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Original Article

4′-hydroxywogonin inhibits oral squamous cell carcinoma progression by targeting Gas6/Axl signaling axis

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KEYWORDS
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Abstract *Background/purpose:* Oral squamous cell carcinoma (OSCC) is one of the most common head and neck malignancies, with current therapeutic strategies often limited by low efficacy and drug resistance. In this study, we investigated the anticancer potential and underlying mechanisms of 4′-hydroxywogonin, a flavonoid extracted from Scutellaria baicalensis, in OSCC.
Materials and methods: OSCC cell lines were treated with 4′-hydroxywogonin, and its anticancer effects were evaluated using cell functional assays. Western blot analysis was performed to examine the regulatory pathways involved, and rescue assays were conducted to validate its mechanism of action.
Results: 4′-Hydroxywogonin selectively inhibited OSCC cell proliferation, migration, and invasion without inducing cytotoxicity in normal human cells. Mechanistic studies revealed that 4′-

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hydroxywogonin downregulated Axl and its ligand Gas6, leading to the suppression of PI3K/AKT signaling, cell cycle checkpoint regulation, and epithelial—mesenchymal transition (EMT)-related proteins, ultimately inducing G1 phase cell cycle arrest and apoptosis. Moreover, the combination of 4′-hydroxywogonin with cisplatin significantly enhanced its inhibitory effects on OSCC.

Conclusion: 4′-Hydroxywogonin exhibits potential as a novel therapeutic agent for OSCC, with the capacity to enhance cisplatin-mediated anticancer effects, highlighting its potential in combination therapy.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common head and neck malignancy, accounting for over 90 % of oral cancers.[1] Each year, approximately 370,000 new cases and 170,000 deaths occur worldwide, with high incidence rates in South Asia, Southeast Asia, and South America due to risk factors such as betel quid chewing, smoking, alcohol consumption, and HPV infection.[2–4] Current OSCC treatment involves surgical resection, chemotherapy, and radiotherapy; however, its high metastatic potential and recurrence rate lead to relapse in ∼50 % of patients within three years.[5,6] Moreover, chemotherapy-related toxicity significantly reduces patients' quality of life, necessitating the development of novel, effective therapies with reduced side effects.

Axl, a receptor tyrosine kinase (RTK) of the TAM family (Tyro3, Axl, and Mer), plays a key role in tumor progression, metastasis, and drug resistance.[7,8] Activated by Growth Arrest-Specific 6 (Gas6), Axl triggers PI3K/AKT, MAPK/ERK, and NF-κB pathways, enhancing tumor survival, proliferation, epithelial—mesenchymal transition (EMT), and immune evasion.[9] Additionally, Axl and Tyro3 regulate phagocytosis and inflammation, suppressing pro-inflammatory cytokines (IL-6, TNF, type I IFNs, IL-12) and promoting an immune-tolerant microenvironment.[10,11] Axl overexpression also correlates with cancer stem cell (CSC) maintenance, further contributing to tumor relapse and therapy resistance.[12,13] Due to its pivotal role in cancer progression, Axl inhibitors such as BGB324 (Bemcentinib) and R428 (BMS-777607) have shown promising preclinical and clinical efficacy, reducing tumor invasion, restoring chemosensitivity, and enhancing anti-tumor immunity.[14,15] Thus, Axl inhibition is an attractive therapeutic strategy, particularly in combination therapy with chemotherapy, immune checkpoint inhibitors, or radiotherapy.

Natural compounds are crucial sources for drug discovery, with clinically approved therapeutics such as penicillin, paclitaxel (Taxol), and camptothecin.[16,17] Scutellaria baicalensis, an herbal medicine widely used in Southeast Asia, contains flavonoids with potential anticancer properties. 4′-Hydroxywogonin, a flavonoid extracted from Scutellaria baicalensis, has been shown to inhibit VEGF-A expression and PI3K/AKT signaling, reducing angiogenesis in colorectal cancer.[18] However, its potential therapeutic effects on OSCC remain unexplored. This study aims to systematically evaluate the anticancer efficacy of 4′-hydroxywogonin and elucidate its underlying molecular mechanisms. By targeting key oncogenic pathways, this research seeks to uncover novel therapeutic opportunities for the development of effective, targeted treatments against OSCC.

Materials and methods

Cell lines, antibodies, plasmids, and reagents

The human OSCC cell lines OECM1 and SAS were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum at 37 °C in a 5 % CO_2 atmosphere. Primary antibodies against Gas6 (#67202), Axl (#8661), phospho-PI3K (#4228), PI3K (#4292), phospho-AKT (#9271), AKT (#9272), p21 (#2947), cyclin D1 (#2978), CDK4 (#12790), cleaved caspase-3 (#9664) were purchased from Cell Signaling Technology, Inc. (Cell Signaling Technology, Danvers, MA, USA). Primary antibodies targeting N-cadherin (#127345), vimentin (#100619), snail (#100754), slug (#128796), caspase-3 (#110543) and β-actin (#109639) were obtained from GeneTex, Inc. (GeneTex, Irvine, CA, USA). Secondary antibodies were procured from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The 4′-hydroxywogonin powder, with a purity exceeding 98 % (verified by HPLC), was purchased from Shanghai BS Bio-Tech Co., Ltd. (Shanghai BS Bio-Tech, Shanghai, China). The pcDNA3.1 vector-based plasmid (pcDNA-Axl) used in this study was constructed by GenScript Co. (GenScript, Piscataway, NJ, USA).

Cell transfection

OECM1 and SAS cell lines were seeded in 6-well plates at a density of 3×10^5 cells/well overnight. The cells were transfected with 1 μg plasmid using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Twenty-four or 48 h later, the cells were subjected to cellular function analysis and western blotting.

Western blot analysis

Cells were treated with different concentrations of 4′-hydroxywogonin for 24 or 48 h, then harvested, washed

twice with PBS, and lysed in 150 μL RIPA buffer containing protease inhibitors. Proteins (30 μg) from the supernatant were separated by SDS-PAGE and analyzed by Western blot to assess proteins regulated by 4′-hydroxywogonin. Immunoreactive bands were visualized using an ECL system (Thermo Fisher Scientific, Waltham, MA, USA) and captured with UVP ChemStudio Imaging Systems (Analytik Jena, Thuringia, Germany). Band intensities were quantified using ImageQuant 5.2 (GE Healthcare, Waukesha, WI, USA).

Cell proliferation assay

Cell growth was assessed using the xCELLigence Real-Time Cell Analyzer (Roche Life Science, Indianapolis, IN, USA) following the manufacturer's protocol.

Cell migration assay

Cells were treated with vehicle or different concentration of 4′-hydroxywogonin for 24hr. The migration ability of cells was assessed using ThinCert Tissue Cell Culture Inserts (Greiner Bio-One, Kremsmuenster, Austria) containing an 8 μm mean pore size membrane, as previously described.[19]

Cell invasion assay

Cell invasion activity was evaluated through a Matrigel-based transwell invasion assay, as previously reported.[19]

Cell apoptosis assay and flow cytometry

Cells treated with varying concentrations of 4′-hydroxywogonin for 24 h were trypsinized, fixed in 100 % ethanol for 10 min, and analyzed for apoptosis using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific) and DiOC6/PI double staining, following the manufacturer's protocol. Apoptotic cells were detected with a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell cycle analysis

Cells subjected to different treatments were harvested by trypsinization, washed with PBS, and fixed in 70 % ethanol on ice for 1 h. Afterward, cells were washed again with PBS and stained with a solution containing 50 μg/mL propidium iodide and 100 μg/mL RNase A. DNA content was analyzed using a FACSCalibur flow cytometer (BD Biosciences), and data were processed with CellQuest Pro software (BD Biosciences).

Data analysis

All data are presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were conducted with SPSS version 16.0 and Microsoft Excel 2007. All tests were two-tailed, and statistical significance was defined as $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

Results

4′-hydroxywogonin inhibits OSCC cell growth, migration, and invasion

To evaluate the inhibitory effects and effective concentrations of 4′-hydroxywogonin on OSCC cells, OECM1 and SAS cell lines were treated with increasing concentrations of the compound, followed by cell proliferation analysis. Results showed that 4′-hydroxywogonin significantly inhibited SAS cell growth at concentrations as low as 10 μM, with increasing potency at higher concentrations. Similar results were observed in OECM1 cells. The IC50 values of 4′-hydroxywogonin were 47.43 μM for OECM1 and 41.44 μM for SAS cells (Fig. 1A).

Given that the high invasiveness of OSCC cells is a major contributor to poor patient prognosis, we further examined whether 4′-hydroxywogonin affected cell migration and invasion using wound healing, transwell migration and invasion assays. OSCC cells treated with 4′-hydroxywogonin exhibited a significant, dose-dependent reduction in migration and invasion capabilities (Fig. 1B–H). Additionally, to assess its cytotoxicity in normal human cells, we treated human foreskin fibroblast (HFF) cells with varying concentrations of 4′-hydroxywogonin. The results indicated that 4′-hydroxywogonin did not significantly affect fibroblast growth except at a high concentration (80 μM), demonstrating its selective anticancer activity against OSCC cells (Fig. 1I and J).

4′-hydroxywogonin suppresses Gas6/Axl expression and inhibits downstream PI3K/AKT signaling

Accumulating evidence has shown that Gas6-Axl signaling is hyperactivated in various malignancies, where it contributes to tumor growth, metastasis, apoptosis resistance, and drug resistance.[20,21] To elucidate the clinical significance of Axl in OSCC, we conducted a comprehensive analysis of The Cancer Genome Atlas (TCGA) database. Our results demonstrated that Axl is markedly overexpressed in the majority of head and neck squamous cell carcinoma (HNSCC) specimens and is significantly associated with disease onset and patient prognosis. Notably, patients with elevated Axl expression exhibited significantly reduced overall survival compared to those with low Axl expression, underscoring a strong correlation between Axl overexpression and head and neck cancer progression (Fig. 2A–C).

To explore the regulatory effects of 4′-hydroxywogonin on Axl and its ligand Gas6 expression, OSCC cells were treated with different concentrations of the compound, followed by Western blot analysis. Results revealed that 4′-hydroxywogonin treatment significantly reduced the protein expression levels of Axl and Gas6 in SAS and OECM1 cells, compared to the vehicle control group. Moreover, PI3K/AKT signaling, a key downstream pathway of Axl activation, was markedly suppressed following 4′-hydroxywogonin treatment, indicating that 4′-hydroxywogonin may exerts its anticancer effects by inhibiting Axl/Gas6 signaling and its downstream pathways in OSCC cells (Fig. 2D–E).

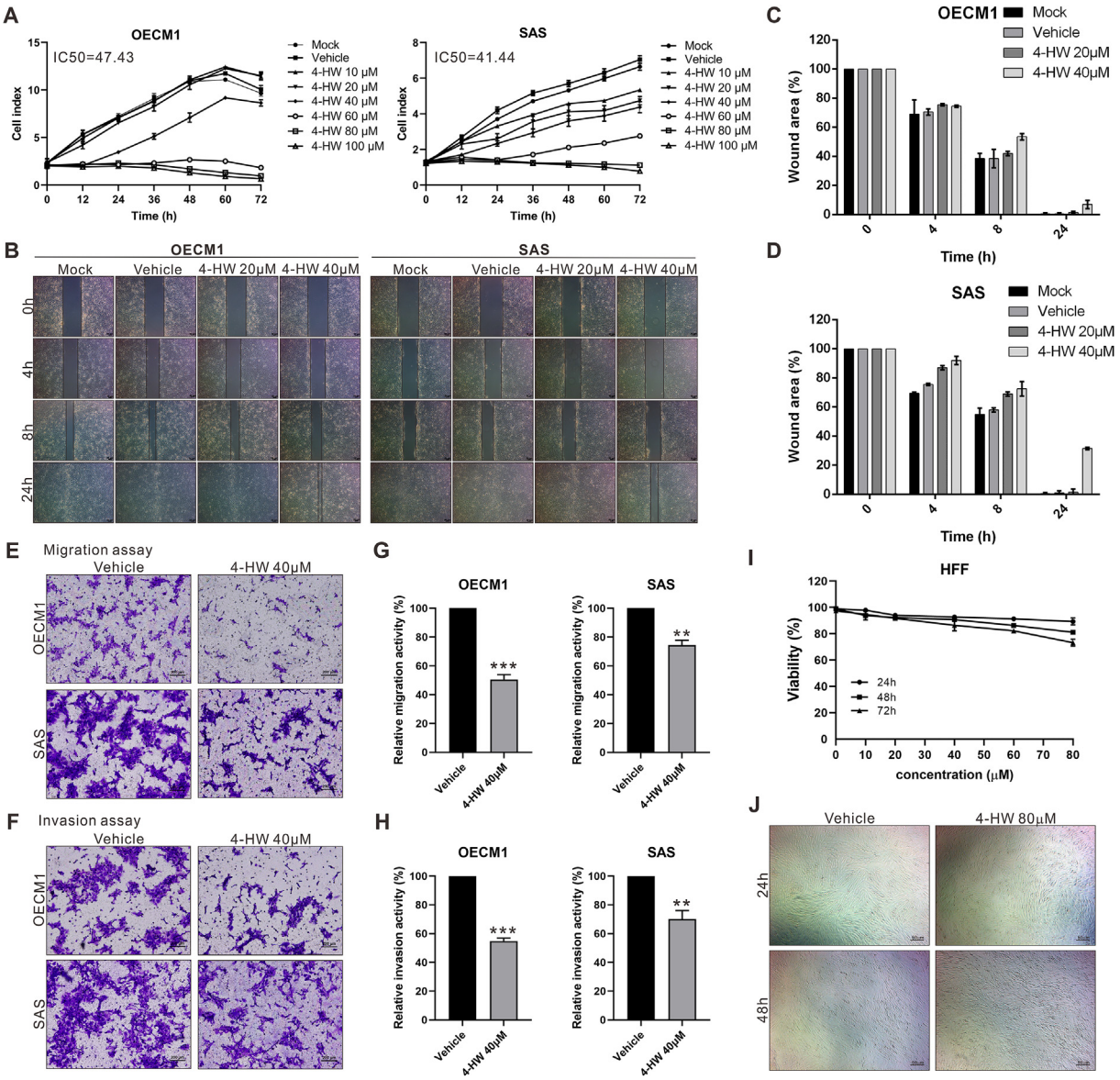

Figure 1 4′-Hydroxywogonin suppresses OSCC cell proliferation, migration, and invasion. (A) Real-time monitoring of OECM1 and SAS cell proliferation following treatment with various concentrations of 4′-hydroxywogonin using the xCELLigence system. (B) Wound healing assay measuring the effect of 4′-hydroxywogonin on OSCC cell motility. The quantitative results are shown in the (C) and (D). (E, F) Transwell migration and invasion assays evaluating the inhibition of OSCC cell motility. The quantitative results are shown in the (G) and (H). (I) Trypan blue assay assessing the viability of human foreskin fibroblasts (HFF) after 4′-hydroxywogonin treatment. (J) Morphological changes in HFF cells after 4′-hydroxywogonin treatment. Significant differences versus vehicle control: $P < 0.01$ (**), $P < 0.001$ (***). Vehicle: cells treated with DMSO. 4-HW: cells treated with 4' -hydroxywogonin. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4′-hydroxywogonin induces cell cycle arrest and EMT suppression in OSCC cells

Gas6-Axl signaling has been implicated in the regulation of cell cycle checkpoint proteins, including p21, cyclin D1, and CDK4, which play a crucial role in cell cycle progression.[22] To determine whether 4′-hydroxywogonin affects OSCC cell cycle progression, flow cytometry analysis was performed. The results demonstrated that OSCC cells treated with 20 μM 4′-hydroxywogonin exhibited G1 cell cycle arrest, which was further enhanced at 40 μM, along with a marked increase in apoptosis compared to the control group (Fig. 3A—D). Furthermore, the results of DiOC6 staining confirmed that 4′-hydroxywogonin induces apoptosis in OSCC cells in a dose-dependent manner (Fig. 3E).

Western blot analysis further revealed that 4′-Hydroxywogonin treatment upregulated p21 expression while downregulating cyclin D1 and CDK4 levels, confirming that 4′-hydroxywogonin inhibits OSCC cell growth by disrupting cell cycle progression (Fig. 3F and G). Additionally, caspase 3 activation was significantly increased in SAS and OECM1

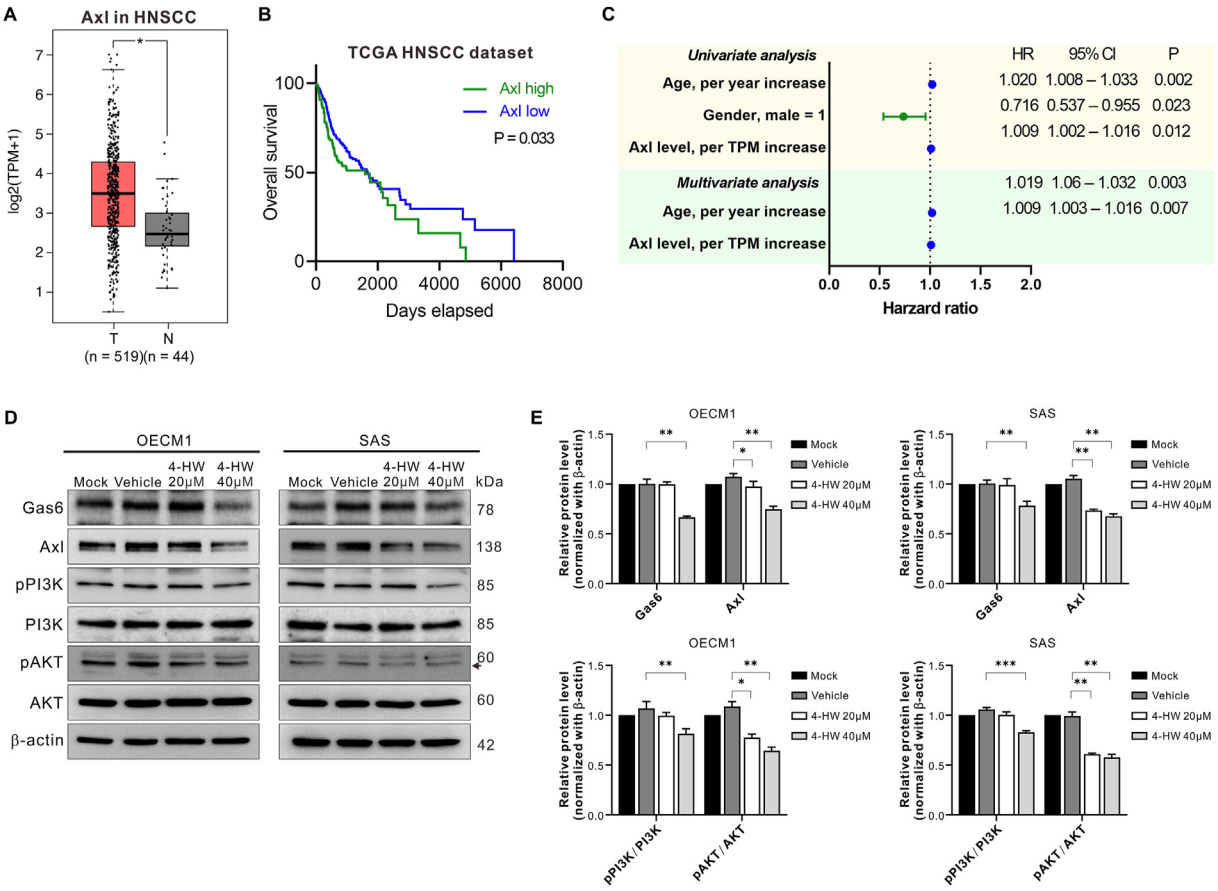

Figure 2 4′-Hydroxywogonin inhibits Gas6/Axl signaling and downstream PI3K/AKT activation. (A) Axl expression in Head and neck squamous cell carcinoma (HNSCC) tumor samples compared with normal samples based on TCGA database. (B) Overall survival analysis of HNSCC patients based on Axl expression levels (TCGA data) (C) Forest plot of the hazard ratio (HR) and 95 % confidence interval (CI) correlating Axl expression with HNSC incidence. (D) Western blot analysis showing the suppression of Gas6/Axl expression and PI3K/AKT pathway activation in OSCC cells after 4′-hydroxywogonin treatment. (E) Quantification of Western blot results from three independent experiments. TPM: transcripts per million. pPI3K: phospho-PI3K. pAKT: phospho-AKT. Significant differences versus vehicle control: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

cells treated with 4′-Hydroxywogonin, further supporting its role in promoting apoptosis (Fig. 3F).

Epithelial-to-mesenchymal transition (EMT) is a prerequisite for cancer metastasis, and previous studies have demonstrated that Axl signaling is a key regulator of EMT.[15,23] To assess whether 4′-Hydroxywogonin impacts EMT, OSCC cells were treated with varying concentrations of the compound, followed by Western blot analysis of EMT-associated proteins. The results showed that vimentin, Snail, Slug, and N-cadherin were significantly downregulated in 4′-hydroxywogonin-treated cells, indicating that 4′-hydroxywogonin suppresses EMT and reduces OSCC metastatic potential (Fig. 4).

4′-hydroxywogonin exerts its anticancer effects by inhibiting Axl signaling

To verify whether the anticancer effects of 4′-hydroxywogonin are mediated through Axl inhibition, a rescue assay was performed. OSCC cells were transfected with vector or an Axl-overexpression construct and subsequently treated with 4′-hydroxywogonin. The results demonstrated that Axl overexpression partially rescued the inhibition of cell proliferation and migration induced by 4′-hydroxywogonin, confirming that Axl suppression is a key mechanism underlying the compound's anticancer activity (Fig. 5).

4′-hydroxywogonin synergizes with cisplatin to enhance OSCC cytotoxicity

To further evaluate the potential of 4′-hydroxywogonin as an adjunct therapy for OSCC, we examined its combinatory effects with cisplatin, a standard chemotherapeutic agent. OSCC cells were treated with 4′-hydroxywogonin alone, cisplatin alone, or both agents in combination, and their inhibitory effects on OSCC cells were analyzed by flow cytometry. The results indicated that the combination of 4′-hydroxywogonin and cisplatin significantly enhanced OSCC cell apoptosis, with a maximum increase of ~3.5-fold compared to monotherapy (Fig. 6A). The results of DiOC6 staining also demonstrated that combined treatment induced higher apoptosis rates compared to single-agent

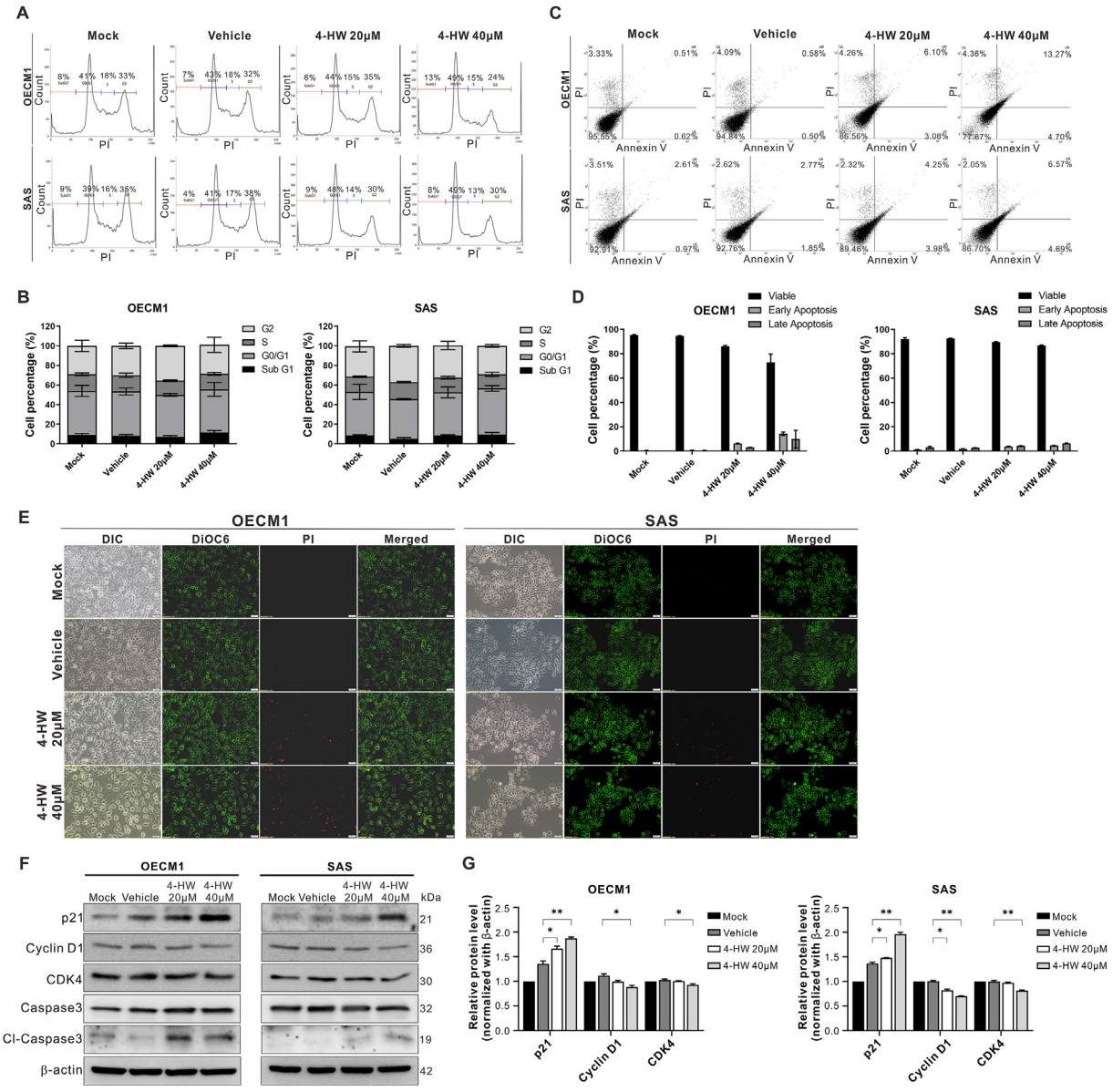

Figure 3 **4′-Hydroxywogonin induces G1 phase arrest and apoptosis in OSCC cells.** (A, C) Flow cytometry analysis of cell cycle distribution and apoptosis in OSCC cells treated with 20 or 40 μM of 4′-hydroxywogonin for 24 h. (B, D) Quantitative analysis of cell cycle arrest and apoptosis. (E) DiOC6/PI double staining to detect apoptotic cells. (F) Western blot analysis of cell cycle checkpoint proteins expression. Cl-Caspase 3: cleaved caspase 3. (G) Quantification of protein expression levels. Significant differences versus vehicle control: $P < 0.05$ (*), $P < 0.01$ (**).

treatment, confirming a synergistic effect between 4′-hydroxywogonin and cisplatin (Fig. 6B).

Discussion

The high metastatic potential and recurrence rate of OSCC contribute to disease relapse in approximately 50 % of patients within a few years after treatment. The Gas6-Axl signaling axis plays a pivotal role in OSCC cell proliferation, metastasis, and drug resistance, making it a promising therapeutic target in cancer treatment.[15] In this study, we demonstrate the anticancer activity of 4′-hydroxywogonin, which exerts its effects by suppressing Axl and Gas6 protein expression and inhibiting downstream signaling pathways, ultimately leading to the inhibition of OSCC cell proliferation, migration, and invasion, as well as the induction of apoptosis (Fig. 6C). Furthermore, the combination of 4′-hydroxywogonin with cisplatin significantly enhanced cytotoxicity against OSCC cells. To the best of our knowledge, this study is the first to validate the inhibitory effects of 4′-hydroxywogonin on OSCC and establish its regulatory impact on the Gas6/Axl signaling pathway.

Axl overexpression has been reported in multiple cancer types and is associated with increased invasiveness, therapy resistance, and poor prognosis.[21,24] Consistent with previous findings, our study revealed that OSCC cells exhibited high Axl and Gas6 expression, which was

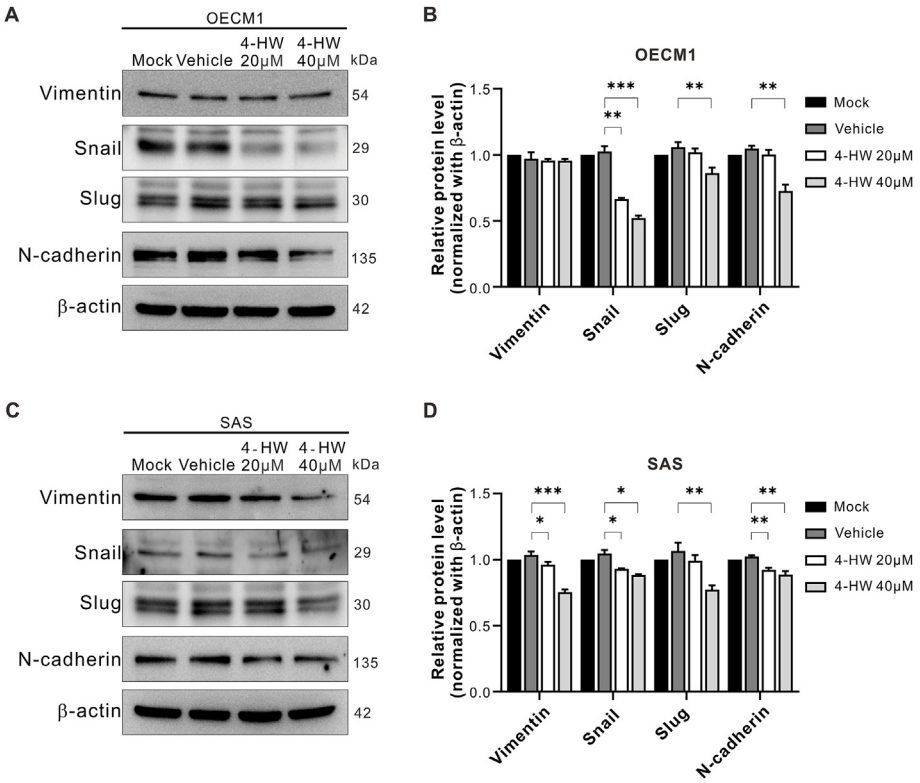

Figure 4 4′-Hydroxywogonin suppresses EMT to inhibit OSCC cell migration and invasion. (A, C) Western blot analysis showing downregulation of EMT-related proteins after treatment. The quantitative results of western blotting were shown in (B, D). Significant differences versus vehicle control: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

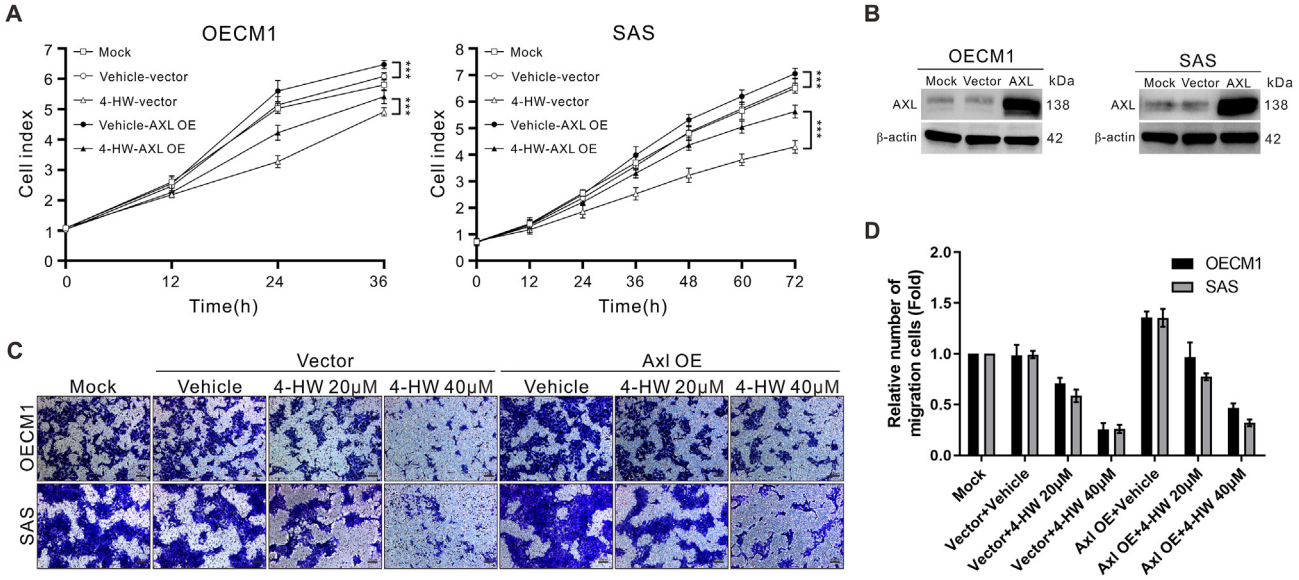

Figure 5 4′-Hydroxywogonin suppresses OSCC cell proliferation and migration via Axl signaling inhibition. (A, C) The inhibitory effects of 4′-hydroxywogonin on cell proliferation and migration were reversed by Axl overexpression. (B) Western blot confirming Axl overexpression in cells transfected with pcDNA-Axl. The quantitative results of cell migration assay were shown in (D).

significantly suppressed upon 4′-hydroxywogonin treatment. Notably, PI3K/AKT pathway activity, a key downstream signaling cascade of Axl, was also inhibited, leading to G1-phase cell cycle arrest and apoptosis induction. The specificity of 4′-hydroxywogonin's anticancer effects was further demonstrated by its minimal cytotoxicity toward normal human fibroblasts, even at higher concentrations. This selectivity is critical, as traditional chemotherapeutic

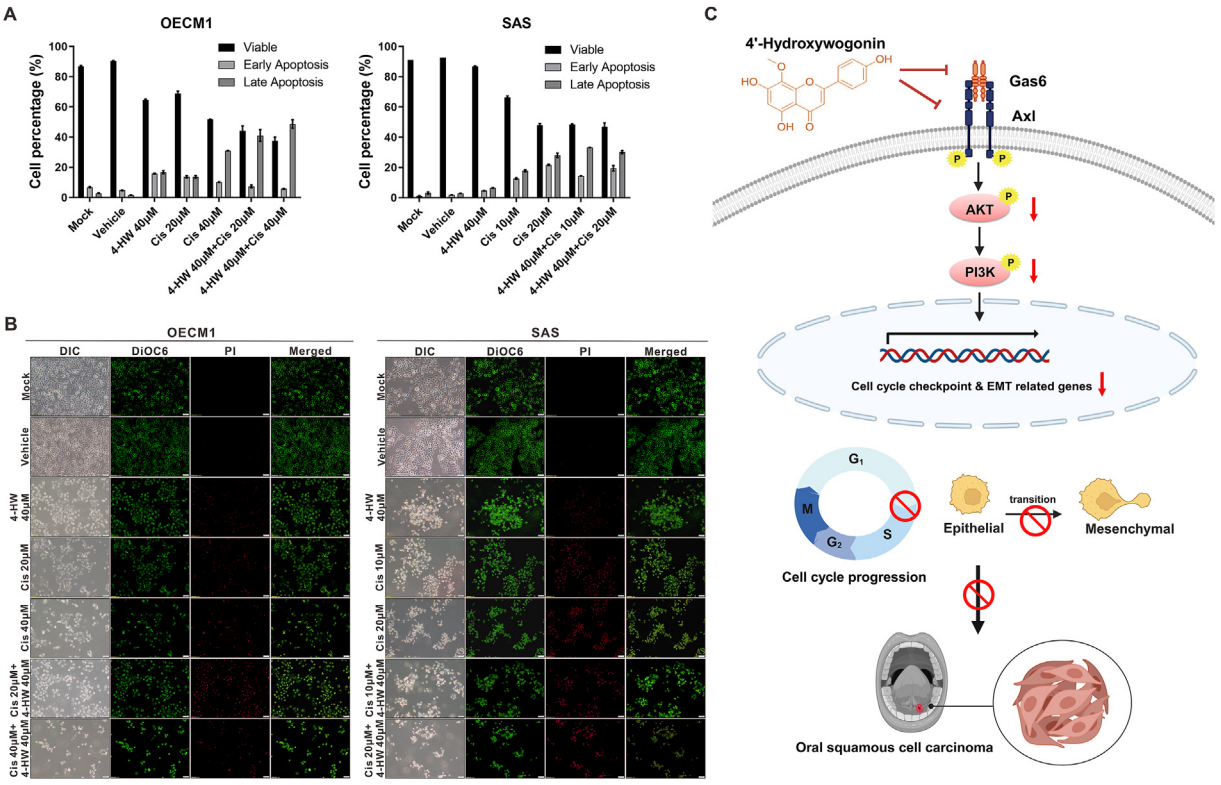

Figure 6 4′-Hydroxywogonin enhances cisplatin-induced cytotoxicity in OSCC cells. OSCC cells were treated with 4′-hydroxywogonin alone or in combination with cisplatin for 24 h. Flow cytometry (A) and DiOC6/PI staining (B) to assess cytotoxic effects. (C) Schematic summary of the proposed anti-OSCC mechanism of 4′-hydroxywogonin (created using Biorender.com, accessed January 2024).

agents often lack tumor specificity, resulting in systemic toxicity and limiting their clinical application. Our findings suggest that 4′-hydroxywogonin exhibits promising selectivity for OSCC cells, potentially reducing the adverse effects commonly associated with conventional treatments.

Beyond OSCC, Axl overexpression has been observed in multiple cancers, including breast, lung, gastric, and prostate cancers, where it correlates with poor patient prognosis. These findings suggest that Axl is not only a prognostic biomarker but also a valuable therapeutic target. Our data indicate that 4′-hydroxywogonin effectively suppresses Axl expression and its downstream signaling in OSCC cells, suggesting that it may have therapeutic potential in other Axl-overexpressing malignancies as well.

Emerging studies indicate that Axl signaling plays a key role in immune evasion by facilitating M2 macrophage polarization and suppressing pro-inflammatory cytokine expression.[25] Given that 4′-hydroxywogonin inhibits Axl signaling, it may also alter the tumor microenvironment (TME) by restoring immune surveillance mechanisms. Future studies should assess immune profiling in OSCC tumors treated with 4′-hydroxywogonin, particularly its impact on tumor-associated macrophages (TAMs), cytotoxic T-cell responses, and immunosuppressive cytokine production. This could reveal additional immune—modulatory properties of 4′-hydroxywogonin, further expanding its therapeutic applications.

Despite being a first-line chemotherapeutic agent, cisplatin-based therapy is often hampered by drug resistance and severe side effects.[26,27] Our findings reveal that 4′-hydroxywogonin significantly enhances cisplatin's cytotoxic effects, leading to a 3.5-fold increase in tumor cell apoptosis induction compared to monotherapy. The synergistic effect of 4′-hydroxywogonin and cisplatin suggests its potential as an adjuvant therapy, allowing for dose reductions of cisplatin while maintaining therapeutic efficacy. This could mitigate cisplatin-induced toxicity, which is a major limitation of long-term chemotherapy. Further studies, including animal models and patient-derived xenografts (PDXs), will be critical in evaluating the translational potential of this combination therapy.

In this study, we primarily investigated the effects of 4′-hydroxywogonin on cell cycle checkpoints and EMT-related gene expression, which partially explain its anticancer mechanism. Given the complexity of OSCC progression, additional molecular targets are likely involved. Moreover, rescue assay results showed that Axl overexpression partially reversed the inhibitory effects of 4′-hydroxywogonin on OSCC cell proliferation and migration, suggesting that additional signaling molecules contribute to its antitumor activity. Further studies are necessary to identify these additional molecular targets and fully elucidate the mechanisms underlying 4′-hydroxywogonin-mediated tumor suppression.

In conclusion, this study provides compelling evidence that 4′-hydroxywogonin exerts potent anticancer effects against OSCC by selectively targeting Axl/Gas6 signaling, leading to tumor growth inhibition, metastasis suppression,

and apoptosis induction. Importantly, its synergistic interaction with cisplatin underscores its potential as an adjuvant therapy to enhance treatment efficacy while minimizing chemotherapy-related toxicity. Furthermore, the selectivity of 4′-hydroxywogonin for cancer cells, combined with its ability to suppress Axl signaling, suggests broader therapeutic applications across multiple Axl-overexpressing malignancies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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