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Original Articles

3,3'-Diindolylmethane inhibits patient-derived xenograft colon tumor growth by targeting COX1/2 and ERK1/2

Xueli Tian[a,b,1], KangDong Liu[a,b,c,d,1], Xueyin Zu[a,b], Fayang Ma[a,b], Zhi Li[c], MeeHyun Lee[a], Hanyong Chen[e], Yan Li[e], Yuzhou Zhao[c], Fangfang Liu[a,b], Naomi Oi[e], Ann M. Bode[e], Zigang Dong[a,d,e,f,**], Dong Joon Kim[a,b,*]

[a] China-US (Henan) Hormel Cancer Institute, Henan, 450008, China
[b] The Pathophysiology Department, The School of Basic Medical Sciences, Zhengzhou University, Zhengzhou, Henan, 450008, China
[c] The Affiliated Cancer Hospital, Zhengzhou University, Zhengzhou, Henan, China
[d] The Collaborative Innovation Center of Henan Province for Cancer Chemoprevention, Zhengzhou, China
[e] The Hormel Institute, University of Minnesota, Austin, MN, 55912, USA
[f] International Joint Research Center of Cancer Chemoprevention, Zhengzhou, China

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ABSTRACT

3,3'-Diindolymethane (DIM) is a dimeric condensation product of indole-3-carbinol (I3C) that is found in broccoli and cabbage. Although DIM has been reported to exhibit anticancer properties against multiple tumor types, the direct target proteins of DIM have not been fully investigated. In the present study, we report that DIM is a novel COX1/2 and ERK1/2 inhibitor that suppresses growth of colon cancer *in vitro* and *in vivo*. To identify possible molecular targets of DIM, 11 potential candidate proteins were validated by an *in vitro* kinase or enzyme assay. We found that DIM directly inhibits COX1/2 and ERK1/2 protein activities *in vitro*. Additionally, the PGE2 production (COX-mediated metabolite) and phosphorylated RSK expression (ERK1/2 direct downstream kinase) were strongly suppressed by DIM in colon cancer cells. The inhibition of cell growth by DIM is dependent on the expression of COX1/2 or ERK1/2 proteins. Notably, oral administration of DIM suppressed patient-derived xenograft colon tumor growth in an *in vivo* mouse model. Overall these results suggest that DIM is a potent and dual COX1/2 and ERK1/2 inhibitor that might be used for chemotherapy against colon cancer.

1. Introduction

Colon cancer is the third most commonly diagnosed cancer and leading cause of cancer-related death in the United States [1]. The development of colon cancer is a multistep process where mutations can occur in the *APC, KRAS,* or *p53* genes leading to uncontrolled cell growth [2,3]. The expression of cyclooxygenase 2 (COX2) and the abundance of its principal metabolic product, prostaglandin E2 (PGE2), are elevated in premalignant and malignant tissues and overexpression of the COX2 protein has been reported in 90% of colon carcinomas and 40% of adenomas, suggesting a crucial role for COX2 in colorectal tumorigenesis [4,5]. COX2 is a major enzyme in the synthesis of prostaglandins and is involved in tumor development, cancer growth, and inhibition of apoptosis [6,7]. Clinical and epidemiologic studies indicated that COX2-selective NSAIDs, such as sulindac, celecoxib, and refecoxib, showed a preventive and/or therapeutic activity in colorectal cancer or familial adenomatous polyposis (FAP) patients [8,9]. COX1 has also been reported to be heavily involved in colon and skin tumorigenesis [10,11]. Additionally, mutant APC^{min} $COX1^{-/-}$ mice show markedly reduced intestinal polyp numbers, indicating that expression of COX1 plays essential roles in the formation of intestinal polyps [6]. A selective COX1 inhibitor strongly suppressed the growth of colon cancer [12,13]. Therefore, COX1/2 are important therapeutic targets in colon cancer.

The extracellular signal–regulated kinases 1/2 (ERK1/2) are major effecters of the RAS/RAF/MEK signaling axis and are substantially

Abbreviations: DIM, 3,3'-; COX1/2, cyclooxygenase 1 and 2; ERK1/2, extracellular signal-regulated kinases 1/2; RSK, ribosomal protein S6 kinase; PDX, patient-derived xenograft

* Corresponding author. China-US (Henan) Hormel Cancer Institute, No, 127 Dongming Road, Zhengzhou, Henan, 450008, China.
** Corresponding author. The Hormel Institute, University of Minnesota, 801 16th Ave NE, Austin, MN, 55912, USA.
E-mail addresses: zgdong@hi.umn.edu (Z. Dong), djkim@hci-cn.org (D.J. Kim).
[1] Xueli Tian and KangDong Liu equally contributed to this work.

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involved in tumorigenesis, cell proliferation, cell survival, and apoptosis [14,15]. Although mutations in ERK1/2 have not been observed in human tumors, ERK1/2 are activated in many cancers, including colon cancer, due to activating mutations of upstream proteins, such as EGFR, KRAS, or PIK3CA [16]. Whereas the RAS, RAF, and MEK families catalyze only a few specific substrates, ERK1/2 activate various substrates, including several kinases and transcription factors [17]. The ribosomal S6 kinase (RSK) is a downstream effector of the ERK1/2 signaling cascade and plays active roles in cell proliferation, anti-apoptosis, and chromatin remodeling [18]. These studies provide strong rationale for the development of ERK1/2 inhibitors for chemopreventive and chemotherapeutic interventions in cancer.

3,3'-Diindolymethane (DIM) is an indole-3-carbinol (I3C) metabolite found in brassica vegetables and has been reported to exert anticancer activities affecting cell proliferation, cell cycle, and apoptosis through the mediation of various signaling molecules [19]. The anticancer activity of DIM has been investigated in several *in vivo* models, including DMBA/TPA skin carcinogenesis, breast cancer xenografts, and in TRAMP prostate mouse models [20–23]. However, the direct target proteins of DIM have not been identified. In the present study, we found that DIM suppresses colon cancer growth by inhibiting COX1/2 and ERK1/2 activities.

2. Materials and methods

2.1. Reagents and antibodies

DIM (purity: > 98% by HPLC) was purchased from Sigma-Aldrich (St Louis, MO, USA) and CNBr-Sepharose 4B beads were obtained from GE Healthcare (Piscataway, NJ, USA). The active V600E mutant BRAF, active EGFR, active CHK1, active CHK2, active MEK1, active MEK2, active AKT1, active p38, active CDK2, inactive MEK1 (V600E mutant BRAF substrate), PLCγ (EGFR substrate), p53 (CHK2 substrate), inactive ERK2 (MEK1 and MEK2 substrate), histone H2B (AKT1 substrate), ATF2 (p38 substrate), and histone H1 (CDK2 substrate) human recombinant proteins for kinase assays were purchased from Millipore (Temecula, CA, USA). LY3214996 (ERK1/2 inhibitor) was from Eli Lilly and Company (Monmouth Junction, NJ, USA). CDC25C was purchased from ENZO (Farmingdale, NY, USA) and the COX fluorescent activity assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). The antibodies to detect total ERK1/2, phosphorylated ERK1/2, total RSK, phosphorylated RSK, cyclin D1, phosphorylated GSK3β, and phosphorylated MKK3/6 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to detect β-actin, p21, and β-catenin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to detect COX1 and COX2 were purchased from Abcam (Cambridge, MA, USA). The ALT and AST activity assay kit was purchased from Nanjing Jiancheng Bioenguneering Institute (Nanjing, China).

2.2. Cell culture

All cell lines were purchased from American Type Culture Collection (ATCC) and were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials were available for each cell line to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for 8 weeks or less. HCT116 and HT29 human colon cancer cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) and 1% antibiotic-antimycotic. HCT15 and DLD1 human colon cancer cells and CCD 841 CoN normal human colon epithelial cells were cultured in RPMI1640 medium supplemented with 10% FBS and 1% antibiotic-antimycotic. SW480 and SW620 human colon cancer cells were cultured in L-15 medium (Leibovitz) supplemented with 10% FBS and 1% antibiotic-antimycotic.

2.3. In vitro kinase assay

The kinase assay was performed according to instructions provided by Upstate Biotechnology (Billerica, MA, USA). Briefly, the reaction was conducted in the presence of 10 μCi of [γ-^{32}P]ATP with each compound in 40 μl of reaction buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. After incubation at room temperature for 30 min, the reaction was stopped by adding 10 μl protein loading buffer and the mixture was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The relative amounts of incorporated radioactivity were assessed by autoradiography.

2.4. Molecular modeling of DIM with COX1, COX2, ERK1, and ERK2

The crystal structures of COX1 and COX2 were obtained from the Protein Data Bank (PDB) [24]. The structures were prepared using the Protein Preparation Wizard in Maestro 9.3. Hydrogens were added consistent with a pH of 7.0. All water molecules were removed. Then the structure was energy minimized with an RMSD cutoff value of 0.3 Å. The chemical structure of DIM was optimized in Maestro 9.3, prepared using LigPrep 2.5, and assigned AMSOL partial atom charge. The program Glide 5.7 was used for ligand docking. The receptor grid was created with the centroid of the crystal ligand as the center of the grid. Flexible docking was performed with the extra precision (XP) mode. The number of poses per ligand was set to 10 in post-docking minimization and at most 5 poses would be output. The other parameters were kept as default. For docking of DIM with ERK1 or 2, the crystal structure of ERK1 and ERK2 were downloaded from the PDB [24]. They were prepared under the standard procedures of the Protein Preparation Wizard (Schrödinger Suite 2016). Hydrogen atoms were added consistent with a pH of 7 and all water molecules were removed. The ATP-binding site-based receptor grids of ERK1 and ERK2 were generated for docking. The DIM compound was prepared for docking by default parameters using the LigPrep program. Then, the docking of DIM with ERK1 and ERK2 was accomplished with default parameters under the extra precision (XP) mode using the program Glide. Herein, we could get the best-docked representative structures.

2.5. In vitro pull-down assay

Colon cancer cell lysates (500 μg) were incubated with DIM-Sepharose 4B (or Sepharose 4B only as a control) beads (50 μl, 50% slurry) in reaction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40, 2 μg/ml bovine serum albumin). After incubation with gentle rocking overnight at 4 °C, the beads were washed 5 times with buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% NP40) and binding was visualized by Western blotting.

2.6. Determination of prostaglandin H synthase (PGHS) activity

PGHS activity was determined by the production of PGE2 using recombinant COX1 or COX2 protein (50 ng) or cell culture medium. Cells (1×10^5 cells per well) were cultured for 24 h in 6-well plates and then treated with various concentrations of DIM (0, 10, 20, 40 or 60 μM). After incubation for 48 h, the amount of PGE_2 released into the medium was measured using the PGE_2 EIA kit following the supplier's instructions. A standard curve with PGE_2 provided with the kit was generated at the same time.

2.7. Anchorage-independent cell growth

Cells (8×10^3 cells per well) suspended in complete growth

medium (RPMI1640 or McCoy's 5A supplemented with 10% FBS and 1% antibiotics) were added to 0.6% agar with different doses of DIM in a base layer and a top layer of 0.3% agar. The cultures were maintained at 37 °C in a 5% CO_2 incubator for 3 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v. 4) program (Media Cybernetics, Rockville, MD, USA).

2.8. Cell cycle analysis

Colon cancer cells were plated into 60-mm culture dishes (1×10^5 cells per dish) and incubated for 24 h in 10% serum-containing media. Cells were treated or not treated with DIM for 24 h in 10% serum-containing media after incubation for 24 h in 1% serum medium. Cells were collected by trypsinization and washed with phosphate buffered saline (PBS) and then fixed in 1 ml of 70% cold ethanol. After rehydration, cells were digested with RNase (100 μg/ml) and stained with propidium iodide (PI, 20 μg/ml). PI staining was accomplished following the product instructions (Clontech, Palo Alto, CA). The cells were analyzed by flow cytometry.

2.9. Annexin V apoptosis assay

Colon cancer cells were plated into 12 well culture dishes (1×10^5 cells per well) and incubated for 24 h in medium containing 10% FBS. Cells were treated with DIM for 72 h in 10% serum-containing media. Cells were collected by trypsinization and washed with phosphate buffered saline (PBS) and then stained with Annexin V (BioLegnd, San Diego, CA, USA) and PI and then apoptosis was analyzed by flow cytometry.

2.10. Invasion assay

Transwell chambers (Corning, Bedford, MA, USA) were coated inside with Matrigel and dried for 30 min at room temperature under sterile conditions. The lower compartment was filled with 600 μl complete medium. Samples containing 5×10^4 cells in 200 μl serum-free medium were added to the upper compartment with or without DIM. The chambers were incubated for 72 h at 37 °C in a 5% CO_2 atmosphere. After incubation, cells that had invaded into the lower compartment or attached to the lower side of the well were counted.

2.11. In vivo studies using the APC^{min+} mouse model

Male C57BL/6J$^{(Min/+)}$ mice were obtained from Jackson Laboratory and maintained under "specific pathogen-free'' conditions according to the guidelines established by the University of Minnesota Institutional Animal Care and Use Committee. APC^{min+} male mice were bred with C57BL/6J APC wildtype female mice. The progeny were genotyped by PCR assay to determine whether they were heterozygous for the *min* allele or were homozygous wildtype. APC^{min+} male or female progeny were randomly assigned to groups after weaning at 3 weeks. Mice (5–6 weeks old) were divided into 3 groups: 1) untreated vehicle group (n = 8); 2) mice treated with 40 mg DIM/kg of body weight (n = 8); and 3) mice treated with 200 mg DIM/kg of body weight (n = 8). DIM (10% DMSO in 20% tween 80) or vehicle was orally administered 5 times a week for 8 weeks.

2.12. Patient-derived xenograft colon tumor growth assay

Female mice with severe combined immunodeficiency (SCID; 6–9 weeks old) were maintained under "specific pathogen-free'' conditions based on the guidelines established by Zhengzhou University Institutional Animal Care and Use Committee. A human tumor specimen of colon cancer tissue was obtained from the Affiliated Cancer Hospital in Zhengzhou University. The colon cancer patient did not receive any chemotherapy or radiotherapy prior to surgery. Tissue histology was confirmed by a pathologist. Prior written informed consent was obtained from the patient. Colon cancer tissues were cut into pieces and implanted into the back of the neck of each mouse. Mice were divided into 2 groups of 8 animals as follows: 1) untreated vehicle group and 2) 40 mg DIM/kg of body weight. DIM or vehicle (10% DMSO in 20% tween 80) was orally administered 5 times per week. Tumor volume was calculated from measurements of 2 diameters of the individual tumor base using the following formula: tumor volume (mm^3) = (length × width × height × 0.52). Mice were monitored until tumors reached 1.0 cm^3 total volume, at which time mice were euthanized and tumors extracted.

2.13. In vivo studies using a metastasis mouse model

Male or female mice (Nu/nu; 6–9 weeks old) were maintained under "specific pathogen-free'' conditions based on the guidelines established by Zhengzhou University Institutional Animal Care and Use Committee. HT29 colon cancer cells were infected with the pCDH-CMV-MCS-EF1-copEGFP-T2A-puro vector (Ziben, Shanghai, China) and incubated for 48 h. After inhalation anesthesia, cells (1×10^6 cells per mice) were injected into the tail vein of nude mice. Mice were divided into 2 groups as follows: 1) untreated vehicle group (n = 10) and 2) 40 mg DIM/kg of body weight (n = 10). DIM or vehicle (10% DMSO in 20% tween 80) was orally administered 5 times per week for 35 days. Fluorescence was measured weekly using an *in vivo* imaging system (IVIS spectrum CT Caliper, PerkinElmer, Massachusetts, USA).

2.14. In vivo acute toxicity assay

Female mice (C57BL/6J; 6–9 weeks old) were maintained under "specific pathogen-free'' conditions based on the guidelines established by Zhengzhou University Institutional Animal Care and Use Committee. Mice were divided into 4 groups as follows: 1) untreated vehicle group (n = 7); 2) 40 mg DIM/kg of body weight (n = 7); 3) 100 mg DIM/kg of body weight (n = 7); and 4) 200 mg DIM/kg of body weight (n = 7). DIM or vehicle (10% DMSO in 20% tween 80) was orally administered for 21 h. Blood samples from each group of mice were collected in heparin-treated tubes. The number of WBCs from whole blood was counted (Prokan PE6800 hematology analyzer, Diamond Diagnostics Inc., Holliston, MA, USA) and the ALT or AST activity from serum was measured at 510 nm.

2.15. Statistical analysis

All quantitative results are expressed as mean values ± S.D. Statistically significant differences were obtained using the Student's *t*-test or by one-way ANOVA. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. DIM is a potent ERK1/2 and COX1/2 inhibitor

Although DIM influences many types of proteins, direct targets of DIM or its specificity have not been determined. Among the potential targets, we selected 10 cancer-related kinases, including V600E mutant BRAF, MEK1, MEK2, ERK2, AKT1, p38, CDK2, EGFR, CHK1, CHK2, and the COX2 enzyme. Next, to identify the direct molecular targets of DIM, we performed an *in vitro* kinase or enzyme assay with DIM. Results indicated that DIM strongly inhibited ERK2 and COX2 activity (Fig. 1A–K).

To confirm the effect of DIM on COX enzyme activity, we performed an *in vitro* enzyme assay using a recombinant COX1 or COX2 protein. Results showed that COX1 or COX2 enzyme activity was significantly inhibited by DIM in a dose-dependent manner (Fig. 2A and B). Next, we investigated whether DIM affected the level of PGE2 production, which

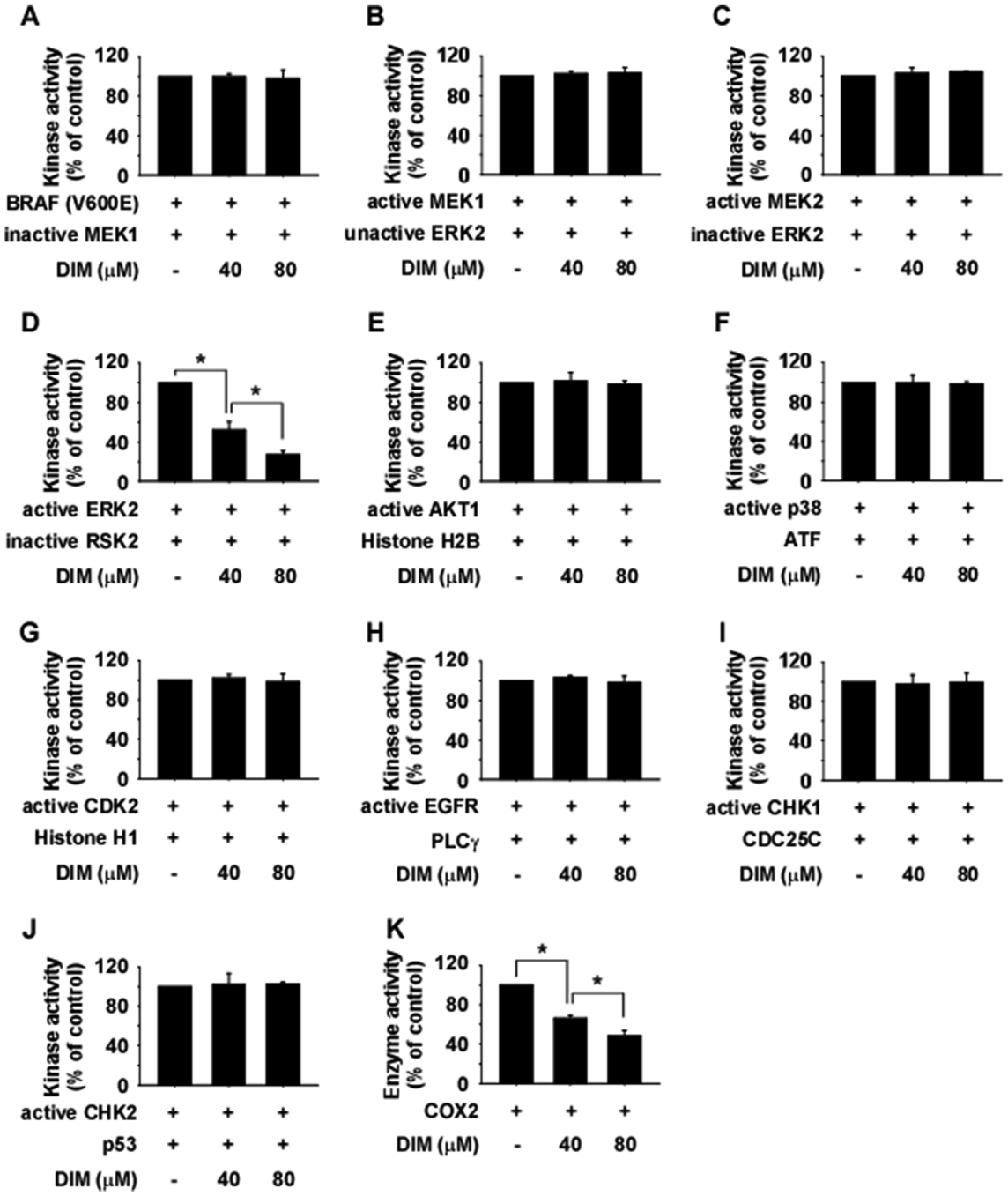

Fig. 1. Effect of DIM on activity of potential protein targets. The effect of DIM on (A) BRAF, (B) MEK1, (C) MEK2, (D) ERK2, (E) AKT1, (F) p38, (G) CDK2, (H) EGFR, (I) CHK1, (J) CHK2, or (K) COX2 activity was determined using the active kinase and its specific substrate for each kinase or production of oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) for COX2 activity. Data are shown as mean values ± S.D. from 3 independent experiments and the asterisk (*) indicates a significant difference ($p < 0.05$).

is a PGH downstream metabolite of COX1/2. HCT15 or HT29 colon cancer cells were treated with DIM for 48 h and PGE2 production was assessed by ELISA. Results indicated that PGE2 level was significantly suppressed by DIM treatment (Fig. 2C). Additionally, we determined whether DIM had an effect on cyclooxygenase 2 (*COX2*) reporter activity in colon cancer cells. *COX2* reporter activity was significantly inhibited by DIM treatment (Fig. 2D). Furthermore, to confirm the effect of DIM on ERK1/2 activity, we performed an *in vitro* ERK1 and ERK2 kinase assay using a recombinant ERK1 or ERK2 protein. Results indicated that DIM effectively inhibited ERK1 and 2 activities (Fig. 2E and F). We next examined the effect of DIM on ERK1/2 and the up- or downstream signaling. HT29 or HCT116 colon cancer cells were treated with DIM for 3 h and expression of ERK1/2 analyzed by Western blotting. Results showed that DIM suppresses phosphorylation of RSK, but does not affect MEK, ERK1/2, GSK3β, or MKK3/6 phosphorylation or β-catenin protein expression (Fig. 2G).

3.2. DIM directly binds with COX1/2 or ERK1/2 proteins

We conducted a molecular docking study between DIM and COX1 and 2 in order to determine its binding orientation, and results suggested that DIM might bind well to COX1 (Fig. 3A, *left panel*) and COX2 (Fig. 3A, *right panel*). To best understand how DIM interacts with ERK1 and ERK2, we docked it at the ATP binding pocket of each kinase,

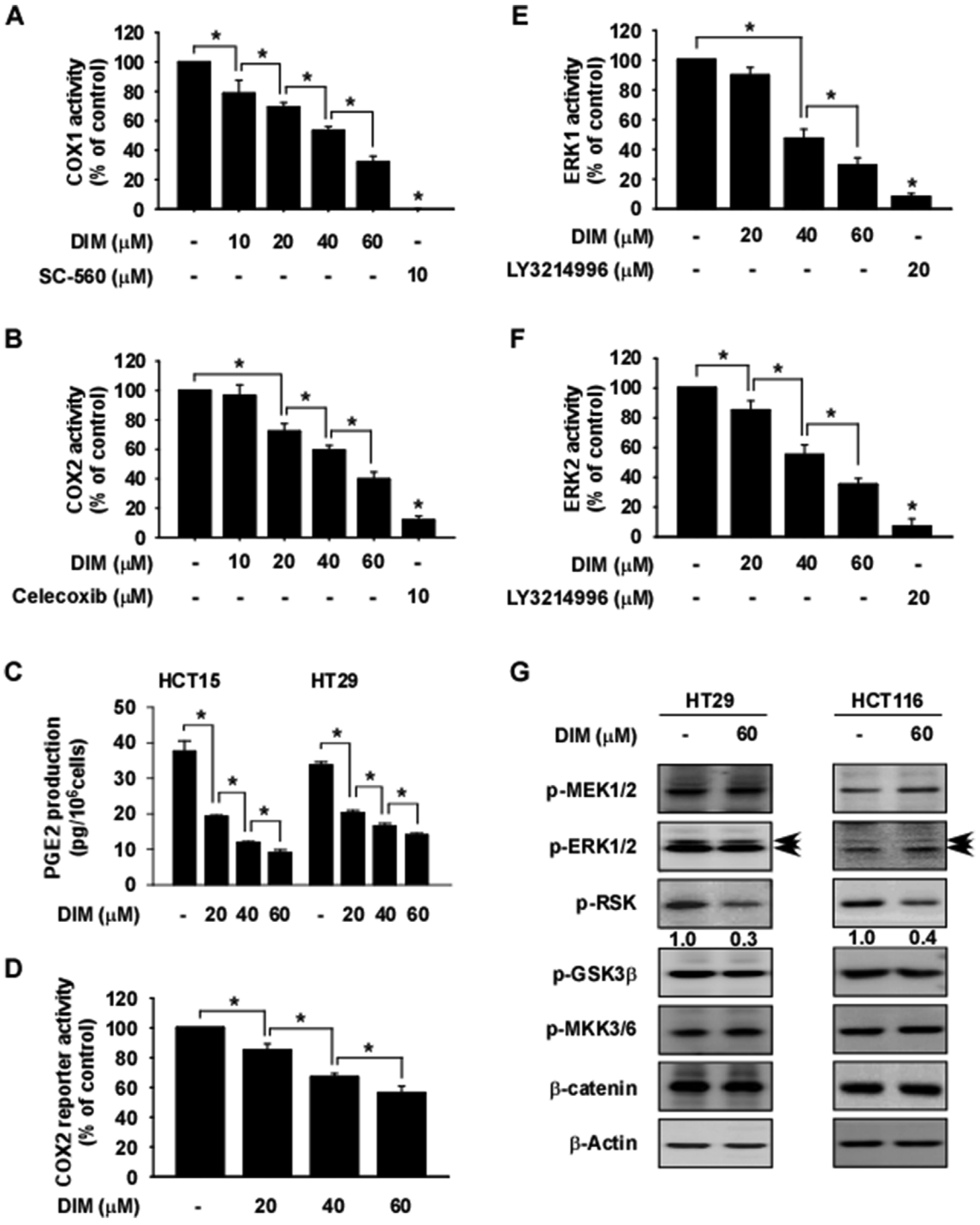

Fig. 2. Effect of DIM on COX1/2 and ERK1/2 activity. The effect of DIM on (A) COX1 or (B) COX2 activity was assessed using an *in vitro* enzyme assay. (C) The effect of DIM on PGE2 production was assessed in HCT15 or HT29 colon cancer cells. Cells were treated with DIM for 48 h and PGE2 production was measured. Detailed methods are described in Materials and methods. (D) The effect of DIM on *COX2* reporter activity was determined. Cells were transfected with a *COX2* reporter plasmid and incubated for 24 h and then treated with DIM for 48 h. Reporter activity was measured using the substrates included in the reporter assay system. The effect of DIM on (E) ERK1 and (F) ERK2 recombinant protein kinase activity was determined by *in vitro* kinase assays. For A-F, data are shown as mean values ± S.D. from 3 independent experiments and the asterisk (*) indicates a significant difference ($p < 0.05$). (G) The effect of DIM on various signaling pathways was determined. Cells were treated with DIM for 3 h. Various signaling proteins were analyzed by Western blotting and similar results were obtained from 3 independent experiments. Band density was measured using the Image J (NIH) software program.

respectively, using several protocols in the Schrödinger Suite 2016. Based on the final computational docking model result, we found that DIM formed several contacts with ERK1 or ERK2 at the respective ATP binding pocket. These results indicate that DIM might be a potential inhibitor of ERK1 and ERK2 (Fig. 3B and C). Note that images were generated with the UCSF Chimera program [25]. To confirm the results of the computer docking, we performed *in vitro* pull-down assays using DIM-conjugated Sepharose 4B beads. These results showed that DIM bound to COX1 and 2 or ERK1/2 (Fig. 3D–F).

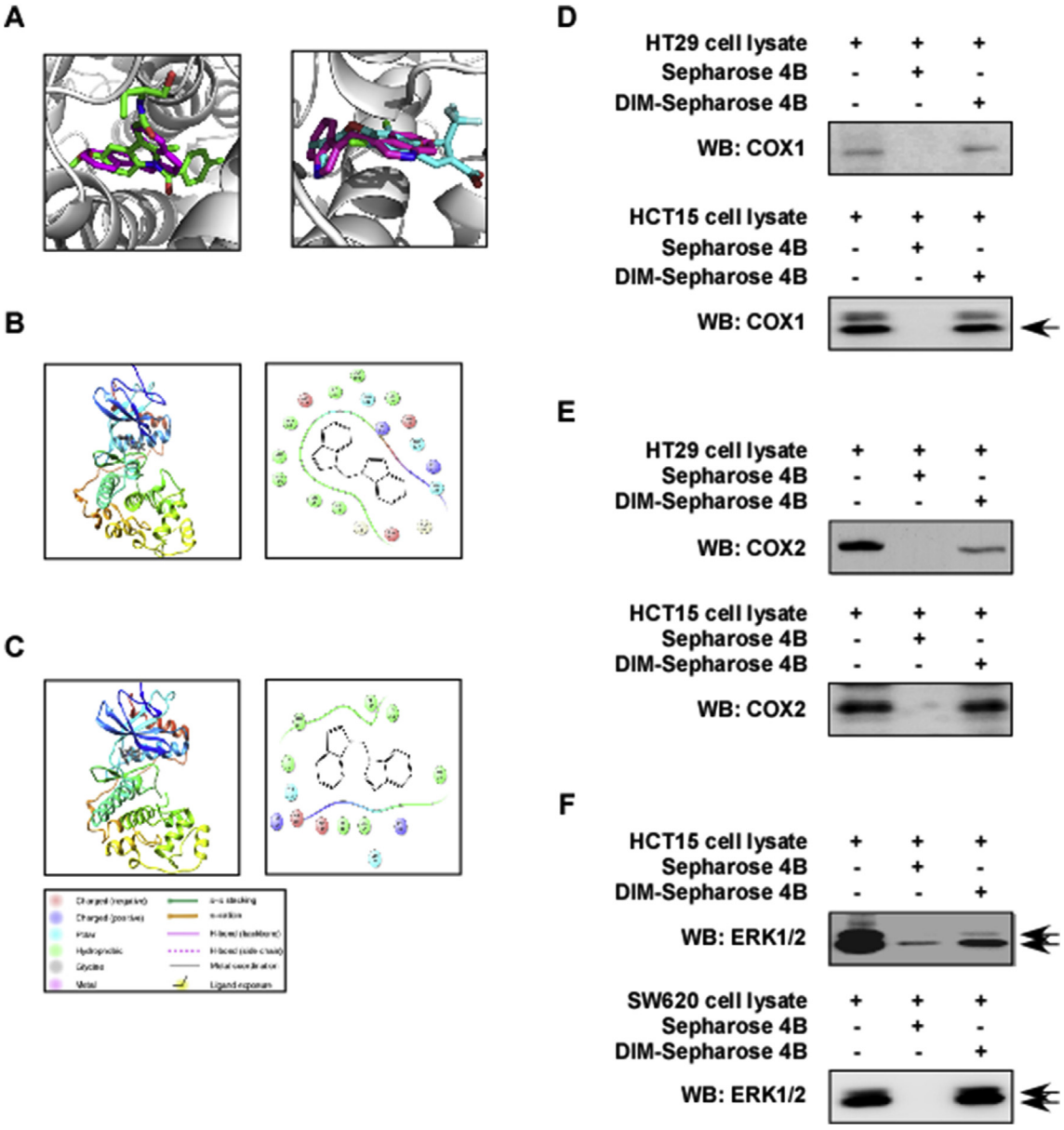

Fig. 3. DIM binds to COX1/2 and ERK1/2. Binding mode of DIM with COX1 (A, *left panel*) or COX2 (A, *right panel*). The carbon atoms of a COX1 or COX2 inhibitor are colored green or cyan. The carbon atoms of DIM are colored magenta. Modeling of DIM binding with ERK1 (B) or ERK2 (C). Left: DIM binding with ERK1 or ERK2 at the ATP binding pocket, Right: Ligand Interaction Diagram (LID) of the binding. ERK1 and ERK2 structures are shown as ribbon representation and DIM is shown as stick. LID legend is shown below. DIM directly binds to COX1 (D), COX2 (E) or ERK1/2 (F). Colon cancer cell lysates (500 μg) were incubated with DIM-conjugated Sepharose 4B beads, or with Sepharose 4B beads alone. The pulled down proteins were analyzed by Western blotting. Similar results were obtained from 3 independent experiments.

3.3. Anticancer activity of DIM

To evaluate the effect of DIM on normal colon cell viability, we treated CCD 841 CoN cells with DIM. Results indicated that DIM had little effect on the viability of these cells (Fig. 4A). Next, we determined whether DIM affects colon cancer cell growth. Cells were treated with DIM at various doses for 24, 48, or 72 h and proliferation was analyzed by MTT assay. Results showed that DIM significantly inhibits colon cancer cell growth (Fig. 4B). Additionally, the cells were treated with DIM for 48 h to determine its effect on cell cycle. Results indicated that DIM induces G1 phase arrest and reduces S phase cell cycle. It also increased p21, a G1 phase marker protein, and decreased cyclin D1, an S phase marker protein (Fig. 4C and D). We also examined the effect of DIM on anchorage-independent colon cancer cell growth and cell invasion. Results indicated that DIM strongly suppressed anchorage-independent growth of these cells (Fig. 4E) and cell invasion (Supplemental Figs. 1A and B). We detected the expression of COX1/2 or ERK1/2 proteins in colon cancer cells, and determined that HT29 cells had the highest expression level of COX1 and COX2 proteins and HCT116 or SW620 cells had the highest phosphorylation level of ERK1/2 proteins (Supplemental Fig. 2). To study the influence of COX1/2 expression or ERK1/2 activation on cancer cell growth, knockdown of COX1 and COX2 in HT29 colon cancer cells or knockdown of ERK1 and ERK2 cells in SW620 colon cancer cells was established (Supplemental Fig. 3A, Supplemental Fig. 4A). Results indicated that growth of colon cancer cells was significantly inhibited by knockdown of COX1/2 or ERK1/2 proteins (Supplemental Figs. 3B and C and Supplemental Figs. 4B and C). We next investigated whether the inhibition of cell growth by DIM is dependent on the expression of the COX1/2 or ERK1/2 proteins. Cells were treated with DIM for 48 h or 3

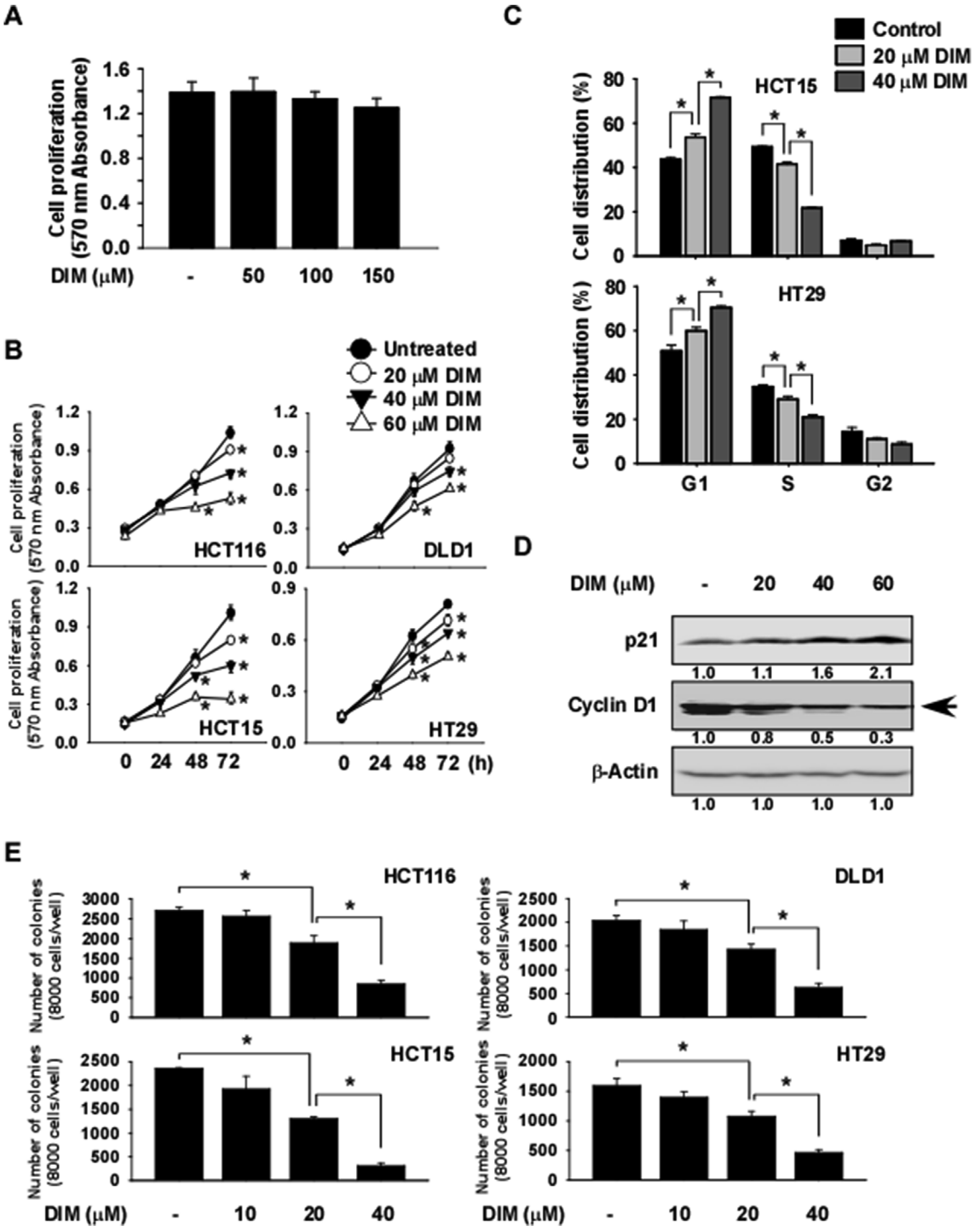

Fig. 4. DIM inhibits the growth of colon cancer cells. (A) The effect of DIM on viability of normal CCD 841 CoN colon cells was assessed. Cells were treated with DIM for 48 h and viability was measured at an absorbance of 570 nm. (B) DIM inhibits colon cancer cell growth in a dose-dependent manner. Cells were treated with DIM at various doses for 24, 48, or 72 h and proliferation was measured by MTT assay. (C) The effect of DIM on cell cycle was determined using HCT15 or HT29 colon cancer cells. Cells were treated with DIM for 48 h in medium supplemented with 10% FBS. Cells were stained with propidium iodide (PI) and cell cycle was analyzed by Fluorescence Activated Cell Sorting (FACS). (D) The effect of DIM on G1 or S phase marker proteins was determined in colon cancer cells. Cells were treated with DIM for 48 h in medium containing 10% FBS and analyzed by Western blotting. For D, similar results were observed from 3 independent experiments. Band density was measured using the Image J (NIH) software program. (E) DIM inhibits anchorage-independent colon cancer cell growth. Colon cancer cells were incubated in 0.3% agar for 2 weeks with DIM. Colonies were counted using a microscope and the Image-Pro PLUS (v.6) computer software program. For A-C and E, data are shown as mean values ± S.D. (N = 3) and the asterisk (*) indicates a significant ($p < 0.05$) difference.

weeks and the results indicated that cells expressing shCOX1 and shCOX2 or shERK1 and shERK2 were resistant to DIM's inhibitory effect on cell growth compared to cells expressing shControl (Supplemental Figs. 3D and E and Supplemental Figs. 4D and E). Therefore, we suggest that COX1/2 and ERK1/2 are the main target proteins of DIM in colon cancer cells.

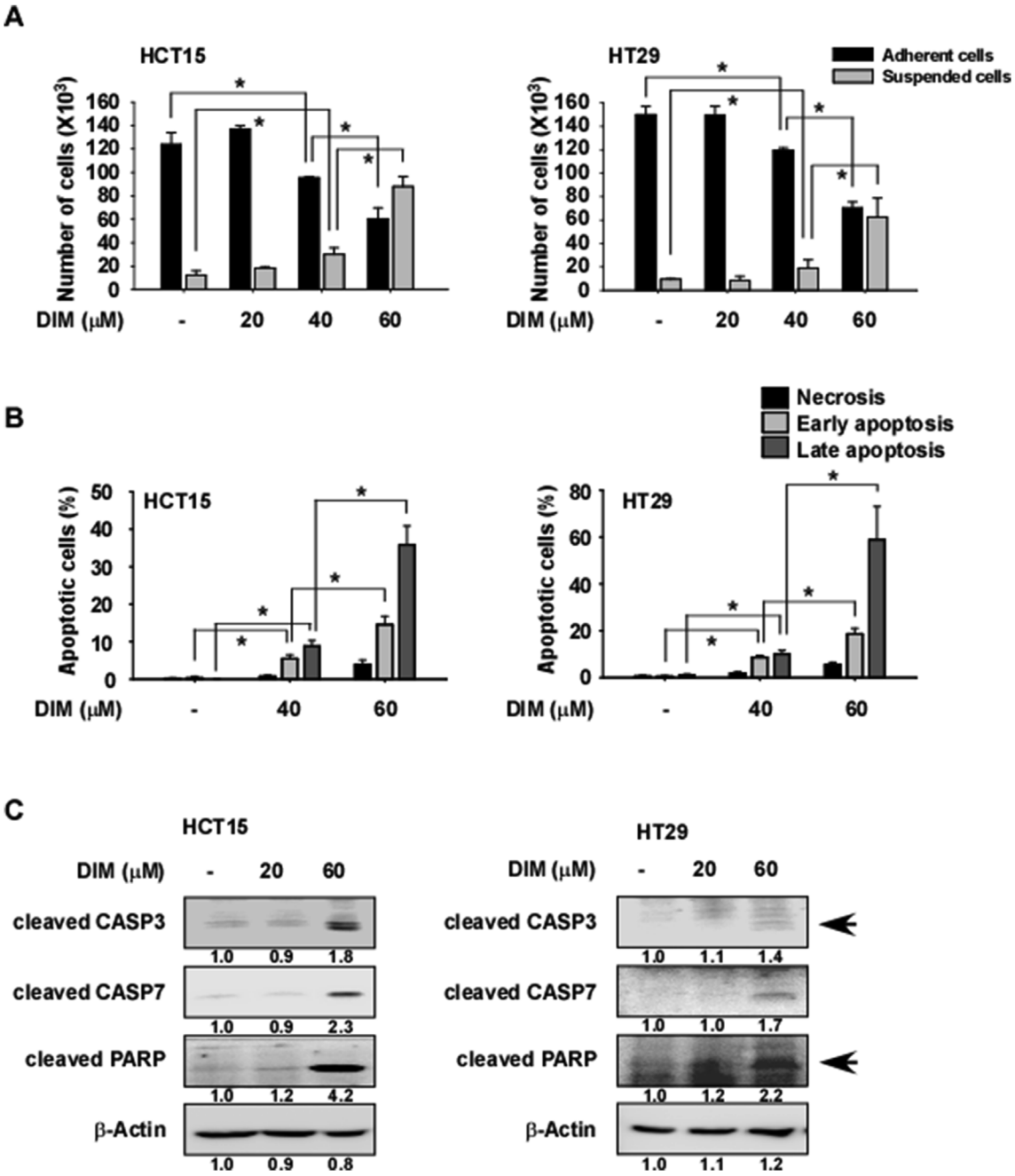

Fig. 5. Effect of DIM on apoptosis of colon cancer cells. (A) DIM induces apoptotic cell death. Cells were seeded in a 12-well plate and treated with DIM for 72 h at the indicated doses. The number of suspended or attached cells was determined using a hematocytometer. (B) DIM induces apoptosis. Cells were seeded with DIM in 10% FBS medium and then incubated for 72 h. Cells were stained with annexin V and propidium iodide (PI) and apoptosis was determined by Fluorescence Activated Cell Sorting (FACS). For A-B, data are shown as mean values ± S.D. (N = 3) and the asterisk (*) indicates a significant ($p < 0.05$) difference. (C) DIM strongly induces apoptotic marker proteins. Cells were treated with DIM for 48 h; and the levels of cleaved caspase 3, 7 and PARP proteins were determined by Western blotting using β-actin as a loading control. Similar results were obtained from 3 independent experiments. Band density was measured using the Image J (NIH) software program.

3.4. DIM induces apoptosis of colon cancer cells

To determine whether DIM could induce apoptosis of colon cancer cells, we measured the viability of DIM-treated or –untreated cells by counting the number of cells found in the suspended (dead) or attached (live) fraction after treatment with different doses of DIM for 72 h. The results indicated that the number of suspended cells was significantly increased in DIM-treated cells compared with control (Fig. 5A, *gray bar*), whereas the number of attached cells was significantly decreased in DIM-treated cells (Fig. 5A, *black bar*). To investigate whether the elevated cell death was due to an increase in apoptosis, we measured Annexin V expression at 72 h after DIM treatment and found a significantly higher level of early and late apoptosis compared to control (Fig. 5B). Apoptosis was also confirmed by measuring the level of proapoptotic marker proteins, including cleaved CASP3, 7 and PARP (Fig. 5C).

3.5. DIM inhibits patient-derived xenograft colon tumor growth in vivo

First, to determine the acute toxicity of DIM *in vivo*, Mice were orally

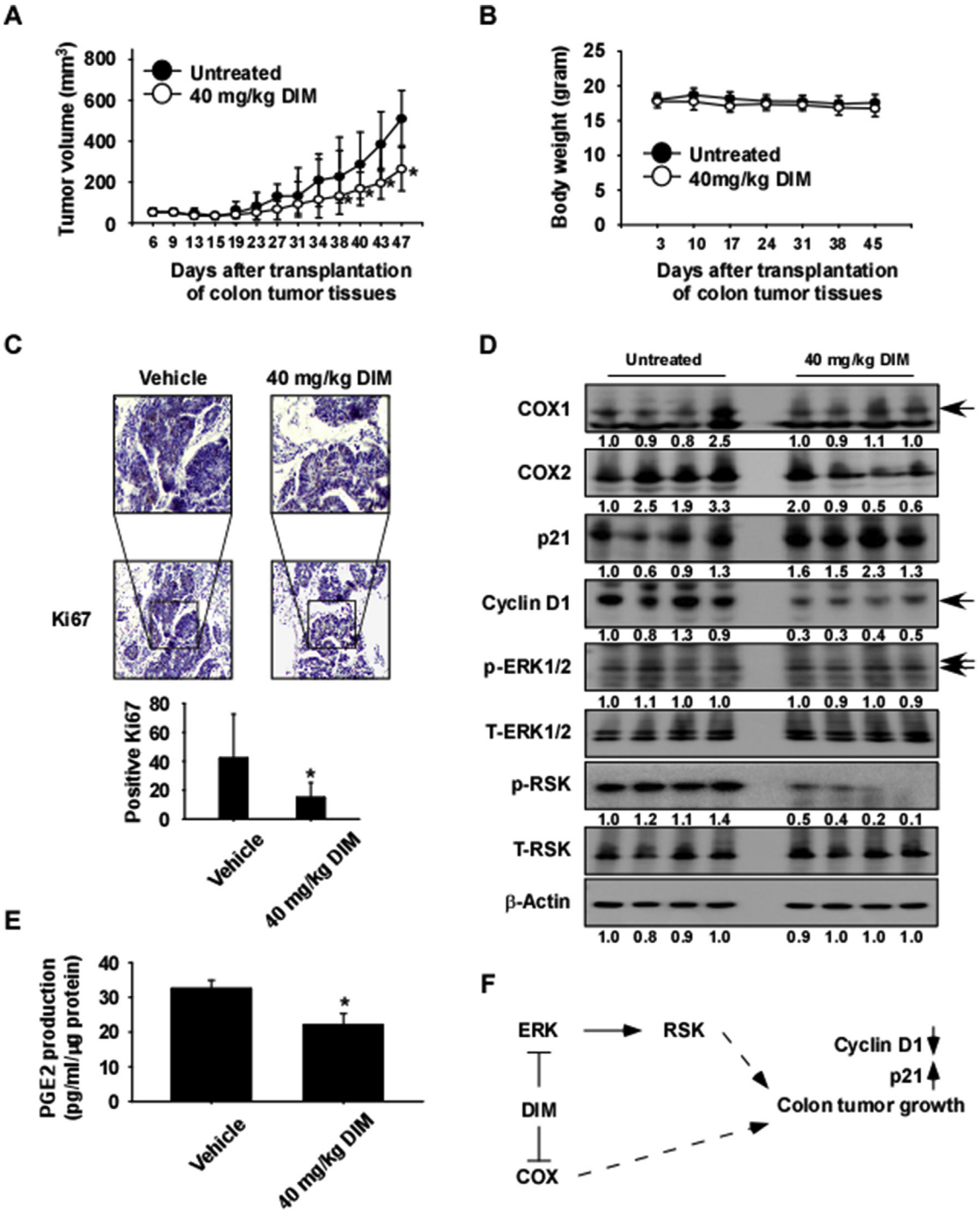

Fig. 6. Effect of DIM on patient-derived xenograft (PDX) colon tumor growth *in vivo*. (A) DIM suppresses PDX colon tumor growth. Mice were orally administered DIM or vehicle 5 times a week for about 7 weeks (47 days). Data are shown as means ± S.E. of values obtained from the experiments. The asterisk (*) indicates a significant difference between tumors from vehicle or DIM-treated mice as determined by *t*-test ($p < 0.05$). (B) DIM has no effect on mouse body weight. Body weights from vehicle or DIM-treated groups of mice were obtained once a week. (C) Immunohistochemical analysis of tumor tissues. Vehicle or DIM-treated groups of tumor tissues were stained with anti-Ki-67 and expression of Ki-67 was visualized by light microscope (*upper panel*). The number of Ki-67-stained cells was determined from immunohistochemistry results (*lower panel*; N = 8; *; $p < 0.05$). (D) DIM affects the expression of COX1/2, ERK1/2, and their downstream signaling proteins in PDX colon tumor tissues. Tumor tissues were analyzed by Western blotting and band density was measured using the Image J (NIH) software program. (E) DIM inhibits PGE2 production in PDX colon tumor tissues. Tumor tissues were analyzed by ELISA. Data are shown as means ± S.E. of values obtained from the experiments. The asterisk (*) indicates a significant difference between tumors from vehicle or DIM-treated mice as determined by *t*-test ($p < 0.05$). (F) Representative diagram of the mechanism of the anticancer activity of DIM.

administrated DIM at 40, 100, or 200 mg/kg or vehicle. Blood samples from each group mice were collected and analyzed after 21 h. Results indicated that the number of white blood cells (WBCs), the activity of alanine transaminase (ALT) and aspartate transaminase (AST) were little changed in mice treated with DIM at 40, 100 or 200 mg/kg compared with the vehicle-treated group (Supplemental Figs. 5A–C). Next, to determine the antitumor activity of DIM *in vivo*, patient-derived colon cancer tissues were implanted into the back of the neck of

athymic nude mice. Mice were orally administrated DIM at 40 mg/kg or vehicle 5 times a week over a period of 42 days. Results indicated that DIM significantly decreased the volume of colon tumor growth relative to the vehicle-treated group (Fig. 6A; $p < 0.05$). Additionally, mice tolerated treatment with DIM without significant loss of body weight similar to the vehicle-treated group (Fig. 6B). We then examined the effects of DIM on the Ki-67 tumor proliferation marker by using immunohistochemistry. The expression of Ki-67 was significantly decreased by treatment with DIM (Fig. 6C). To validate the results of the *in vivo* PDX model, we investigated the effect of DIM on the production of the COX metabolite, PGE2, by ELISA and downstream targets of ERK1/2 by Western blot analysis of PDX colon tumor samples. The expression of cyclin D1 and phosphorylation of RSK, a direct downstream substrate of ERK1/2, were strongly inhibited and the expression of p21 was increased in the DIM-treated group (Fig. 6D). Additionally, PGE2 production was significantly inhibited by DIM treatment compared to vehicle treatment (Fig. 6E). We suggest that inhibition of ERK1/2 and COX1/2 by DIM exerts multiple effects on tumor progression, cancer growth and apoptosis by modulating the ERK/RSK signaling axis or the prostaglandin signaling pathway (Fig. 6F).

4. Discussion

DIM is a natural plant-derived compound that has been extensively studied and has demonstrated anticancer activity by inducing cell cycle arrest leading to apoptosis in several types of cancers [26–29]. Materials from natural sources are assumed to have relatively low toxicity and have been studied for use in clinical applications [30]. Although DIM has been reported to demonstrate anticancer properties *in vitro* and *in vivo*, the mechanistic targets of DIM have not been clearly identified.

In this study, we searched for direct target proteins contributing to the DIM-induced anticancer properties in colorectal cancer cells. We found that DIM could inhibit the growth of colon cancer cells by directly targeting ERK1/2 and COX1/2 proteins and confirmed the anticancer activity of DIM *in vitro* and *in vivo*.

Previous reports suggested that both ERK1/2 and COX1/2 regulate or mediate antiproliferative events [31,32]. One mechanism occurs through ERK1/2-mediated down-stream pro-apoptotic proteins, such as p90RSK [33]. The D-domains of p90RSK could bind the CD domain in ERK2 to mediate apoptosis [34]. DIM possibly inhibits cell proliferation by competitively blocking the docking site of ERK1/2, which is similar with a reported ERK1/2 inhibitor [33]. Furthermore, COX2 is also involved in apoptotic processes through several pathways by regulating PGE2 metabolism [35]. COX2/PGE2 might induce an increased expression of Bcl-2 and thus suppress apoptosis through the Ras/MEK/ERK1/2 pathway [36]. More evidence showed that, COX2/PGE2 also could promote cell survival by indirectly transactivating the nuclear peroxisome PARP through a PI3−K/AKT-dependent mechanism [37]. Another isoform, COX1 was also reported to be a potential target in cancer therapy [38]. The COX1 inhibitor, SC-560, could mediate apoptosis in Huh-6 or HA22T HCC cells by activating caspase-3 and -7 as well as decreasing expression of anti-apoptotic proteins such as survivin and XIAP [39]. Selective inhibition of COX1 could reduce the malignant characteristics through cooperation with the EGFR pathway [13]. Consistent with these reports, DIM is a potent inhibitor of ERK1/2 and COX1/2 that induces apoptosis (Fig. 5).

Cell cycle arrest reportedly is almost always accompanied by apoptosis after treatment with anticancer reagents. CDK inhibitors and cyclin proteins are key factors in cell cycle progression [40–42]. If ERK1/2 activity is inhibited chemically or by a dominant-negative ERK1/2, cyclin D1 expression is decreased, because sustained ERK1/2 activity is necessary for regulating cyclin D1 expression at the G1 phase [43]. Because the localization of ERK1/2 to the nucleus is necessary for cell cycle re-entry [44], sustained ERK1/2 activity could direct the expression between the cytoplasm and nucleus [45]. In addition, inactivated ERK1/2 could stimulate the *p21* promoter and increase the p21 expression level in a p53-dependent manner [46]. Also, COX inhibitors exert antitumor effects through the up-regulation of p21 as well as the down-regulation of cyclin D1 [47]. Based on these results (Fig. 4C and D), DIM clearly induces G1 phase cell cycle arrest through regulating p21 and cyclin D1 expression by targeting ERK1/2 and COX1/2.

Based on the application of data from clinical trials using DIM (NCT00888654; NCT00305747; NCT00450229; NCT01391689) and conversion calculation to an equivalent dose in animals [48], we determined a dose of DIM at 40 mg/kg body weight as appropriate for the *in vivo* mouse study. We next investigated whether DIM could have acute toxicity. Interestingly, the highest dose of DIM (200 mg/kg B.W.) did not affect the number of WBCs or the activity of AST and ALT (Supplemental Figs. 5A–C). Additional studies are planned to further characterize DIM and to determine long term toxicological responses.

To determine whether DIM affected colon cancer metastasis, HT29 colon cancer cells were injected into a tail vein and then mice were orally administrated DIM or vehicle for 35 days. We monitored the growth of the metastatic tumor by *in vivo* imaging and observed fluorescence (HT29 colon cancer cells) in the lung, liver, colon, bladder, and bone (Supplemental Fig. 6A). Results indicated that DIM could not inhibit colon cancer metastasis (Supplemental Figs. 6B–D). Furthermore, we examined whether DIM showed preventive effects on colon carcinogenesis using *APC* $^{min+}$ mice, a model of familial adenomatous polyposis. However, DIM had little effect on polyp number or size compared with the vehicle group (Supplementary Figs. 7A and B). To determine the therapeutic effect of DIM on colon tumor growth, we used a colon patient-derived xenograft model. Interestingly, the volume of colon PDX tumors was decreased dramatically (Fig. 6A). The *APC* $^{min+}$ mice usually develop tumors of the gastrointestinal tract under the activation of the *Wnt* pathway [49], which is an applicable model for studying the effect of a preventive agent. In the PDX mouse models, tumor tissues from patients are directly grafted into an immune-deficient animal, which more accurately maintains the genetic information of the human tumor [50]. Based on this *in vivo* study, we conclude that DIM could act effectively as a therapeutic agent, but not as a preventive or anti-metastatic agent against colon cancer *in vivo*.

The results of the present study showed that DIM might be a potent anticancer drug, especially against colon cancer. Several clinical trials focusing on treatment with DIM in prostate and breast cancer have already been completed, but the effect of DIM has not been studied clinically in colon cancer. Due to the high expression of COX1/2 in colon cancer, DIM might be a better therapeutic drug for treatment of colon cancer. In summary, the results presented here show that DIM worked well to inhibit colon cancer growth both in *vivo* and in *vitro* by directly targeting COX1/2 and ERK1/2, which might offer another strategy in the clinic.

Author contribution

X.T. performed the *in vitro* experiments and assisted with the cell based and *in vivo* experiments; X.Z, M.L and N.O assisted with the cell based assays; Z.L and Y.Z. assisted with the *in vivo* experiments; X.Z, F.M. and F.L. performed the *in vivo* experiments; H.C and Y.L. performed the computer modeling; K.L. and A.M.B. supervised the *in vivo* experimental design, data analysis and manuscript editing; Z.D. supervised the overall experimental design; D.J.K. supervised designed experiments, provided the idea and prepared the manuscript.

Competing financial interest statement

None of the authors have any competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.031.

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