

Curcumin Suppresses Proliferation of Colon Cancer Cells by Targeting CDK2

Tae-Gyu Lim^{1,5}, Sung-Young Lee^{1,3}, Zunnan Huang¹, Do Young Lim¹, Hanyong Chen¹, Sung Keun Jung^{1,2}, Ann M. Bode¹, Ki Won Lee^{2,4}, and Zigang Dong¹

Abstract

Curcumin, the yellow pigment of turmeric found in Southeast Indian food, is one of the most popular phytochemicals for cancer prevention. Numerous reports have demonstrated modulation of multiple cellular signaling pathways by curcumin and its molecular targets in various cancer cell lines. To identify a new molecular target of curcumin, we used shape screening and reverse docking to screen the Protein Data Bank against curcumin. Cyclin-dependent kinase 2 (CDK2), a major cell-cycle protein, was identified as a potential molecular target of curcumin. Indeed, *in vitro* and *ex vivo* kinase assay data revealed a dramatic suppressive effect of curcumin on CDK2 kinase activity. Furthermore, curcumin induced G₁ cell-cycle arrest, which is regulated by CDK2 in HCT116 cells. Although the expression levels of CDK2 and its regulatory subunit, cyclin E, were not changed, the phosphorylation of retinoblastoma (Rb), a well-known CDK2 substrate, was reduced by curcumin. Because curcumin induced cell-cycle arrest, we investigated the antiproliferative effect of curcumin on HCT116 colon cancer cells. In this experiment, curcumin suppressed HCT116 cell proliferation effectively. To determine whether CDK2 is a direct target of curcumin, CDK2 expression was knocked down in HCT116 cells. As expected, HCT116 sh-CDK2 cells exhibited G₁ arrest and reduced proliferation. Because of the low levels of CDK2 in HCT116 sh-CDK2 cells, the effects of curcumin on G₁ arrest and cell proliferation were not substantially relative to HCT116 sh-control cells. From these results, we identified CDK2 as a direct target of curcumin in colon cancer cells. *Cancer Prev Res*; 7(4); 466–74. ©2014 AACR.

Introduction

Curcumin or diferuloylmethane is a yellow pigment and a constituent of turmeric extracted originally from *Curcuma longa* (Linn.). In China and India, curcumin is widely used as a coloring and flavoring agent. The chemotherapeutic

and chemopreventive effects of curcumin have been reported previously (1–7). In addition, oral intake of curcumin reportedly showed a beneficial effect against precancerous lesions in phase I and II clinical trials (8, 9). Furthermore, some investigators have suggested several target proteins and signaling pathways affected by curcumin, including the AMPK-COX-2 (10), *c-jun*—NH₂—kinase (JNK; ref. 11), E2F4 (12), and cyclin D1-CDK4 signaling pathways (13). Although numerous proteins have been suggested as targets of curcumin, CDK2 has not been reported as a direct target of curcumin in colon cancer.

Aberrant growth is a characteristic of cancer cells (14). Research data clearly indicate that cancer cell proliferation is much greater and less controlled than normal cell growth (15). Cyclin-dependent kinases (CDK), which are serine/threonine protein kinases, are essential kinases mediating the cell cycle (16). Thus far, nine CDKs have been identified and each regulates specific points of the cell cycle (16, 17). For example, G₁ is mediated by CDK4, CDK6, and CDK2; the S phase is regulated by CDK2; and the G₂–M phase is controlled by CDK1 (16, 17). The kinase activity of CDKs is regulated by cyclins and different CDKs are required by various cyclins for their kinase activity (18). In the G₁-phase, cyclins D1, D2, and D3 interact with CDK4 and CDK6 (19). In the G₁- to S-phase transition, cyclin E binds to CDK2 (20, 21) and during the S-phase, the cyclin A/CDK2 complex regulates cell cycle (21). To promote the M-phase, cyclin A

Authors' Affiliations: ¹The Hormel Institute, University of Minnesota, Austin, Minnesota; ²WCU Biomodulation Major, Department of Agricultural Biotechnology and Center for Food and Bioconvergence; ³WCU, Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Sciences and Technology, Seoul National University; ⁴Advanced Institutes of Convergence Technology, Seoul National University, Suwon; and ⁵Research Institute of Bio Food Industry, Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang, Republic of Korea

Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

T.-G. Lim and S.-Y. Lee contributed equally to this work.

Normal human colon epithelial cells (HCEC) comprise an immortalized epithelial cells derived from human colon biopsies with properties similar to normal colon epithelial cells.

Corresponding Authors: Zigang Dong, The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912. Phone: 507-437-9600; Fax: 507-437-9606; E-mail: zgdong@hi.umn.edu; and Ki Won Lee, Seoul National University, Seoul, Republic of Korea. Phone: 82-2-880-4661; Fax: 82-2-878-6178; E-mail: kiwon@snu.ac.kr

doi: 10.1158/1940-6207.CAPR-13-0387

©2014 American Association for Cancer Research.

binds to CDK1 in the late G₂ and early M-phase (22, 23). Deregulation of growth is characteristic of cancer cells and cell-cycle proteins are highly activated in these cells (24). Cyclin E-dependent kinase activity was reported to be substantially higher in colorectal cancers and CDK2 expression levels are also high in colorectal adenomas (25). We found that three colon cancer cell lines, HCT116, HCT15, and DLD-1, show dramatically increased expression of CDK2 compared with "normal" (see footnote on the first page) human colon epithelial cells (HCEC). This suggests that CDK2 could be a potential therapeutic target for colon cancer treatment.

Herein, we used computational modeling to identify CDK2 as a potential target of curcumin. Both *in vitro* and *ex vivo* kinase assay data confirmed that curcumin effectively suppressed CDK2 kinase activity. Because CDK2 is highly activated in colon cancer, we selected the HCT116 colon cancer cell model to evaluate the effects of curcumin on cell cycle. We found that curcumin caused G₁ cell-cycle arrest, which is regulated by CDK2. Furthermore, curcumin exhibited a dramatic antiproliferation effect on three different colon cancer cell lines and knocking down expression of CDK2 confirmed that CDK2 is a direct target of curcumin. To our knowledge, this is the first report to show that CDK2 is a direct target of curcumin and could be a novel target of curcumin in preventing or treating colon cancer.

Materials and Methods

Chemicals

Curcumin and FBS were purchased from Sigma-Aldrich. McCoy's 5A was obtained from Thermo Fisher Scientific. Penicillin/streptomycin were purchased from Invitrogen. Antibodies against CDK2, CDK4, phosphorylated retinoblastoma (Rb; Thr821), and cyclin E were obtained from Santa Cruz Biotechnology and the antibody against phosphorylated c-Myc was purchased from Cell Signaling Technology. Secondary antibodies to detect rabbit and mouse immunoglobulin G (IgG) and conjugated to horseradish peroxidase (HRP) were obtained from Zymed.

Cell culture

All cell lines were obtained from American Type Culture Collection. The cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained for a maximum of 8 weeks. The frozen vials were available for each cell line to ensure that all cell-based experiments were performed on cells that had been tested and were in culture for 8 weeks or less. HCECs were cultured in basal media (HyClone) supplemented with EGF (25 ng/mL), insulin (10 µg/mL), gentamicin sulfate (50 µg/mL), transferrin (2 µg/mL), hydrocortisone (1 µg/mL), sodium selenite (5 nmol/L), and 2% Cosmic Calf serum (HyClone). HCT116 human colon cancer cells were cultured in McCoy's 5A medium supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin. HCT15 and DLD-1 human colon cancer cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Shape screening

Shape screening was performed using the phase module (26, 27) in Schrödinger Suite 2011 (28) by comparing the volumetric similarity between curcumin and each compound from the Protein Data Bank (PDB) ligand database. The structure of curcumin was downloaded from the PubChem Compound database (<http://www.ncbi.nlm.nih.gov/pccompound>) and further processed using the ConfGen module (29) in Maestro (30). The best three-dimensional conformation of the lowest potential energy from ConfGen was selected as the query molecule structure. The ligand database was downloaded from the PDB (31) in March 2010. Shape screening was performed directly with this database without further modification. During the screening process, polar hydrogens were included and one alignment per ligand was retained. Only the molecules with a ShapeSim (i.e., the overall similarities between curcumin and screened compounds) score of less than 0.7 were eliminated.

Reverse docking

To enhance the reliability of the evaluation of the potential targets of curcumin from shape screening, reverse docking using the Glide module (32, 33) in Schrödinger was also used. For this calculation, only the potential kinase targets were considered. The crystal structure of each protein kinase bound with the potentially identified ligand from the PDB was downloaded. Each raw PDB structure was then converted into an all-atom, fully prepared receptor model structure using Protein Preparation Wizard module in Maestro. The docking receptor grid of each potential target was prepared using Glide's Receptor Grid Generation (32, 33). Docking of the curcumin molecule into each receptor grid was performed using Glide in the XP (Extra Precision) mode (34). All generated conformations of curcumin from ConfGen were used for rigid-protein docking. In all other respects, the default settings were used. The best scoring energy was selected from the best docking of different initial conformations of curcumin to each kinase target.

Molecular modeling

The docking structure of curcumin and CDK2 was generated by the Induced Fit Docking (IFD) protocol (35) in the Schrödinger Suite 2011. All default settings were used, except that the XP (Extra Precision) scoring function was used (34). Briefly, the grid box and center were settled by default using the ligand in the ATP-binding site and no constraints were defined. During the Glide docking process, the van der Waals radii of protein and ligand were scaled by a factor of 0.5. In addition, only the residues within 5 Å of the ligand were refined for Prime active site optimization. The binding pose with the lowest score was considered as the correct binding structure and used as the representative docking structure.

CDK1, CDK2, and CDK4 kinase assays

The CDK1, CDK2, and CDK4 kinase assays were performed using active recombinant CDK1, CDK2, and CDK4

enzymes (Millipore) and evaluated the ability of each kinase to transfer the radiolabeled phosphate from [γ - 32 P] ATP according to the manufacturer's instructions. Briefly, active recombinant CDK1, CDK2, or CDK4 (100–200 ng) and curcumin were incubated at 30°C for 15 minutes in assay buffer [20 mmol/L MOPS (pH 7.2), 25 μ mol/L β -glycerol phosphate, 5 mmol/L EGTA, 1 mmol/L sodium orthovanadate, and 1 mmol/L dithiothreitol (DTT)]. As a substrate myelin basic protein (20 μ g) was added to each mixture and then incubated at 30°C for 15 minutes with a [γ - 32 P] ATP solution in a magnesium acetate-ATP cocktail buffer (Upstate Biotechnology Inc.). Then the reaction mixture was transferred onto p81 paper, which was then washed three times for 5 minutes each with 0.75% phosphoric acid. The radiolabeled phosphate was measured using a scintillation counter.

For the *ex vivo* CDK2 immunoprecipitation and kinase assays, 20 μ L of Protein A/G Plus Agarose was added to cell lysates containing 600 μ g protein and then incubated for 1 hour at 4°C. After centrifugation at 12,000 rpm for 5 minutes at 4°C, an antibody against CDK2 (30 μ g) was added to the supernatant fraction of the mixture, and then rocked overnight at 4°C. These mixtures were centrifuged at 12,000 rpm for 5 minutes at 4°C and washed twice. Curcumin was added at various concentrations and the kinase assay was performed in a manner similar to the *in vitro* kinase assay.

Western blot analysis

HCT116 colon cancer cells were cultured for 48 hours, and to synchronize the cell cycle, the cells were incubated in serum-free McCoy's 5A media for 12 hours. After synchronization, the cells were treated or not treated with various concentrations of curcumin for 30 minutes. Then the cells were disrupted with lysis buffer [10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol and protease inhibitor cocktail tablet]. The protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories) as described by the manufacturer. The proteins were separated electrophoretically using a 10% SDS-PAGE. After separation, the proteins were transferred to an Immobilon-P membrane (Millipore Corporation). The membrane was blocked with 5% fat-free milk for 1 hour and incubated with the specific primary antibody at 4°C overnight. After hybridization with an HRP-conjugated secondary antibody, the protein bands were detected using a chemiluminescence detection kit (GE healthcare).

Preparation of curcumin-Sepharose 4B beads

CNBr-activated Sepharose 4B beads, used in the current experiments, bind and immobilize chemicals at available hydroxyl (–OH) groups. Curcumin has three hydroxyl groups and therefore can be easily coupled with Sepharose beads. For activation, the Sepharose 4B beads (0.3 g) were washed with 30 mL of 1 mmol/L HCl three times for 5 minutes each by gentle inversion and then incubated

with 3 mg of curcumin or dimethyl sulfoxide (DMSO) in coupling buffer [0.1 mol/L NaHCO₃ (pH 8.3) and 0.5 mol/L NaCl (pH 8.3)] at 4°C overnight. The mixtures were washed five times with coupling buffer and incubated with blocking buffer [0.1 mol/L Tris–HCl (pH 8.0)] at 4°C overnight. The excess of uncoupled curcumin was removed by washing buffer [0.1 mol/L acetate buffer (pH 4.0)] and 0.1 mol/L Tris–HCl buffer (pH 8.0) containing 0.5 mol/L NaCl (pH 8.0) three times and then resuspended in 1 mL of PBS.

Cell-based pull-down assay

HCT116 colon cancer cells were disrupted with lysis buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.5% Nonidet P-40, 0.02 mmol/L PMSF, and 1 \times protease inhibitor mixture] and proteins were extracted. Proteins (500 μ g) were incubated with Sepharose 4B beads (as a negative control) or curcumin-Sepharose 4B beads (100 μ L of 50% slurry) in reaction buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% Nonidet P-40, 2 mg/mL bovine serum albumin (BSA), 0.02 mmol/L PMSF, and 1 \times protease inhibitor mixture] at 4°C overnight. The mixture was washed five times with 1 mL of washing buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% Nonidet P-40, and 0.02 mmol/L PMSF] and proteins bound to the beads were analyzed by Western blotting.

ATP and curcumin competition assay

Recombinant CDK2 (200 ng) was incubated with ATP (0, 1, 10, or 100 μ mol/L) at 4°C for 1 hour. Then, 100 μ L of Sepharose 4B (negative control) or curcumin-Sepharose 4B beads were added to the mixture and incubated at 4°C overnight. The samples were then washed with washing buffer (above) and proteins were analyzed by Western blotting.

Cell proliferation assay

To evaluate the effect of curcumin on proliferation, HCT116 cells were seeded (4×10^3 cells/well) in 96-well plates for 24 hours. Cells were then treated with curcumin for 24, 48, or 72 hours. Cell proliferation was analyzed using CellTiter 96 AQueous One Solution (Promega) by incubating with 20 μ L of MTS solution for 1 hour at 37°C in a 5% CO₂ incubator. The absorbance was read at 492 nm.

Cell-cycle analysis

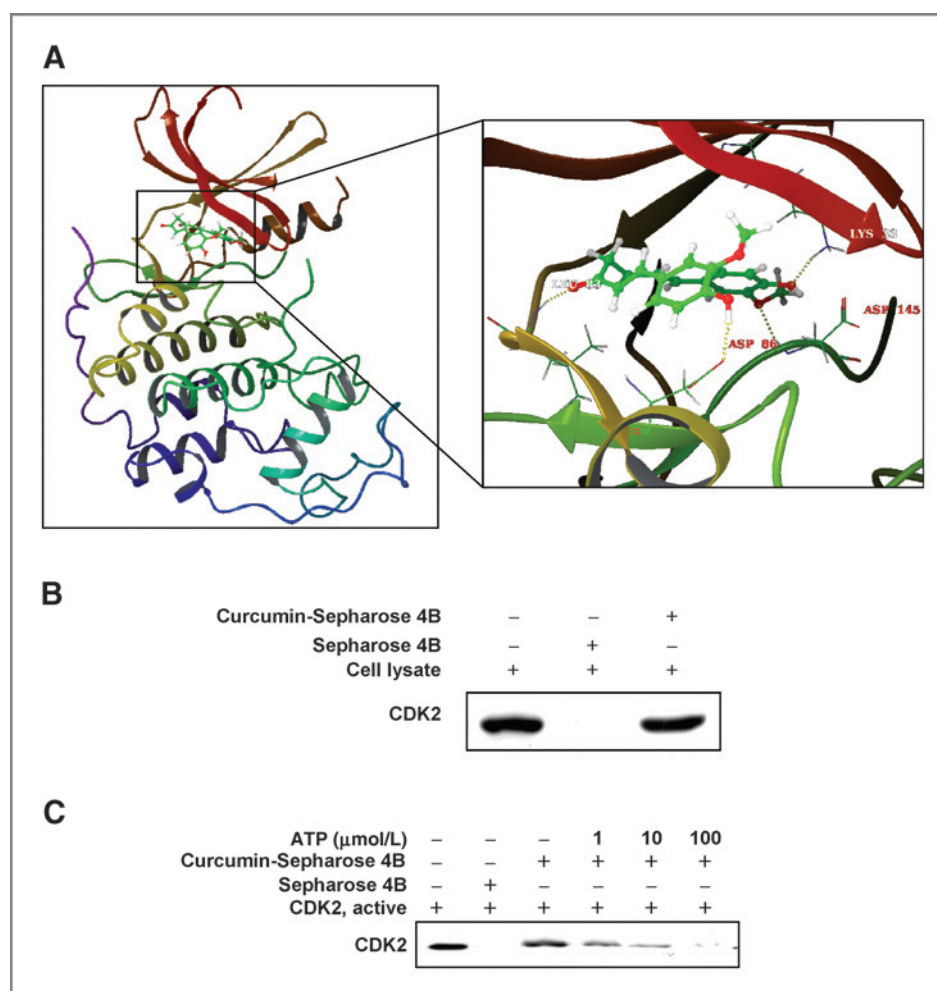
HCT116 cell cycle was evaluated using a published method (36) with slight modifications. After arresting the cells in the G₀-phase, the cells were treated with various concentrations of curcumin for 9 hours. After fixing with ethanol, cells were stained with propidium iodide and the cell-cycle phase was determined by flow cytometry.

Results

Computer modeling and binding between curcumin and CDK2

To identify potential targets of curcumin, we performed both ligand- and structure-based *in silico* screening using the computer modeling methods described in Materials and

Figure 1. Curcumin directly binds to CDK2 in an ATP-competitive manner. **A**, the interaction between curcumin and several residues in the ATP-binding pocket of CDK2. Curcumin forms hydrogen bonds with Lys33, Leu83, Asp86, and Asp145, respectively. Note: the α -helices are drawn as cylinders and the β -strands as arrows. Curcumin is shown in stick model and protein residues are shown in line model. **B**, curcumin directly binds with CDK2 in HCT116 colorectal cancer cells. The binding of curcumin with CDK2 in HCT116 cells was detected by immunoblotting with a specific CDK2 antibody: lane 1 (input control), whole-cell lysates from HCT116 cells; lane 2 (control), lysates from HCT116 cells; and lane 3, whole-cell lysates from HCT116 cells precipitated with curcumin-Sepharose 4B beads. **C**, curcumin competes with ATP for binding with CDK2. Active CDK2 was incubated with ATP at different concentrations (0, 1, 10, or 100 $\mu\text{mol/L}$) with 50 μL of curcumin-Sepharose 4B beads or 50 μL of Sepharose 4B (as a negative control) beads in reaction buffer. After washing, the pulled down CDK2 proteins were detected by Western blotting.



Methods. On the basis of the structure of curcumin, we first screened the PDB ligand library to identify compounds that showed a shape similarity score above 0.7. The proteins co-crystallized with the potentially identified ligands could possibly bind to curcumin. To enhance the reliability of the evaluation of these potential targets of curcumin identified from shape screening, we then performed structure-based reverse docking studies using curcumin and the crystal structures of these proteins and discovered CDK2 as a potential kinase target of curcumin.

To predict the binding conformation between curcumin and CDK2, we performed flexible-ligand flexible-protein docking using the IFD module (35). IFD has an advantage over the regular Glide docking (33) because of its ability to capture the ligand-induced conformational changes in the receptor active site (37). From the IFD, the binding affinity between curcumin and CDK2 was predicted to be very good with a score of -12.69 kcal/mol. The computational model of the curcumin-CDK2 complex is shown in Fig. 1A. Curcumin formed four hydrogen bonds with CDK2—two are involved with the backbone atoms of the hinge loop residue Leu83 and the activation loop residue Asp145, and the other two are formed with the side chain atoms of Asp86 and

Lys33. In addition, several residues around the ATP-binding pocket, including Ile10, Val64, Phe80, Phe82, Leu83, and Leu134, showed some hydrophobic interactions with the carbons of curcumin (data not shown). These computational results indicate that curcumin should exhibit ATP-competitive inhibitory effects against the CDK2 kinase.

Because the computer modeling data suggested that curcumin could bind to the ATP pocket of CDK2 (Fig. 1A), we evaluated the binding between curcumin and CDK2 in HCT116 colorectal cancer cell lysates. CDK2 was pulled down by curcumin-Sepharose 4B beads, but was not detected in Sepharose 4B beads (Fig. 1B). To confirm that curcumin binds to the ATP pocket of CDK2, various concentrations of ATP were premixed with a recombinant CDK2 protein before the addition of curcumin-Sepharose 4B or Sepharose 4B beads. The binding between CDK2-curcumin was dose-dependently reduced by ATP (Fig. 1C). The results indicate that curcumin directly binds to the ATP pocket of CDK2.

Curcumin selectively suppresses CDK2 kinase activity

The effect of curcumin on CDK2 kinase activity was evaluated using *in vitro* kinase assays. The kinase assay

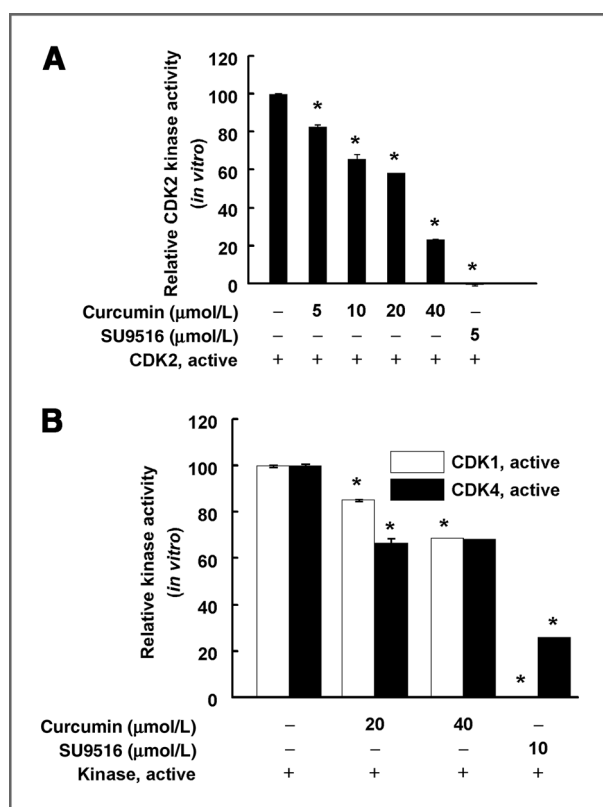


Figure 2. Curcumin effectively suppresses CDK2 kinase activity *in vitro* compared with CDK1 or 4 kinase activity. The effect of curcumin on CDK2 (A) or CDK1 and CDK4 (B) *in vitro* kinase activity was examined as described in Materials and Methods. Data are represented as mean \pm SD as determined from three independent experiments. The asterisk (*) indicates a significant ($P < 0.001$) decrease in kinase activity compared with untreated control.

data revealed that curcumin suppressed CDK2 kinase activity in a dose-dependent manner. Curcumin at 5, 10, 20, or 40 $\mu\text{mol/L}$ reduced CDK2 kinase activity by 17.5%, 34.3%, 58.2%, or 77.0%, respectively (Fig. 2A). SU9516, a well-known CDK2 inhibitor, also decreased CDK2 kinase activity (positive control). To investigate the effect of curcumin on CDK2 in HCT116 colorectal cancer cell lysates, CDK2 kinase was immunoprecipitated using a specific CDK2 antibody. Curcumin showed a similar inhibitory effect on immunoprecipitated CDK2 kinase activity (Supplementary Fig. S1). In addition, the effect of curcumin on CDK1 and CDK4 kinase activity was measured. The inhibitory effect of curcumin on CDK1 and CDK4 kinase activity was less than the effect on CDK2. Even curcumin at 40 $\mu\text{mol/L}$ did not decrease activity below 60% (Fig. 2B). Taken together, curcumin strongly affects CDK2 kinase activity compared with CDK1 or CDK4 kinase activity.

Curcumin decreases proliferation of colon cancer cells

CDK2 is an important protein in the cell cycle. The cyclin E/Cdk2 complexes induce mammalian cells to enter the S-phase by phosphorylating specific substrates such as Rb.

Thus, many chemotherapeutic and chemopreventive agents have been developed targeting CDK2 (38). In particular, cyclin E-dependent kinase activity and the kinase activity and expression level of the CDK2 protein are substantially higher in colorectal cancers and in 90% of colorectal adenomas (38). Here, we investigated the endogenous expression level of CDK2 in colorectal cancer cells (HCT116, HCT15, and DLD-1) and "normal" (see footnote on the first page) HCECs. Our results (Fig. 3A) show that the expression level of CDK2 is much higher in colon cancer cells, including HCT116, HCT15, and DLD-1 cells, compared with HCECs. Thus, we hypothesized that curcumin could suppress proliferation of colon cancer cells by inhibiting CDK2 kinase activity. The effect of curcumin was evaluated on the three colon cancer cell lines and results indicated that curcumin substantially reduced proliferation of HCT116 (Fig. 3B), HCT15 (Fig. 3C), and DLD-1 (Fig. 3D) colon cancer cells. Curcumin did not exhibit any significant effects on the proliferation or viability of "normal" (see footnote on the first page) HCECs (data not shown).

Curcumin induces G₁ cell-cycle arrest and reduces phosphorylation of Rb in HCT116 colorectal cancer cells

Because curcumin inhibited CDK2 kinase activity and decreased proliferation of colon cancer cells, we hypothesized that curcumin might affect the G₁ cell-cycle phase, which is regulated by CDK2. Curcumin induced G₁ cell-cycle arrest in a dose-dependent manner (Fig. 4A). The well-known CDK2 inhibitor, SU9516, was used as a positive control. In addition, phosphorylation of Rb was reduced by curcumin (Fig. 4B). Because the expression levels of CDK2, CDK4, and cyclin E were not changed by curcumin, the effect of curcumin on Rb is likely due to the inhibitory effect of curcumin on CDK2 kinase activity.

The effect of curcumin is reduced in HCT116-sh-CDK2 cells

Many proteins have been reported as a target of curcumin. However, no reports demonstrate that CDK2 is a direct protein target of curcumin. To confirm that CDK2 is a direct target of curcumin, we knocked down the expression of CDK2 in HCT116 colon cancer cells. Cells expressing sh-CDK2 HCT116 cells showed a greater number of cells in G₁-phase compared with sh-Mock cells (Fig. 5A). However, curcumin had less effect on inducing cell-cycle arrest in sh-CDK2 cells compared with sh-Mock (Fig. 5B). sh-CDK2 HCT116 cells also exhibited a greater reduction in proliferation (Fig. 6A) and in addition, curcumin had less effect on proliferation in these cells (Fig. 6B). Next, the phosphorylation of Rb, a substrate of CDK2, was compared between sh-Mock HCT116 and sh-CDK2 HCT116 cells. Curcumin could suppress Rb phosphorylation in cells expressing sh-Mock, whereas the effect was much less in sh-CDK2 cells (Fig. 6C). Taken together, we suggest that CDK2 is involved in the antiproliferative effects of curcumin in colon cancer cells.

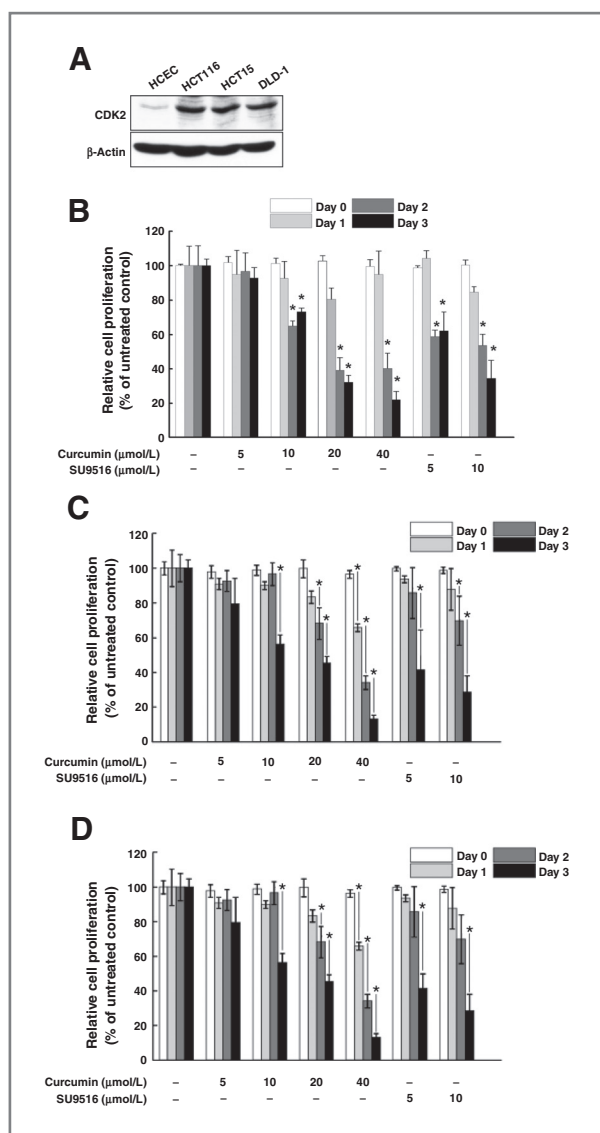


Figure 3. CDK2 is highly expressed in colon cancer cells and curcumin selectively suppresses HCT116 cell proliferation. A, the CDK2 expression level is much higher in colon cancer cells (HCT116, HCT15, and DLD-1) compared with "normal" (see footnote on the first page) colon cells (HCEC). Cells were harvested and CDK2 expression was visualized using a specific antibody. Curcumin reduces proliferation of HCT116 (B), HCT15 (C), and DLD-1 (D) colon cancer cells. Each colon cancer cell line was seeded (1×10^3 cells/well) in 96-well plates and then cells were treated with curcumin (0, 5, 10, 20, or 40 $\mu\text{mol/L}$) at 6 hours after seeding. Cell growth was determined at 1, 2, or 3 days using the MTS assay as described in Materials and Methods. Data are represented as the mean \pm SD as determined from three independent experiments. The asterisk (*) indicates a significant ($P < 0.001$) decrease in activity compared with untreated control.

Discussion

Curcumin is a compound that targets multiple molecular and cellular pathways *in vitro* as well as in animal models, suggesting that curcumin is an attractive compound for chemoprevention or chemotherapy. However, the molec-

ular mechanism of curcumin activity against cancer cells is not understood. In the current study, we showed an interaction between curcumin and CDK2 through the ATP pocket of CDK2. In addition, we demonstrated an inhibitory effect of curcumin on CDK2 activity. Similar to SU9516, a known CDK2 inhibitor, curcumin also could inhibit CDK1/4 activity (Fig. 2B). However, the inhibitory effect of curcumin on CDK1 was less effective than that against CDK2. Although the inhibitory effect of curcumin on CDK4 seemed to be fairly substantial, it did not act in a dose-dependent manner. Computational docking model data consistently showed that the binding affinity of CDK2 on curcumin was stronger than its binding with CDK1 or 4 (Supplementary Fig. S2). Taken together, we considered CDK2 as a more specific molecular target of curcumin than CDK1 or 4.

On the basis of the idea that cell-cycle proteins are activated in cancer cells, we found that CDK2 expression in three colon cancer cell lines, HCT116, HCT15, and DLD-1, was much higher than in "normal" (see footnote on the first page) HCECs (Fig. 3A). Thus, the specific antiproliferative effect of curcumin on cancer cells is likely due to the

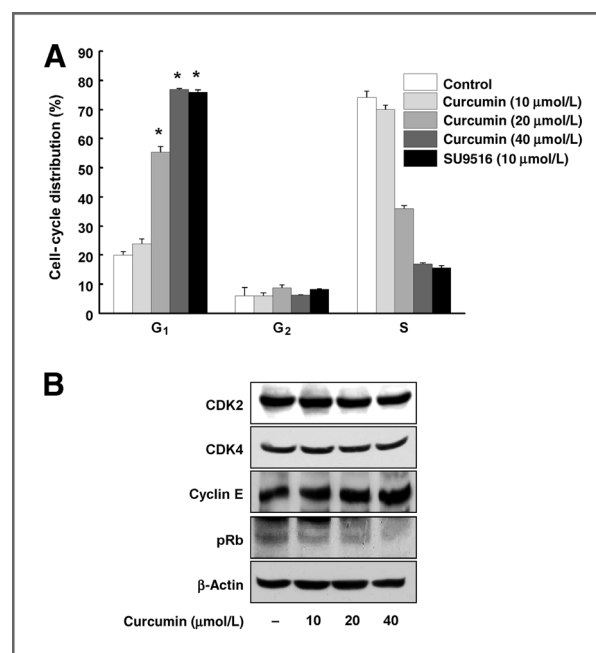


Figure 4. Curcumin induces G_1 arrest in HCT116 colon cancer cells. A, the effect of curcumin on cell cycle was investigated using flow cytometry. HCT116 colon cancer cells were cultured until they reached confluence in 96-well plates and then synchronized in G_0 -phase by serum deprivation. After 9 hours of treatment with curcumin (0, 10, 20, or 40 $\mu\text{mol/L}$) or SU9516 (10 $\mu\text{mol/L}$), cell-cycle phases were analyzed. Data are represented as mean \pm SD as determined from three independent experiments and the asterisk (*) indicates a significant difference ($P < 0.001$) compared with untreated group. B, to determine the effect of curcumin on cell-cycle proteins, Western blotting was performed using specific antibodies. After synchronizing the cells for 12 hours, cells were treated with the indicated concentration of curcumin for 30 minutes. Data are representative of three independent experiments that gave similar results.

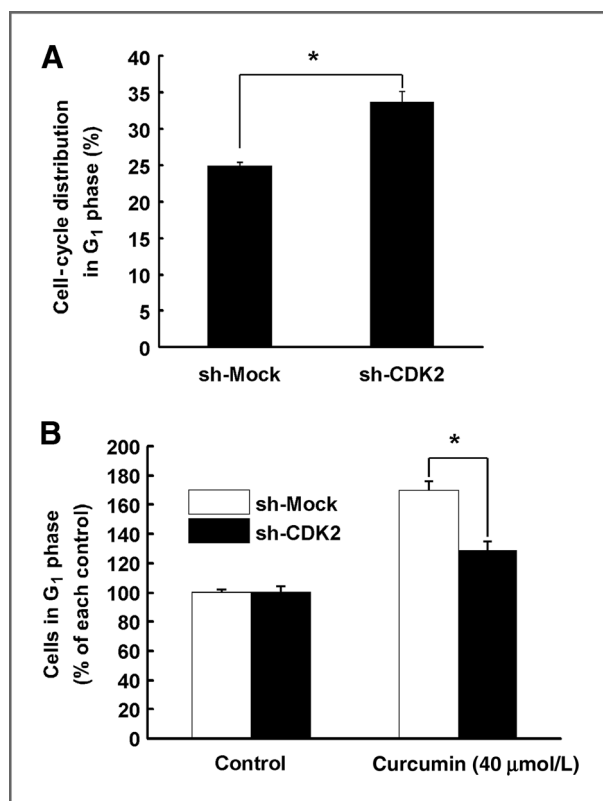


Figure 5. The effect of curcumin on cell cycle in sh-CDK2-transfected HCT116 colon cancer cells. **A**, HCT116 cells stably expressing knockdown of CDK2 were established and G₁-phase was arrested in sh-CDK2-transfected HCT116 cells. Cell cycle was analyzed using flow cytometry. **B**, the effect of curcumin on G₁-phase arrest was less in sh-CDK2-transfected HCT116 colon cancer cells compared with sh-Mock-transfected cells. After 9 hours of curcumin treatment, cell-cycle phases were analyzed. Data are shown as mean ± SD as determined from three independent experiments and the asterisk (*) indicates a significant ($P < 0.001$) difference compared with sh-Mock-transfected HCT116 cells.

high CDK2 expression level. To support this hypothesis, we used knockdown CDK2 HCT116 cells to show that curcumin could induce G₁ arrest and inhibit proliferation. The effect of curcumin was relatively lower in sh-CDK2 HCT116 cells compared with sh-Mock HCT116 cells. This suggested that the inhibitory effect of curcumin is dependent on the level of CDK2 expression. Although curcumin has a CDK2-dependent inhibitory effect on G₁ arrest and cell growth, it substantially inhibited cell growth (Fig. 6B) and phosphorylation of Rb (Fig. 6C) and induced G₁ arrest (Fig. 5B) in sh-CDK2 cells. Moreover, the effect of sh-CDK2 on G₁ arrest (Fig. 5A) is much less than that of curcumin (Fig. 4A). These results indicate that curcumin has CDK2-dependent effects as well as CDK2-independent effects on G₁-phase and cell growth of HCT116 cells. Currently, curcumin targets multiple molecular and cellular pathways in various cancers. In the present study, we did not address the CDK2-independent effects of curcumin or try to identify the molecule involved in the CDK2-independent effects of curcumin. This question remains to be investigated.

Previous studies have reported several targets of curcumin's anticarcinogenic effects, including NF-κB (39), STAT3 (40), EGF receptor (EGFR; ref. 41), phosphoinositide 3-kinase (PI3K; ref. 42), and extracellular signal-regulated kinase-1/2 (ERK1/2; ref. 43). One group suggested that curcumin inhibits human multiple myeloma cell proliferation by

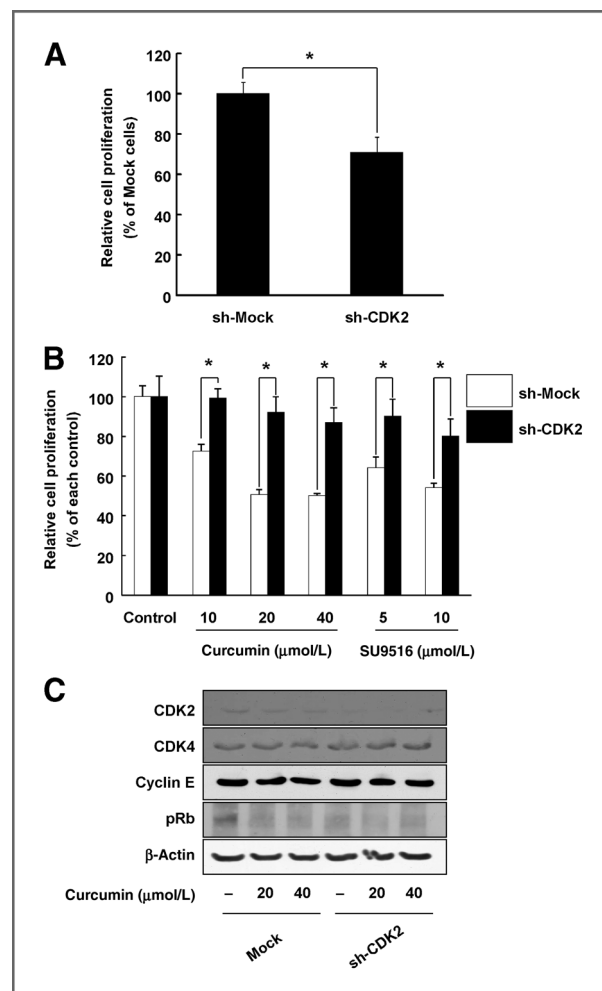


Figure 6. The effect of curcumin on proliferation and cell-cycle protein expression in sh-CDK2-transfected HCT116 colon cancer cells. **A**, proliferation was relatively lower in sh-CDK2-transfected HCT116 cells compared with sh-Mock-transfected HCT116 cells. HCT116 cells (1×10^3 cells) were seeded in 96-well plates and after 72 hours, proliferation was measured using the MTS assay as described in Materials and Methods. **B**, the effects of curcumin on cell proliferation were less in sh-CDK2-transfected HCT116 cells compared with sh-Mock-transfected cells. sh-Mock- and sh-CDK2-transfected HCT116 cells were treated with curcumin (0, 10, 20, or 40 μmol/L) and growth was determined at 3 days using the MTS assay as described in Materials and Methods. Data are represented as mean ± SD as determined from three independent experiments. For **A** and **B**, the asterisk (*) indicates a significant difference ($P < 0.001$) compared with sh-Mock-transfected HCT116 cells. **C**, curcumin had no effect on cell-cycle proteins in sh-CDK2-transfected HCT116 colon cancer cells. sh-Mock- and sh-CDK2-transfected HCT116 cells were treated with the indicated concentrations of curcumin for 30 minutes. Cell-cycle protein expression was determined using specific antibodies. Data are representative of three independent experiments that gave similar results.

interfering STAT3 phosphorylation (40). Others reported that curcumin (up to 10 $\mu\text{mol/L}$) reduced EGFR and insulin-like growth factor-I receptor (IGF-IR) phosphorylation (41). However, no one has reported a direct target of curcumin.

Here, we report CDK2 as a direct target of curcumin, which could contribute to curcumin's positive preventive or therapeutic effects against colorectal cancer. However, to verify the direct binding mode between curcumin and CDK2, point mutation studies and X-ray crystallographic studies will be needed. In addition, although *in vitro* data support an antiproliferative effect of curcumin, *in vivo* studies will be required for full confirmation of the effect of curcumin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T.-G. Lim, S.-Y. Lee, Z. Huang, K.W. Lee, Z. Dong

Development of methodology: Z. Huang, K.W. Lee, Z. Dong

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.-G. Lim, S.-Y. Lee, H. Chen, S.K. Jung, K.W. Lee, Z. Dong

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.-G. Lim, S.-Y. Lee, Z. Huang, H. Chen, A.M. Bode, K.W. Lee, Z. Dong

Writing, review, and/or revision of the manuscript: T.-G. Lim, S.-Y. Lee, Z. Huang, H. Chen, A.M. Bode, K.W. Lee, Z. Dong

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.Y. Lim, A.M. Bode, K.W. Lee, Z. Dong

Study supervision: K.W. Lee, Z. Dong

Acknowledgments

The authors thank Nicki Brickman at The Hormel Institute, University of Minnesota for assistance in submitting our article.

Grant Support

This work was supported by The Hormel Foundation, NIH grants CA027502 (to Z. Dong), CA120388 (to Z. Dong), R37 CA081064 (to Z. Dong), and NIESH ES016548 (to Z. Dong), the National Leap Research Program (No. 2010-0029233; to K.W. Lee) through the National Research Foundation, the Global Frontier Project grant (NRF-M1AXA002-2012M3A6A4054949; to K.W. Lee) of National Research Foundation funded by the Ministry of Education, Science and Technology of Korea, and WCU (World Class University) program (R31-10056; to K.W. Lee) through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 1, 2013; revised February 3, 2014; accepted February 7, 2014; published OnlineFirst February 19, 2014.

References

- Kuttan G, Kumar KB, Guruvayoorappan C, Kuttan R. Antitumor, anti-invasion, and antimetastatic effects of curcumin. *Adv Exp Med Biol* 2007;595:173-84.
- Thangapazham RL, Sharma A, Maheshwari RK. Beneficial role of curcumin in skin diseases. *Adv Exp Med Biol* 2007;595:343-57.
- Chen A, Xu J, Johnson AC. Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. *Oncogene* 2006;25:278-87.
- Shehzad A, Khan S, Shehzad O, Lee YS. Curcumin therapeutic promises and bioavailability in colorectal cancer. *Drugs Today* 2010;46:523-32.
- Ryu MJ, Cho M, Song JY, Yun YS, Choi IW, Kim DE, et al. Natural derivatives of curcumin attenuate the Wnt/beta-catenin pathway through down-regulation of the transcriptional coactivator p300. *Biochem Biophys Res Commun* 2008;377:1304-8.
- Huang MT, Smart RC, Wong CQ, Conney AH. Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res* 1988;48:5941-6.
- Huang MT, Lysz T, Ferraro T, Abidi TF, Laskin JD, Conney AH. Inhibitory effects of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Res* 1991;51:813-9.
- Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* 2001;21:2895-900.
- Carroll RE, Benya RV, Turgeon DK, Vareed S, Neuman M, Rodriguez L, et al. Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. *Cancer Prev Res* 2011;4:354-64.
- Lee YK, Park SY, Kim YM, Park OJ. Regulatory effect of the AMPK-COX-2 signaling pathway in curcumin-induced apoptosis in HT-29 colon cancer cells. *Ann N Y Acad Sci* 2009;1171:489-94.
- Collett GP, Campbell FC. Curcumin induces c-jun N-terminal kinase-dependent apoptosis in HCT116 human colon cancer cells. *Carcinogenesis* 2004;25:2183-9.
- Kim KC, Lee C. Curcumin induces downregulation of E2F4 expression and apoptotic cell death in HCT116 human colon cancer cells; involvement of reactive oxygen species. *Korean J Physiol Pharmacol* 2010;14:391-7.
- Mukhopadhyay A, Banerjee S, Stafford LJ, Xia C, Liu M, Aggarwal BB. Curcumin-induced suppression of cell proliferation correlates with down-regulation of cyclin D1 expression and CDK4-mediated retinoblastoma protein phosphorylation. *Oncogene* 2002;21:8852-61.
- Nguyen-Ba G, Vasseur P. Epigenetic events during the process of cell transformation induced by carcinogens (review). *Oncol Rep* 1999;6:925-32.
- Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif* 2003;36:131-49.
- Nurse P, Masui Y, Hartwell L. Understanding the cell cycle. *Nat Med* 1998;4:1103-6.
- Hartwell LH, Kastan MB. Cell cycle control and cancer. *Science* 1994;266:1821-8.
- Pines J. Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem J* 1995;308(Pt 3):697-711.
- Sherr CJ. G₁ phase progression: cycling on cue. *Cell* 1994;79:551-5.
- Dulic V, Lees E, Reed SI. Association of human cyclin E with a periodic G₁-S phase protein kinase. *Science* 1992;257:1958-61.
- Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G₁-to-S phase transition. *Mol Cell Biol* 1995;15:2612-24.
- King RW, Jackson PK, Kirschner MW. Mitosis in transition. *Cell* 1994;79:563-71.
- Arellano M, Moreno S. Regulation of CDK/cyclin complexes during the cell cycle. *Int J Biochem Cell Biol* 1997;29:559-73.
- Tubiana M. [Kinetics of cell proliferation and cancer: introduction (author's transl)]. *Bull Cancer* 1978;65:407-15.
- Cam WR, Masaki T, Shiratori TY, Kato N, Okamoto M, Yamaji Y, et al. Activation of cyclin E-dependent kinase activity in colorectal cancer. *Dig Dis Sci* 2001;46:2187-98.
- Phase version 3.3. New York: Schrödinger, LLC; 2011.
- Dixon SL, Smondryev AM, Knoll EH, Rao SN, Shaw DE, Friesner RA. PHASE: a new engine for pharmacophore perception, 3D QSAR model development, and 3D database screening: 1. Methodology and preliminary results. *J Comput Aided Mol Des* 2006;20:647-71.
- Schrödinger Suite Schrödinger, LLC; 2011.
- Confgen version 2.3. New York: Schrödinger, LLC; 2011.
- Maestro version 9.2. New York: Schrödinger, LLC; 2011.

31. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. *Nucleic acids research* 2000;28:235–42.
32. Glide version 5.7. New York: Schrödinger, LLC; 2011.
33. Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* 2004;47:1739–49.
34. Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, et al. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J Med Chem* 2006;49:6177–96.
35. Schrödinger Suite 2011 induced fit docking protocol; Glide version 5.7, Schrödinger, LLC, New York, NY, 2011; Prime version 3.0. New York: Schrödinger, LLC; 2011.
36. Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst* 1997;89:1881–6.
37. Sherman W, Day T, Jacobson MP, Friesner RA, Farid R. Novel procedure for modeling ligand/receptor induced fit effects. *J Med Chem* 2006;49:534–53.
38. Liu H, Liu K, Huang Z, Park CM, Thimmegowda NR, Jang JH, et al. A chrysin derivative suppresses skin cancer growth by inhibiting cyclin-dependent kinases. *J Biol Chem* 2013;288:25924–37.
39. Singh S, Aggarwal BB. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. *J Biol Chem* 1995;270:24995–5000.
40. Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. *J Immunol* 2003;171:3863–71.
41. Reddy S, Rishi AK, Xu H, Levi E, Sarkar FH, Majumdar AP. Mechanisms of curcumin- and EGF-receptor related protein (ERRP)-dependent growth inhibition of colon cancer cells. *Nutr Cancer* 2006;55:185–94.
42. Squires MS, Hudson EA, Howells L, Sale S, Houghton CE, Jones JL, et al. Relevance of mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/protein kinase B (PI3K/PKB) pathways to induction of apoptosis by curcumin in breast cells. *Biochem Pharmacol* 2003;65:361–76.
43. Lev-Ari S, Vexler A, Starr A, Ashkenazy-Voghera M, Greif J, Aderka D, et al. Curcumin augments gemcitabine cytotoxic effect on pancreatic adenocarcinoma cell lines. *Cancer Invest* 2007;25:411–8.