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Santamarine Synergizes With Cisplatin via ROS/JNK Axis to Selectively Induce Apoptosis and DNA Damage in Oral Cancer Cells In Vitro

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ABSTRACT

Santamarine (SAMA), a *Michelia compressa*-derived sesquiterpene lactone, exhibits ROS-mediated anticancer activity; however, its potential mechanisms and synergy with cisplatin in oral cancer have not been explored. This study investigated SAMA and cisplatin's combined effects and underlying ROS/MAPK mechanisms of action on oral cancer cells. The combination significantly reduced viability (ATP assay) in oral cancer Ca9-22 and CAL 27 cells compared with either agent alone, with synergistic indices confirming enhanced antiproliferation. Moreover, this combination markedly suppressed colony formation and cell migration. SAMA/cisplatin induced subG1 accumulation and G2/M arrest, elevated cellular and mitochondrial ROS, and promoted apoptosis, as evidenced by increased annexin V positivity and caspase-3, -8, and -9 activation, while DNA damage markers γ H2AX and 8-OHdG were also markedly elevated. In contrast, normal oral S-G cells showed minimal cytotoxic damage and mechanical changes, suggesting selectivity. Mechanistically, JNK inhibition rescued the antiproliferative response and suppressed apoptosis, caspase activation, and DNA damage. Pretreatment with the ROS scavenger NAC attenuated all test effects, confirming ROS dependence. Together, these findings demonstrate that SAMA synergizes with cisplatin to selectively enhance oxidative stress, apoptosis, and DNA damage in oral cancer cells via ROS-dependent JNK signaling, highlighting its potential as a cisplatin adjunct.

Hsin-I Lu and Kuan-Liang Chen are contributed equally to this work.

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1 | Introduction

Oral cancer ranks among the top 10 most common cancers worldwide (Rivera 2015). Although radiotherapy and chemotherapy can improve treatment outcomes, benefits are often limited by their associated adverse effects (Riva et al. 2022; Silverman 1999). However, accumulating evidence shows that combined therapies can overcome specific resistance mechanisms and produce clinical benefit (Lopez and Banerji 2017). Moreover, combination therapies can, in some cases, reduce overall toxicity and improve safety by allowing dose reduction for individual agents, exploiting complementary mechanisms, and achieving pharmacologic synergy in the treatment of diseases (Liu et al. 2025) including cancers (Glasmacher et al. 2025; Qiu and Li 2025).

Sesquiterpene lactones display antiproliferative effects across multiple cancer types. Several members of this class can generate reactive oxygen species (ROS), including carpespene A (Yan et al. 2023) and santamarine (SAMA) (Lu et al. 2024), which induce ROS-mediated apoptosis in liver and oral cancer cells. ROS can promote apoptosis by modulating mitogen-activated protein kinase (MAPK) pathways (Shiau et al. 2022; Woo et al. 2013; Zhong et al. 2016), including c-Jun N-terminal kinase (JNK) (An et al. 2020), extracellular signal-regulated kinase (ERK) (Acosta-Casique et al. 2023), and p38 (Dong et al. 2016), links which justify a focused investigation of ROS production and MAPK responses in cancer models treated with sesquiterpene lactones.

Recently, several synthetic compounds (Świtalska et al. 2022) and natural products (Shan et al. 2022) have been investigated in vitro as adjuncts to conventional anticancer drugs. Cisplatin is routinely used as the systemic agent given concurrently with radiotherapy for locoregionally advanced oral cancer (Gupta et al. 2022), and some natural products have been shown to enhance cisplatin sensitivity (Dasari et al. 2022; Sun et al. 2019); for example, ginsenosides exhibit anticancer potency, attenuating cisplatin's side effects (Li et al. 2024). Hence, the potential synergies of other natural products with cisplatin need to be investigated to enhance oral cancer treatment.

Certain sesquiterpene lactones, such as carpespene A (Yan et al. 2023) and costunolide (Li et al. 2022), have been reported to sensitize liver and oral cancer cells to cisplatin. Santamarine (SAMA) is a naturally occurring sesquiterpene lactone characterized by an α -methylene- γ -lactone moiety, a structural feature commonly associated with the biological activity of sesquiterpene lactones. Previous studies have shown that SAMA exhibits antiproliferative activity in several cancer types, including liver (Mehmood et al. 2017), cervical (Zhang et al. 2021), lung (Wu et al. 2017), and oral (Lu et al. 2024) cancer cells. These properties suggest that SAMA may act as a potential chemosensitizing agent, and its combination with cisplatin may enhance anticancer efficacy while potentially allowing dose reduction of cisplatin, thereby reducing treatment-related toxicity. However, an EndNote search (retrieved 30 Dec. 2025) identified no combined SAMA/cisplatin studies. Although SAMA can modulate ROS in specific cancer cells, its effects on the MAPK signaling pathway remain unexplored. Moreover, the impact of oxidative stress and MAPK on combined SAMA/

cisplatin treatment is unclear, particularly for oral cancer cells, and the safety of the combination is also unknown.

Accordingly, we examined the combined effects of SAMA/cisplatin on oral cancer cells and investigated the MAPK-related mechanisms behind their enhanced antiproliferative activity, alongside performing experiments on normal oral cells.

2 | Materials and Methods

2.1 | Drug Information

SAMA was isolated from *Michelia compressa* and validated by NMR as described in our previous study (Lu et al. 2024). Cisplatin (Selleckchem) was dissolved in 1× PBS. Inhibitors of ROS (*N*-acetylcysteine (NAC), 10 mM pretreatment (Sigma-Aldrich)) and MAPKs (p38 (SB203580, 10 μ M), JNK (SP600125, 5 μ M), and ERK1/2 (PD98059, 2 μ M) (Abmole BioScience)) were pretreated for 1 h before SAMA treatment.

2.2 | Cell Cultures, Viability, and Combination Index

Human oral cancer cell lines Ca9-22 and CAL 27 were obtained from the JCRB Cell Bank (Osaka, Japan) and ATCC (Manassas, VA, USA), respectively. Smulow-Glickman (S-G) gingival epithelial cells (Hsieh et al. 2020; Kasten et al. 1989; Kasten et al. 1990) served as normal controls. Culture conditions were as previously described (Shiau et al. 2022).

Cell viability following single or combined treatments was measured using an ATP detection kit (PerkinElmer Life Sciences) and normalized to vehicle-treated controls (100% viability). Fractional inhibition was calculated as $1 - \text{viability fraction}$. Combination index was calculated using Bliss synergy score based on the Bliss independence model (Bliss 1939). The expected inhibition was calculated as $E_{\text{Bliss}} = A + B - A \times B$, where A and B represent the fractional inhibition induced by each single agent (SAMA or cisplatin). The observed inhibition was obtained from the combination treatment (SAMA and cisplatin). The Bliss synergy score (ΔBliss) was defined as the difference between observed and expected inhibition. Values of ΔBliss equal to, greater than, and less than 0 correspond to additive, synergistic, and antagonistic effects, respectively.

2.3 | Colony Formation

Cells were seeded into 6-well plates at a density of 3000 cells per well and allowed to attach overnight. Cells were then treated with the indicated treatments or vehicle control and cultured for 7 days. At the end of the incubation period, colonies were fixed with 4% paraformaldehyde for 15 min and stained with Coomassie blue (Li et al. 2024).

2.4 | Wound Healing

Cells were seeded into 6-well culture plates and grown to 90%–100% confluence, after which a straight scratch was

created using a sterile 200- μ L pipette tip. Detached cells were removed by washing with PBS, and cells were cultured in serum-free medium and treated with the indicated or vehicle control. Images were captured at 0 h and indicated time points using a microscope. Wound areas were quantified using ImageJ software, and wound closure was calculated as the percentage of wound area closed relative to the initial wound area at 0 h (Peng et al. 2020).

2.5 | Cell Cycle

Cells fixed in 75% ethanol were stained with 7-aminoactinomycin D (7-AAD; 1 μ g/mL) for 30 min (Biotium) (Vignon et al. 2013). DNA-bound fluorescence was measured on a Guava easyCyte flow cytometer (Luminex) and used for cell cycle analysis.

2.6 | Oxidative-Stress-Associated Responses (ROS, MitoSOX, and Mitochondrial Membrane Potential (MMP))

ROS, as indicators of cellular oxidative stress, were assessed by flow cytometry as follows. Non-fixed cells were incubated with DCFH-DA (Liu et al. 2020; Shiau et al. 2022) for 30 min according to the manufacturer's instructions, and the fluorescence generated by the DCFH-DA reaction was measured to quantify ROS levels. Mitochondrial oxidative stress was assessed using MitoSOX and MMP probes: non-fixed cells were incubated with 5 μ M of MitoSOX Red dye (Sigma-Aldrich) for 30 min (Sgarbi et al. 2018) and 50 nM of DiOC₂(3) (Invitrogen) for 20 min (Yen et al. 2014), with their fluorescence signals detected by flow cytometry.

2.7 | Apoptosis Response (Annexin V/7AAD)

Annexin V/7AAD staining was used to evaluate apoptosis. Following the manufacturer's instructions, unfixed cells were incubated with an Annexin V/7AAD solution (Strong Biotech) to detect general apoptosis response, and fluorescence intensities were measured by flow cytometry (Fan et al. 2020; Shiau et al. 2022).

2.8 | Apoptotic Caspase-3/8/9 Signaling (Flow Cytometry)

Caspase-3, -8, and -9 activations were measured by using their specific peptide substrates—PhiPhiLux-G1D2, CaspaLux8-L1D2, and CaspaLux9-M1D2 (OncoImmunin)—at a 1:1000 dilution (Chou et al. 2018; Lee et al. 2017). Unfixed cells were incubated with the probes at 37°C for 1 h; upon induction of apoptosis, activated caspases cleaved the peptides, generating green-fluorescent products that were detected by flow cytometry.

2.9 | Apoptotic Caspase-3/8/9 Signaling (Luminescence)

Promega's Caspase-Glo 3/7, -Glo 8, and -Glo 9 reagents were used to assess caspase cleavage activity by luminescence.

Assays were carried out following the manufacturer's protocol, and luminescent signals for caspase-3/7, -8, and -9 were recorded with a Berthold Technologies luminometer (Chuang et al. 2025).

2.10 | Western Blotting for Apoptotic Caspase-3/8/9 and p-JNK/JNK

We loaded 36 μ g of protein per lane onto a 10% SDS-PAGE gel. Proteins were then transferred to PVDF membranes and blocked overnight in 5% skim milk. Apoptotic markers were examined by Western blotting with a Cell Signaling apoptosis sampler kit targeting cleaved caspase-3, -9, and -8, as described earlier (Chien et al. 2021). p-JNK and JNK were detected with primary antibodies (1:1000, Cell Signaling Technology) followed by a 1:10,000 secondary antibody.

2.11 | DNA Damage Flow Cytometry (γ H2AX and 8-Hydroxy-2'-deoxyguanosine (8-OHdG))

DNA damage markers γ H2AX and 8-OHdG were detected as described in (Shiau et al. 2022). Fixed cells were incubated with anti- γ H2AX primary antibody (Santa Cruz; 1:500), washed, stained with an Alexa Fluor 488-conjugated secondary antibody (Cell Signaling) together with 7-AAD for 30 min, and analyzed by flow cytometry for Alexa Fluor 488 signal. Fixed cells were stained with FITC-conjugated anti-8-OHdG (Santa Cruz) without a secondary antibody and also analyzed by flow cytometry.

2.12 | Statistical Analysis

Statistical significance was evaluated with JMP12 (SAS Institute). For multiple comparisons, one-way ANOVA followed by Tukey's HSD test was used. JMP12 assigned lowercase letters to each treatment to indicate significant differences; treatments that did not share letters were considered significantly different ($p < 0.05$). Experiments were performed in triplicate, and data are presented as mean $\pm$ standard deviation.

3 | Results

3.1 | Selective Antiproliferation of SAMA/Cisplatin in Oral Cancer Cells

The SAMA/cisplatin combination reduced viability (24 h ATP assay) to 36.15% in Ca9-22 cells and 41.29% in CAL 27 cells (Figure 1A), significantly lower values than with cisplatin (81.85% and 75.49%) or SAMA alone (49.40% and 89.82%) ($p < 0.05$), indicating an enhanced antiproliferative effect of the combined treatment. To further evaluate the interaction between SAMA and cisplatin, Bliss independence analysis was performed. The combination indices (Δ Bliss) for Ca9-22 and CAL 27 were 0.23 and 0.15, respectively, indicating synergism (positive Δ Bliss values). Thus, SAMA/cisplatin produces superior antiproliferative effects in oral cancer cells compared with either single agent. In contrast, the combination (7.5 μ g/mL SAMA/4 μ g/mL cisplatin) did not induce cytotoxicity in normal oral S-G cells (Figure 1A), suggesting a favorable safety profile for normal cells at these concentrations.

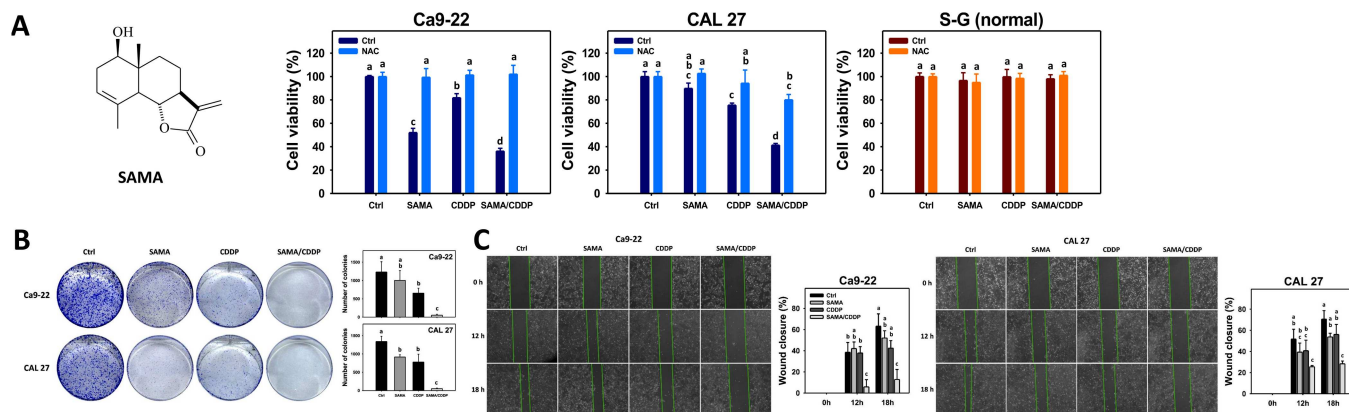


FIGURE 1 | Cell viability and migration assays following SAMA and/or cisplatin treatment. (A) Cell viability assay (ATP detection). Oral cancer cell lines (Ca9-22, CAL 27) and normal S-G cells were exposed for 24 h to one of four conditions—vehicle control (0.1% DMSO), SAMA (7.5 $\mu\text{g/mL}$), cisplatin (CDDP; 4 $\mu\text{g/mL}$), or the SAMA/CDDP combination (7.5 and 4 $\mu\text{g/mL}$)—either with or without a 1-h NAC pretreatment (10 mM). (B) Colony formation assay. Cells were exposed to the same conditions for 7 days. (C) Cell migration assay (wound healing). Cells were exposed to the same conditions for 0, 12, and 18 h. Data = mean $\pm$ SD ($n = 3$). Treatments labeled with non-overlapping letters are significantly different from each other ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test) within the same cell type. In the example of CAL 27 cells, the cell viabilities of the control, SAMA, CDDP, and SAMA/CDDP ("a, c, b, d") were statistically distinct, as indicated by their lettering not overlapping.

To assess the role of oxidative stress in SAMA/cisplatin treatment, cells were pretreated with the ROS scavenger NAC, which significantly increased viability across all single and combined treatments compared with non-NAC controls (Figure 1A, $p < 0.05$), indicating that the enhanced antiproliferative effect of SAMA/cisplatin is ROS-dependent.

To evaluate the long-term inhibitory effects of SAMA/cisplatin on oral cancer cell proliferation, a colony formation assay was performed in Ca9-22 and CAL 27 cells (Figure 1B). Treatment with SAMA or cisplatin alone significantly reduced the number of colonies compared with the control group. Notably, the combination treatment produced a significantly greater inhibitory effect than either SAMA or cisplatin alone, suggesting a synergistic suppression of clonogenic survival in oral cancer cells.

3.2 | Anti-Migration of SAMA/Cisplatin in Oral Cancer Cells

Furthermore, to determine whether the SAMA/cisplatin combination affects cell migratory capacity, wound healing assays were performed in Ca9-22 and CAL 27 oral cancer cells (Figure 1C). In both cell lines, SAMA or cisplatin alone moderately reduced wound closure at 12 and 18 h compared with the control group. In contrast, the combined treatment markedly suppressed wound closure at both time points. These results indicate that the SAMA/cisplatin combination effectively inhibits the migratory ability of oral cancer cells.

3.3 | Selective Cell Cycle Arrest by SAMA/Cisplatin in Oral Cancer Cells

Treatment with SAMA/cisplatin or cisplatin alone markedly elevated the subG1, S, and G2/M phases in Ca9-22 and CAL

27 relative to SAMA treatment alone and controls ($p < 0.05$) (Figure 2), accompanied by decreased G1 proportions; by contrast, normal cells exhibited only slight cell cycle distortion.

To assess whether oxidative stress mediates the cell cycle effects of SAMA/cisplatin, cells were pretreated with NAC. This decreased the subG1 population and increased G2/M for both the individual and combined treatments relative to untreated groups, revealing that SAMA/cisplatin drives subG1 and G2/M accumulation through ROS (Figure 2).

3.4 | Selective Induction of Oxidative Stress by SAMA/Cisplatin in Oral Cancer Cells

Given that NAC attenuated the potentiated antiproliferation and cell cycle alterations induced by SAMA/cisplatin, oxidative stress in oral cancer cells merits further in-depth examination.

For cellular oxidative stress, SAMA/cisplatin produced a higher proportion of ROS-positive oral cancer cells than either agent alone (Figure 3A). Examination of mitochondrial oxidative stress revealed that the combination also yielded significantly greater MitoSOX-positive and MMP-negative percentages than single treatments ($p < 0.05$) (Figure 3B,C). By contrast, these parameters changed only minimally in normal S-G cells.

To evaluate oxidative stress, cells were pretreated with the ROS scavenger NAC, which significantly reduced the proportion of ROS-positive cells and lowered MitoSOX positivity (%) while decreasing the MMP loss (%) for both single and combined treatments versus non-pretreated groups ($p < 0.05$) (Figure 3A–C), indicating that SAMA/cisplatin induces both cellular and mitochondrial oxidative stress in oral cancer cells via a ROS-dependent mechanism.

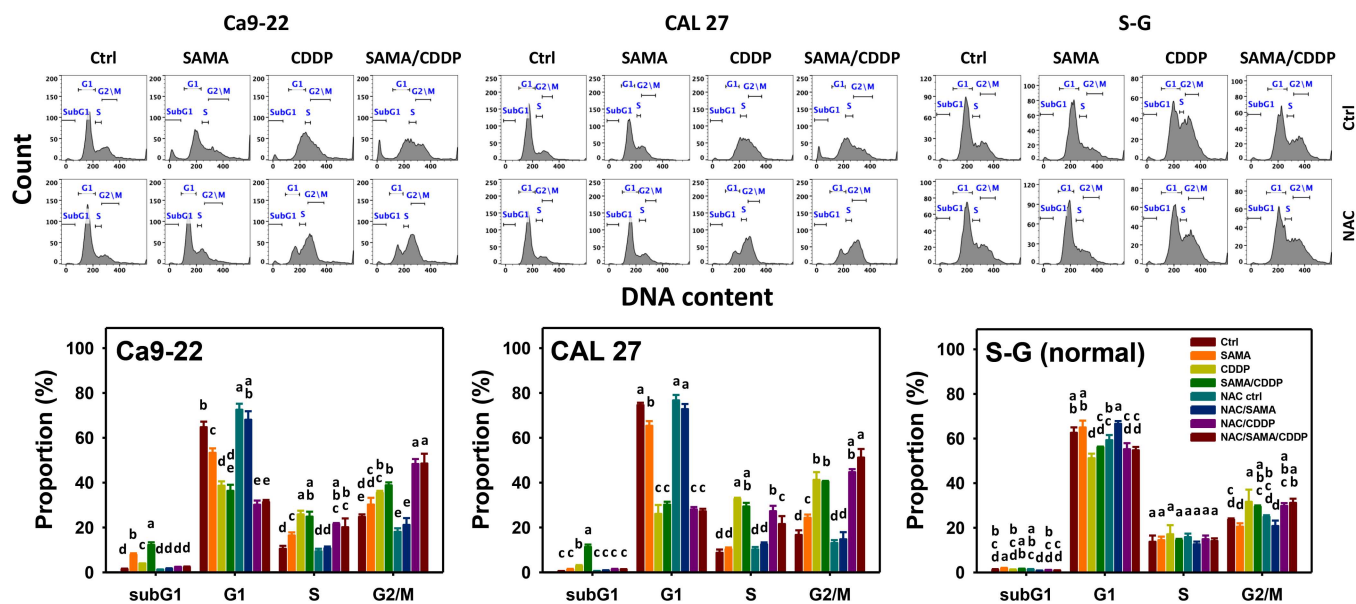


FIGURE 2 | Cell cycle assays following SAMA and/or cisplatin treatment. Oral cancer cell lines (Ca9-22, CAL 27) and normal S-G cells were exposed for 24 h to one of four conditions—vehicle control (0.1% DMSO), SAMA (7.5 $\mu\text{g/mL}$), cisplatin (CDDP; 4 $\mu\text{g/mL}$), or the SAMA/CDDP combination (7.5 and 4 $\mu\text{g/mL}$)—either with or without a 1 h NAC pretreatment (10 mM). Data = mean $\pm$ SD ($n = 3$). Treatments labeled with non-overlapping letters are significantly different from each other ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test) within the same cell type.

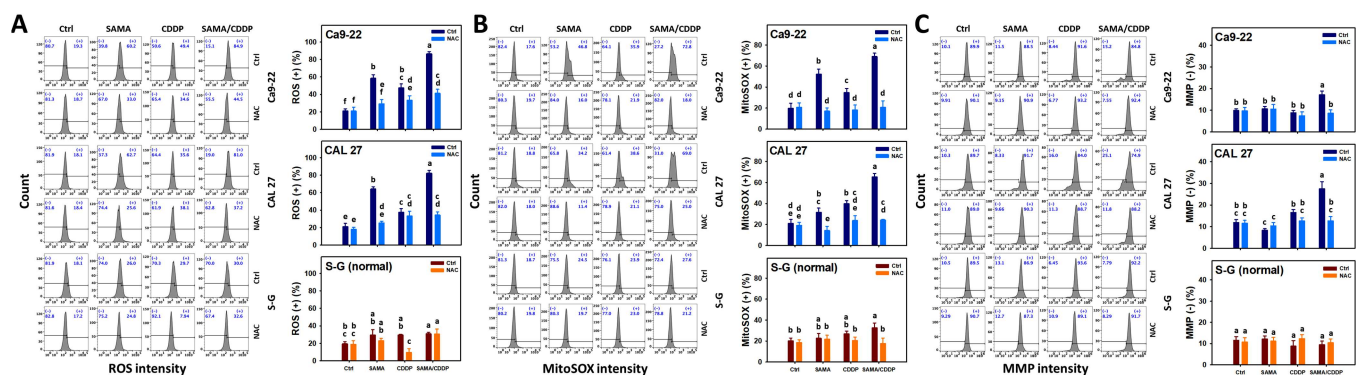


FIGURE 3 | ROS, MitoSOX, and MMP assays following SAMA and/or cisplatin treatment: (A) ROS, (B) MitoSOX, (C) MMP. Oral cancer cell lines (Ca9-22, CAL 27) and normal S-G cells were exposed for 24 h to one of four conditions—vehicle control (0.1% DMSO), SAMA (7.5 $\mu\text{g/mL}$), cisplatin (CDDP; 4 $\mu\text{g/mL}$), or the SAMA/CDDP combination (7.5 and 4 $\mu\text{g/mL}$)—either with or without a 1-h NAC pretreatment (10 mM). Data = mean $\pm$ SD ($n = 3$). Treatments labeled with non-overlapping letters are significantly different from each other ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test) within the same cell type.

3.5 | Selective Apoptosis (Annexin V) by SAMA/Cisplatin in Oral Cancer Cells

SAMA/cisplatin produced significantly higher annexin V-positive signals in Ca9-22 and CAL 27 than either single treatment ($p < 0.05$) (Figure 4A), indicating enhanced apoptosis; by contrast, only a slight increase was observed in normal S-G cells. NAC pretreatment significantly reduced annexin V-positive percentages for both single and combined treatments versus non-pretreated groups ($p < 0.05$) (Figure 4A), indicating that SAMA/cisplatin promotes apoptosis through a ROS-dependent mechanism.

3.6 | Selective Activation of Caspase-3, -8, and -9 by SAMA/Cisplatin in Oral Cancer Cells

To confirm apoptotic signaling, we performed Western blotting and flow cytometry. Cleaved caspase-3, -8, and -9 levels were

significantly upregulated by SAMA/cisplatin compared with single treatments ($p < 0.05$) (Figure 4B), and although the levels in the combined treatment group were considerably greater than with cisplatin alone ($p < 0.05$), the differences were slight in Ca9-22 cells but high in CAL 27 cells. By contrast, only a slight increase was observed in normal S-G cells.

Consistent with Western blotting, caspase-3/8/9 activation correlates with caspase-3/8/9 (+) intensity in flow cytometry. SAMA/cisplatin produced significantly higher caspase-3/8/9 (+) percentages than the single treatment or control treatments ($p < 0.05$) (Figure 5A–C), whereas only a minor increase was seen in normal S-G cells. This suggests that SAMA/cisplatin triggers both extrinsic and intrinsic apoptosis in oral cancer cells.

NAC pretreatment was used to evaluate the role of oxidative stress in caspase activation by SAMA/cisplatin. The ROS

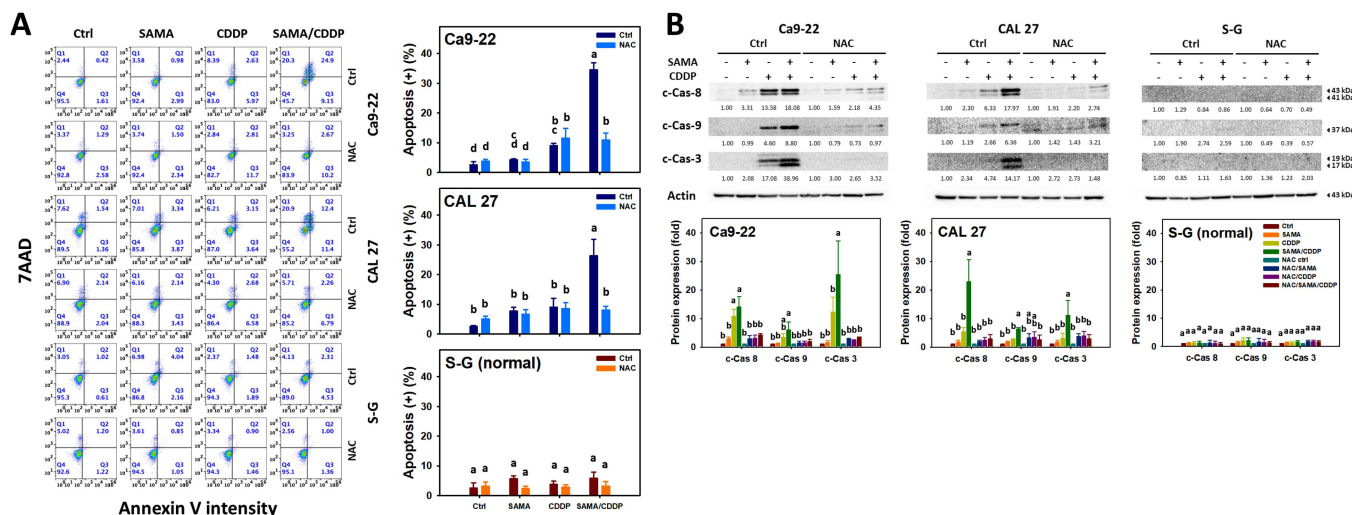


FIGURE 4 | Annexin V staining and Western blotting following SAMA and/or cisplatin treatment: (A) Annexin V/7AAD flow cytometry, (B) Western blotting (caspase-3, -8, and -9). Oral cancer cell lines (Ca9-22, CAL 27) and normal S-G cells were exposed for 24 h to one of four conditions—vehicle control (0.1% DMSO), SAMA (7.5 $\mu\text{g/mL}$), cisplatin (CDDP; 4 $\mu\text{g/mL}$), or the SAMA/CDDP combination (7.5 and 4 $\mu\text{g/mL}$)—either with or without a 1 h NAC pretreatment (10 mM). Data = mean $\pm$ SD ($n = 3$). Treatments labeled with non-overlapping letters are significantly different from each other ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test) within the same cell type.

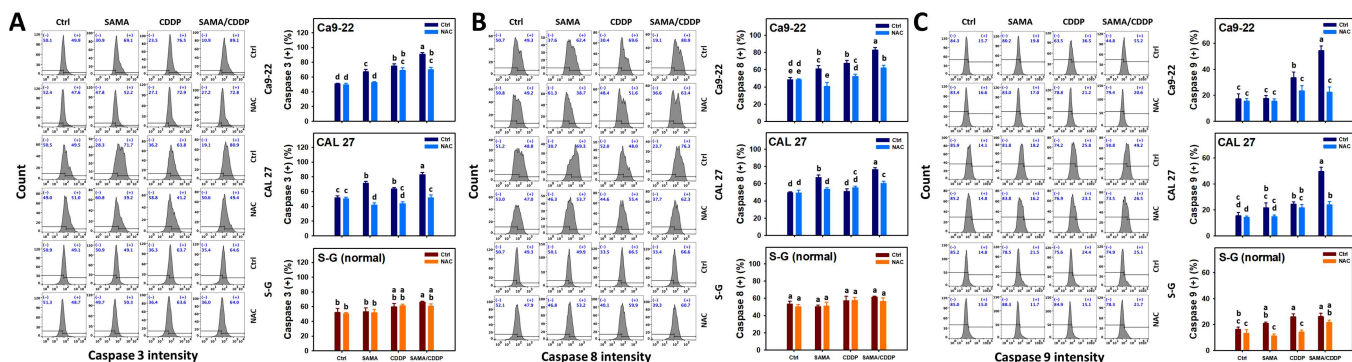


FIGURE 5 | Flow cytometric analysis of caspase-3, -8, and -9 activation following SAMA and/or cisplatin treatment: (A–C) caspases-3, -8, and -9. Oral cancer cell lines (Ca9-22, CAL 27) and normal S-G cells were exposed for 24 h to one of four conditions—vehicle control (0.1% DMSO), SAMA (7.5 $\mu\text{g/mL}$), cisplatin (CDDP; 4 $\mu\text{g/mL}$), or the SAMA/CDDP combination (7.5 and 4 $\mu\text{g/mL}$)—either with or without a 1 h NAC pretreatment (10 mM). Data = mean $\pm$ SD ($n = 3$). Treatments labeled with non-overlapping letters are significantly different from each other ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test) within the same cell type. The percentages of cells positive for caspases-3, -8, and -9 are denoted as caspase-3 (+) (%), caspase-8 (+) (%), and caspase-9 (+) (%), respectively.

scavenger significantly reduced caspase-3, -8, and -9 positivity in both single and combined treatments compared with non-NAC groups ($p < 0.05$) (Figures 4B and 5A–C), demonstrating that SAMA/cisplatin enhances caspase activation in oral cancer cells through a ROS-dependent mechanism.

3.7 | Selective Induction of DNA Damage by SAMA/Cisplatin in Oral Cancer Cells

Given that oxidative stress leads to DNA damage (Barzilai and Yamamoto 2004), we assessed the impact of SAMA/cisplatin on this outcome. After 24 h, the combination markedly elevated γH2AX (%) relative to single treatments ($p < 0.05$) (Figure 6A). Furthermore, 8-OHdG positivity was significantly higher in the combination group than with single treatments ($p < 0.05$) (Figure 6B). In contrast, normal S-G cells exhibited only slight changes in γH2AX and 8-OHdG positivity.

To assess the involvement of oxidative stress in DNA damage, cells were pretreated with NAC, which markedly lowered γH2AX and 8-OHdG positivity under both single and combined treatments relative to untreated groups ($p < 0.05$) (Figure 6A,B), demonstrating that SAMA/cisplatin enhances DNA damage in oral cancer cells via ROS.

3.8 | Triggering ROS/JNK Axis With SAMA/Cisplatin in Oral Cancer Cells

To assess MAPK involvement in SAMA/cisplatin's anti-proliferative activity, cells were pretreated with p38i, JNKi, and ERKi. Only JNKi substantially rescued the antiproliferative effect, while p38i and ERKi had little impact (Figure 7A), with JNKi's ability to counteract this response underscoring the importance of p-JNK. Indeed, SAMA/cisplatin elevated p-JNK levels in oral cancer cells compared to treatment with either

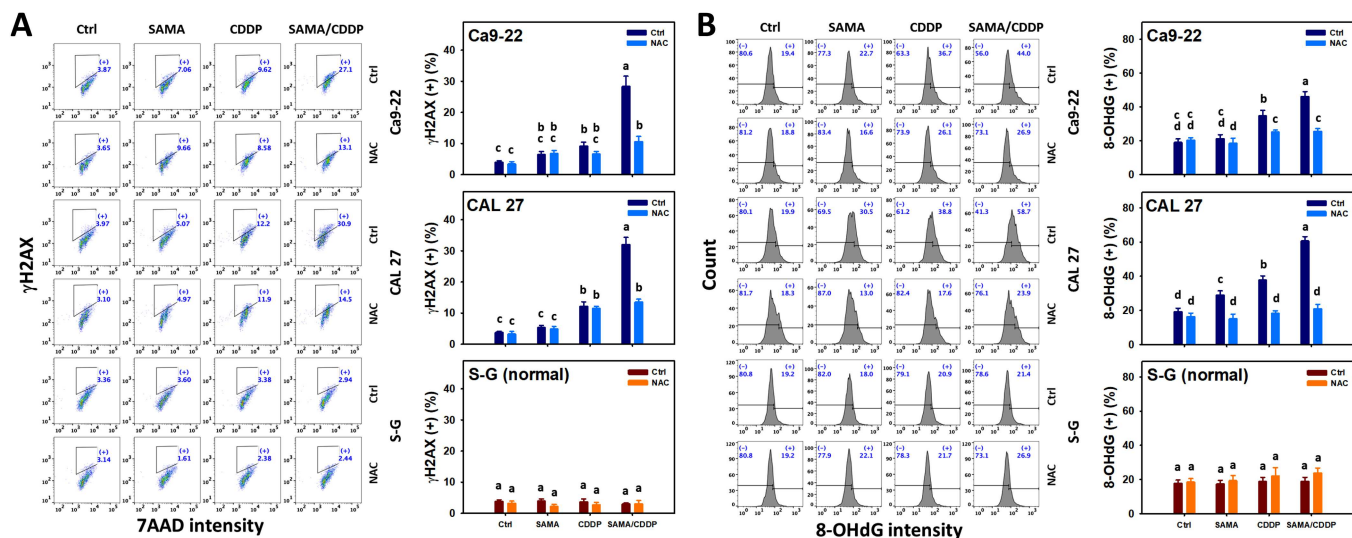


FIGURE 6 | γ H2AX (A) and 8-OHdG (B) assays following SAMA and/or cisplatin treatment. Oral cancer cell lines (Ca9-22, CAL 27) and normal S-G cells were exposed for 24 h to one of four conditions—vehicle control (0.1% DMSO), SAMA (7.5 μ M), cisplatin (CDDP; 4 μ M), or the SAMA/CDDP combination (7.5 and 4 μ M)—either with or without a 1 h NAC pretreatment (10 mM). Data = mean $\pm$ SD ($n = 3$). Treatments labeled with non-overlapping letters are significantly different from each other ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test) within the same cell type. The percentages of γ H2AX- and 8-OHdG-positive cells are denoted as γ H2AX (+) (%) and 8-OHdG (+) (%), respectively.

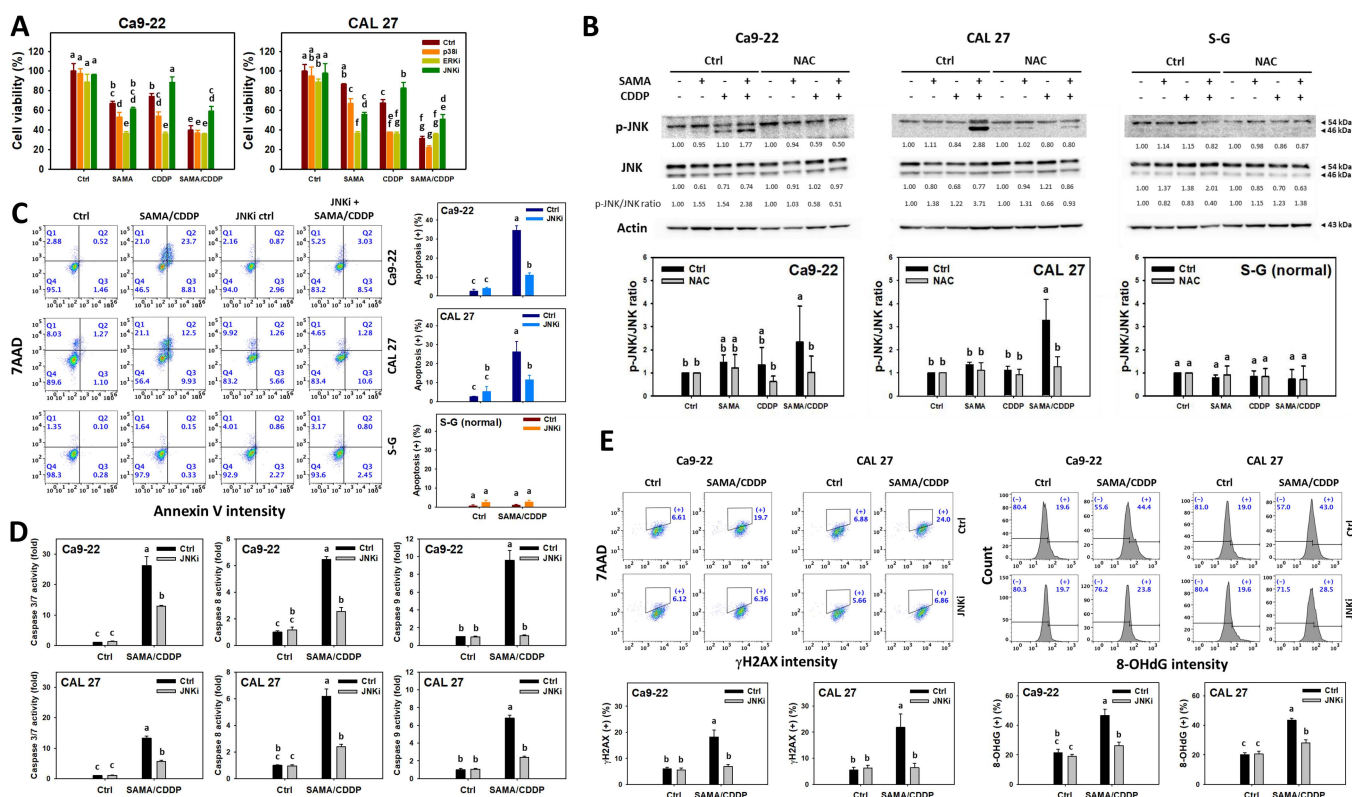


FIGURE 7 | Role of MAPK in regulating cell viability, apoptosis, caspase-3/8/9 activation, and DNA damage. (A) Impact of MAPK inhibitors on cell viability (SAMA or/and CDDP). Oral cancer cells (Ca9-22, CAL 27) were exposed for 24 h to one of four conditions—vehicle control (0.1% DMSO), SAMA (7.5 μ M), cisplatin (CDDP; 4 μ M), or the SAMA/CDDP combination (7.5 and 4 μ M)—either with or without a 1 h MAPK inhibitor pretreatment (p38i (10 μ M), ERKi (2 μ M), and JNKi (5 μ M)). (B) Impact of NAC on p-JNK and JNK. All cells were exposed for 24 h to one of four treatments—control, SAMA, CDDP, and SAMA/CDDP—either with or without a 1 h NAC (10 mM) pretreatment. (C–E) Impact of JNK inhibitor on apoptosis (annexin V), caspase-3, -8, and -9 activations (luminescence assay), and γ H2AX/8-OHdG levels. Cell treatments were similar to (A), but only JNKi was used. Data = mean $\pm$ SD ($n = 3$). Treatments labeled with non-overlapping letters are significantly different from each other ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test) within the same cell type.

agent alone, while in contrast, normal S-G cells showed only minor changes (Figure 7B). Notably, this p-JNK induction in oral cancer cells was suppressed by NAC.

To examine JNK involvement in SAMA/cisplatin-induced apoptosis, caspase activation, and DNA damage, cells were pretreated with JNKi. In oral cancer cells, JNKi reduced the elevated annexin V positivity induced by the combination treatment compared with the single agents. In contrast, normal S-G cells showed only minor changes (Figure 7C). JNKi also diminished the enhanced activation of caspase-3/7, -8, and -9 (Figure 7D), as well as the increased levels of DNA damage markers γ H2AX and 8-OHdG (Figure 7E).

4 | Discussion

Although several sesquiterpene lactones have been reported to sensitize cancer cells to cisplatin (Li et al. 2022; Yan et al. 2023), SAMA's cisplatin sensitization ability has not been previously examined. We examined the antiproliferative effects of SAMA/cisplatin on oral cancer cells and explored the involvement of ROS-driven JNK signaling.

4.1 | The Role of ROS in Sesquiterpene Lactones/Cisplatin Combined Treatments

Numerous synthetic agents and plant-derived compounds have been shown to synergize with cisplatin to inhibit tumor cell growth by increasing ROS production, an elevation which likely contributes to the reported actions of cisplatin (Kleih et al. 2019), synthetic chemicals (Wang et al. 2022), and natural products (Chen et al. 2023). Examples include SK2 (a dioxabicyclo[3.3.1]nonane tricycle) (Wang et al. 2022) and chrysin (Chen et al. 2023), both of which intensify cisplatin-induced apoptosis in oral cancer cells by promoting oxidative stress.

Many sesquiterpene lactones induce ROS. Carpespene A (Yan et al. 2023) and SAMA (Lu et al. 2024) cause ROS-dependent apoptosis in liver and oral cancer models. Costunolide similarly elevates MitoSOX, potentiating cisplatin-induced apoptosis and antiproliferative effects in oral cancer cells by cooperatively raising MitoSOX levels beyond those seen with single agents (Li et al. 2022).

In this study, SAMA combined with cisplatin produced more substantial antiproliferative effects on oral cancer cells in a ROS-dependent manner, as shown by their reversal with NAC pretreatment (Figure 1). Additionally, the combination increased oxidative stress—evidenced by ROS and MitoSOX generation and MMP loss—which was mitigated by NAC (Figure 3). These results indicate that oxidative stress is a central mediator of the enhanced anticancer activity observed with combined sesquiterpene lactone and cisplatin treatment, supporting further exploration of ROS-targeted strategies to improve cisplatin efficacy.

Notably, SAMA/cisplatin inhibits oral cancer cells while sparing normal oral cells (Figure 1), indicating selective antiproliferative activity with potential safety advantages.

4.2 | SAMA/Cisplatin Enhances G2/M Arrest, Apoptosis, and DNA Damage to a Greater Extent Than Either Treatment Alone

Various anticancer therapies increase oxidative stress, leading to apoptosis (Huang et al. 2018; Hung et al. 2016; Shih et al. 2014), caspase activation (Shiau et al. 2022), and DNA damage (Hung et al. 2016; Wu et al. 2018). DNA damage causes G2 arrest, as reported in ochratoxin A-treated immortalized human gastric epithelial cells. G2/M arrest can progress to apoptosis, as observed in prunetin-treated liver cancer cells (Abusaliya et al. 2024). Moreover, cisplatin causes interstrand DNA crosslinks, and SAMA/cisplatin elevates oxidative stress in oral cancer cells. Accordingly, it is crucial to assess G2/M arrest, DNA damage, and apoptosis following SAMA/cisplatin treatment.

Consistently, SAMA/cisplatin induces greater G2/M arrest in oral cancer cell lines (Ca9-22 and CAL 27) than in normal cells. This effect is ROS-dependent, as shown by reversal with NAC pretreatment (Figure 2). The combination also increases apoptosis (annexin V) (Figure 4A), activates both intrinsic and extrinsic caspases (caspases-3, -8, and -9) (Figures 4B and 5), and elevates DNA damage markers (γ H2AX and 8-OHdG) (Figure 6) to a larger extent in oral cancer cells than in normal cells; these responses are similarly reversed by NAC. Therefore, the enhanced G2/M arrest, apoptosis, and DNA damage induced by SAMA/cisplatin in oral cancer cells are attributable to oxidative stress.

4.3 | SAMA/Cisplatin Triggers Antiproliferation, Apoptosis, and DNA Damage in Oral Cancer Cells Through the ROS-JNK Axis

ROS also modulates MAPK signaling, which governs cancer cell death (Fan et al. 2020; Park 2013; Sheng et al. 2020) as MAPK family members—JNK (An et al. 2020), p38 (Dong et al. 2016), and ERK (Acosta-Casique et al. 2023)—can influence apoptosis in cancer cells.

SAMA has been reported to modulate MAPK signaling in noncancerous studies. For example, it inhibits LPS-induced NO production in mouse microglia by suppressing JNK and ERK phosphorylation (Wei et al. 2024), and it exerts anti-adipogenic and anti-UVA photoaging effects in differentiated preadipocytes and human dermal fibroblasts by inhibiting p38 and JNK phosphorylation (Oh et al. 2021). Most noncancer studies, therefore, report SAMA-mediated MAPK inhibition.

In the present study, a JNK inhibitor restored viability in SAMA/cisplatin-treated oral cancer cells more effectively than inhibitors of other MAPKs (Figure 7). SAMA/cisplatin induced p-JNK more strongly in oral cancer cells than either agent alone, with only minor effects in normal cells, and NAC attenuated this induction. These results indicate that SAMA/cisplatin selectively activates p-JNK in oral cancer cells in a ROS-dependent manner (Figure 7B). Moreover, ROS have been shown to suppress protein phosphatases, including dual-specificity phosphatases (DUSPs), through oxidation of their catalytic cysteine residues, thereby prolonging JNK activation (Kamata et al. 2005). Thus, NAC may attenuate JNK

phosphorylation by relieving ROS-mediated inhibition of DUSPs, facilitating JNK dephosphorylation. The involvement of DUSPs in regulating JNK activation for SAMA/cisplatin treatment warrants further investigation.

The role of p-JNK in triggering apoptosis in oral cancer but not normal cells was also confirmed by annexin V assay (Figure 7C). Moreover, the SAMA/cisplatin-induced caspase activation (3/7, 8, and 9) in oral cancer cells was also attenuated by the JNK inhibitor. Likewise, isoalantolactone, another sesquiterpene lactone, enhances cisplatin-induced ROS production, JNK activation, and apoptosis in prostate cancer cells, effects that are reduced by NAC. Together, the data support a model in which ROS-driven JNK activation amplifies cisplatin cytotoxicity when combined with sesquiterpene lactones. These reports also suggest that SAMA's effects on MAPK signaling are cell-context dependent, varying between cancerous and non-cancerous cells, and warrant further investigation.

Moreover, SAMA/cisplatin-induced double-stranded breaks and oxidative DNA damage, as assessed by γ H2AX and 8-OHdG, were attenuated by the JNK inhibitor, suggesting that SAMA/cisplatin has selective antiproliferative effects on oral cancer cells through ROS and JNK signaling regulation.

4.4 | Potential Intracellular Target Proteins of SAMA/Cisplatin

In a molecular docking assay, SAMA was reported to target thioredoxin reductase 1 (TrxR1) (Zhang et al. 2021). Cisplatin can react with thioredoxin-1 (Trx1) and thereby attenuate cisplatin-induced ROS (Wang et al. 2024). This effect is consistent with the role of TrxR1, which reduces oxidized Trx1 to its reduced dithiol form and thus enables Trx1 to function as a protein disulfide antioxidant. Together, the TrxR1–Trx1 system represents a plausible target for SAMA and cisplatin, but this hypothesis requires further experimental validation.

4.5 | The Rationale and Limitation for the Combination of SAMA and Cisplatin

SAMA is a sesquiterpene lactone that contains an α -methylene- γ -lactone moiety, which confers electrophilic reactivity toward protein thiols and underlies its biological effects. Sesquiterpene lactones are generally lipophilic, with poor aqueous solubility, which favors membrane permeability but complicates formulation and reduces oral bioavailability (Barrios 2018). While cisplatin is effective against oral cancer, it can cause dose-limiting toxicities (Madhulaxmi et al. 2017).

Combining SAMA with cisplatin could be advantageous because combination therapy often allows lower doses of each agent while maintaining or enhancing antitumor activity, which may mitigate cisplatin-related side effects and reduce the absolute exposure needed for SAMA, easing formulation and bioavailability challenges. Together, these features make the SAMA–cisplatin combination a rational strategy for improving oral cancer therapy. The synergistic effect and drug safety of the combination were validated in oral cancer and normal cell models in the current study.

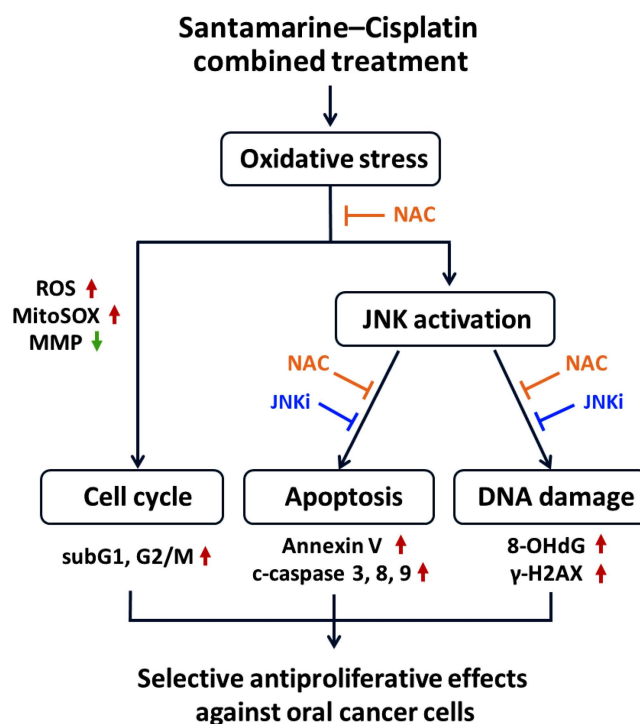


FIGURE 8 | Overview of the increased antiproliferative activity and underlying mechanisms of SAMA/cisplatin in oral cancer cells. The SAMA/cisplatin combination elevates oxidative stress more than the single treatments, resulting in JNK activation. This ROS–JNK signaling axis drives apoptosis and DNA damage, and pretreatment with ROS or JNK inhibitors attenuated these effects. These responses are pronounced in oral cancer cells but minimal in normal oral cells, yielding selective antiproliferative activity with limited toxicity to normal cells.

However, these findings warrant in vivo validation of the safety and antitumor efficacy of the SAMA/cisplatin combination in relevant animal models to support translational development.

5 | Conclusions

Although SAMA is already recognized for its antiproliferative activity against oral cancer cells (Lu et al. 2024), this study is the first to evaluate its safety and combined effects with the chemotherapeutic agent cisplatin in this context. We found that the SAMA/cisplatin combination potentiates antiproliferative effects in oral cancer cells via increased cellular and mitochondrial oxidative stress and JNK-dependent signaling (Figure 8), while sparing normal cells. Specifically, the combined treatment enhanced apoptosis, activated both extrinsic and intrinsic caspases, and elevated markers of DNA damage in a ROS–JNK-dependent manner. Therefore, these results indicate that SAMA enhances cisplatin efficacy via the ROS–JNK axis, supporting further preclinical evaluation of this combination for oral cancer therapy.

Author Contributions

Hsin-I Lu: conceptualization, data curation, formal analysis, writing – original draft. **Kuan-Liang Chen:** conceptualization, methodology. **Chung-Yi Chen:** conceptualization, methodology, writing – original draft. **Ching-Yu Yen:** methodology. **Ammad Ahmad Farooqi:**

conceptualization. **Tsu-Ming Chien:** methodology, writing – original draft. **Jiiang-Huei Jeng:** methodology. **Bing-Hung Chen:** conceptualization, supervision, writing – review and editing. **Hsueh-Wei Chang:** conceptualization, supervision, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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