Calcitriol and Cisplatin Combination Decreases Expression of MAPK2 and NF-kB/p65 Induce Apoptosis in Oral Squamous Carcinoma Cells

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Abstract

Objective to evaluate the effect of calcitriol and cisplatin combination on MAPK2, p65, and NF-κB in OSCC CAL27 cell line.

Using real-time polymerase chain reactions (PCR), we investigated the anti-proliferative and apoptotic effect of calcitriol-cisplatin combination on oral squamous cell carcinoma (OSCC) CAL27 cells by evaluating the expression MAPK2, NF-κB and its p65 subunit.

The combination of calcitriol and cisplatin 36.91 ppm was more effective in decreasing MAPK2, p65, and NF-κB expression compared to cisplatin and calcitriol groups. The treatment of combined calcitriol and cisplatin on OSCC CAL27 cells led to decrease MAPK2, p65 and NF-κB expression resulting in inhibit cells proliferation and induce apoptosis.

In CAL27 cells, the combination of calcitriol and cisplatin inhibits cell proliferation and induces apoptosis more potently than either substance alone, via downregulation of MAPK2, p65, and NF-κB. These effects of calcitriol demonstrate its potential as an effective adjuvant therapy for OSCC.

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Introduction

Oral cancer is a significant global public health concern, but its epidemiology varies considerably between populations. Oral cancer incidence differs geographically, with higher rates reported in Southeast Asia, parts of Eastern Europe, and parts of Sub-Saharan Africa, among others.[1] Alcohol consumption and tobacco use, including smoking and smokeless tobacco, are well-established risk factors for oral cancer.[2–4] According to GLOBOCAN 2020, the prevalence of oral cancer over a five-year period in Indonesia ranks 16th compared to all other cancers.[5] Numerous studies have been conducted in specific areas to overcome oral cancer, including epidermal growth factor receptor, tumor invasion, epithelial-mesenchymal transition, angiogenesis, apoptosis, and metastasis.[6–10]

Oral squamous cell carcinoma (OSCC) is the most common oral cancer found in the world. In OSCC progression, the mitogen activated kinase pathway was activated due to prostaglandin E2 release in inflammation.[11] These mitogen activated kinase (MAPK1 and MAPK2), play crucial roles in cancer proliferation[12], survival[13], invasion[14], metastasis[15], and angiogenesis[16]. According to Nie et al., MAPK2 play more critical role than MAPK1 in EBR-induced apoptotic H2O2 accumulation.[17]

NF-κB is also regulated by MAPK to promote the progression of cancer.[18] The NF-κB transcription factors family consists of several subunits, including p65 (RelA), p50, p52, RelB, and c-Rel.[19] The p65 subunit is one of the principal NF-κB pathway components. In its

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inactive state, NF-κB is sequestered in the cytoplasm by binding with inhibitory proteins IκBs (Inhibitor of kappa B). Upon activation by various stimuli (including pro-inflammatory cytokines, growth factors, mitogens, microbial components and stress agents), the IκB proteins are phosphorylated and degraded, resulting in the release and nuclear translocation of the NF-κB complex. [20,21]

In OSCC patients, the level of vitamin D has been found lower compared normal individual.[22–24] Moreover, vitamin D deficiency has been found correlated with a variety of cancers. [22,25] Information on the effects of calcitriol (1,25(OH)2D3) on OSCC is still limited in certain pathway, and its mechanism remains unclear. In this study, we investigated the effects of calcitriol on oral squamous cell carcinoma cell lines, particularly CAL27 cells. In addition, we investigated the regulation of calcitriol uptake by cancer cells based on the involvement of MAPK2, p65 and NF-κB as apoptotic regulators, thereby supporting the possibility of calcitriol as an adjuvant therapy for oral squamous cell carcinoma.

Materials and methods

Cell Lines

The CAL27 cell culture was obtained from American Type Culture Collection CRL-2095 (ATCC; Manassas, Virginia, USA), which contained human tongue squamous cell carcinoma.[26] The cell line was cultivated to the appropriate density (70%) in Dulbecco's modified Eagle medium supplemented with fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C with 5% CO_2. For experiments with calcitriol, cells were plated in a 12-well plate and treated with calcitriol at varying concentrations and time. The control cells were only treated with medium.

Calcitriol

Vitamin D3 activated 7-dehydrocholesterol Calcitriol (catalog number PHR1237) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Calcitriol was diluted to a concentration of 5 mg/100 µL in 2% ethanol.

Determine IC50 of Calcitriol and Cisplatin

In a volume of 100 µL, CAL27 cells (1.7×102 cells/well) were inoculated in a 96-well plate. After 24 hours, cells were treated with calcitriol at 125 (0.325 µM), 62.5 (0.1625 µM), 31.25 (0.08 µM), 15.63 (0.04 µM), and 7.81 ppm (0.02 µM) concentrations. In addition, 60 ppm (0.19 µM) cisplatin was used as a positive control, 2% ethanol as negative control, DMEM as media only control, and DMEM containing cells untreated for 24 hours were used as negative controls. Before measuring absorbance at 570 nm, all samples were incubated in Presto blue reagent (ThermoFisher Scientific A13262, Waltham, MA, USA) for two hours. The cell viability was determined by dividing the absorbance of the sample and control, which had been subtracted from the absorbance of the control and multiplied by 100%. The IC50 was analyzed using four non-linear parametric regressions. IC50 values were 9.996 ppm (33.20 µM) for cisplatin, 44.872 ppm (107.70 µM) for calcitriol and 36.916 ppm (100.10 µM) for the combination of cisplatin and calcitriol.[27]

Antibody	Primer sequence	Bp
MAPK2_qh	(F)GAGTCCGCGGTCCTCTCTCGT (R)CCTCGCGGTCACATAGCAGTCG	115
p65_qh	(F)CTCCGCGGGCAGCATCC (R)CATCCCGGCAGTCCTTTCCTACAA	170
NF-κB_qh	(F)GCACCCTGACCTTGCCTATTT (R)TCCCAGGCGCCTTGTGAAGC	184

Table 1. Primers for RT-PCR

Cell Treatment Groups

The effects of various treatment groups were examined using a 24-well plate. The experimental design consisted of three primary groups: the cisplatin group, which served as a positive control; the calcitriol group, which received calcitriol treatment alone; and a calcitriol and cisplatin combination treatment group. A concentration of 9.99 ppm (parts per million) was used for the cisplatin group. The calcitriol group was administered calcitriol at varying concentrations, including 4.48 ppm, 11.21 ppm, 22.43 ppm, and 44.87 ppm. In the combination treatment group, various concentrations of calcitriol and cisplatin were administered, including 3,69 ppm, 9.22 ppm, 18.45 ppm, and 36.91 ppm.

Primers of MAPK2, p65 and NF-κB

Antibodies againts were MAPK2, p65 and NF-κB were obtained from PT. Genetika Science Indonesia for (Table 1).

Cell Treatment for RT-PCR

Approximately 10^6 cells were cultured a 24-well plate until 80% confluence, after which they were treated with varying concentrations of

calcitriol. The adherent cells were rinsed with ice-cold phosphate-buffered saline (PBS) (Gibco 18912014) after a 24-hour treatment. A 100 100 µL TRIzol reagent (ThermoFisher Scientific 15596026) per well was added, and the plates were scraped. Aspirated TRIzol-cell lysates were deposited in a microtube. After adding 25 µL of chloroform (Merck 102445, Burlington, MA, USA), the tube was vigorously stirred for 15 seconds. The samples were centrifuged for five minutes at 10,000 rpm. Using a micropipette, the clear aqueous phase was removed and separated into another tube. A 550 µL of isopropanol (Merck 109634.2500) was added to the aqueous phase and carefully mixed. The solution is left five minutes at room temperature. At minimum 20 minutes, the solution was centrifuged at 14,000 rpm. Then, samples were put on ice. After removing the isopropanol, 1 mL of 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water was added and gently mixed. The solution is centrifuged for five minutes at 9,500 rpm. After removing the ethanol and allowing the pellets to to air dry, 15 µL of DEPC-treated water was added to the RNA pellet. The RNA was then measured with a multimode reader (TECAN M200Pro, Männedorf, Switzerland). The 260/280 nm absorbance ratio should be between 1.8 and 2.0.

Microscopic Examination

Using an Olympus microscope, cell structures were examined and the cell death was recorded using 200 and 400 magnification (EVOS XL Core Imaging System, ThermoFisher Scientific, USA).

Statistical Analysis

A t-test was used to analyze the data. Differences were considered significant when $p<0.05$. At least three experiments were performed on each experiment.

Ethical Clearance

All experimental laboratoric procedure was approved by the Ethical Committee of Universitas Padjadjaran no. 162/UN6.KEP/EC/2022.

Results

Calcitriol inhibit CAL27 cell proliferation

Compared to controls, treatment of cells with calcitriol at various concentrations for 24 hours resulted in morphological changes (Figure 1). Changes in cellular morphology indicative of cell death were observed. Figure 1 displays near-infrared imaging using PSVue to depict apoptotic cells. The results demonstrate analogous cellular apoptosis in response to calcitriol and cisplatin, validating the morphological changes depicted in Figure 1.

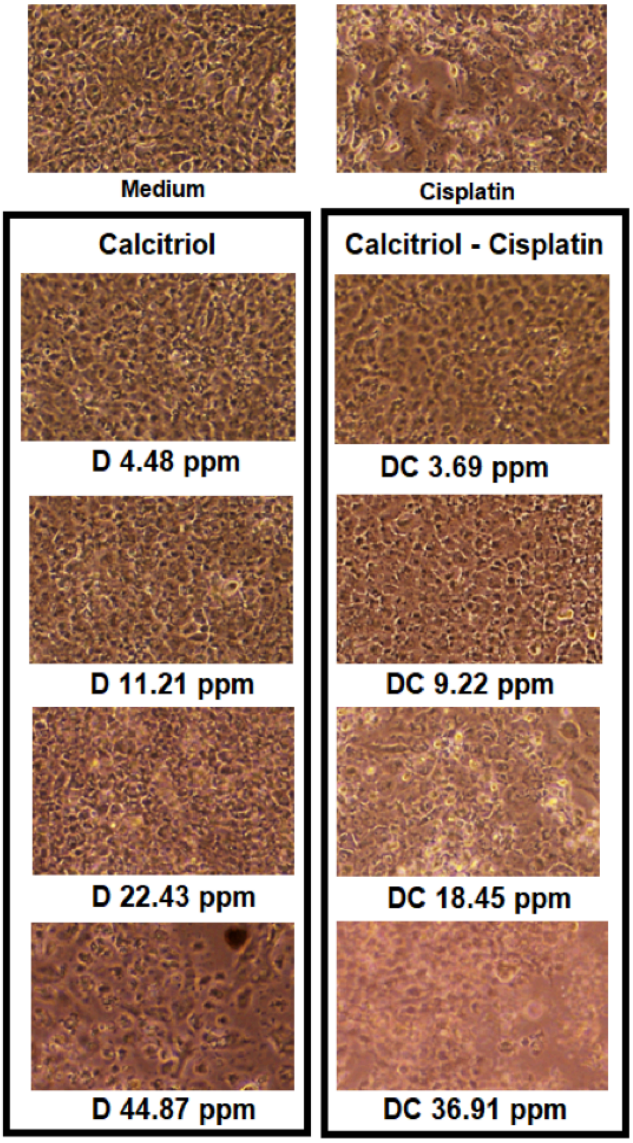

Figure 1. Morphological changes between treatment groups in CAL27 cell line. Medium negative control; Cisplatin positive control; D calcitriol; DC combination of calcitriol and cisplatin.

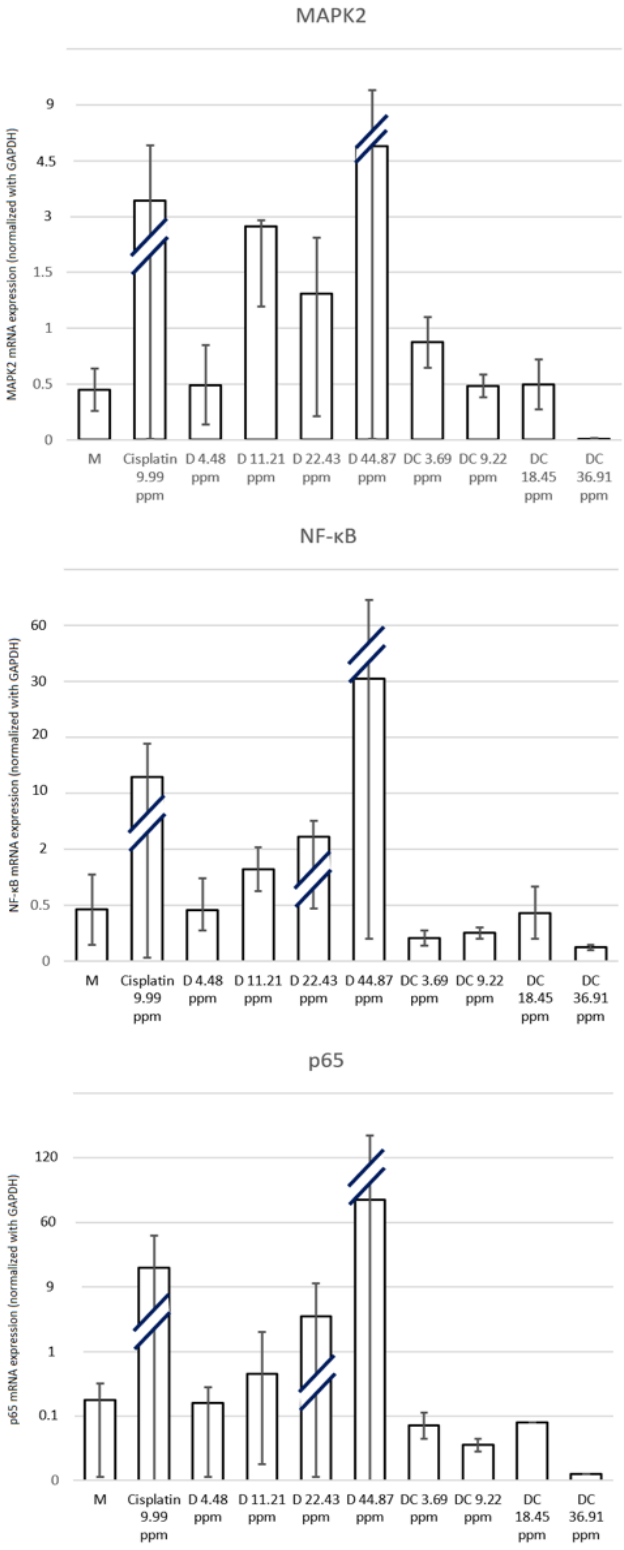

Figure 2. Dose-dependent of calcitriol inhibits CAL27 proliferation. MAPK mitogen activated kinase; NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells; M medium negative control; Cisplatin positive control; D calcitriol; DC combination of calcitriol and cisplatin.

Calcitriol upregulated apoptosis

Calcitriol upregulated expression of MPK-2, p65 and NF-κB (Figure 2). In contrast, combination calcitriol-cisplatin may decreased MPK-2, p65 and NF-κB expressions in all groups compared to calcitriol group. The combination of calcitriol and cisplatin at 36.91 ppm has a profound effect on all markers compared to the calcitriol group, even though there was no statistical significance in any of the groups ($p>0.05$).

Discussion

Apoptosis is a programmed cell death, and essential for maintaining tissue homeostasis and eliminating abnormal cells, including in oral cancer.[28] This a highly regulated process involving a sequence of molecular events orchestrated by complex signaling pathways.[28,29] Apoptotic cells are characterized morphologically by loss of adherence, cell shrinkage, condensed chromatin and cytoplasm.[30] The morphology of oral squamous cell carcinoma in our study revealed typical round-shaped cells, indicating an apoptotic event (Figure 1). Based on doses of calcitriol, Meier et al reported that dose dependent calcitriol would inhibit the in vitro proliferation of oral squamous cell carcinoma.[31] The stage of intervention and duration of calcitriol treatment against oral carcinogenesis have been demonstrated to be efficacious in all preclinical oral squamous cell carcinoma stages.[32] The binding of calcitriol to VDR increases apoptosis and differentiation while decreasing proliferation and inflammation.[33] In our study, the combination of calcitriol and cisplatin decreased MPK2, p65 and NF-κB expression, resulting in apoptosis of oral squamous cell carcinoma cell line.

Several critical molecular players, including MPK2, p65, and NF-κB, are involved in the regulation of apoptosis in oral cancer. MPK2 belongs to the MAPK family, which has been implicated in pro- and anti-apoptotic signaling pathways.[34] It can modulate apoptosis through the phosphorylation and activation of downstream targets, such as transcription factors and pro-apoptotic proteins.[35] p38MAPK activation serves a crucial role in H2O2-induced apoptosis of HLE cells.[36] Compared to cisplatin alone, the combination of calcitriol and cisplatin decreased MPK2 expression in our study. The reduction in p65 and NF-B further supported this

phenomenon. Additionally, the p65 subunit of the NF-κB complex is also essential driver of apoptosis.[37] NF-κB activation, including nuclear translocation of p65, can promote cell survival and inhibit apoptosis by upregulating anti-apoptotic genes transcriptionally.[38] However, p65 can have pro-apoptotic effects under certain conditions by cooperating with other factors or engaging in crosstalk with apoptotic signaling pathways.[39] The intricate interaction between MPK2, p65, and NF-κB in the regulation of apoptosis emphasizes the need to comprehend the precise mechanisms and contextual factors that determine their pro- or anti-apoptotic functions in oral cancer cells.

The limitation of the present study is that we used a limited number of markers to evaluate the effects of calcitriol alone or in its combination with cisplatin on the oral squamous cell carcinoma cell line. Numerous potential markers may play a role in cancer cell proliferation, therefore additional research is required to decipher the complex relationships and signaling networks involving these molecules in order to develop targeted therapies for oral cancer that effectively modulate apoptosis.

Conclusions

In this study, combination calcitriol and cisplatin was found to induce apoptosis in oral squamous cell carcinoma by decreasing MAPK2, p65 and NF-κB expression. The combination of cisplatin and calcitriol may enhance the direct anticancer effect on oral cancer cells. Our research revealed a novel perspective and novel approaches for the treatment of oral cancer.

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Declaration of Interest

The authors report no conflict of interest.

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