DA affects the nuclear localization of NF-κB in a concentration-dependent manner in SCC9 cells. We also analyzed the expression of c-Fos, c-Jun, SP1, and IκBα through Western blotting [23]. 40 

µM DA treatments significantly reduced c-Fos, c-Jun, and SP1 expression and inhibited IκBα phosphorylation (Fig. 4C and D). The catalytic activities of both IκB kinase (IKK) α and IKKβ critically contribute to IκB phosphorylation and NF-κB activation. IKKα and IKKβ serve as the catalytic subunits of the kinases, and IKKγ serves as the regulatory subunit [29,30]. Accordingly to Fig. 4E and F, even at a low concentration (10 

µM), DA not only reduced total IKKα/β/γ expression but also inhibited their phosphorylation. Combined treatment of DA with IKK 16, selective IKK inhibitor, further reduced MMP-2 expression compared with those treated with DA alone (Fig. 4G and H). Thus, DA suppresses MMP-2 expression by inhibiting IKKα/β/γ expression, thus affecting NF-κB and AP-1 expression.

3.5. DA suppresses invasion and migration abilities of SCC9 cells by mediating phosphorylation of ERK1/2, p38, and JNK

Mitogen-activated protein kinases (MAPKs), including JNK, p38, and ERK, have been demonstrated to be crucial in cell migration [31,32]. To elucidate their underlying mechanism, the effects of DA on the expression of MAPK and PI3K/Akt pathway components were investigated through Western blotting. We observed that DA treatment activated ERK1/2, p38, and JNK1/2; this effect decreased in a dose-dependent manner (Fig. 5A and B). According to densitometry analyses of blots, compared with control, 40 

µM DA treatments reduced the phosphorylation of ERK1/2, p38, and JNK1/2 to 37%, 20%, and 42% at 24 h, respectively. To further elucidate whether ERK1/2, p38, and JNK1/2 activation affects cell migration and invasion, U0126, SB203580, and SP600125 were added, respectively. As shown in Fig. 5C, combined treatment of DA with specific inhibitors further reduced MMP-2 activity compared with the single treatments. We also investigated the roles of p38, Akt, ERK1/2, and JNK1/2 in the DA-mediated suppression of MMP-2 expression. SCC9 cells pretreated with U0126, SB203580, or SP600125 exhibited a considerably decreased MMP-2 expression compared with those treated with DA alone (Fig. 5D–F). To further determine whether DA suppresses cell migration and invasion by inhibiting the phosphorylation of ERK1/2, p38, and JNK1/2, we investigated its effects on MAPK-specific inhibitors in SCC9 cells. We used wound closure assay to determine the effects of 12- and 24-h DA treatments on the migration of SCC9 cells (Fig. 6A). Compared with those treated only with DA, the number of SCC9 cells migrating into the wound decreased considerably for DA treatment combined with MAPK-specific inhibitors. Thus, the migration and invasion assay results indicated that the combined treatment of the DA and MAPK-specific inhibitors considerably reduced SCC9 cell migration (Fig. 6B–D, upper panel) and invasion (Fig. 6B–D, lower panel) compared with single DA treatments. According to these data, the ERK1/2, p38, and JNK pathways are involved in the DA-mediated suppression of MMP-2 activity and expression in SCC9 cells as well as their migration and invasion.

3.6. DA hinders epithelial–mesenchymal transition in SCC9 cells

Epithelial–mesenchymal transition (EMT) is a key developmental process often activated during cancer invasion and metastasis [33]. Epithelial cells lose their polarity and cell–cell adhesion but gain migratory and invasive properties to become mesenchymal stem cells [34]. EMT occurs during wound healing, organ fibrosis, and metastasis initiation in cancer progression [35]. EMT activation involves more pleiotropic signals, as in the case of reactive oxygen species produced in response to MMP exposure [36,37]. The effects of DA on EMT were examined by treating SCC9 cells with different concentrations of DA for 24 h and analyzing EMT-related proteins. As shown in Fig. 7A and B, DA significantly induced the expression of E-cadherin and claudin-1. In addition, the expression of EMT-related proteins, including Vimentin, ZO-1, Zeb-1, Twist-1, Snail, Slug, N-cadherin, and β-cadherin, decreased in the DA-treated SCC9 cells. The initiation of metastasis requires invasion, which is enabled through EMT [38]. Epithelial and mesenchymal cells differ in phenotype and function. We treated SCC9 cells with different concentrations of DA for 24 h and...
observed for morphological changes. The microscopy data indicated that DA-treated SCC9 cells exhibited typical epithelial morphology compared with the spindle-shaped, mesenchymal morphology of control SCC9 cells (Fig. 7C). We also assessed the EMT status through immunofluorescence. Compared with control cells, DA-treated SCC9 cells demonstrated upregulation of the epithelial marker E-cadherin (green) and downregulation of the mesenchymal marker Vimentin (red; Fig. 7D and E).

3.7. Significant anti-metastatic effects of DA in HSC-3 orthotopic graft model

To examine the effects of DA on tumor growth, the antitumor effects of DA were evaluated in vivo. Tumor volumes were determined using caliper measurements every 6 days. The control-group animals, transplanted with HSC-3 cancer cells, presented a progressive increase in their tumor volumes. At day 24, the mean tumor volumes and weights of 40 mg/kg DA-treated mice and vehicle-treated mice did not differ significantly (Fig. 8A and B); in addition, no significant change was noted in their body weights (Fig. 8C). To determine the efficacy of DA in reducing the in vivo metastasis ability, the control and experimental mice were sacrificed at the end of all experiments; their lungs were removed and analyzed for tumors metastases. The number of mice with lung metastasis was lower in the DA-treated group than in the control group (Fig. 8D). In IHC analysis for cell proliferation, the number of Ki67-positive tumor cells slightly decreased after DA treatment compared with the control (Fig. 8E). In addition, MMP-2 expression in tumor specimens decreased in the DA-treated group compared with the control group (Fig. 8F). These results indicated that in vivo DA treatment had an anti-metastatic effect on HSC-3 cancer cells in mice.

4. Discussion

Metastasis involves a complex series of steps, in which cancer cells leave the original tumor site and migrate to other body parts through the bloodstream, the lymphatic system, or direct extension [39]. Metastatic tumors are extremely common in the late stages of cancer. Treatment for metastatic cancer aims to decelerate the growth or spread of cancer. In general, metastatic cancer requires systemic therapy, such as chemotherapy and hormone therapy. Other treatments may include biological therapy, radiation therapy, surgery, or a combination of these [40]. At present,
in addition to chemotherapeutic drugs, identifying novel approaches for chemoprevention by using nontoxic natural substances is of great interest and is being widely investigated [41]. *A. paniculata*, is widely cultivated in many Asian countries and has been demonstrated to have anticancer, anti-HIV, antinfluenza virus, and cardioprotective properties [7–9]; andrographolide and DA are two of its principal components and contribute toward its therapeutic properties. However, the mechanisms underlying the anti-metastatic activities of DA in OSCC remain unclear. Our study results suggest that a distinct mechanism causes this DA-mediated anti-metastatic effect.

First, although DA did not reduce the viability of SCC9 cells, it inhibited their migration and invasion. In our previous study, after a 72-h DA treatment reduced the viability of SAS and OECM-1 cells to respectively 85% and 53% at 25 μM and 71% and 31% at 50 μM [25]. In the present study, DA did not affect SCC9 cell viability in serum (Fig. 1) or serum-free (Fig. 3A) conditions. The results revealed that the effect of DA on cell survival is specific. At a low concentration (40 μM), DA can suppress the migration and invasion ability of cells, particularly cancer cell lines.

Second, the uPA/MMPs cascade, particularly MMP-2 [30–34] and MMP-9 [34–39], is involved in the malignancy of cancers. The expression of MMP-2 and vascular endothelial growth factor is closely related to invasion and metastasis [30,31]. MMP2 activation is mediated by NF-κB in different cancers [27,28]. The NF-κB/Rel transcription factor complex with the inhibitory IκB proteins remains in an inactive state. IκBα activation results in ubiquitin-mediated proteasome-dependent degradation of IκBα and the release and nuclear translocation of active NF-κB dimers [23]. The key regulatory step in this pathway involves the activation of the high-molecular-weight IKK complex, whose catalysis is generally performed by a heterodimeric kinase comprising IKKα and IKKβ subunits [30]. Figs. 3 and 4 indicate that DA suppresses MMP-2 expression by inhibiting IKKα/β/γ expression and affects NF-κB and AP-1 expression.

Third, MAPK pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery controlling fundamental cellular processes such as growth, proliferation, differentiation, migration, and apoptosis. A study indicated that 17β-estradiol-downregulated human LoVo colon cancer cell migra-
tion and MMP-2 and MMP-9 expression are markedly inhibited by p38 MAPK inhibitors alone [42]. The blockade of the RhoA-JNK-c-Jun-MMP2 cascade by atorvastatin reduces osteosarcoma cell invasion [43]. Magnolin, a natural compound abundant in Magnolia flos, inhibits cell migration and invasion by targeting the ERKs/RSK2 signaling pathway [44]. The present study reveals that the ERK1/2, p38, and JNK pathways are involved in the DA-mediated suppression of MMP-2 activity and expression SCC9 cells, as well

Fig. 4. Effect of NF-κB, SP-1 and AP-1 transcription factors in DA-treatment SCC9 cells. (A, C, E) SCC9 cells were treated with DA for 24 h and then the nuclear fraction was prepared as described in Section 2. Representative results of NF-κB, c-Fos, c-Jun, SP-1 and phosphorylation of IkBα, IkBα/βγ protein levels by Western blot analysis. (B, D, F) Quantitative results of protein levels which were adjusted with the internal control C23 or GAPDH protein level. (G) SCC9 cells were pretreated with IKK 16 for 1 h followed by treatment with DA for 24 h and then subjected to western blotting to analyze the expression of MMP-2. (H) Quantitative results of protein levels which were adjusted with the internal control β-actin protein level. The values represented the mean ± SD from 3 determinations per condition repeated 3 times. *P < 0.05 as compared with the untreated cells. #P < 0.05 as compared with the only DA-treated cells.
as their migration and invasion (Figs. 5 and 6). Our present results are consistent with those of the aforementioned studies.

The initiation of metastasis requires invasion, which is enabled through EMT. EMT and mesenchymal–epithelial transition represent the initiation and completion of the invasion–metastasis cascade, respectively [38]. A critical molecular feature of EMT is the downregulation of E-cadherin expression. E-cadherin acts de facto as a tumor suppressor, inhibiting invasion and metastasis [45]. Vimentin is the major cytoskeletal component of mesenchymal cells [46], and is often used as a marker of mesenchymal-derived cells or cells undergoing EMT during both normal development and metastatic progression [47]. In the present study, DA-treated SCC9 cells exhibited typical epithelial morphology compared with the spindle-shaped mesenchymal morphology of control SCC9 cells (Fig. 7C). Moreover, DA significantly induced E-cadherin and claudin-1 expression and reduced that of Vimentin, Zo-1, Zeb-1, Twist-1, Snail, Slug, N-cadherin, and β-cadherin in SCC9 cells (Fig. 7). Thus, these findings suggest that DA can inhibit oral cancer invasion and metastasis.

In conclusion, this is the first study verifying the anti-metastatic effect of DA in SCC9 cells. Our findings demonstrate that DA inhibits cell invasion and migration by regulating the activities of metastasis-associated proteases, the natural invasion and migration inhibitors, and inhibits the phosphorylation of ERK1/2, p38, and JNK. DA also inhibits the expression of IKKα/β/γ and reduces that of NF-κB and AP-1, resulting in the downregulation of MMP-2 expression and inhibition of metastasis. DA is potentially useful for developing preventive and therapeutic agents for cancer metastasis.

**Author contributions**


**Conflict of interest**

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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**Fig. 5.** DA inhibits MMP-2 expression through inhibition of ERK1/2, p38 and JNK1/2 in SCC9 cells. (A) Cells were treated with different concentrations of DA for 24 h. The levels of phosphorylation of Akt, ERK1/2, p38 and JNK1/2 were investigated by western blotting. (B) Quantitative results of Akt, ERK1/2 and JNK1/2 and p38 protein level which were adjusted with β-actin protein level. Results are shown as mean ± SD. *P < 0.05 compared with the control. (C) SCC9 cells were pretreated with U0126 (20 μM), SP600125 (20 μM) or SB203580 (20 μM) for 1 h followed by treatment with DA for 24 h in serum free medium and then subjected to gelatin zymography to analyze the activity of MMP-2. The values represented the mean ± SD from 3 determinations per condition repeated 3 times. *P < 0.05 as compared with the untreated cells. **P < 0.05 as compared with the only DA-treated cells. (D–F) SCC9 cells were pretreated with U0126 (20 μM), SB203580 (20 μM) or SP600125 (20 μM) for 1 h followed by treatment with DA for 24 h and then subjected to western blotting to analyze the expression of MMP-2. The values represented the mean ± SD from 3 determinations per condition repeated 3 times. *P < 0.05 as compared with the untreated cells. **P < 0.05 as compared with the only DA-treated cells.
Fig. 6. Effect of DA, ERK1/2, p38 and JNK inhibitors on in vitro wound closure, cell migration and invasion in SCC9 cells. (A) SCC9 cells were pre-treated with U0126, SB203580 or SP600125 for 1 h and then incubated in the presence or absence of DA for 12, 24 h and then the cells were then subjected to detect in vitro wound closure. (B–D) Cell migration (upper plane) and invasion assay (down plane). The migration and invasion abilities of SCC9 cells were quantified by counting the number of cells that invaded to the underside of the porous polycarbonate as described in the Section 2. The values represented the mean ± SD from 3 determinations per condition repeated 3 times. *P < 0.05 as compared with untreated cells. #P < 0.05 as compared with the cells treated with only DA (40 μM) or only inhibitors.

Fig. 7. Effects of DA on the EMT were examined by treating SCC9 cells. (A) SCC9 cells were treated with different concentrations of DA for 24 h and then subjected to western blotting. (B) Quantitative results of protein level which were adjusted with β-actin protein level. Results are shown as mean ± SD. *P < 0.05 compared with the control. (C) SCC9 cells were treated with different concentrations of DA for 24 h and then subjected to observe morphology by microscopy. (D) SCC9 cells were treated with different concentrations of DA for 24 h and then subjected to observe EMT status by immunofluorescence staining. DA-treatment SCC9 cells express upregulation of epithelial marker, E-cadherin (green) and downregulation of mesenchymal markers, Vimentin (red), compared to control cells. Original magnifications: 200×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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