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Original Article

Daidzein enhances cisplatin sensitivity and inhibits migration of oral squamous cell carcinoma through modulating mitogen-activated protein kinase signaling pathway

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Abstract *Background/purpose:* Oral squamous cell carcinoma (OSCC), a prevalent head and neck malignancy, is associated with poor survival rates in advanced stages. According to the American Society of Clinical Oncology (2023), the 5-year survival rate is 86 % for localized OSCC but drops to 69 % and 40 % for regional and distant metastases, respectively, underscoring the critical role of metastasis in treatment failure. Despite advances, few chemotherapeutic agents effectively target metastatic OSCC. Daidzein (DZ), a plant-derived isoflavone, has demonstrated anti-metastatic properties in breast and colon cancers.
Materials and methods: This study evaluated DZ's therapeutic potential in OSCC, focusing on its effects on proliferation, migration, invasion, and epithelial–mesenchymal transition (EMT) markers, as well as its ability to enhance cisplatin (Cis) sensitivity.

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Results: Molecular docking showed DZ binds strongly to MMP-2 and MMP-9, with binding energies of −8.87 kcal/mol and −8.96 kcal/mol, respectively. In vitro, DZ dose-dependently inhibited OSCC cell proliferation and significantly reduced anchorage-independent growth, invasion, and migration. When combined with Cis, DZ exerted a synergistic inhibitory effect on metastatic properties. Mechanistically, DZ suppressed MMP-2 and MMP-9 expression, reduced ERK1/2 and p38 phosphorylation in the MAPK pathway, and modulated EMT-associated markers.

Conclusion: In conclusion, DZ suppresses MMP-2 and MMP-9 expression, inactivates MAPK signaling (ERK1/2 and p38), and inhibits EMT, thereby reducing OSCC migration and invasion. Its biphasic effects on Cis cytotoxicity highlight the potential for optimized combination therapies to prevent OSCC dissemination and metastasis.

Introduction

Oral squamous cell carcinoma (OSCC), accounting for over 90 % of oral cancer cases, represents a significant global health burden with approximately 389,000 new cases and 180,000 deaths reported in 2022.[1] The disease develops through malignant transformation of epithelial cells in the oral cavity and oropharynx, affecting various tissues including the lips, tongue, gums, palate, and cheeks. Its etiology is multifactorial, with primary risk factors including chronic oral irritation from betel quid chewing, smoking, human papillomavirus (HPV) infection, alcohol consumption, and certain dietary habits such as excessive intake of spicy foods.[2] While surgical resection combined with radiotherapy and chemotherapy remains the primary treatment modality, advanced or inoperable cases rely heavily on chemotherapy. However, conventional chemotherapeutic approaches are associated with significant adverse effects, including cardiotoxicity, ototoxicity, and cognitive impairment.[3—6] Despite advancements in therapeutic strategies, the 5-year survival rate for OSCC remains dismal, particularly in metastatic cases, underscoring metastasis as a leading cause of mortality (ASCO, 2023).

The metastatic process in OSCC involves complex molecular mechanisms, including local invasion, intravasation, and migration through blood and lymphatic vessels. This process is critically dependent on the interaction between tumor cells and the extracellular matrix (ECM), with matrix metalloproteinases (MMPs) playing essential roles.[7—10] Additionally, EMT significantly contributes to metastasis by enhancing tumor cell motility and invasiveness. Therefore, therapeutic approaches targeting these molecular pathways, particularly those exhibiting minimal toxicity, represent a promising direction in OSCC treatment.

Natural compounds, especially flavonoids derived from herbal medicine, have demonstrated considerable potential in cancer treatment through their ability to modulate carcinogenic pathways, induce apoptosis, and inhibit cancer cell migration and proliferation.[11] Among these compounds, the isoflavone daidzein (DZ) has shown particular promise, demonstrating significant anticancer activity in various malignancies, including breast and colon cancers, through downregulation of MMP-2, MMP-9, and ERK signaling.[12,13] The structural characteristics of DZ, particularly its C4 oxygen group and C2—C3 double bond, contribute to its anti-angiogenic properties, which are crucial for inhibiting tumor growth and metastasis.[14,15]

Despite these promising findings in other cancer types, the effects of DZ on OSCC migration and invasion remain inadequately explored. Therefore, this study aimed to investigate the anti-metastatic potential of DZ in human OSCC cells, with a specific focus on its ability to inhibit migration and invasion through modulation of key signaling pathways. Furthermore, we examined the potential synergistic effects between DZ and Cis, a standard chemotherapeutic agent, to develop more effective therapeutic strategies for OSCC treatment. Understanding these mechanisms could provide valuable insights for developing novel therapeutic approaches that combine the anticancer properties of natural compounds with conventional chemotherapy, potentially improving treatment outcomes while minimizing adverse effects.

Materials and methods

Daidzein

Daidzein (DZ, purity ≥98 %) was obtained from Thermo Fisher Scientific, Waltham, MA, USA and a 10 mM stock solution was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. The final DMSO concentration in all treatments was <1 %. Cisplatin (cis-Diamine platinum (II) dichloride) was dissolved in phosphate-buffered saline (PBS) at 5 mM and stored at −20 °C.

Molecular docking simulation

Molecular docking was conducted as previously described. DZ's 3D structure was retrieved from PubChem and converted into a PDB file using PyMOL (Schrödinger, Inc, New York, NY, USA). The crystal structures of MMP-2 (PDB ID: 1QIB), MMP-9 (PDB ID: 1L6J), caspase-3 (PDB ID: 1RE1), and caspase-8 (PDB ID: 1QTN) were obtained from the Protein Data Bank (Piscataway, NJ, USA). Using AutoDockTools 1.5.7, water molecules were removed from the PDB files, hydrogen bonds were added, and the files were saved as PDBQT. Docking was performed with AutoDock Vina, where

the docking box was centered on the receptor, covering the entire protein. The binding energy (kcal/mol) was used as an indicator of receptor-ligand affinity. Docking results were visualized in PyMOL.

Cell culture

Smulow-Glickman human gingival epithelial (SG) and human gingival carcinoma (Ca9-22) cell lines were cultured in DMEM with 10 % FBS and 1 % PSA at 37 °C with 5 % CO2. The human tongue squamous cell carcinoma (SAS) cell line was cultured in DMEM/F12 with 10 % FBS and 1 % PSA under the same conditions.

Cell viability assay

Cells (1×10^4/well) were seeded in a 96-well plate and incubated at 37 °C for 24 h. The culture medium was replaced with medium containing Cis and DZ, and cells were cultured for 24 h. PrestoBlue™ (10 %) was added, and after 2 h, absorbance was measured at 570 nm using a spectrophotometer to determine relative cell viability.

Wound healing assay

A linear wound was created in a monolayer of cells cultured in a 12-well plate. After washing with PBS, cells were cultured in serum-free medium with DZ and Cis for 24 h. Wound closure was photographed at 0 and 24 h, and the percentage of closure was calculated using Image J.

Transwell migration and Matrigel invasion assays

Transwell assays were performed in 24-well plates. SAS and Ca9-22 cells (1×10^5/well) were seeded in serum-free medium in the upper chamber, and medium with 10 % FBS was added to the lower chamber. Cells were treated with DZ and Cis for 24 h, fixed with methanol, stained with 0.1 % crystal violet, and the absorbance at 590 nm was measured. For Matrigel invasion assays, cells (1×10^6/well) were seeded in the upper chamber pre-coated with Matrigel.

Western blot analysis

Protein samples were mixed with 4X loading buffer, heated at 95 °C for 5 min, and separated by SDS-PAGE (8—10 % gel). After transfer to a nitrocellulose membrane, blocking was done with 5 % skimmed milk in TBST for 1 h. Membranes were incubated with primary antibody overnight at 4 °C, followed by secondary antibody incubation at room temperature for 1 h. Signal was detected using a luminescence system and analyzed with Image J software.

Statistical analysis

Data were analyzed using JMP 16.2.0 and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). All experiments were repeated at least three times. Statistical significance was determined using Student's t-test or one-way ANOVA, with Tukey's post hoc test, and was considered significant at $P < 0.05$. Results are expressed as mean ± SD.

Results

To evaluate the anticancer effects of DZ, we first examined its cytotoxicity on normal oral cells (SG) and OSCCs (Ca9-22 and SAS). SAS and Ca9-22 exhibiting moderate and high metastatic potentials respectively, are valuable models for investigating the mechanisms underlying OSCC metastasis, including cell migration, invasion, and angiogenesis.[16] The results showed a dose-dependent decrease in OSCC viability as the concentration of DZ increased (Fig. 1B and C). At higher concentrations (>50 μM), OSCC viability was significantly reduced (Fig. 1B and C), while SG cell viability remained above 80 % (Fig. 1A). These findings suggest that DZ has the potential to selectively inhibit cancerous cells. Next, we investigated whether DZ could enhance the chemosensitivity of OSCCs. Cells were treated with 100 μM DZ in combination with Cis at various concentrations. The results showed that 100 μM DZ significantly potentiated the cytotoxicity of low-dose Cis (3.125—12.5 μM) on OSCC viability compared to Cis treatment alone (Fig. 1D—F). However, at higher concentrations of Cis (25—100 μM), DZ significantly restored cell viability (Fig. 1D—F).

Next, to assess whether DZ could inhibit the migratory properties of OSCCs, we first determined the half-maximal inhibitory concentration (IC_{50}) of Cis on OSCC viability. The results demonstrated that 25—100 μM DZ significantly suppressed wound-healing migration in both Ca9-22 and SAS cells. Notably, while Cis inhibited wound closure in SAS cells (Fig. 2B and D) and caused significant cell death (Fig. 2B), no significant increase in detached cells was observed in Ca9-22 cells treated with 25—100 μM DZ (Fig. 2A and C). Thus, the inhibitory effect of DZ on wound closure ability may not be solely attributable to its cytotoxicity.

To further confirm the effects of DZ on OSCC metastatic properties, we employed a Transwell system to evaluate the impact of combined DZ and Cis treatment on OSCC migration and invasion. The results revealed that the inhibitory effect of Cis on cell migration and invasion was weaker compared to its effect on wound healing (Fig. 3). Importantly, 100 μM DZ effectively reduced the migration and invasion abilities of both Ca9-22 and SAS cells (Fig. 3). Furthermore, the addition of Cis did not significantly enhance the inhibition of migration and invasion when OSCCs were exposed to 50 or 100 μM DZ (Fig. 3).

Epithelial cancer cells undergoing EMT lose intercellular contacts and gain increased migratory capacity. In the Transwell migration assay, OSCC colonies exposed to DZ exhibited a more clustered morphology compared to untreated controls (Figs. 3A and 4B). Consistent with this observation, DZ treatment significantly increased the expression of the epithelial marker E-cadherin (Fig. 4A and B) while suppressing the expression of mesenchymal markers such as vimentin (Fig. 4C and D) and Twist (Fig. 4E and F) in OSCCs. Although the combination of DZ and Cis appeared to augment DZ-mediated inhibition of EMT, no statistically significant difference was observed (Fig. 4).

The MAPK pathway, including ERK1/2, p38, and JNK, is known to play a crucial role in driving EMT in head and neck

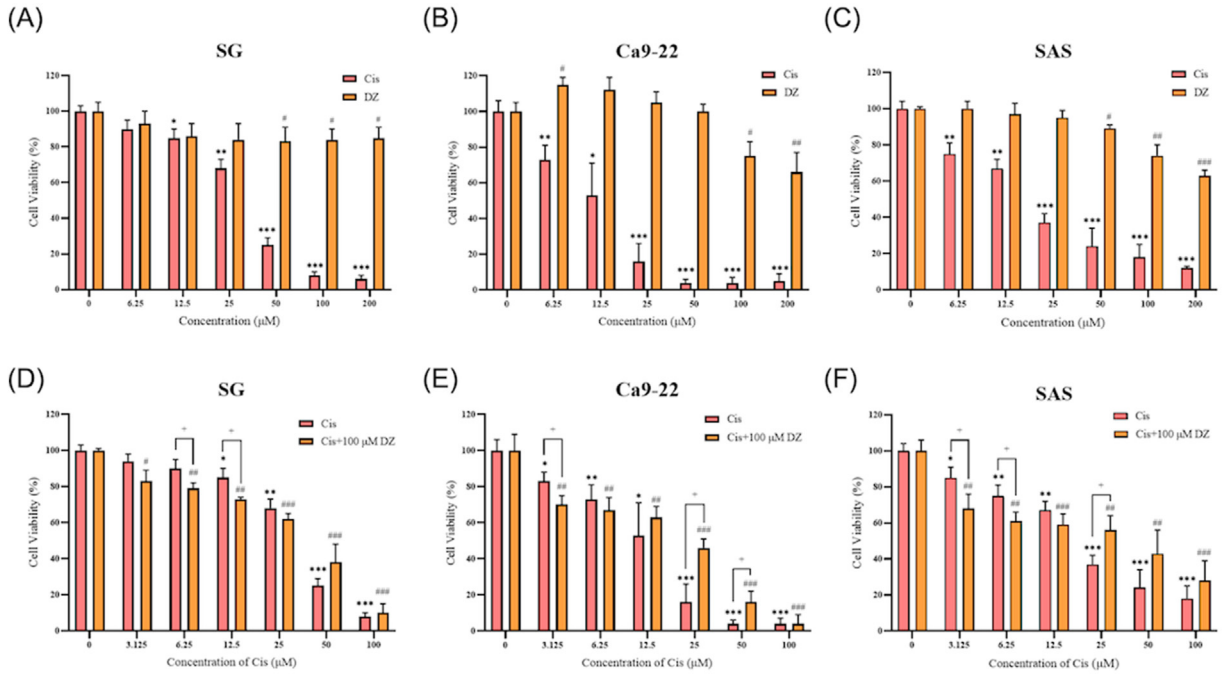

Figure 1 Cisplatin (Cis) and daidzein (DZ) inhibit cell viability. The SG (A), Ca9-22 (B), and SAS (C) cell lines were treated with varying concentrations (0–200 μM) of Cis and DZ for 24 h, and cell viability were assessed. A synergistic effect was observed at low doses of Cis when co-administered with 100 μM DZ, whereas antagonistic effects were evident at higher doses of Cis + DZ (D–F). Data are presented as mean ± SD from at least three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to Cis control group. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with DZ control group. +$P < 0.05$ compared to respective Cis only group.

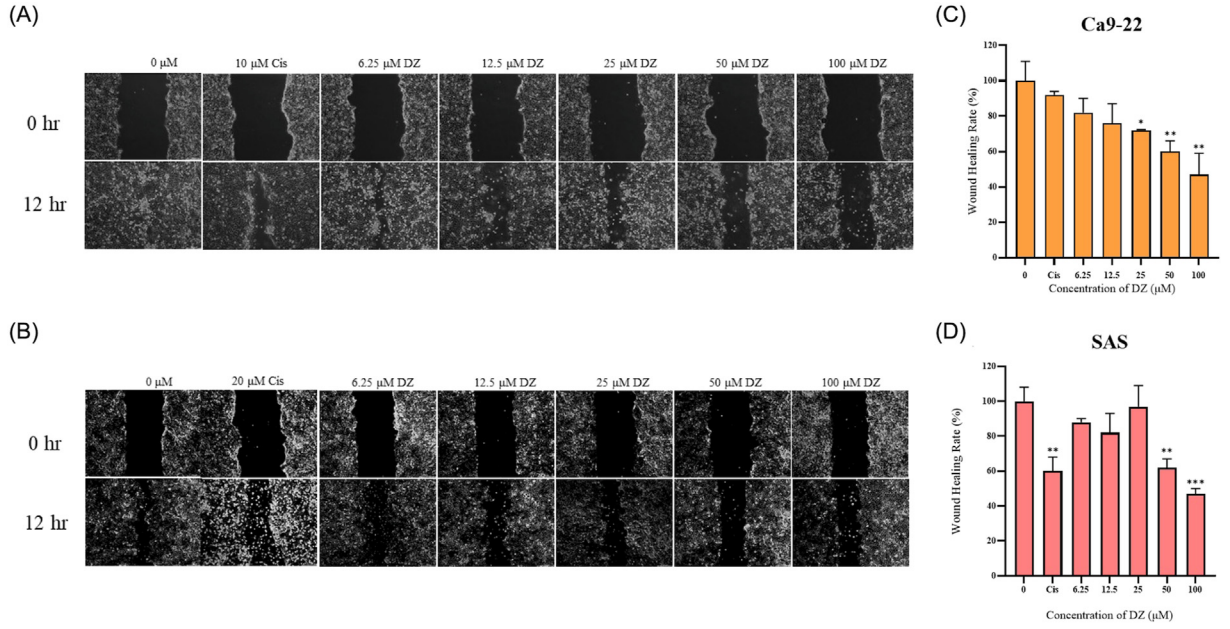

Figure 2 Daidzein (DZ) inhibits wound healing in oral cancer cells. Wound healing assays were conducted on Ca9-22 (A, C) and SAS (B, D) cells treated with increasing concentrations of Cis and DZ for 12 h. Representative images illustrate wound healing abilities, with cell morphologies visualized under a microscope (scale bar, 100 μm). Results are derived from at least three independent experiments, with statistical significance indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to untreated controls.

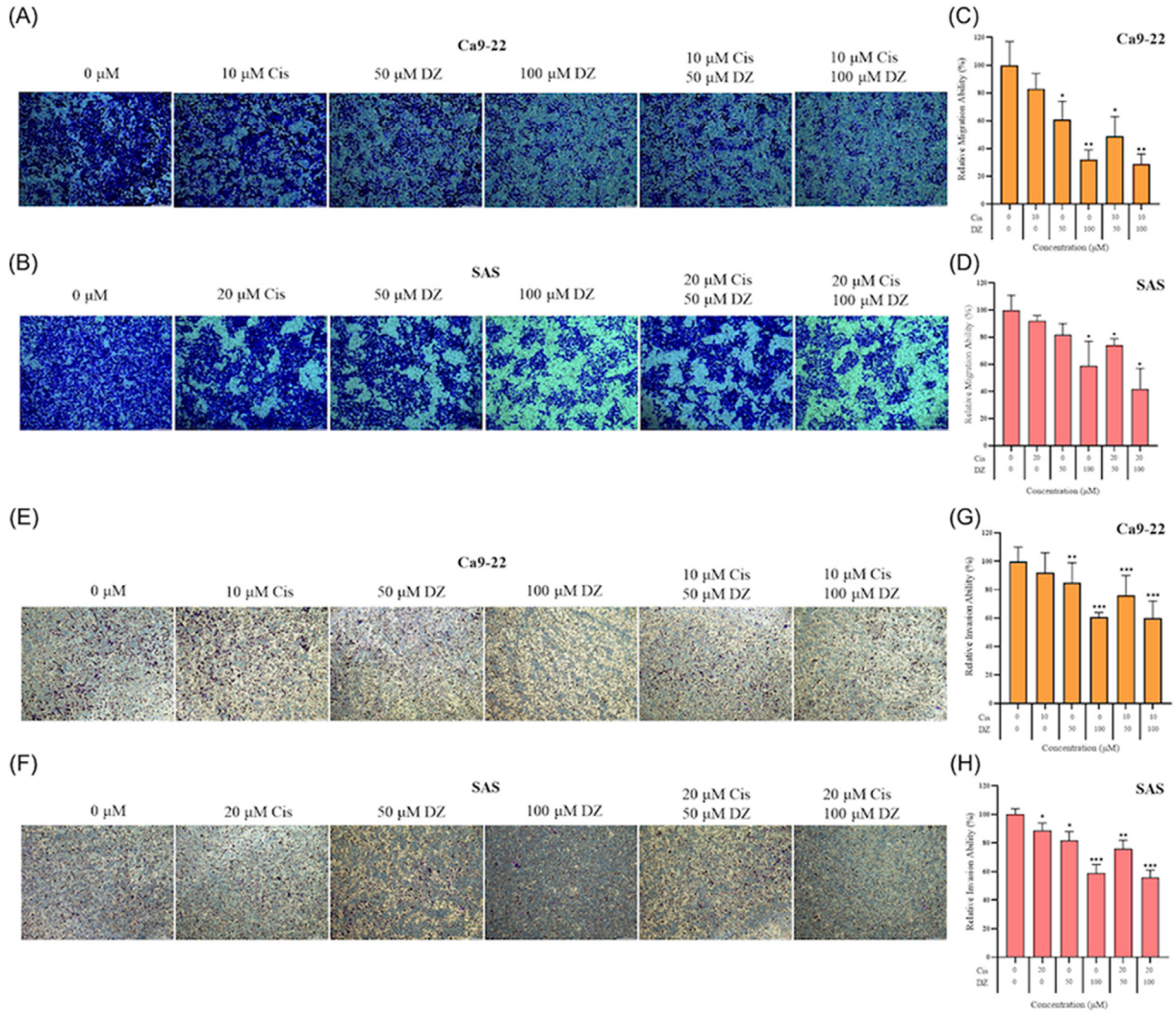

Figure 3 Daidzein (DZ) inhibits migration and invasion abilities in oral cancer cells. Migration and invasion abilities in oral cancer cells were evaluated using Transwell assays following 24-h treatments with increasing concentrations of Cis and DZ. Representative images and histograms illustrate migration (A—D) and invasion (E—H) abilities in Ca9-22 and SAS cells. Morphologies were imaged under a microscope (scale bar, 100 μm). Results are derived from at least three independent experiments, with statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated controls.

cancer cells. To investigate the effects of DZ and Cis on MAPK signaling activity, we examined the phosphorylation of ERK1/2, p38, and JNK in OSCCs. Both DZ and Cis treatments significantly reduced the levels of phosphorylated ERK1/2 (p-ERK1/2) in Ca9-22 and SAS cells (Fig. 5A and B). While a decrease in phosphorylated p38 (p-p38) levels was observed in both cell lines treated with 50 and 100 μM DZ, a statistically significant reduction was only detected in Ca9-22 cells treated with 100 μM DZ (Fig. 5E and F). Additionally, the combination of DZ and Cis further reduced p-ERK1/2 and p-p38 levels compared to DZ alone (Fig. 5A, B, E, and F). In contrast, no significant changes in JNK phosphorylation were detected following treatment with DZ, Cis, or their combination (Fig. 5C and D).

To elucidate how DZ regulates MAPK activity and migratory properties in OSCCs, molecular docking simulations were conducted. The results identified MMP-2 and MMP-9 as potential targets of DZ (Table 1 and Fig. 6). MMP-2 was found to form hydrogen bonds with DZ through LEU164 and ALA165, yielding a binding energy of −8.87 kcal/mol. Similarly, MMP-9 formed a hydrogen bond with DZ via GLU416, with a binding energy of −8.96 kcal/mol. Consistent with these findings, treatment with 50 and 100 μM DZ significantly reduced MMP-2 and MMP-9 levels in both Ca9-22 and SAS cells (Fig. 7). Although the combination of DZ and Cis appeared to enhance the inhibitory effects on MMP-2 and MMP-9, no statistically significant difference was observed.

Discussion

Previous studies have demonstrated that DZ significantly inhibits cell migration and invasion while exerting minimal effects on cancer cell viability and proliferation.[17,18] Our research revealed that DZ at concentrations of 25—100 μM effectively suppressed two-dimensional migration ability in OSCCs, showing a clear dose-dependent relationship.

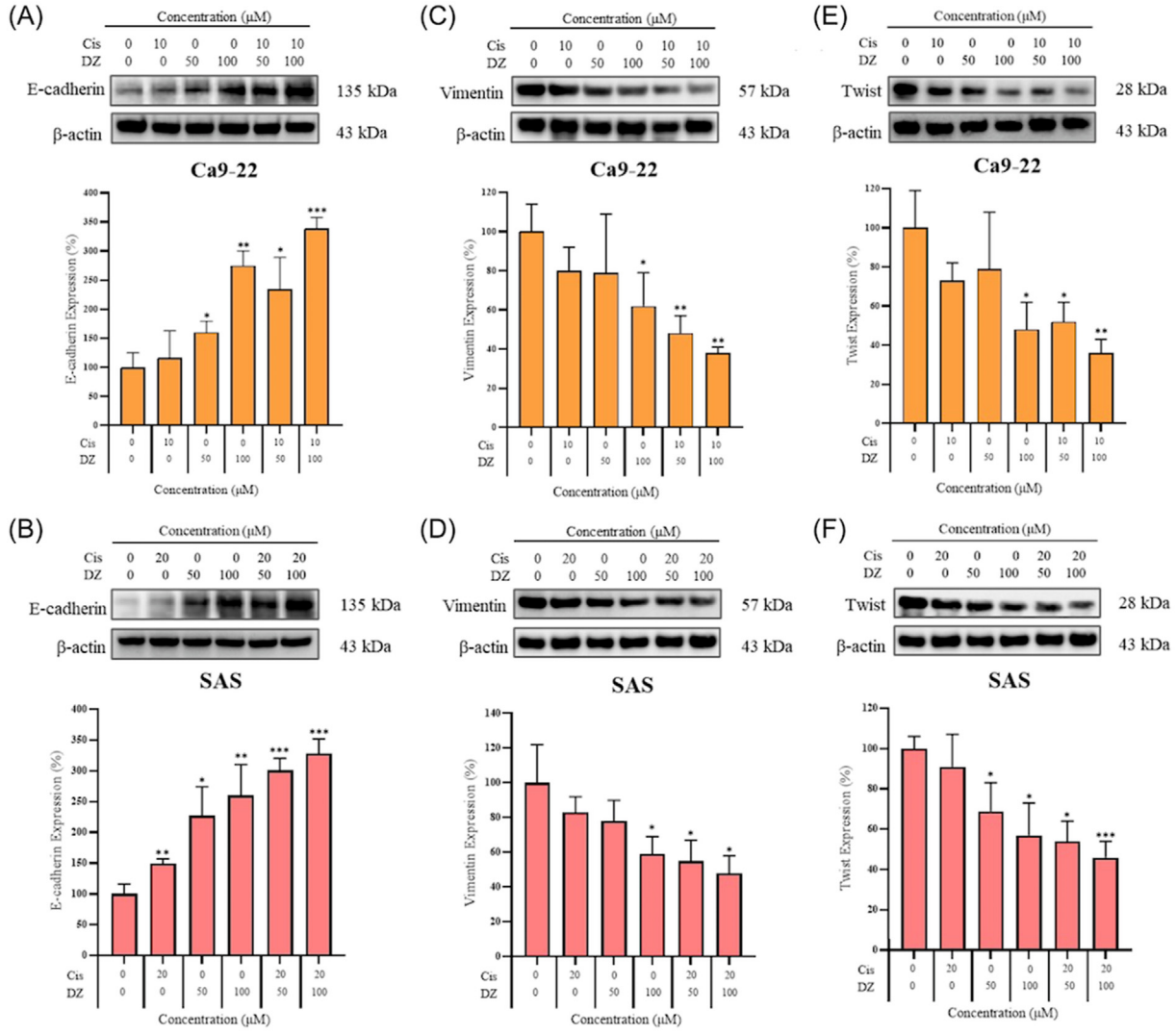

Figure 4 Daidzein (DZ) affects epithelial-mesenchymal transition (EMT)-related protein expression in oral cancer cells. Western blot analysis was performed to examine EMT markers, including E-cadherin (A, B), vimentin (C, D), and twist (E, F), in Ca9-22 and SAS oral cancer cells following 24-h treatments with DZ and Cis. Histograms depict the expression levels of these proteins, quantified using ImageJ software. Results are representative of at least three independent experiments, with statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated controls.

Furthermore, at concentrations of 50 and 100 μM, DZ significantly inhibited both vertical migration and invasion capabilities of OSCCs in the Transwell system. These findings strongly suggest DZ's potential as an anti-metastatic agent against OSCC. Given that lymph node invasiveness represents a critical factor in OSCC progression and poor patient prognosis,[19] the observed synergistic effect between DZ and low-dose Cis on OSCC cell viability presents an intriguing therapeutic opportunity. This combination strategy could potentially allow for reduced Cis dosages while effectively restraining tumor spread and preventing malignant progression. DZ enhanced the cytotoxic effects of low-dose Cis by inhibiting MAPK pathways, thereby amplifying sublethal damage. However, at high-dose Cis, the extensive oxidative stress induced overwhelmed DZ's MAPK inhibition, allowing its antioxidant and cytoprotective effects to dominate and promote cell survival.

EMT plays a crucial role in cancer progression, where cancer cells lose their epithelial characteristics and acquire mesenchymal phenotypes, leading to enhanced migration and invasiveness.[19] EMT phenotypes are predominantly expressed in cancer cells located at the invasive leading edges of primary tumors, where interaction with the tumor microenvironment creates favorable conditions for invasion. Transcriptomic analyses of head and neck SCC cohorts have identified higher EMT scores as predictive factors for nodal metastases and advanced N stage.[19] In our investigation, DZ-treated OSCCs formed more distinct colonies compared to the dispersed pattern of untreated cells, suggesting enhancement of the epithelial phenotype. This observation was further supported by increased expression of the epithelial marker E-cadherin and suppressed expression of mesenchymal markers Twist and Vimentin, indicating DZ's role in inhibiting EMT in OSCC.

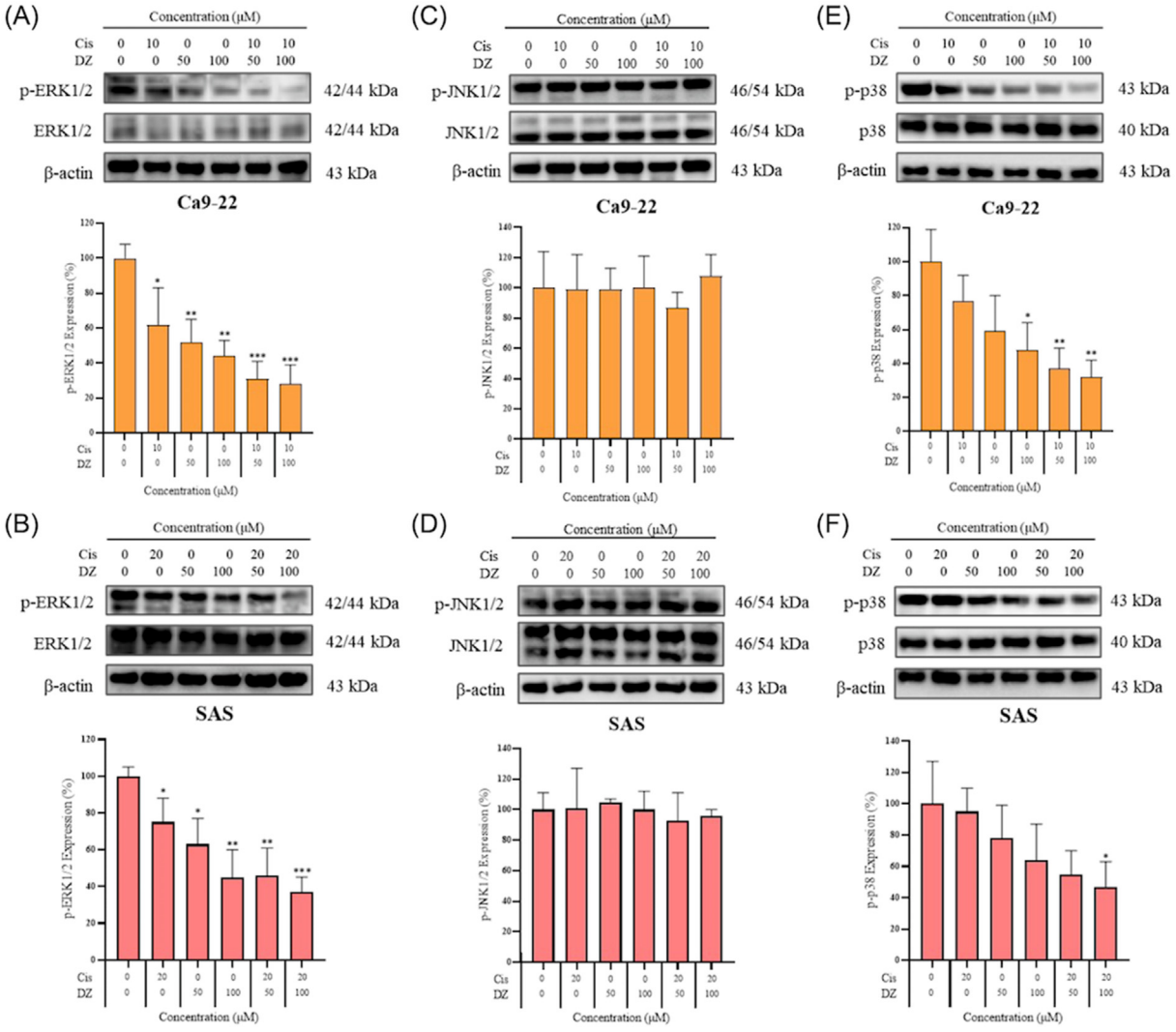

Figure 5 Daidzein (DZ) modulates mitogen-activated protein kinase (MAPK) signaling pathway in oral cancer cells. Western blot analysis was used to assess the expression of MAPK signaling, including total-/p-ERK1/2 (A, B), total-/p-JNK1/2 (C, D), and total-/p-p38 (E, F), in Ca9-22 and SAS cells after 24-h treatments with DZ and Cis. Protein expression levels were quantified based on at least three independent experiments, with statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated controls.

Table 1 Quantification of molecular docking interactions between daidzein (DZ) and active site of MMP-2 and MMP-9.

Ligand	Protein	Interacting residue	Nature of bonding	Interacting distance (Å)	Binding energy (kcal/mol)
DZ	MMP-2	LEU164	Hydrogen bond	1.966	−8.87
		ALA165	Hydrogen bond	2.109	
	MMP-9	GLU416	Hydrogen bond	2.067	−8.96

Abbreviations: DZ: Daidzein.

Previous research has shown that TNFα-induced EMT in OSCC cells can be blocked by inhibiting MAPK signaling pathways, including ERK, JNK, and p38.[20] Tan and colleagues demonstrated that pre-treatment with 50 μM DZ significantly inhibited the LPS-induced pro-inflammatory response in mouse macrophages by reducing ERK and p38 activity, without affecting JNK10.[21] Consistent with these findings, our study showed that 50 and 100 μM DZ significantly reduced phosphorylation of ERK1/2 and p38 in OSCC, while total and phosphorylated JNK remained unaltered. This suggests that DZ targets specific kinases within the ERK and p38 pathways, possibly due to structural differences in the kinase active sites or variations in DZ's binding affinity to these kinases. Further investigation into DZ's

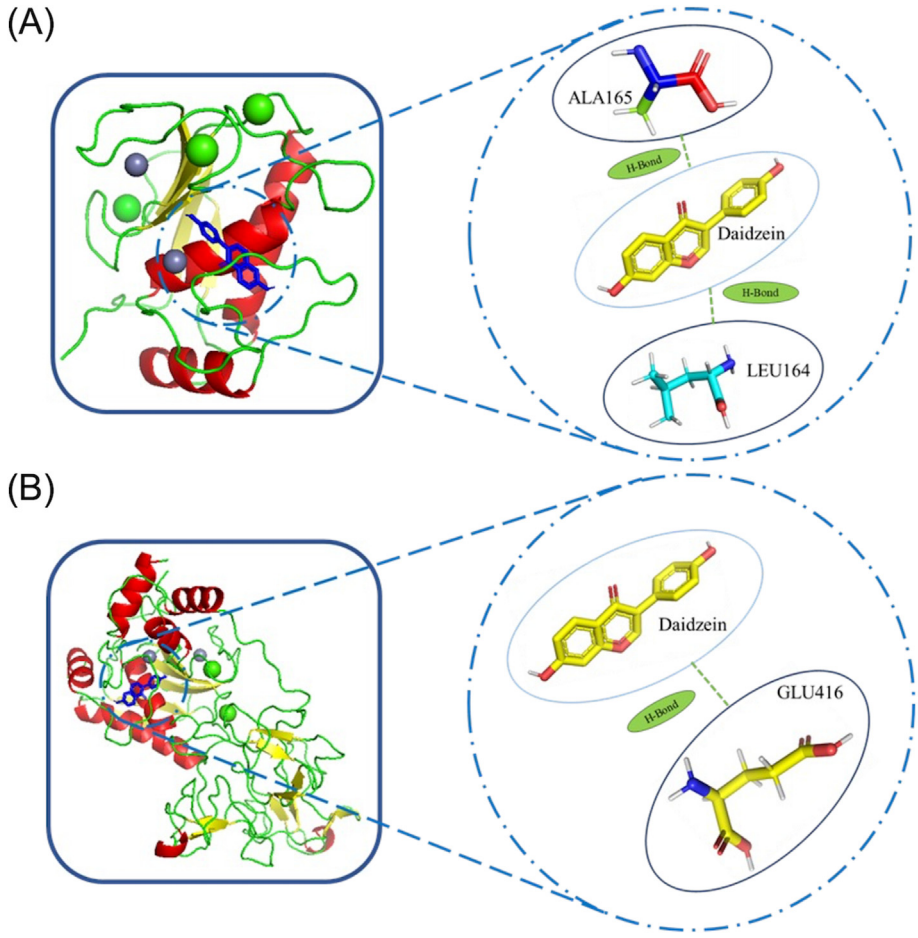

Figure 6 Molecular docking of Daidzein (DZ) with matrix metalloproteinase (MMP)-2 and MMP-9. Visualization of molecular docking results showing DZ binding to MMP-2 (A) and MMP-9 (B). Hydrogen bonds are illustrated as dashed green lines, indicating interactions within the active sites. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

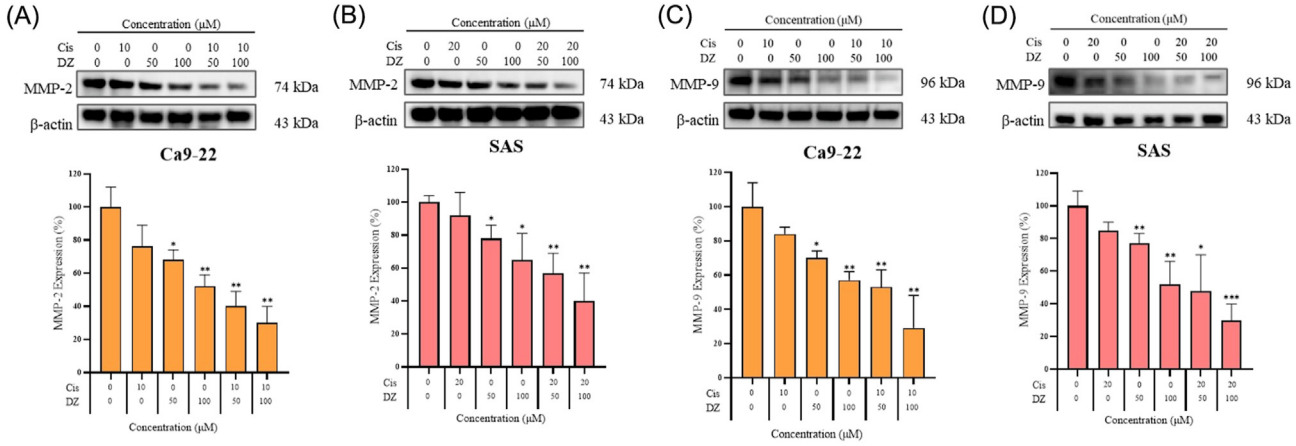

Figure 7 Daidzein (DZ) affects matrix metalloproteinase (MMP)-2 and MMP-9 expression in oral cancer cells. Western blot analysis was conducted to evaluate the expression of MMP-2 (A, B) and MMP-9 (C, D) in Ca9-22 and SAS cells after 24-h treatments with DZ and Cis. Protein expression levels are shown in histograms, with quantification based on at least three independent experiments, with statistical significance indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated controls.

interactions with specific components of the MAPK pathways is needed to understand the precise mechanisms underlying this selectivity.[21]

DZ and its glucoside form, daidzin, are primary isoflavones found in soy.[21] Research has revealed that daidzin down-regulated the PI3K/Akt/mTOR pathway by inhibiting superoxide dismutase (SOD)-2 in A549 cells, thereby suppressing EMT and cellular migration and invasion.[22] Notably, H_2O_2 treatment restored the EMT phenotype and cellular migration/invasion capabilities in SOD2-knockdown tongue OSCCs,[23] suggesting SOD2-mediated H_2O_2 production as a key EMT driver. Given the inconsistent evidence regarding DZ's effects on SOD2 expression,[24,25] further investigation is warranted to determine whether DZ inhibits EMT in OSCCs partly through SOD2 suppression.

MMPs secretion and subsequent ECM remodeling are crucial for cancer cell invasion and metastasis.[26] Multiple studies have demonstrated DZ's ability to reduce MMPs expression through inhibition of upstream signaling.[13,27,28] Liu et al. showed that p38 activity and MMP-2 expression in aortic tissues from angiotensin II (AngII)-induced abdominal aortic aneurysm mice were down-regulated following DZ administration.[27] Bao et al. reported DZ's inhibition of TNFα-induced MMP-9 expression and enzymatic activity, along with reduced migration and invasion in breast cancer cells.[28] In colon cancer cells, Salama et al. showed that MMP-9 inhibition following DZ treatment was associated with a reduction in ERK and AKT activity.[13] In this study, our findings suggest a mechanism by which DZ directly binds to suppress MMP-2 and MMP-9 expression (Figs. 6—7 and Table 1). This aligns with previous evidence on plant-derived bioflavonoids. For instance, Agraval et al. demonstrated that fisetin, another bioflavonoid, inhibits MMP-2 and MMP-9, subsequently reducing downstream ERK and β-catenin pathway activation. This inhibition blocked cigarette smoke extract-induced EMT, invasion, and migration in human airway epithelial cells.[29] Similarly, the DZ-induced inactivation of ERK1/2 and p38 observed in this study, as well as its inhibition of EMT phenotypes in OSCC cells (Figs. 4 and 5), may be partially mediated through the suppression of MMP-2 and MMP-9.

However, alternative mechanisms have been proposed. Oh et al. suggested that DZ acts as an agonistic ligand for the retinoic acid receptor, reducing its transcriptional activity and thereby decreasing MMP-9 expression in HaCaT cells.[30] Thus, further studies are required to delineate the precise molecular pathways by which DZ inhibits MMP-2 and MMP-9 expression in OSCC cells. Our results support a mechanism whereby DZ suppresses MMP-2 and MMP-9 expression through direct binding. This finding aligns with previous research showing that inhibition of MMP-2 or MMP-9 attenuates the metastatic properties of OSCCs and lymphatic invasion of OSCC tumors.[31,32] Transcriptome analysis of metastatic and non-metastatic OSCC tumors has demonstrated that low E-cadherin expression and high MMP-2/9 expression significantly correlate with advanced cancer stage.[33] Taken together, these findings underscore the potential of DZ to inhibit OSCC tumor progression, especially by preventing OSCC tumor dissemination and metastasis.

In conclusion, our study reveals that DZ suppresses MMP-2 and MMP-9, leading to MAPK signaling inactivation (ERK1/2 and p38) and subsequent inhibition of EMT, ultimately reducing OSCC cell migration and invasion. The biphasic effects of DZ on Cis cytotoxicity suggest that optimizing the dosages of both compounds could provide a promising therapeutic strategy to prevent OSCC dissemination. These findings collectively support DZ's potent antitumor effect, particularly in preventing OSCC invasion and metastasis.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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