



Dual inhibition of MMP-2 and actin dynamics by a novel bis-chalcone: an anticancer strategy for oral squamous cell carcinoma

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Abstract

Oral squamous cell carcinoma (OSCC), accounts for approximately 90% of oral cancer cases, and due to its aggressive nature and limited treatment options remains a significant clinical challenge. In the pursuit of novel therapeutic agents, bis-chalcones (a subclass of chalcones) have emerged as promising candidates with multipronged anticancer potential. In this study, we synthesized and evaluated a novel bis-chalcone (B2OCH₃), for its cytotoxic, anti-migratory, anti-invasive, and anti-adhesive activities, as well as potential mechanisms of action. The compound was obtained via Claisen–Schmidt condensation and spectroscopically characterized. Cytotoxicity was assessed using MTT, pulse treatment, and trypan blue exclusion assays. Morphological changes were examined with panoptic staining and immunofluorescence. Clonogenic, migration, adhesion, and invasion assays were performed to assess anti-metastatic potential. Molecular docking and gene/protein expression analyses were conducted by quantitative PCR and Western blot. B2OCH₃ exhibited potent cytotoxicity against HSC-3, SCC-4, and CAL-27 OSCC cell lines, with HSC-3 being the most sensitive. The compound demonstrated selectivity for cancer cells as compared with non-tumor HS5 and HaCaT cells. Reduced viability was accompanied by mitotic figures and apoptotic bodies. B2OCH₃ significantly inhibited colony formation, migration, invasion, and adhesion in HSC-3 cells. Morphological analysis revealed modulation of actin filament organization and reduced filopodia formation. Molecular docking indicated MMP-2 and F-actin affinity, and was corroborated by molecular assays confirming protein modulation for both. Our findings identify B2OCH₃ as a promising therapeutic candidate for OSCC, highlighting its potential to simultaneously target both MMP-2 activity and actin cytoskeleton regulation.

Keywords Symmetrical bis-chalcone · Oral squamous cell carcinoma · Cell migration · Cell invasion · Metalloproteinases

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Abbreviations

OSCC	Oral squamous cell carcinoma
MMP	Matrix metalloproteinase
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
PDB	Protein data bank
qPCR	Quantitative polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
EDTA	Ethylenediaminetetraacetic acid

SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
RMSD	Root mean square deviation

Introduction

Oral squamous cell carcinoma (OSCC) ranks as the sixth most prevalent cancer worldwide and constitutes a major subset of head and neck malignancies (Fatima et al. 2024). Epidemiological data from the Oral Cancer Foundation indicate a steadily rising incidence, with over 300,000 new cases diagnosed globally per year. Although significant progress has been achieved in current therapies, recurrence remains a frequent clinical event and continues to be strongly correlated with poor survival outcomes (Haque et al. 2019).

A central determinant of this prognosis is the metastatic potential of OSCC. Metastasis involves a series of biological processes through which malignant cells dissociate from the primary lesion, acquire migratory and invasive properties, penetrate vascular structures, disseminate systemically, and eventually establish secondary foci (Welch and Hurst 2019; He et al. 2025). The clinical impact is profound. Patients with metastatic OSCC exhibit a markedly reduced five-year survival rate of less than 6.3% (Sung et al. 2021). Effective treatment will require an understanding of the events that drive metastasis and the development of strategies to counteract the phenomenon.

Medicinal chemistry offers an avenue for the discovery of innovative compounds that can modulate metastatic behavior (Araújo et al. 2024). Chalcones constitute a class of compounds which exhibit a broad spectrum of biological activities against various diseases. Their chemical structure, characterized as a privileged scaffold, provides versatility for the design and development of new drugs (Zhuang et al. 2017). In addition, due to their ability to interact with multiple active sites, chalcones display affinity for various enzymes and molecular targets. Consequently, they have emerged as promising therapeutic agents, exhibiting antioxidant, anti-inflammatory, anti-neurodegenerative, anticancer, antiviral, and antiparasitic properties (Zhang et al. 2017; Constantinescu and Lungu 2021).

Our interest has been primarily directed toward bis-chalcones, compounds that contain two chalcone units within a single molecule. Bis-chalcones represent an extraordinary chemical scaffold with multifaceted pharmacological properties. Owing to this, they are considered highly attractive compounds and have been studied in a wide spectrum of potential biological applications (Olender et al. 2024). Numerous bis-chalcone derivatives have been investigated as antimicrobial, anticonvulsant, antidiabetic, antioxidant, antitubercular, anti-HIV, anti-amoebic, and anti-inflammatory agents, as well as their carbonic anhydrase

and AChE inhibitor activity (Pereira et al. 2023). Chalcone-based structures have demonstrated anticancer activity (Modzelewska et al. 2006; Sharma et al. 2010; Fathi et al. 2021).

Though various bis-chalcone derivatives have been shown to possess anticancer activity, including inhibition of proliferation, colony formation, and even migratory/invasive properties (Burmaoglu et al., 2021; Nian et al., 2024), none have been specifically evaluated in OSCC. For example, boronic acid bis-chalcones display activity in breast cancer cells (Modzelewska et al. 2006). Asymmetric bis-chalcones inhibit colony formation and induce apoptosis via the FGFR1/ERK pathway in gastric cancer (Nian et al., 2024). Our study compound (B2OCH₃) uniquely targets OSCC, affecting phenotypes linked to adhesion and migration.

In light of these activities, bis-chalcone scaffold-based molecules hold significant promise for applications in medicinal chemistry. Bis-chalcones appear to be potential candidates for the development of new drugs for the treatment of various diseases, including cancer. Although numerous studies have reported the synthesis of bis-chalcones as well as their diverse biological roles, a full understanding of their cytotoxic effects and their impact on processes associated with tumor metastasis remains limited. In this context, our study stands out as the first to systematically investigate the impact of a novel bis-chalcone (B2OCH₃) on key events of metastatic behavior in HSC-3 cells. The present work aimed to synthesize and evaluate the effects of B2OCH₃ and modulation of tumor aggressiveness, with emphasis on migration and adhesion processes, using both in vitro and in silico approaches.

Materials and methods

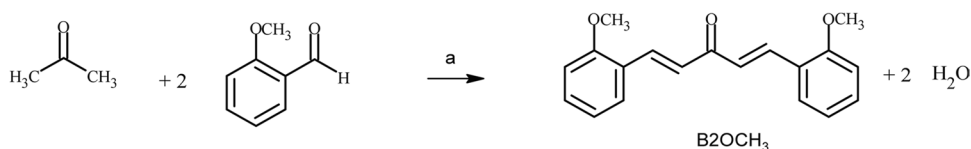
Synthesis and physicochemical characterization of bis-chalcone B2OCH₃

Bis-chalcone (B2OCH₃) was synthesized by Claisen-Schmidt condensation as described previously by (Batovska et al. 2007), with slight modifications. The corresponding benzaldehyde (1.5 mmol) was dissolved in methanol (1 mL) and acetone (0.73 mmol), and 1.5 mL of 6 M NaOH solution was added. After 15 min of stirring at room temperature, the precipitate was filtered and crystallized from methanol. The Fig. 1 outlines chalcone synthesis.

The chemical structure of (1E,4E)–1,5-Bis(2-methoxyphenyl)–1,4-pentadien-3-one (B2OCH₃) was confirmed by IR and ¹H NMR spectral techniques. The melting point (M.p.) was measured, and elemental analyses (C, H) were performed.

(1E,4E)–1,5-Bis(2-methoxyphenyl)–1,4-pentadien-3-one (B2OCH₃): Yellow crystals, yield 81%. M.p.: 119–120

Fig. 1 Synthesis of chalcone B2OCH₃. (a) NaOH (6 M), MeOH, 15 min at room temperature



°C. IR (KBr) cm^{-1} : 3031, 2840, 1670, 1615, 750. ^1H NMR (500 MHz – CDCl_3) δ : 8.08 (d, 2H, $J=15.7$ Hz, H β), 7.63 (dd, 2H, $J=7.7, 1.8$ Hz, H6), 7.38 (ddd, 2H, $J=7.3, 1.7, 1.2$ Hz, H5), 7.19 (d, 2H, $J=15.7$ Hz, H α), 7.00 (t, 2H, $J=7.7$ Hz, H3), 6.94 (d, 2H, $J=8.4$ Hz, H4), 3.92 (s, 6H, $-\text{OCH}_3$). Anal. Calcd. for $\text{C}_{19}\text{H}_{18}\text{O}_3$: C, 77.53; H, 6.16. Found: C, 78.1; H, 6.2%. A coupling constant of 15.7 Hz for the vinyl H-atoms confirmed the (E)-configuration (spectra provided in Supplementary Material, Figs. S1 and S2). The melting point, IR, and ^1H NMR data are consistent with previously reported values (Adams et al. 2004; Liang et al. 2009).

Cytotoxicity assay

The cytotoxic effects were evaluated using the MTT assay (Mosmann 1983; De Sá et al. 2023). Human oral squamous cell carcinoma (OSCC) cell lines HSC-3, SCC-4, and CAL-27 were used as cancerous models, whereas HS-5 (human bone marrow stromal cells) and HaCaT (immortalized human keratinocytes) served as non-cancerous control models. Cells in the exponential growth phase were plated in 96-well plates at a density of 7×10^4 cells/mL.

After 24 h of incubation, the cells were exposed to B2OCH₃ at concentrations ranging from 0.01 to 50 μM . The cells were then incubated at 37 °C for periods of 24, 48, and 72 h. Dimethyl sulfoxide (DMSO) served as the control. Following treatment, MTT solution (0.5 mg/mL) was added to each well and incubated for 3 h. The resulting MTT-formazan product, dissolved in DMSO, was quantified by measuring absorbance at 595 nm using a microplate reader. An intermittent exposure protocol was applied in which cells were treated with B2OCH₃ for 3, 6, 12, or 24 h to evaluate the impact of different exposure durations on viability. Following compound removal, cells were incubated for a total of 72 h to capture both immediate and delayed effects on proliferation and survival (Almeida et al. 2019).

Trypan blue

Cell viability was assessed using the trypan blue exclusion assay after incubating HSC-3 cells (7×10^4 cells/mL) with cytotoxic (3.25 and 6.5 μM) and subcytotoxic (0.25 and 0.5 μM) concentrations of B2OCH₃, as determined by the prior cytotoxicity assay. After 48 h, a 90 μL aliquot of the cell suspension was mixed with 10 μL of 0.4% trypan blue. Viable and nonviable cells were counted in a Neubauer chamber (De Sá et al. 2023).

Morphological analysis

HSC-3 cells treated with B2OCH₃ at concentrations of 3.25 and 6.5 μM for 24 h were examined under light microscopy. To observe morphological changes, after incubation, the cells were harvested, transferred onto cytocentrifuge slides, fixed with methanol for 30 s, and stained using the Rapid Panoptic staining kit to visualize the nucleus and cytoplasm. The cells were then analyzed under a light microscope at 400 $\times$ magnification.

Clonogenic assay

HSC-3 cells were plated at a density of 3×10^2 cells per well in sterile 6-well plates and incubated at 37 °C with 5% CO_2 for 24 h. Following this incubation, subcytotoxic concentrations of B2OCH₃ (0.25 and 0.5 μM) were applied to the wells and incubated for an additional 24 h. The cells were then washed with phosphate-buffered saline (PBS) and subsequently cultured in medium supplemented with 10% FBS for five more days under the same conditions. After this period, the medium was removed, and the cells were fixed with a methanol–acetic acid solution (3:1) for 5 min, followed by staining with a 2% crystal violet solution in methanol for 5 min.

Anchorage-independent growth assay

In a 12-well plate, a 500 μL layer of 0.5% agarose was first added to establish the base layer. Another 500 μL was then used for the top layer, containing human oral squamous cell carcinoma cells, treated or untreated with 0.25 and 0.5 μM B2OCH₃, suspended in 0.3% agarose. To prevent evaporation, 200 μL of medium was placed on top of the agarose layers. The plates were incubated for approximately 21 days to allow colony formation. Following this incubation, the colonies were stained with MTT solution (5 mg/mL) for 45 min. Images were captured and subsequently analyzed using image analysis software.

Matrigel invasion assay

The migration potential of HSC-3 cells was evaluated using a Boyden transwell system with 8 μm pore-sized filters. After 12 h treatment with B2OCH₃ (0.25 and 0.5 μM), cells were placed in the upper chamber, while the lower chamber was filled with 0.5 mL medium containing 20%

FBS. Following an incubation period of either 24 or 10 h, the cells were stained with crystal violet. Any non-migratory cells remaining on the upper filter surface were carefully removed, and the cells that migrated to the underside of the filter were counted under a light microscope. For the invasion assay, transwell filters (8 µm pore size, 0.33 cm² area) were coated with 50 µL of Matrigel diluted to 25 mg/50 mL using cold, filtered medium without FBS and allowed to polymerize at 37 °C before seeding the cells. After 48 h of incubation, the invading cells were stained and counted as described previously.

Cell adhesion assay

The adhesion assay was performed with modifications based on the previously described method (Chen 2012). First, 10 µg/mL type I collagen was used to coat the wells for 1 h, followed by two washes with PBS and a blocking step using 1% bovine serum albumin (BSA) in PBS for 1 h. Cells were plated in 24-well plates at a concentration of 2×10^5 cells/mL in serum-free DMEM and treated with B2OCH₃ at 0.25 and 0.5 µM. After 2 h of incubation at 37 °C, the supernatant was carefully discarded, and the wells were rinsed twice with PBS. The cells were fixed using a 0.1% triarylmethane solution, stained with 0.1% xanthene and 0.1% thiazine, and the excess dye was removed by washing. The stained cells were then analyzed and imaged using an inverted optical microscope.

Scratch assay

Approximately 9×10^4 HSC-3 cells were plated in each well of a 24-well plate and allowed to grow until they reached 85–95% confluence. Afterward, the medium was removed, and a scratch was introduced at the base of each well using a 200 µL pipette. The wells were washed with PBS to eliminate debris, and fresh DMEM medium containing 0.25% FBS, along with either the vehicle (Ø) or varying concentrations of B2OCH₃ (0.25 and 0.5 µM), were added. Images were captured at regular intervals (0, 24, and 48 h) using a digital inverted light microscope. Finally, the percentage of migration was calculated using the following formula: $[(\text{Scratch area}_{0\text{ h}}) - (\text{Scratch area}_{24/48\text{ h}})] \times 100 / (\text{Scratch area}_{0\text{ h}})$.

Migration assay on spheroid model

Each well of a 96-well plate was prepared with 65 µL of 1% agarose at the bottom, and 1×10^4 HSC-3 cells were seeded per well. The cells formed spheroids, which were cultured for 7 days. Once the spheroids were established, they were transferred to a 6-well plate. After the spheroids adhered to the surface, they were treated with either the vehicle (Ø)

or various concentrations of B2OCH₃ (0.25 and 0.5 µM). Periodic imaging was performed using a digital inverted light microscope.

Immunofluorescence assay

The HSC-3 cells, treated with the vehicle (Ø) or 0.5 µM concentration for 3 and 6 h, were fixed using 100% cold methanol and made permeable with 0.5% Triton X-100 in PBS for 30 min at room temperature. Subsequently, the cells were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The cells were then incubated overnight at 4 °C with anti-α-tubulin conjugated to a fluorophore, in the dark. After washing once with PBS, the cells were stained with phalloidin conjugated to a fluorescent dye for 1 h. Following staining, the slides were mounted with an antifade mounting medium containing DAPI for 1 h at room temperature. Images were taken using a fluorescent microscope at $\times 400$ magnification.

Molecular docking

The 3D structures of the target proteins were retrieved from the Protein Data Bank (PDB) (<https://www.rcsb.org/>) (Berman et al. 2000), with the following PDB IDs: 2ZWH (F-actin), 8H78 (MMP-2), and 6ESM (MMP-9). Protein preparation was performed in Chimera v.13.1 (Pettersen et al. 2004) by removing water molecules and other groups, such as ions. The ligand 1,5-Bis(2-methoxyphenyl) penta-1,4-dien-3-one was obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Docking simulations were conducted using AutoDock Vina (Trott and Olson 2010), with ligands and proteins prepared in AutoDock Tools v.1.5.6 (Morris et al. 2008). Receptors were treated as rigid and ligands as flexible. Hydrogens were added, Gasteiger charges calculated, and non-polar hydrogens merged (Gasteiger and Marsili 1980). Method validation was performed by re-docking crystallographic ligands, yielding RMSD ≤ 2.0 Å using PyRx (Ferrari and Patrizio 2021; Repasky et al. 2012). Docking poses and overlap between co-crystallized and re-docked ligands were analyzed in Discovery Studio 2.0. The x, y, and z coordinates of the computational poses defined the active site region (Table 1 of the supplementary material). Docking parameters included 50 modes, exhaustiveness of 24, and a grid box size of 30 points per axis. The best binding energy values (ΔG_{bind}) were used to assess interaction efficiency (Rocha et al. 2018).

Quantitative PCR

Total RNA from HSC-3 cells treated with B2OCH₃ (0.5 µM) or a negative control was extracted, and cDNA was synthesized from 1 µg of RNA. Quantitative PCR (qPCR)

was performed with specific primers for GAPDH, MMP-2, and MMP-9. Primer sequences are listed in Table 2 of the supplementary material. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Western blot analysis

Protein extraction was performed from HSC-3 cells treated with vehicle or B2OCH₃ (0.25 and 0.5 μ M) for 48 h. Protein concentration was determined using the Bradford assay. Equal amounts of protein (30 μ g) were separated on SDS-PAGE gels and transferred onto membranes. Primary antibodies against GAPDH, Pan-actin, and MMP-2 were incubated overnight at 4 °C, followed by HRP-conjugated secondary antibodies for 2 h at room temperature. Detection was performed using a chemiluminescent substrate, and bands were visualized with a gel imaging system.

Statistical analysis

Statistical analyses were performed, and IC₅₀ values were obtained by non-linear regression. The results of three independent experiments are represented as the mean $\pm$ standard error of the mean (S.E.M). Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Tukey's post-test, with significance defined as $*p < 0.05$.

Results

Cytotoxicity of B2OCH₃ and its impact on colony formation of HSC-3 cells

Initially, the cytotoxic effects of B2OCH₃ were evaluated in human oral squamous cell carcinoma and stromal cells at 24, 48, and 72 h of incubation. As shown in Fig. 2A and B, B2OCH₃ reduced tumor cell viability under all tested conditions, with no additional increase in cytotoxicity after 24 h. The IC₅₀ values calculated ranged from 6.5 to 7.0 μ M (Fig. 2B). In HS5 and HaCaT cells, no toxicity was observed (IC₅₀ exceeding the highest tested concentration of 50 μ M), indicating that B2OCH₃ exhibits selectivity toward HSC-3 cells (Fig. 2B). Doxorubicin, used as positive control, presented significant toxicity against HSC-3 cells (IC₅₀ ranging from 0.21 to 0.29 μ M) (Table 3 of the supplementary material), being 24 times more potent than B2OCH₃ at 24 h incubation. However, doxorubicin seemed less selective than the bis-chalcone, as it was also toxic to stromal cells. The selectivity index (IC₅₀ for stromal cells/IC₅₀ for oral carcinoma cells) for doxorubicin after 24 h of incubation was 2.14. For B2OCH₃, the selectivity index was estimated at

7.14 using the highest tested concentration in stromal cells (50 μ M) (Fig. 3 of the supplementary material), as it was not possible to determine the IC₅₀ values.

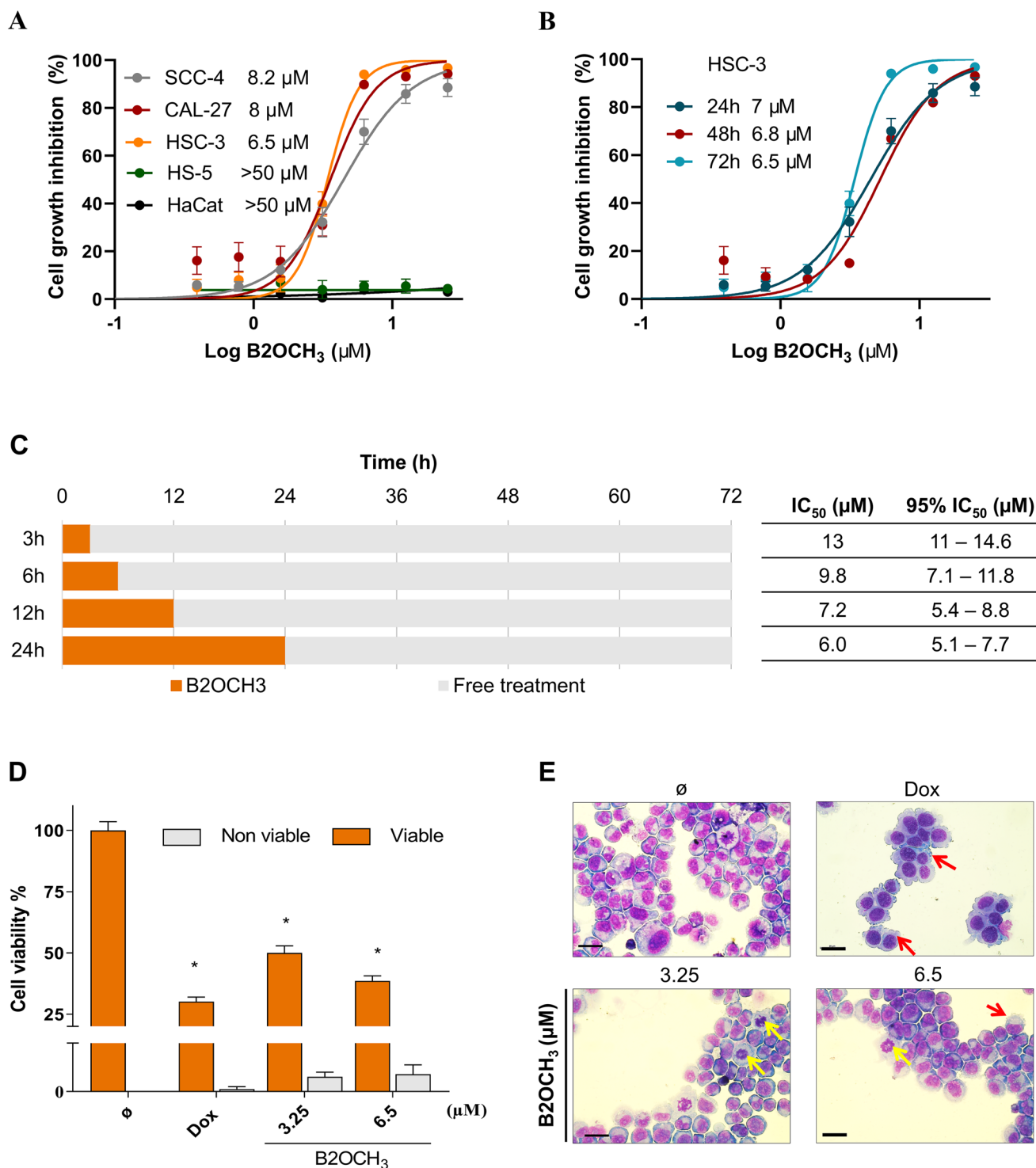
A 3 h pulse treatment with B2OCH₃ was sufficient to significantly decrease cell viability measured after a total incubation time of 72 h (Fig. 2C). When the total incubation time was reduced to 24 h (even with continuous exposure to the drug), the cytotoxic effects were reduced (Fig. 2C). Trypan blue staining results also demonstrated a significant reduction in the total number of cells after treatment with B2OCH₃ (Fig. 2D). Morphological analysis with rapid panoptic staining revealed the presence of mitotic figures, and a reduction in cell density and cell bodies at a concentration of 6.5 μ M after treatment with B2OCH₃ (Fig. 2E).

As B2OCH₃ induced marked reductions in cell viability after only 3 h of exposure, with sustained effects even after 72 h in drug-free medium, and morphological analyses revealed changes consistent with mitotic arrest, we hypothesized that these effects might not be restricted to direct cytotoxicity. We therefore investigated whether these effects might also impact functional events related to tumor progression, such as cell migration and adhesion.

Sub-cytotoxic concentrations of 0.25 and 0.5 μ M were selected based on the IC₅₀ values for subsequent experiments with the bis-chalcone, ensuring minimal cell death and allowing a clearer assessment of the biological response. According to literature reports, concentrations below the IC₅₀ values are commonly employed in in vitro studies with structurally related chalcones, reinforcing the experimental suitability and biological relevance of these conditions (Jansson-Löfmark et al. 2020; Araújo et al. 2024). The trypan blue exclusion assay confirmed that neither concentration selected affected HSC-3 cell viability (Fig. 3A), and clonal growth was inhibited by exposure to B2OCH₃ at both concentrations tested. B2OCH₃ reduced the colony area by 24% at 0.25 μ M and by 63.4% at 0.5 μ M (Fig. 3B). In the anchorage-dependent assay, the compound suppressed colony formation by 68% at 0.25 μ M and by 88.5% at 0.5 μ M (Fig. 3C).

Effects of B2OCH₃ on stages of tumor progression and cell morphology

To investigate whether B2OCH₃ influences tumor progression-related events in HSC-3 cells, we performed adhesion, invasion, and migration assays. Treatment with B2OCH₃ at sub-cytotoxic concentrations (0.25 and 0.5 μ M) significantly inhibited adhesion of HSC-3 cells to type I collagen (Fig. 4A). Specifically, 0.25 μ M and 0.5 μ M B2OCH₃ reduced adhesion to 350.9 ± 35.0 and 270.8 ± 30.0 cells per field, respectively, as compared with 900.2 ± 35.3 cells per field for the control group (Fig. 4B). Morphological



alterations were observed in cells exposed to these concentrations as well.

Given that invasion is a crucial factor in cancer progression and metastasis, we investigated whether the compound B2OCH₃ affected cancer cell invasion using Matrigel® coated Transwell® chambers. Consistent with the data

from adhesion and migration assays, B2OCH₃ significantly inhibited the invasion of oral squamous cell carcinoma (Fig. 4A). In HSC-3 cells treated with 0.25 and 0.5 μM B2OCH₃, the invasion ratio relative to the control group was respectively reduced to 24% and 22% (Fig. 4C). These findings demonstrate that B2OCH₃ significantly suppressed

Fig. 2 Cytotoxic activity of B2OCH₃ in non-tumor and tumor cells. **A**) According to the MTT assay, B2OCH₃ reduces cell viability in tongue squamous cell carcinoma cell lines (HSC-3, SCC-4, and CAL-27) and shows selectivity toward non-tumor stromal (HS-5) and keratinocyte (HaCaT) cells. Dose-response curves of cytotoxicity kinetics after 72 h of treatment ($n=3$ independent experiments, each performed in triplicate). **B**) Dose-response curves for B2OCH₃ treatment for 24, 48, and 72 h in HSC-3 cells, assessed by the MTT assay ($n=3$ independent experiments, each performed in triplicate). **C**) Intermittent exposure (pulse) assay. The bar graph illustrates the exposure schedule of HSC-3 cells to B2OCH₃ (orange bars), followed by incubation periods in drug-free medium (gray bars), designed to evaluate the effects of transient drug exposure on cell recovery and viability. Dose-response curves for B2OCH₃ treatment for 24, 48, and 72 h in HSC-3 cells, assessed by the MTT assay ($n=3$ independent experiments, each performed in triplicate). **D**) Percentage of viable HSC-3 cells in control and B2OCH₃-treated groups (3.25 and 6.5 μ M), determined by the trypan blue exclusion assay ($n=3$ independent experiments, each performed in triplicate). **E**) Optical microscopy of HSC-3 cells after 24 h of incubation with B2OCH₃ (3.25 and 6.5 μ M). Red arrow: bleb formation; yellow arrow: mitotic figures. Scale bar 20 μ m. Data are expressed as mean $\pm$ SEM. IC₅₀ values were calculated by nonlinear regression using GraphPad Prism software (version 8.0), and 95% confidence intervals (95% CIs) were obtained from at least three independent experiments performed in triplicate. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$)

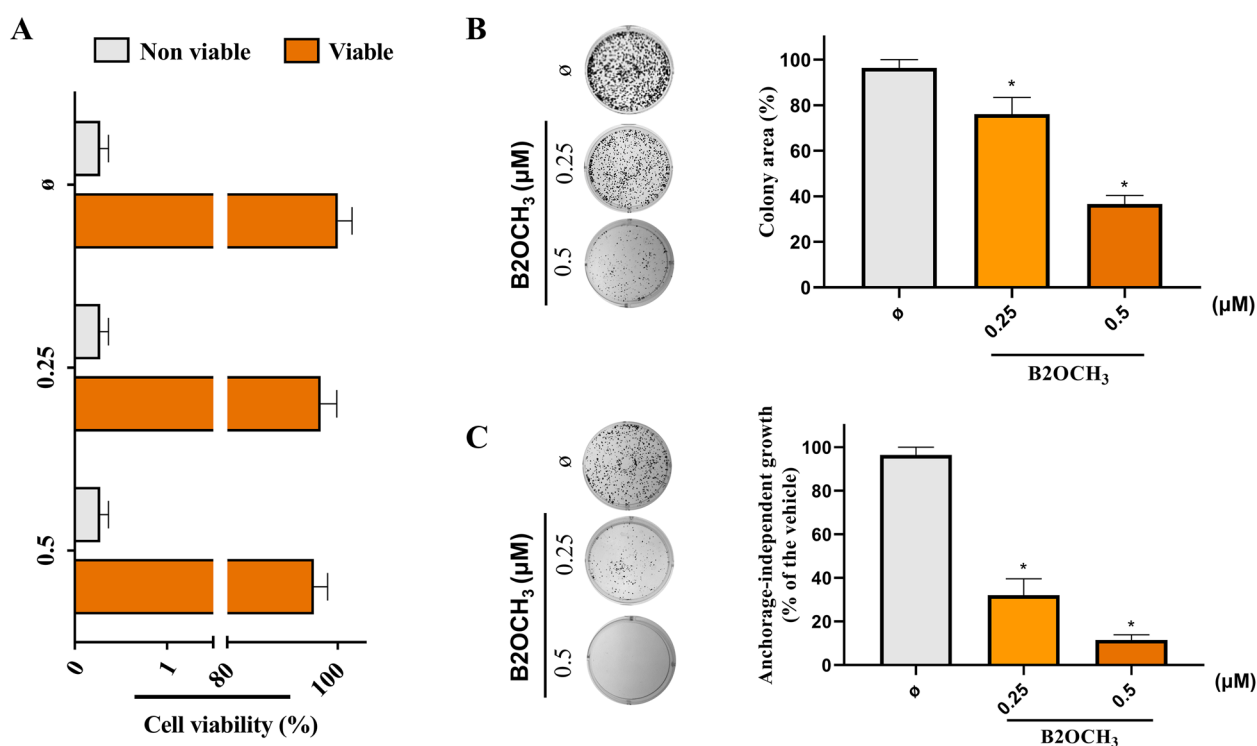


Fig. 3 Effects of subcytotoxic concentrations of B2OCH₃ on HSC-3 cells. **A**) Percentage of viable HSC-3 cells in control and B2OCH₃-treated groups (0.25 and 0.5 μ M), assessed by Trypan blue exclusion assay ($n=3$ independent experiments, each performed in triplicate). **B**) Effect of B2OCH₃ on colony formation in HSC-3 cells; representative image from triplicates and corresponding quantification of colony area ($n=3$ independent experiments). **C**) Anchor-

the invasive capacity of HSC-3 cells through Matrigel® in a dose-dependent manner.

To further investigate the effects of B2OCH₃ on cellular processes associated with tumor progression, immunofluorescence analysis was performed to assess whether exposure to non-cytotoxic concentrations of the compound induces alterations in cell morphology. Untreated HSC-3 cells exhibited an elongated morphology with cytoplasmic extensions, indicating adhesion to the coverslip, along with a typical interphase microtubule network spanning the entire cell (Fig. 4D). In contrast, B2OCH₃-treated cells displayed a rounded morphology with shrunken cell bodies, suggesting impaired adhesion and spreading ability. The treatment modified the cytoskeletal organization by altering the architecture of actin filaments and microtubules, resulting in pronounced cell retraction, as indicated by the white arrows. Notably, a reduction in filopodia formation was observed in the treated groups (Fig. 4D).

The scratch closure on the cell monolayer was calculated based on the scratched area at time zero and at 24 and 48 h of exposure to either vehicle or treatment (Fig. 5A). The compound at concentrations of 0.25 and 0.5 μ M, significantly

age-independent growth of HSC-3 cells treated with vehicle or B2OCH₃ for 21 days in soft agar. Bar graphs show mean $\pm$ standard error of the mean (SEM) from three independent experiments ($n=3$ independent experiments). Bar graphs show mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$). Effect size is expressed as percentage inhibition relative to the control

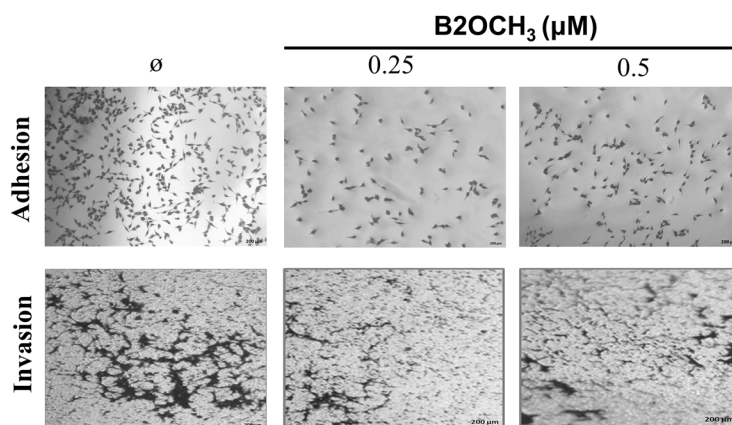
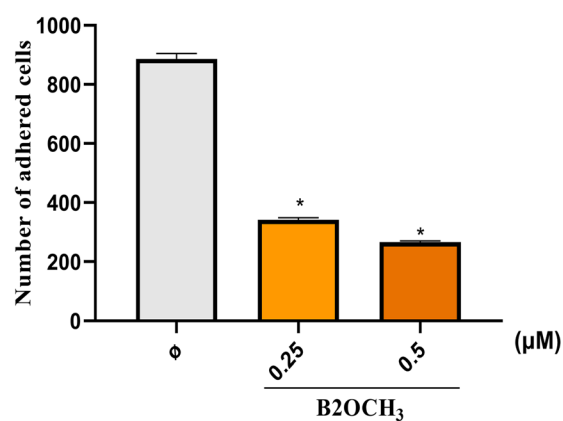
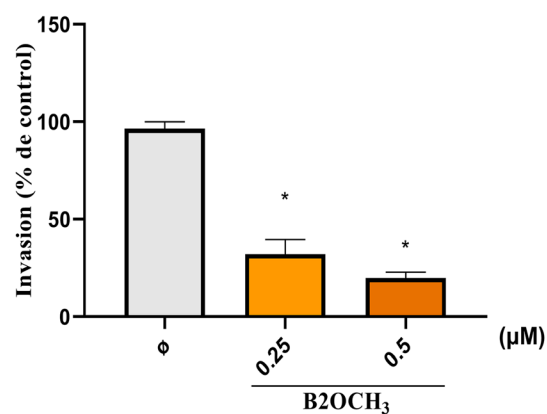
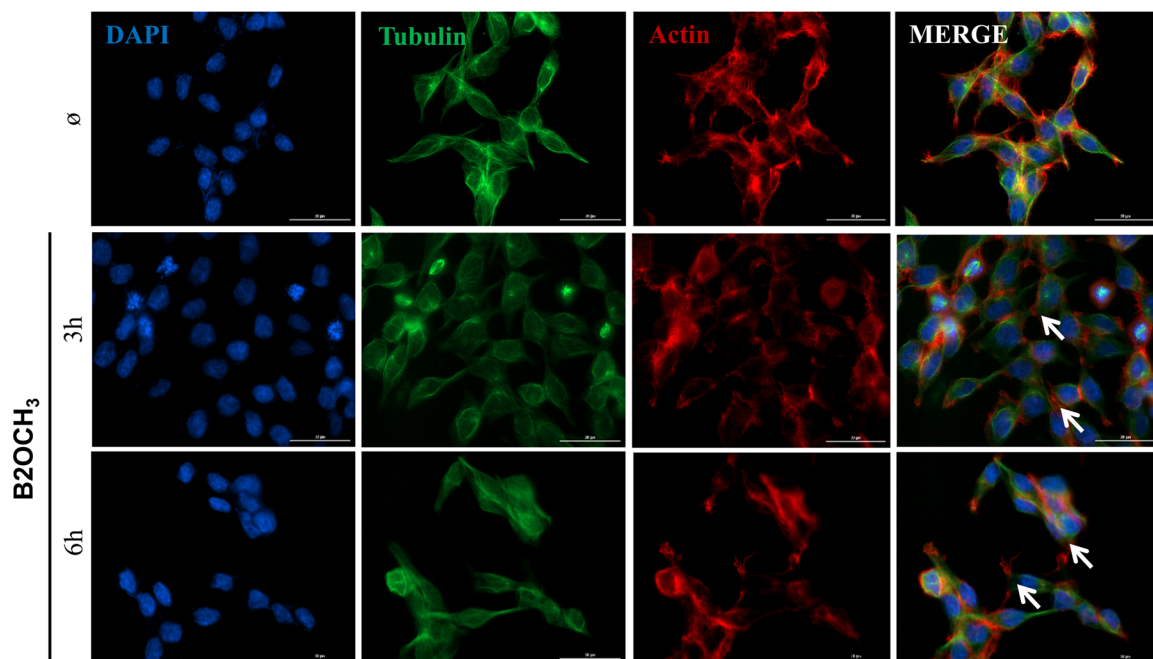
A**B****C****D**

Fig. 4 Effects of B2OCH₃ on adhesion, invasion, and cytoskeletal alterations in HSC-3 cells. **A**) Representative images of HSC-3 cell adhesion to type I collagen and invasion assay (crystal violet staining). **B**) Quantification of cell adhesion to type I collagen, based on two representative fields per replicate (3 independent experiments, each performed in triplicate). **C**) Quantification of cell invasion, expressed as a percentage relative to the control group (3 independent experiments, each performed in triplicate). **D**) Immunofluorescence images showing nuclei (DAPI, blue), microtubules (α -tubulin, green), and actin filaments (F-actin, red) after 3 and 6 h of exposure to B2OCH₃ (0.5 μ M). Arrows indicate cytoskeletal changes. For each condition, four quadrants per well were photographed and analyzed. Data are expressed as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$)

reduced the migration percentage compared to the negative control in the HSC-3 cell line. The results demonstrated that after 24 h of incubation, the percentage of cell migration when exposed to B2OCH₃ was 24% at 0.25 μ M and 22% at 0.5 μ M, while untreated cells presented a percentage of 48% (Fig. 5B). The results also revealed that cells treated with 0.25 μ M and 0.5 μ M exhibited respective migration rates of 38% and 11.5% after continuous incubation for 48 h (Fig. 5C). The negative control presented a migration rate of 96.2% (Fig. 5C).

Cell migration was also assessed using a three-dimensional model of HSC-3 cells, and revealed that B2OCH₃ treatment inhibits spheroid cell migration (as compared to the negative control spheroids) (Fig. 5D, E and F).

B2OCH₃ promotes interaction and changes in molecular markers involved in tumor progression

To explore the potential molecular interactions of B2OCH₃, we conducted *in silico* analyses focusing on proteins implicated in metastatic progression, including actin and matrix metalloproteinases. These analyses served as a hypothesis-generating approach, with subsequent experimental validation prioritizing targets showing functional modulation.

Molecular docking analysis revealed favorable binding of B2OCH₃ to actin (A), MMP-2 (B), and MMP-9 (C), with predicted binding free energies of -7.7 , -8.3 , and -6.5 kcal·mol⁻¹, respectively. In all complexes, B2OCH₃ occupied well-defined binding pockets and established stabilizing interactions with residues located in functionally relevant regions of each protein.

Among the evaluated targets, MMP-2 exhibited the strongest predicted interaction, which was subsequently corroborated by experimental evidence showing reduced mRNA (Fig. 6D) and protein levels following B2OCH₃ treatment (Fig. 6E). Actin also displayed a favorable docking profile and was experimentally validated by decreased protein expression and marked disruption of the actin

cytoskeletal network, as demonstrated by Western blot (Fig. 6F) and immunofluorescence analyses (Fig. 4D).

Although MMP-9 showed a thermodynamically favorable binding energy *in silico*, its gene expression was not significantly modulated under the experimental conditions tested (Fig. 6D). Overall, these results support MMP-2 and actin as the primary experimentally validated molecular targets of B2OCH₃.

When complexed with actin, hydrogen bond interactions were observed with Asn101(A), and hydrophobic bonds with Ala180(A), Thr179(A), Val181(A), Asn258(B), Met259(B), Lys352(B), Leu255(B), Ala250(B), Lys254(B), Leu248(B), and Asn249(B), as shown in (Fig. 5A of the supplementary material). In MMP-2, B2OCH₃ interacts through hydrophobic contacts with (A), Val118(A), Tyr143(A), His121(A), Leu117(A), Thr146(A), Phe149(A), Ile142(A), Ala140(A), Thr144(A), Pro135(A), Ala137(A), Pro141(A), and Leu83(A) (Fig. 5B of the supplementary material). In MMP-9, a hydrogen bond was found at Tyr248(A), and other additional bonds at Ala189(A), Gln227(A), Arg249(A), Val223(A), Tyr245(A), His226(A), Met247(A), Ala242(A), Leu188(A), Leu243(A), Glu241(A), Thr251(A), Pro255(A), Leu222(A), Gly186(A), and Leu187(A) for MMP-9 (Fig. 5C of the supplementary material).

Taken together, these findings provide experimental support for MMP-2 as a primary molecular target and indicate modulation of actin-associated cytoskeletal organization.

Discussion

This study describes the biological effects of a novel symmetrical bis-chalcone (B2OCH₃) in *in vitro* models of oral squamous cell carcinoma (OSCC). Consistently, our findings indicate that (1) B2OCH₃ exhibits potent cytotoxic activity against the HSC-3, SCC-4, and CAL-27 tumor cell lines, with remarkable selectivity over the non-tumoral HS5 cell line; (2) it significantly reduces the clonogenic potential and anchorage-independent growth of HSC-3 cells; (3) it impairs migration and invasion in both 2D and 3D models, in addition to inhibiting cell adhesion; and (4) at the molecular level, the compound decreases MMP-2 expression and reduces pan-actin protein levels. Taken together, these results support B2OCH₃ as a promising preclinical lead compound with the potential to advance into studies employing more complex biological models. Although the available data are derived primarily from *in vitro* and *in silico* approaches, they provide a solid foundation for subsequent investigations in more advanced experimental systems.

In the context of current therapies for oral OSCC, the *in vitro* selectivity index enables comparison with

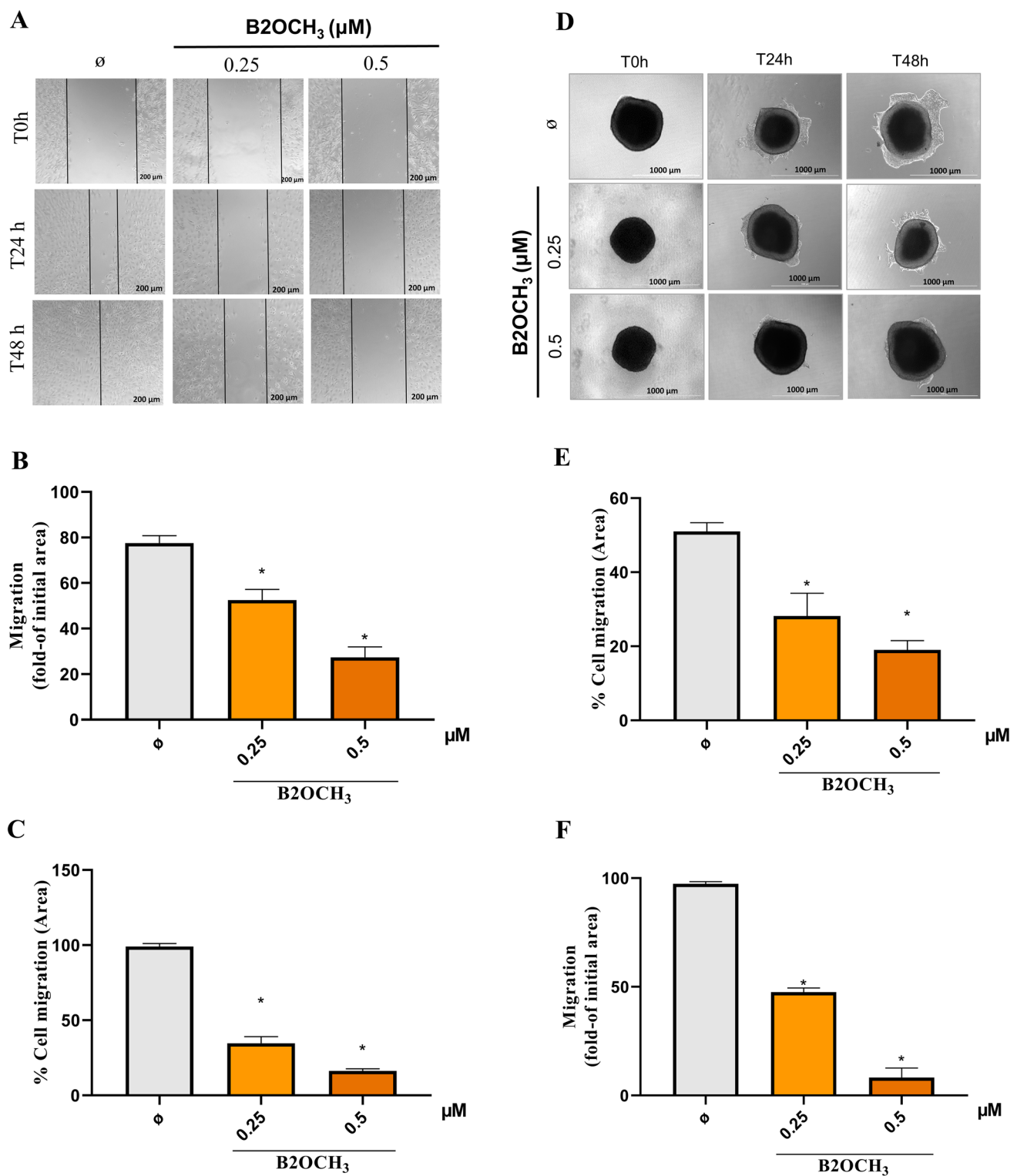


Fig. 5 A) Representative images of the scratch wound-healing assay showing HSC-3 cell migration after treatment with vehicle or B2OCH₃ (0.25 and 0.5 μ M) at 0, 24, and 48 h. Scale bar: 200 μ m. Quantification of scratch closure after 24 h (B) and 48 h (C) of treatment. D) Representative images of the spheroid-based migration assay of HSC-3 cells treated with vehicle or B2OCH₃ (0.25 and

0.5 μ M) for 24 and 48 h. Scale bar: 1000 μ m. Quantification of the spheroid model after 24 h (E) and 48 h (F) of treatment. Data are expressed as mean $\pm$ SEM from three independent experiments ($n = 3$, each performed in triplicate). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$)

chemotherapeutic agents commonly used in clinical practice, such as cisplatin, 5-fluorouracil, paclitaxel, and docetaxel, which typically display low micromolar IC_{50} values in tumor cells but also affect non-tumor cells under in vitro conditions (Eloraby et al. 2024; Sati et al. 2024; Cabral et al. 2025). Within this framework, B2OCH₃ exhibited a differential response between tumor and non-tumor cells across the tested concentration range. Importantly, the effective subcytotoxic concentrations fall within ranges reported for compounds at early preclinical stages; however, their pharmacological relevance remains to be validated in more complex biological models.

In HSC-3 cells, the effects occurred after a 3 h pulse treatment (at minimum) and lasted for several days, as demonstrated by the MTT assays (72 h). While speculative, this observation may provide initial insights into pharmacokinetic behavior, however this underscores the need for dedicated in vivo studies (De Almeida et al. 2019).

Previous studies have reported antiproliferative effects of bis-chalcone analogs in cancer cells (Yang et al. 2020; Abubakar et al. 2024). In line with these observations, B2OCH₃ exhibited greater selectivity toward HSC-3 cells than toward the non-cancerous HS5 cell line, supporting its evaluation as a selective scaffold for therapeutic development. Based on these findings, we further investigated the effects of this compound on cancer cell biology. Among the established hallmarks of cancer, the ability to invade and metastasize is particularly critical, as metastatic disease remains a major cause of cancer-related morbidity and mortality (Hanahan 2022; Gerstberger et al. 2023).

Tumor metastasis is a complex, multifactorial process involving cell adhesion, invasion, and motility. For anti-metastatic therapy, targeting and disrupting any of these steps is a promising strategy (Pearson 2019). The capacity of cancer cells to form colonies reflects their ability to survive independently and adapt to diverse environmental conditions, offering insights into their tumorigenic potential following metastasis (Lambert et al. 2024). In this study, B2OCH₃ was found to reduce the size of HSC-3 colonies, even at subcytotoxic concentrations. This finding is particularly noteworthy given the highly aggressive nature of HSC-3 cells, which are widely used to evaluate compounds targeting cell migration and invasiveness. Further, B2OCH₃ effectively inhibited anchorage-independent colony formation in the soft agar assay. Resistance to anoikis, a form of apoptosis induced by detachment from the extracellular matrix, is a critical adaptation that facilitates cancer cell dissemination and represents a key process in metastatic cancers, including advanced tongue squamous cell carcinoma (Adeshakin et al. 2021).

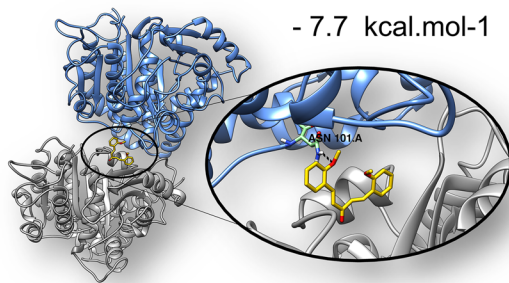
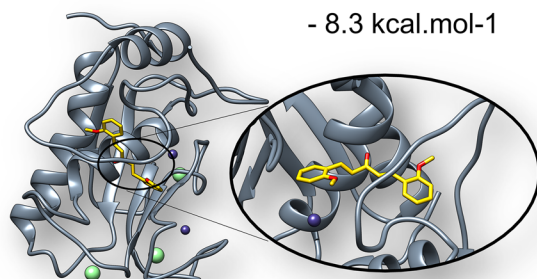
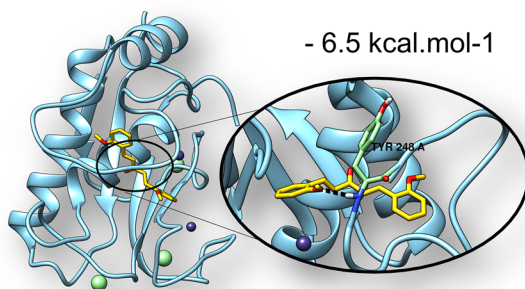
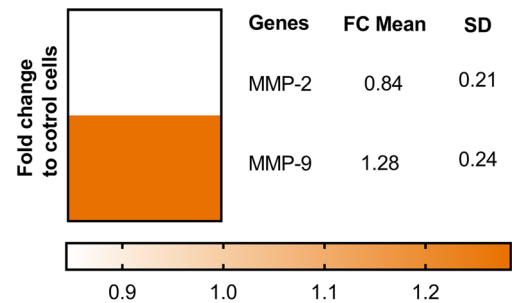
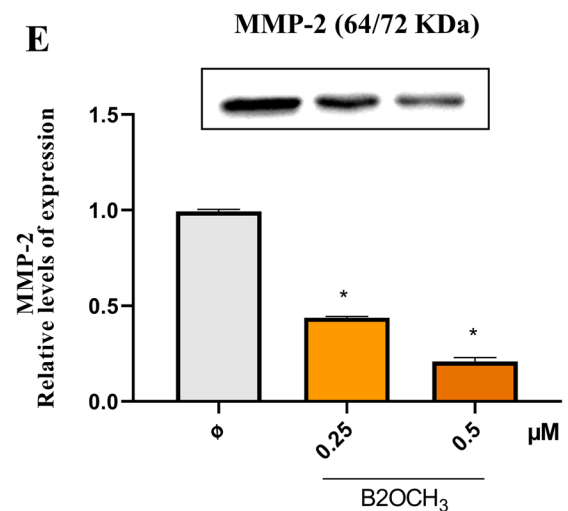
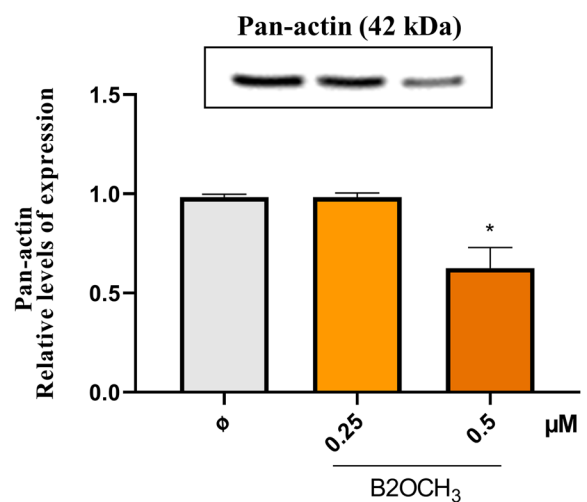
During the functional analyses, B2OCH₃ significantly inhibited both the migration and invasion of tongue cancer cells in 2D and 3D cellular models, primarily through

modulation of key proteins involved in tumor progression (including actin and MMP-2). These processes require dynamic alterations in cell morphology, migratory capacity, adhesion to the extracellular matrix, and the ability to invade surrounding tissues. By disrupting these mechanisms, B2OCH₃ may impair critical cellular processes associated with metastatic behavior in vitro (Hamidi and Ivaska 2019; Lima et al. 2023; Crossley et al. 2024). Successful cell migration depends on the coordinated assembly of adhesion structures and the organization of actin filaments. Evidence indicates that actin stress fiber organization is critical for cell stiffening during invasion and also contributes to the expansion of premalignant cancer cells (Mancini et al. 2024).

Immunofluorescence analysis showed that B2OCH₃ treatment led to disorganization of the actin cytoskeleton and a reduction in cell body length in tongue cancer cells. These changes were associated with altered adhesion and reduced migratory and invasive behavior in vitro, particularly in the HSC-3 cell line, which represents a highly aggressive tongue cancer subtype. The observed decrease in adhesion, migration, and invasion may be related to metalloproteinase activity, specifically the binding and negative regulation of MMP-2 following exposure to B2OCH₃. MMP activity is controlled by a complex network of molecular pathways within the tumor microenvironment and is influenced by multiple signaling molecules (Rajendran 2024; Shi et al. 2024). Consistent with these findings, inhibitory effects on MMP-2 have also been reported for other chalcone-based compounds in in vitro models (De Souza et al. 2022). Given the central role of matrix metalloproteinases in tumor progression, targeting MMP-2 interactions with cell surface molecules remains an area of active therapeutic interest (Lenci et al. 2021; Kim et al. 2023).

Although B2OCH₃ exposure was associated with marked cytoskeletal disorganization and reduced formation of actin-based protrusions, the present data do not establish a direct cause–effect relationship between actin modulation and the impaired migratory and invasive behavior. Actin remodeling may represent a downstream consequence of altered cell–ECM interactions and reduced MMP-2 expression, both of which are known to influence focal adhesion dynamics and cytoskeletal organization (Eren et al. 2022). Alternatively, actin-associated pathways may be directly sensitive to B2OCH₃, contributing to the observed phenotype. Given the absence of direct target engagement or rescue experiments, these mechanisms cannot be distinguished in the current study. Therefore, actin modulation is interpreted as a biologically relevant correlate of reduced migration rather than as a confirmed primary molecular target.

Our results demonstrated that B2OCH₃ treatment markedly reduced filopodia formation, a feature we associated with decreased motility in HSC-3 cells. Immunofluorescence

A**B****C****D****E****F**

analysis also indicated that this loss of filopodia is likely linked to changes in adhesion dynamics and cytoskeletal rearrangements. Given the central role of filopodia in cytoskeletal organization, their loss is expected to influence cell architecture and contribute to the observed phenotypic changes (Janiszewska et al. 2020).

In addition to these structural changes, a clear shift in cell morphology was observed. While untreated HSC-3 cells displayed a spindle-like phenotype, B2OCH₃-treated cells exhibited a more rounded shape. Cell morphology is intrinsically associated with the underlying cytoskeletal framework and biophysical properties of cells, including

Fig. 6 Molecular interactions of B2OCH₃ with actin, MMP-2, and MMP-9, and their effects on gene and protein expression. (A–C) Tree and two-dimensional representations of the molecular interactions between the compound B2OCH₃ and the proteins actin (A), MMP-2 (B), and MMP-9 (C), indicating the binding site, molecular interactions and binding energy values (kcal mol⁻¹) for each molecular complex. (D) Heat map showing the relative variation in MMP-2 and MMP-9 gene expression in HSC-3 cells treated with B2OCH₃. Gene expression analysis was performed using samples from three independent experiments (*n* = 3). Orange shades indicate increased mRNA levels, while lighter shades indicate decreased expression. (E–F) Protein levels of pan-actin (E) and MMP-2 (F) in HSC-3 cells treated with B2OCH₃. Membranes were reprobed with the antibody to detect total protein GAPDH. Quantitative data are expressed as mean ± SEM from three independent experiments (*n* = 3), each experiment performed in triplicate. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (**p* < 0.05)

their mechanical characteristics (Zhao et al. 2020). Notably, metastatic cancer cells from various tumor origins often present cytoskeletal remodeling, which enhances their deformability and promotes invasive behavior. Thus, the morphological changes observed following B2OCH₃ treatment may reflect biomechanical alterations associated with disrupted cytoskeletal organization and modulation of adhesion protein expression (Xiao et al. 2019; Li et al. 2021).

Reductions in focal adhesion are not only associated with integrin inhibition but may also reflect alterations in downstream signaling pathways mediated by the extracellular matrix (ECM) (Pang et al. 2023). In this study, B2OCH₃ was associated with decreased pan-actin expression in HSC-3 cells. The actin cytoskeleton is known to contribute to cell motility through the formation of dynamic structures such as lamellipodia and filopodia. Taken together, our *in silico* and *in vitro* data suggest that modulation of actin-related cytoskeletal organization may be associated with the reduced migratory behavior observed after B2OCH₃ exposure.

This study employed an experimental *in vitro* model of metastatic tongue squamous cell carcinoma to investigate key aspects of cancer cell biology. The results demonstrate that B2OCH₃ induces morphological, molecular, and functional alterations in HSC-3 cells under controlled experimental conditions. Specifically, the compound exhibited selective activity *in vitro*, impaired tumor cell growth, and reduced adhesion, migration, and invasion, concomitant with modulation of MMP-2 expression and actin-associated cytoskeletal organization.

Despite the strength and coherence of the *in vitro* findings, this study has inherent limitations, as all analyses were restricted to cellular models. Nevertheless, the results provide valuable insights and reinforce the potential relevance of B2OCH₃, supporting further investigation into its safety, pharmacological profile, and pharmacokinetic behavior.

Conclusion

Our study provides evidence that B2OCH₃ exerts multiple biological effects in an *in vitro* model of tongue squamous cell carcinoma, leading to reduced cell viability and impairment of cellular processes associated with the metastatic phenotype, accompanied by morphological and molecular alterations. The data indicate that B2OCH₃ displays a selective profile and modulates pathways related to MMP-2 expression and actin-associated cytoskeletal organization, as supported by gene expression, protein analyses, and cytoskeletal imaging.

Overall, these findings demonstrate that B2OCH₃ influences key cellular mechanisms associated with oral squamous cell carcinoma aggressiveness in experimental cell models.

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Author contributions R.E.S., B.O.A., M.P.O., M.C.M., A.D.M.B.G., K.L., and A.B.V. performed the experiments, wrote the manuscript, and designed the figures. J.A.R., J.D.B.M.F., C.N.P., J.A.M.N., L.V.C.L., and A.J.A. conceived the original idea and supervised the project. All authors reviewed and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability All source data for this work (or generated in this study) are available upon reasonable request.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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