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# The dietary phytochemical 3,3′-diindolylmethane induces G2/M arrest and apoptosis in oral squamous cell carcinoma by modulating Akt-NF-κB, MAPK, and p53 signaling

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ABSTRACT

In light of the growing incidence of oral cancer in Taiwan, this study is aimed at investigating the antitumor activity of 3,3′-diindolylmethane (DIM), an active metabolite of the phytochemical indole-3-carbinol (I3C), in oral squamous cell carcinoma (OSCC). DIM exhibited substantially higher antiproliferative potency than I3C in three OSCC cell lines with $IC_{50}$ values in SCC2095, SCC9, and SCC15 cells, respectively, of 22 versus 168 μM, 25 versus 176 μM, and 29 versus 300 μM. Flow cytometric analysis and Comet assay indicated that DIM suppressed the viability of SCC2095 cells by inducing apoptosis and G2/M arrest. Western blot analysis of various signaling markers revealed the ability of DIM to target pathways mediated by Akt, mitogen-activated protein (MAP) kinases, nuclear factor (NF)-κB, and p53, of which the concerted action underlined its antitumor efficacy. The concomitant inactivation of Akt and MAP kinases in response to DIM facilitated the dephosphorylation of the proapoptotic protein Bad at Ser-136 and Ser-112, respectively. Through endoplasmic reticulum (ER) stress, DIM stimulated the activation of p53 via Ser-15 phosphorylation, leading to increased expression of the BH3-only proapoptotic Bcl-2 members Puma and Noxa. Together, these changes decreased the mitochondrial threshold for apoptosis. G2/M arrest might be attributable to the suppressive effect of DIM on the expression of cyclin B1 and cdc25c. As many downstream effectors of the Akt-NF-κB pathway, including glycogen synthase kinase 3β, IκB kinase α, and cyclooxygenase-2, have been shown to promote oral tumorigenesis, the ability of DIM to inhibit this signaling axis underscores its chemopreventive potential in oral cancer.



## 1. Introduction

The growing incidence of oral cancer has emerged as an important public health issue in Taiwan as well as South and Southeast Asia due to the prevalent habit of betel quid chewing coupled with smoking and drinking [1]. More than two million people have the betel quid chewing habit which is associated with approximately 80% of oral cancer cases. Even with the use of chemotherapeutic agents including platinum, 5-fluorouracil, taxane, ifosfamide, and methotrexate, their therapeutic efficacy is often compromised by the development of drug resistance during the course of tumor progression, leading to poor clinical outcomes [2]. To reduce the incidence of oral cancer, development of effective, nontoxic chemopreventive agents becomes an urgent issue for patients with oral epithelial dysplasia to reduce the incidence of oral cancer by blocking carcinogen-induced oral tumorigenesis.

The use of dietary phytochemicals in cancer prevention has received much attention because of the pleiotropic effects of these agents on multiple carcinogen-activated oncogenic pathways, and equally important, excellent safety profiles [3,4]. Among various phytochemicals with chemopreventive potential, our research focused on indole-3-carbinol (I3C), one of the active phytonutrients of cruciferous vegetables (e.g., broccoli, cabbage, and cauliflower) [5,6]. Substantial evidence indicates that increased consumption of cruciferous vegetables helps to reduce the risk for some types of cancer [7–10], and that this chemopreventive effect is, in part, attributable to the antiproliferative activity of I3C [5,6]. However, a major issue with I3C is its intrinsic instability in acidic milieu to undergo acid-catalyzed dehydration and polymerization to generate a series of oligomeric products, of which the most noteworthy is 3,3′-diindolylmethane (DIM) [11]. This acid lability severely restricts the plasma concentrations of I3C that can be achieved, rendering its pharmacokinetic behavior unpredictable. For example, a phase I trial in women showed that I3C was not detectable in plasma following oral doses even up to

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1200 mg [12]. The other drawbacks of I3C include poor antitumor potency and cellular uptake, and hepatotoxicity [6].

DIM, a major metabolite contributing to I3C's *in vivo* antiproliferative activities (structures, Fig. 1A), has been the focus of numerous investigations in different types of cancer cells [13–15] in light of its many desirable features for clinical translation, including higher potency, pleiotropic mechanisms of action, oral bioavailability, and favorable pharmacokinetic behaviors [11]. In this study, we investigated the efficacy of DIM and the possible mechanisms underlying its antitumor activity in oral squamous cell carcinoma (OSCC).

## 2. Materials and methods

### 2.1. Reagents

I3C and DIM were purchased from Sigma-Aldrich (St. Louis, MO). For *in vitro* experiments, these agents at various concentrations were dissolved in DMSO, and were added to cells in medium with a final DMSO concentration of less than 0.1%. Rabbit polyclonal antibodies against various biomarkers were obtained from the following sources: p-$^{473}$Ser Akt, p-$^{308}$Thr Akt, p-$^{202/204}$Thr/Tyr ERK, p-$^{183/185}$Thr/Tyr JNK, JNK, cyclin D1, cyclin B1, p-$^{15}$Ser p53, p53, cyclin-dependent kinase (CDK) 6, cdc25c, p19, p-$^{180/182}$Thr/Tyr p38, IKKα, p-$^{176}$Ser IKKα, GSK3β, p-$^{112}$Ser Bad, p-$^{136}$Ser Bad, IκB, XIAP, and RelA (Cell Signaling Technologies, Beverly, MA); Akt, ERK, p27, p21, Bax, p-$^{9}$Ser GSK3β, COX-2, c-Myc, Noxa, Puma, and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); survivin (R&D Systems, Minneapolis, MN); β-actin (Sigma-Aldrich). The enhanced chemiluminescence (ECL) system for detection of immunoblotted proteins was from GE Healthcare Bioscience (Piscataway, NJ). SB203580, PD98059, Wortmannin, other chemicals and biochemistry reagents were obtained from Sigma–Aldrich unless otherwise mentioned.

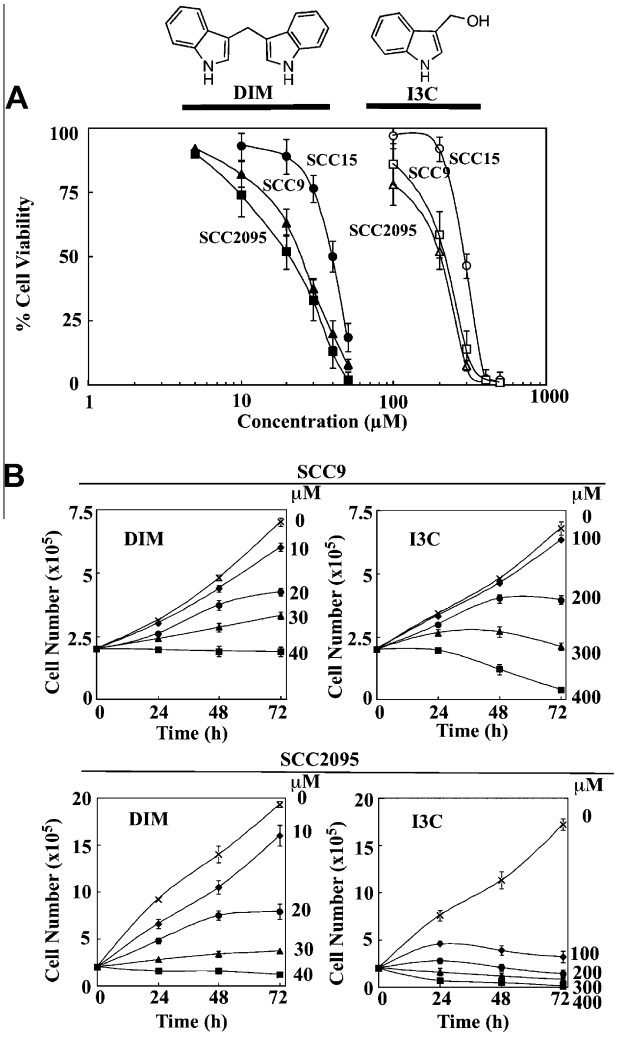


**Fig. 1.** Antiproliferative effects of DIM and I3C in oral cancer cells. (A) Dose-dependent suppressive effects of DIM and I3C on the viability of SCC9, SCC15, and SCC2095 oral cancer cells. Cells were treated with DIM or I3C at the indicated concentrations in 5% FBS-supplemented DMEM/F12 medium for 48 h, and cell viability was determined by MTT assays. *Points*, mean; *bars*, SD ($n = 6$). (B) Dose-dependent suppressive effect of DIM versus I3C on the number of SCC9 and SCC2095 cells. Cells were seeded onto six-well plates (200,000 cells/well) and exposed to the test agent at the indicated concentrations in 5% FBS-supplemented DMEM/F12 medium. At different time intervals, cells were harvested, and counted using a Coulter Counter. Values were obtained from triplicates.

### 2.2. Cell culture

SCC9, SCC15, and SCC2095 human OSCC cells were kindly provided by Professor Susan R. Mallery (The Ohio State University). Cells were cultured in DMEM/F12 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco), 5 mg/ml of penicillin, 10 mg/ml of neomycin and 5 mg/ml streptomycin. All cells were cultured at 37 °C in a humidified incubator containing 5% $CO_2$.

### 2.3. Cell viability analysis

The effect of test agents on cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [5] assay in 6 replicates. Cells ($5 \times 10^3$) were seeded and incubated in 96-well, flat-bottomed plates in 10% FBS-supplemented DMEM/F12 for 24 h, and were exposed to various concentrations of test agents dissolved in DMSO in 5% DMEM/F12 for different time intervals. The medium was removed, replaced by 200 μL of 0.5 mg/ml MTT in 5% FBS-DMEM/F12, and cells were incubated in the carbon dioxide incubator at 37 °C for 2 h. Medium was removed and the reduced MTT dye was solubilized in 200 μL/well DMSO. Absorbance was determined with a Synergy HT (Bio-Tek) at 570 nm.

### 2.4. Cell proliferation assay

Cells ($2 \times 10^5$ per well) were seeded in 6-well plates and allowed to attach for 24 h. Then, the cells were treated in triplicate with the indicated concentrations of test agent or DMSO vehicle in 5% FBS-containing DMEM/F12. At different time intervals, cells were counted to evaluate the effects of test agents on the number of viable cells using a Z1 Coulter counter (Model Z, D/T, Beckman Coulter).

### 2.5. Flow cytometry analysis

$5 \times 10^4$ Oral cancer cells were plated and treated with the indicated concentration of DIM for 48 h with 5% FBS-supplemented DMEM/F12. The cells were harvested at the end of treatment and the pellet was washed twice in ice-cold phosphate-buffered saline (PBS). The cells were then fixed in 70% cold ethanol for 4 h at 4 °C followed by spinning at 1200 rpm for 5 min and re-suspending in ice-cold PBS containing 2% PBS. The cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and analyzed by using BD FACSAria flow cytometer (Becton, Dickinson and Company) and cell cycle analysis was performed using the multicycler software.

### 2.6. Comet assay

DIM-treated or etoposide-treated cells ($2 \times 10^5$) were pelleted and resuspended in ice-cold PBS. The resuspended cells were mixed with 1.5% low-melting point agarose. This mixture was loaded onto a fully frosted slide that had been precoated with 0.7% agarose, and a coverslip was then applied to the slide. The slides were submerged in prechilled lysis solution (1% Triton X-100, 2.5 M NaCl, and 10 mM EDTA, pH 10.5) for 1 h at 4 °C. After the slides had been soaked with prechilled unwinding and electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) for 20 min, they were subjected to electrophoresis for 30 min at 0.5 V/cm (20 mA). After electrophoresis, the slides were stained with propidium iodide (PI) (2.5 μg/mL), and nuclei images were visualized and captured at 200 × magnification by a fluorescence microscope.

### 2.7. Western blot

Drug-treated cells were collected, washed with ice-cold PBS, and resuspended in lysis buffer [20 mM Tris–HCl (pH 8), 137 mM NaCl, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin at 10 μg/mL, and aprotinin at 10 μg/mL]. Soluble cell lysates were collected after centrifugation at 1500*g* for 5 min. Equivalent amounts of protein (60–100 μg) from each lysate were resolved in 10% SDS–polyacrylamide gels. Bands were transferred to nitrocellulose membranes and blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST) and incubated overnight with the corresponding primary antibodies at 4 °C. After washing with PBST three times, the membrane was incubated at room temperature for 1 h with the secondary antibody with PBST and visualized by the ECL.

### 2.8. Fluorescence staining for confocal imaging

Cells ($2 \times 10^5$/3 mL) were plated on cover slips in each well of a six-well plate. The cells were treated with 25 μM DIM for 48 h with or without 20 nM tumor necrosis factor-α (TNF-α) for 30 min. Cells were fixed in 2% paraformaldehyde for 30 min at room temperature, and permeabilized with 0.1% Triton X-100 for 20 min. After blocking with 1% bovine serum albumin (BSA) for 1 h, cells were incubated with rabbit anti-human nuclear factor (NF)-κB antibody (1:1000, Cell Signaling Technologies, Beverly, MA) overnight at 4 °C, followed by incubation with anti-rabbit IgG (1:5000) for 1 h at room temperature. Cells were washed with TBST for three times and then covered before undergoing Confocal Microscope Detection System, Leica TCS SP2 (Leica Biosystems Nussloch GmbH, Heidelberg, Germany) examination.

### 2.9. Statistical analysis

All data are presented as mean ± S.D. obtained from three independent experiments. Statistical differences were calculated using Student's *t*-test, with the following symbols of significance level: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

## 3. Results

### 3.1. Differential antitumor effects of DIM versus I3C in OSCC cells

The dose-dependent suppressive effect of DIM on cell viability was compared to I3C by MTT assays in three OSCC cell lines: SCC2095, SCC9, and SCC15. DIM exhibited higher potency relative to I3C with the respective $IC_{50}$ values as follows: for SCC2095, 22 and 168 μM; for SCC9, 25 and 176 μM; for SCC15, 39 and 300 μM (Fig. 1A). Moreover, cell counting assays revealed a different mode of growth inhibition between these two agents. As shown, DIM at a concentration of 40 μM did not cause a significant decrease in the number of SCC9 and SCC2095 cells at 72 h of exposure, which contrasts with a sharp reduction of cell number with I3C treatment at a concentration of 400 μM (Fig. 1B).

### 3.2. DIM induces G2/M cell cycle arrest and apoptosis in OSCC cells

Flow cytometric analyses of DIM-treated SCC2095 cells indicated a dose-dependent increase in the sub-G1 and G2/M phases (Fig. 2A). DIM-induced apoptosis was also confirmed by the Comet assay, which showed that DIM at 15 and 30 μM for 2 h induced a concentration-dependent increase of DNA strand breaks in SCC2095 cells (Fig. 2B). Cells treated with etoposide at 30 μM were used as a positive control.

Furthermore, we demonstrated the ability of DIM to target multiple signaling pathways governing survival and cell cycle progression in SCC2095 cells, including those mediated by Akt, mitogen-activated protein (MAP) kinases, NF-κB, and endoplasmic reticulum (ER).

### 3.3. Inhibition of Akt and MAP kinases

Reminiscent with that reported in other cancer types [16,17], Western blot analysis suggested that DIM mediated a dose-dependent suppression of Akt signaling as evidenced by decreased phosphorylation of Akt at both Ser-473 and Thr-308 sites and its downstream targets GSK3β and IKKα (Fig. 3A). Moreover, this Akt dephosphorylation was accompanied by parallel decreases in the phosphorylation levels of ERKs, p38, and JNK. In order to confirm that DIM exerts the downregulation of p38 and ERKs, we further sought to determine if inhibition of p38 or ERKs activity could lead to the similar activity of the DIM-mediated inhibition. As shown in Fig. 3B, treatment of SCC2095 cells with the ERKs inhibitor PD98059 (30 μM) or the p38 inhibitor SB203580 (40 μM) suppressed the phosphorylation of p38 and ERKs in a manner similar to that of DIM (30 μM).

As Akt and these MAP kinases mediate the phosphorylation of the proapoptotic protein Bad at Ser-136 [18] and Ser-112 [19], respectively, we demonstrated that DIM dose-dependently facilitated Bad dephosporylation at both sites, which plays a crucial role in inducing apoptosis by facilitating its dissociation from the binding protein 14–3-3 [20].

### 3.4. Blockade of NF-κB signaling

Evidence indicates that DIM inhibited NF-κB signaling through two distinct mechanisms. First, DIM caused the dose-dependent accumulation of the NF-κB inhibitor IκB in SCC-2095 cells (Fig. 4A), presumably resulting from the aforementioned DIM-mediated inactivation of IKKα (Fig. 3A). Second, DIM also exhibited a unique ability to suppress the expression of the RelA/p65 subunit of NF-κB (Fig. 4A). Through this concerted mechanism, DIM antagonized the effect of TNFα on activating RelA nuclear translocation (Fig. 4B). The inhibition of NF-κB transcriptional activity was also evidenced by changes in the expression levels of NF-κB-regulated gene products, including the downregulation of the antiapoptotic proteins Bcl-2, survivin, XIAP, the transcription factor c-Myc, and the cyclooxygenase (COX)-2, and the concomitant upregulation of the proapoptotic protein Bax (Fig. 4A).

### 3.5. Modulation of the expression of cell cycle-regulatory proteins

To shed light onto the mechanism by which DIM mediated G2/M arrest, we examined the dose-dependent drug effects on various

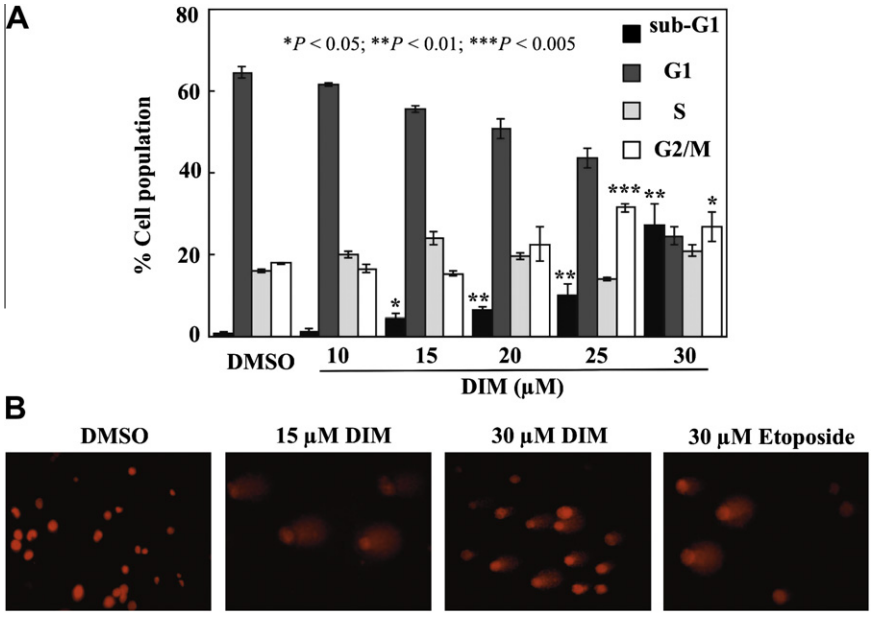


**Fig. 2.** Effects of DIM on apoptosis and G2/M arrest in SCC2095 cells. (A) Histogram showing the dose-dependent effect of DIM on cell cycle distribution. SCC2095 cells were treated with various concentrations of DIM, followed by propidium iodide staining and flow cytometry at 48 h. An untreated control was considered to be representative of the percentage of cells in each phase of the cell cycle. Data are presented as the mean ± S.D. and are representative of an average of three independent experiments per concentration. (B) Effects of DIM for 2 h on apoptosis assessed by determining the chromosomal DNA integrity using the Comet assay. Cells exposed to etoposide at 30 μM were used as a positive control.

cell cycle regulators in SCC2095 cells (Fig. 5A). As shown, DIM facilitated multifold increases in the expression levels of the CDK inhibitors p21, p27, and p19 in a dose-dependent manner, while down-regulating the expression of cyclin D1, CDK6, and the G2/M cell cycle progression proteins cyclin B1 and cdc25c. It is noteworthy that expression of these genes is transcriptionally regulated by NF-κB and/or c-Myc. Together, these protein expression data correlated with the dose-dependent effect of DIM on G2/M cell cycle arrest (Fig. 2A).

### 3.6. Endoplasmic reticulum (ER) stress

DIM dose-dependently increased the expression of the ER stress biomarker GADD153, accompanied by parallel increases in the phosphorylation of p53 at Ser-15 and the expression of the p53 targets Puma and Noxa (Fig. 5B). In previous reports, the PI3 K signaling pathway is closely linked with p53-related cell cycle regulation, and DIM targets PI3 K signaling [21,22]. To confirm this mechanistic link, SCC2095 cells were co-treated with DIM and wortmannin, a PI3 K inhibitor. As shown in Fig. 5C, the expression levels of p-p53, p53 and p21 in the presence of wortmannin decreased in SCC2095 cells. p53 activation by DIM was inhibited substantially by wortmannin. This finding is consistent with recent reports that ER stress induced apoptosis through the activation of p53 signaling [23,24].

## 4. Discussion

In this study, we demonstrated that DIM mediated an antiproliferative effect in OSCC cells by targeting multiple signaling pathways mediated by Akt, NF-κB, MAP kinases, ER stress, and p53. This pleiotropic mechanism gives rise to changes in the activation status or expression of a broad spectrum of signaling effectors, including GSK3β, IKKα, IκB, the Bcl-2 members Bcl-2, Bax, Bad, Puma, and Noxa, the inhibitor of apoptosis protein (IAP) family members survivin and XIAP, c-Myc, COX-2, and many cell cycle-regulatory proteins, leading to G2/M cell cycle arrest and apoptosis (Fig. 6). It is well recognized that Akt activation is a significant prognostic indicator for OSCC [25] and that activation of NF-κB promotes oral cancer invasion [26]. Many signaling events downstream of the Akt-NF-κB pathway, such as phosphorylating inactivation of GSK3β by Akt activation [27], upregulation of COX-2 expression through NF-κB activation [28], and activation of IKKα [29] have also been shown to promote the development of oral cancer. Moreover, recent evidence indicates that chronic exposure of oral fibroblasts and keratinocytes to subtoxic betel nut extracts led to activation of Akt and NF-κB [30,31], suggesting a mechanistic link between the Akt-NF-κB signaling axis and betel quid chewing/smoking-induced oral carcinogenesis. Thus, the unique ability of DIM to modulate these clinically relevant targets underlies its potential to be developed as a chemopreventive agent for the large betel quid-chewing population.

Although SCC9 and SCC25 cells have been reported to express negligible amounts of p53 transcripts and undetectable levels of p53 protein, respectively [32], the functional status/expression level of p53 remains unclear in SCC2095 cells. In this study, we showed that DIM treatment induced the expression of p53 in SCC2095 cells, which, however, contrasts a recent report that DIM induced cell cycle arrest in prostate cancer cells irrespective of p53 status [15]. A possible explanation for this discrepancy is that components of the NF-κB signaling network interact with p53 at multiple levels [33,34]. Recent results suggest that some chemotherapeutic agents and natural plant products repress the function of the NF-κB pathway while still activating p53 [7,35]. In addition to NF-κB and Akt pathways, MAPK has received increasing attention as a target for cancer prevention and therapy. The MAPK pathway consists of a three-tiered kinase core where an MAP3 K activates an MAP2 K that activates an MAPK (ERK, JNK, and p38), resulting in the activation of NF-κB, cell growth, and cell survival [36]. Our results demonstrated that all MAPKs were down-regulated by DIM which suggested that MAPK signaling is among the signaling pathways mediated by DIM.

In addition to DIM in the present study, several other phytochemicals have also been reported to exhibit chemopreventive

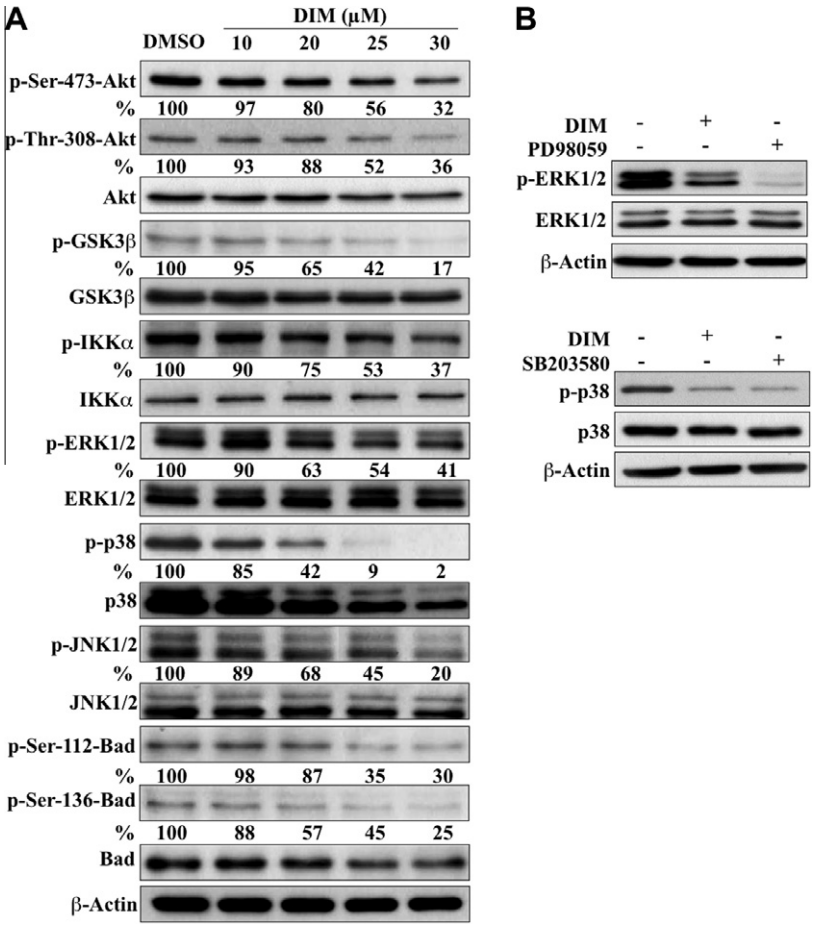


**Fig. 3.** (A) Western blot analysis of the effects of DIM on the phosphorylation of Akt at Ser-473 and Thr-308 and its downstream targets GSK3β and IKKα, the MAP kinases ERK1/2, p38, and JNK1/2, and the proapoptotic protein Bad at Ser-112 and Ser-136 in SCC2095 cells. (B) Suppressive effects of DIM (30 μM) vis-à-vis the ERKs inhibitor PD98059 (30 μM) (upper panel) or the p38 inhibitor SB203580 (40 μM) (lower panel) on the phosphorylation of ERKs and p38. Cells were exposed to the test agent at the indicated concentrations in 5% FBS-supplemented DMEM/F12 medium for 48 h. The values in Fig. 3A denote percentage changes as determined by the relative intensity of protein bands of treated samples to that of the respective DMSO vehicle-treated control after normalization to the respective internal reference β-actin. Each value represents the average of three independent experiments.

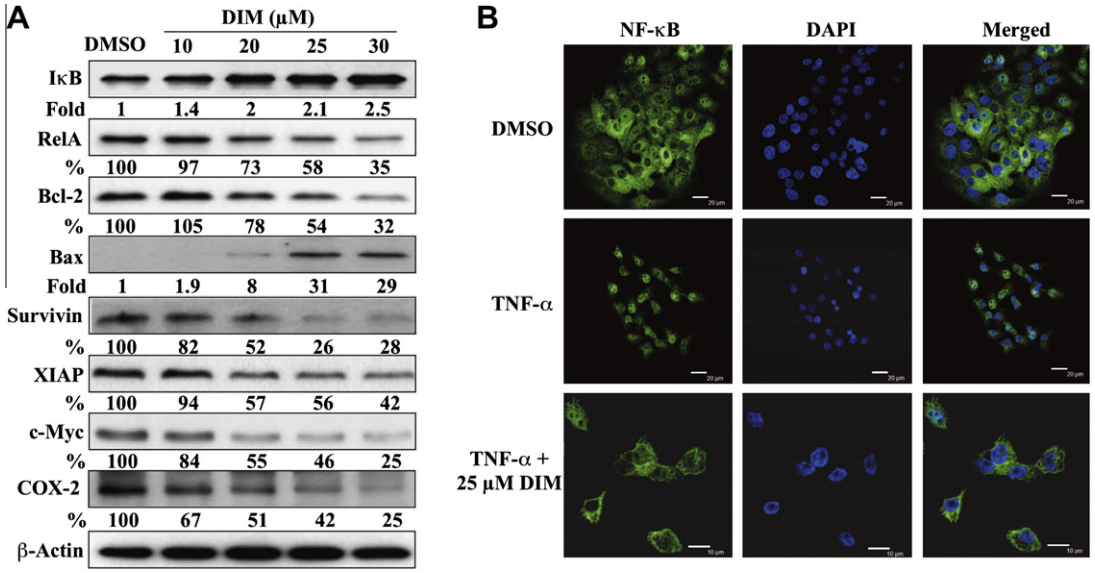


**Fig. 4.** Suppressive effect of DIM on NF-κB signaling. (A) Western blot analysis on the expression levels of the NF-κB inhibitor IκB, NF-κB/RelA, and the target gene products of NF-κB, including Bcl-2, Bax, survivin, XIAP, c-Myc, and COX-2. Cells were exposed to DIM at the indicated concentrations in 5% FBS-supplemented DMEM/F12 medium for 48 h. The values denote fold increases or percentage changes as determined by the relative intensity of protein bands of treated samples to that of the respective DMSO vehicle-treated control after normalization to the respective internal reference β-actin. Each value represents the average of three independent experiments. (B) Effect of DIM (25 μM) on antagonizing the TNF-α activated NF-κB nuclear translocation in SCC2095 cells.

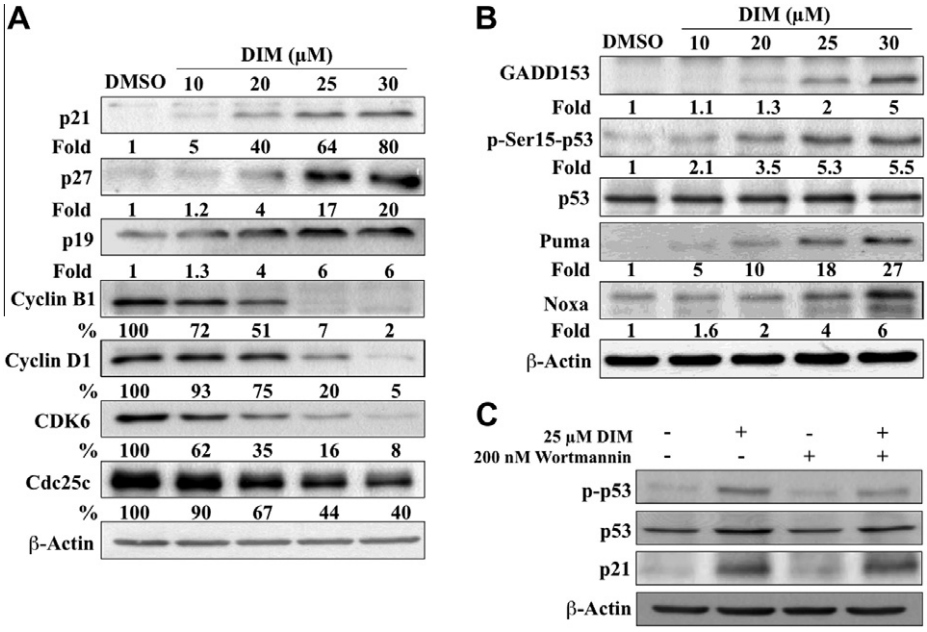


**Fig. 5.** Western blot analysis of the effects of DIM on the expression and/or phosphorylation of (A) cell cycle-regulatory proteins, including the CDK inhibitors p21, p27, and p19, cyclin B1, cyclin D1, CDK6, and cdc25c, and (B) GADD153, p53, Puma, and Noxa in SCC2095 cells. (C) Western blot analysis of the effects of DIM on the expression levels of p-p53, p53, and p21. Cells were exposed to DIM at the indicated concentrations in 5% FBS-supplemented DMEM/F12 medium for 48 h. The values denote fold increases or percentage changes as determined by the relative intensity of protein bands of treated samples to that of the respective DMSO vehicle-treated control after normalization to the respective internal reference β-actin. Each value represents the average of three independent experiments.

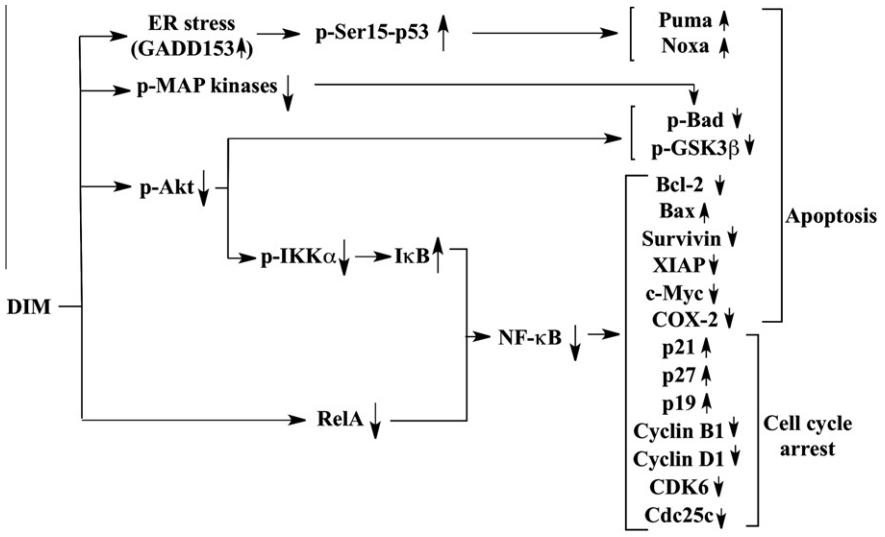


**Fig. 6.** Proposed diagrams depicting the effects of DIM on the Akt-NF-kB signaling axis, MAP kinases, and ER stress. The interplay between these signaling networks at different cellular levels results in the ability of DIM to induce G2/M arrest and apoptosis in OSCC cells.

potential for OSCC, including curcumin [37], freeze-dried black raspberry ethanol extracts (RO-ET) [38], resveratrol [39], quercetin [39], and tea polyphenols [40]. As each of these phytochemicals display a unique mode action, it is worthwhile to investigate the possibility of synergism between DIM and these agents to obtain optimal antitumor efficacy in OSCC. For example, RO-ET has been shown to block carcinogen-induced activation of NF-κB through the inhibition of MAP kinases [41], which is different from the effect of DIM on NF-κB activation through the suppression of Akt phosphorylation and RelA expression. For a mechanistic perspective, DIM and RO-ET might synergize in suppressing OSCC cell proliferation through a concerted action on NF-κB inhibition.

According to the NCI ClinicalTrials.gov homepage, there are at least nine clinical trials of DIM in healthy volunteers or patients with different types of cancers, which demonstrates the translational interest and value of DIM in cancer treatment/prevention. The findings of the present study further suggest that DIM might represent a promising chemopreventive agent for the large population of betel quid chewing people in endemic areas, of which the proof-of-concept animal study is currently underway.

## Conflict of interest statement

The authors declare no competing financial interests.

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