Caffeic acid phenethyl ester induces growth arrest and apoptosis of colon cancer cells via the β-catenin/T-cell factor signaling

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Caffeic acid phenethyl ester, an active component of propolis, has been implicated in the regulation of cell growth and apoptosis, although the exact mechanism of this activity has not been elucidated. In this study, we explored the effects of caffeic acid phenethyl ester on growth, cell cycle, apoptosis and β-catenin/T-cell factor signaling in human colon cancer cells. Using two human sporadic colon cancer cell lines (HCT116 and SW480), we assayed for cell growth inhibition, cell cycle and apoptosis induction. We also assayed for β-catenin and downstream target genes (cyclin D1 and c-*myc*) mRNA and protein expression by reverse transcriptase-polymerase chain reaction and Western blot analysis. β-Catenin localization was detected by indirect immunofluorescence. β-Catenin/T-cell factor transcriptional activity was determined by transient transfection and reporter gene assay. Caffeic acid phenethyl ester completely inhibited growth, and induced G_1 phase arrest and apoptosis in a dose-dependent manner in both HCT116 and SW480 cells. Treatment of human colon cancer cells with apoptotic concentrations of caffeic acid phenethyl ester resulted in a dose-dependent and time-dependent loss of total β-Catenin protein, associated with decreased nuclear β-catenin. Caffeic acid phenethyl ester reduced the expression of cyclin D1 and c-*myc* in a dose-dependent and time-dependent manner. We proved that caffeic acid phenethyl ester markedly suppressed the transcriptional activity of β-catenin/T-cell factor in both HCT116 and SW480 cells depending on the concentration of caffeic acid phenethyl ester. These results indicate that caffeic acid phenethyl ester is an excellent inhibitor of β-catenin/T-cell factor signaling in colon cancer cell lines and suggest that caffeic acid phenethyl ester merits further study as an agent against colorectal cancers. *Anti-Cancer Drugs* 17:753–762 © 2006 Lippincott Williams & Wilkins.

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Introduction

β-Catenin performs dual functions, which include a crucial role in cell–cell adhesion and the Wnt/*Wingless* signaling pathway [1]. Originally identified as a cytoplasmic protein that interacts with cell adhesion molecules, such as E-cadherin, β-catenin was found to be the mammalian homologue of *armadillo*, a segment polarity gene involved in the *Wingless* pathway in *Drosophila* [2]. Its localization in two subcellular compartments corresponds well to these two functions. Several cellular proteins have been identified as directly or indirectly interacting with β-catenin [1,3]. In normal and unstimulated cells, the majority of β-catenin protein is present in cell–cell junctions with very little in cytoplasmic or nuclear fractions, due to the rapid turnover of β-catenin promoted by the adenomatous polyposis coli (APC)/glycogen synthase kinase-3β (GSK-3β)/Axin complex. In the presence of Wnt signal, however, GSK-3β activity is inactivated, leading to the accumulation of cytoplasmic and, subsequently, nuclear β-catenin, and the activation of β-catenin/T-cell factor (Tcf) downstream target genes, such as c-*myc*, cyclin D1, fibroblast growth factor 18 and epidermal growth factor receptor [4–8].

Activated β-catenin/Tcf signaling by accumulation of β-catenin in the nucleus has been implicated in human carcinogenesis. This accumulation may result from the mutation of either the β-catenin gene (CTNNB1) itself or the tumor-suppressor gene, APC. Actually, the APC gene or serine–threonine phosphorylation sites for the GSK-3β within exon 3 of the β-catenin gene are mutated in many cancer cells including colorectal cancer (CRC), melanoma, hepatocellular carcinoma and gastric carcinoma, and the transcriptional activity of β-catenin is

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upregulated in these cancer cells [9–11]. At least 60% of sporadic CRC contain one APC mutation and almost half of them show abnormalities in both APC alleles, and approximately half of the sporadic CRC and CRC cell lines lacking APC mutations were shown to possess somatic mutations in the β-catenin gene [12–14]. Moreover, nuclear translocation of β-catenin in CRC is significantly associated with tumor progression and poor survival [15,16]. Therefore, control of β-catenin and/or control of downstream Tcf target gene expression represents an ideal target for anticancer therapeutics and chemoprevention [17–19]. There exist, however, few β-catenin inhibitors.

Caffeic acid phenethyl ester (CAPE) is an active component of propolis obtained from honeybee hives. It has antioxidant, anti-inflammatory, anticarcinogenic and immunomodulatory activities in diverse systems. Several investigators have demonstrated that CAPE has an antiproliferative effect, an apoptosis-inducing effect against various tumor cell lines [20–25], and it also reduce CRC cells invasion and metastasis *in vitro* and *in vivo* [26–28]. In addition, CAPE inhibits the formation of aberrant crypts induced by azoxymethane *in vivo*, which are relevant to colon carcinogenesis [29]. Dietary intake of CAPE also decreases tumor formation and expression of β-catenin protein in the enterocytes of the Min/+ mouse, which suggests that CAPE can suppress APC-associated intestinal carcinogenesis [30]. Therefore, we investigated the effects of CAPE on growth, cell cycle, apoptosis and β-catenin/Tcf signaling in HCT116 (mutant CTNNB1, wild-type APC) and SW480 (mutant APC, wild-type CTNNB1) colon cancer cells.

Materials and methods

Chemicals and cell culture

CAPE, dimethyl sulfoxide (DMSO), propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St Louis, Missouri, USA). The human sporadic colon cancer cell lines HCT116 and SW480 were purchased from the American Type Culture Collection (Manassas, Virginia, USA). The cells were cultured in RPMI 1640 medium supplemented with penicillin G (100 U/ml), streptomycin (100 U/ml) and 10% fetal calf serum at 37°C in a 5% CO_2 humidified atmosphere. Cells were routinely subcultured using 0.25% (w/v) trypsin/ethylene diaminetetraacetic acid solution. CAPE was prepared as 80 g/l stock solutions in DMSO. Control flasks or plates contained DMSO at an equivalent dilution (0.1% v/v) to that in cultures containing CAPE, which had not effect on cells.

Cell growth inhibition assay

Logarithmically growing HCT116 and SW480 cells were plated at a density of 4000 cells/well into a 96-well plate. After 24 h, cells were treated with CAPE at various concentrations (0, 2.5, 5, 10, 20, 40 and 80 μg/ml) for 24, 48, 72 or 96 h. Then, 20 μl MTT (5 g/l) was added to each well and incubated for an additional 4 h, and then culture media were discarded followed by addition of 0.15 ml DMSO and vibration for 10 min. The absorbance was measured at 490 nm using a model 550 microplate reader. Six replicate experiments were performed for each cell line. Percent absorbance relative to control was plotted as a linear function of drug concentration. The 50% inhibitory concentration (IC_{50}) was identified as the concentration of drug required to achieve 50% growth inhibition relative to untreated control populations. IC_{50} value was determined using a CalcuSyn software [31].

Cell cycle analysis by flow cytometry

HCT116 and SW480 cells were serum-starved for 24 h and then treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml) for 24 h. At the end of the treatment, the floating cells were collected by centrifugation, whereas adherent cells were harvested by trypsin–ethylene diaminetetraacetic acid solution to produce a single-cell suspension. The cells were then pelleted by centrifugation and washed twice with phosphate-buffered saline (PBS). Then the cell pellets were suspended in 0.5 ml PBS and fixed in 5 ml ice-cold 70% ethanol at 4°C. The fixed cells were spun by centrifugation and the pellets were washed with PBS. After resuspension with 1 ml PBS, the cells were incubated with RNase A (20 μg/ml) and PI (50 μg/ml) for 1 h at 37°C in the dark. The stained cells were analyzed using a FACSCalibur flow cytometer in combination with Modefit software (Becton Dickinson, Franklin Lakes, New Jersey, USA). Three replicate experiments were performed for each cell line.

Flow cytometry analysis with the Annexin-V/propidium iodide assay

HCT116 and SW480 cells were treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml) for 24 h. The apoptotic index was assessed by flow cytometry using Annexin-V–FITC kit (Roche, Indianapolis, USA) following the manufacturer's instructions. Data acquisition and analysis were performed in a FACSort cytometer (Becton Dickinson) using CellQuest Software (Franklin Lakes, New Jersey, USA). For each analysis, 20 000 events were acquired on a forward and side scatter gate. Three replicate experiments were performed for each cell line.

Reverse transcriptase-polymerase chain reaction

HCT116 and SW480 cells were treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml) for 24 or 48 h. Total RNA was prepared from HCT116 and SW480 cells with Tripure RNA Purification Kit (Roche) according to the manufacturer's instructions. Reverse transcription (RT) was performed with Moloney-murine leukemia virus reverse transcriptase (Promega, San Luis Obispo, California, USA) according to the manufacturer's protocol. To amplify 226-bp β-catenin, 354-bp cyclin D1, 219-bp c-*myc*

and 667-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments, the sequences of PCR primers (Sangon, Shanghai, China) were as follows: for β-catenin sense (5′-ATTTGATGGAGTTGGACATGGC-3′), antisense (5′-CCAGCTACTTGTTCTTGAGTGAA GG-3′); for cyclin D1 sense (5′-TCTAAGATGAAGGAGA CCATC-3′), antisense (5′-GCGGTAGTAGGACAGGAA GTTGTT-3′); for c-*myc* sense (5′-CAAGAGGCGAACAC ACAACGTCT-3′), antisense (5′-AACTGTTCTCGTCG TTTCCGCAA-3′) and for GAPDH sense (5′-CCCATCA CCATCTTCCAGGAGCG-3′), antisense (5′-AGATGGA GGAGTGGGTGTCGCTGT-3′). The samples were first denatured at 94°C for 2 min, followed by 28 polymerase chain reaction (PCR) cycles, each with temperature variations as follows: 94°C for 60 s, 54°C–62°C (54°C for β-catenin, 59°C for c-*myc*, 60°C for GAPDH and 62°C for cyclin D1) for 50 s and 72°C for 50 s. The last cycle was followed by an additional extension incubation of 10 min at 72°C. Analysis of amplification was accomplished on 1.5% agrose gel containing 0.2 μg/μl ethidium bromide and visualized under UV transilluminator. The densitometric analysis of PCR products was performed by computer software using a GS-800 Imaging Densitometer (Bio-Rad, Hercules, California, USA) and standardized to GAPDH product. Three replicate experiments were performed.

Western blot analysis

HCT116 and SW480 cells were treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml) for 24 or 48 h. In the end, the attached cells and floating cells were extracted in lysis buffer using standard methods. Western blot was carried out using standard techniques. Briefly, equivalent protein concentrations of 50 μg in each sample were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins transferred onto polyvinylidine diflouride membranes. After blocking with 5% nonfat dried milk, the membranes were incubated with the appropriate dilution of primary mouse monoclonal antibodies against human β-catenin, cyclin D1, c-*myc* and β-actin. The β-catenin antibody (Sigma) was used at a 1:1000 dilution. The cyclin D1 antibody was used at a 1:500 dilution (Santa Cruz, California, USA). The c-*myc* antibody (Santa Cruz) was used at a 1:500 dilution. The β-actin antibody (Sigma) was used at a 1:1000 dilution. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) (Pierce, Rockford, Illinois, USA). The proteins were detected by an enhanced chemiluminescence detection system (Pierce) and light emission was captured on Kodak X-ray films. Three replicate experiments were performed.

Indirect immunofluorescence for β-catenin localization

Cells grown on glass coverslips were treated for 24 or 48 h with a range of CAPE (0, 2.5, 5 and 10 μg/ml), under standard culture conditions as described above. Cells were fixed in 100% methanol at −20°C for 10 min and washed twice in PBS. Monolayers were incubated with β-catenin antibody (Sigma) in PBS plus 1% (w/v) dried skimmed milk powder overnight at 4°C. Omission of the respective primary antibody was used as a negative control. Monolayers were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Pierce) in 1% dried skimmed milk in PBS for 30 min at 37°C. Confocal microscopy was performed using a Leica TCS SP laser scanning confocal microscope. Three replicate experiments were performed.

Transfection and reporter gene assay

To evaluate the Tcf/Lef transcriptional activity, we used a pair of luciferase reporter constructs, TOPflash and FOPflash (Upstate Biotechnology, Lake Placid, New York, USA). TOPflash contains three copies of the Tcf/Lef binding site upstream of the thymidine kinase minimal promoter and FOPflash contains a mutated Tcf/Lef binding site. pRL-TK luciferase reporter gene plasmid (Promega) was always cotransfected to normalize for transfection efficiency. Transient transfection was performed using Fugene 6 (Roche) according to the manufacturer's instruction. Cells were transiently transfected by one of these luciferase reporters and pRL-TK. Three hours after transfection, cells were treated with different concentrations of CAPE (0, 2.5, 5 or 10 μg/ml) for 24 or 48 h. Luciferase activity was measured with the Dual luciferase reporter assay system (Promega). Three replicate experiments were performed.

Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical significance of differences between two groups was determined using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

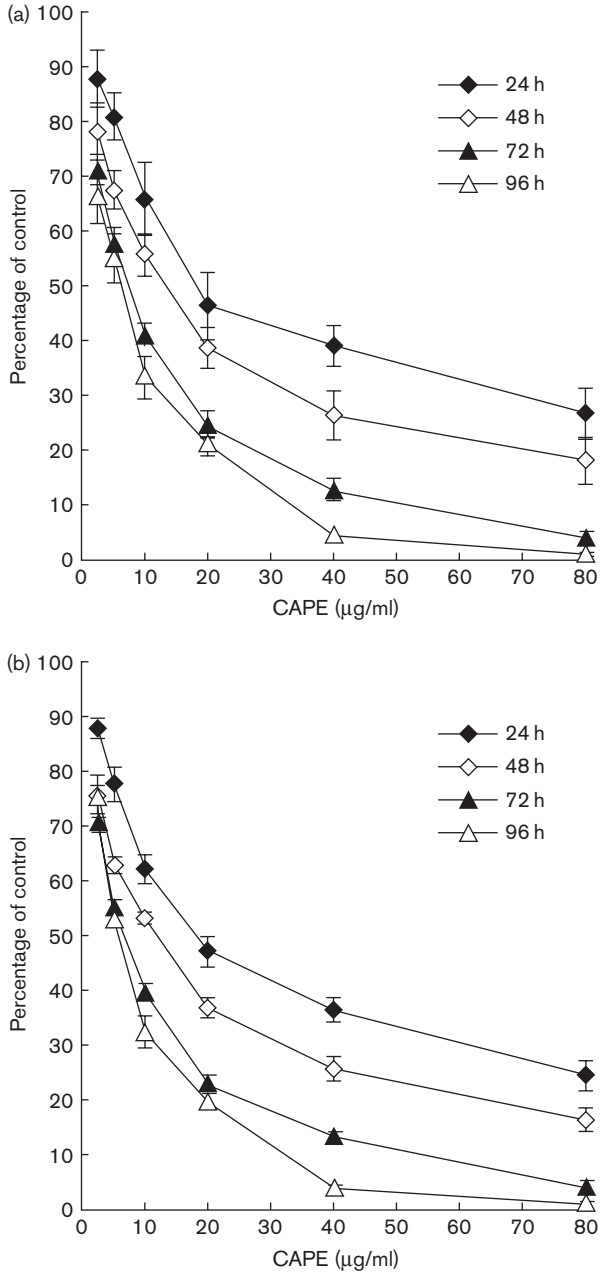
Caffeic acid phenethyl ester inhibits human colon cancer cells growth *in vitro*

HCT116 and SW480 cells were treated with CAPE at various concentrations for 24–96 h, and the cell viability was determined as described above by MTT assay. As shown in Fig. 1, CAPE inhibited the growth of both HCT116 and SW480 cells in a dose-dependent and time-dependent manner. IC_{50} values for HCT116 cells at 24, 48, 72, 96 h after CAPE treatment were 22.45, 12.07, 6.47 and 5.36 μg/ml, respectively. IC_{50} values for SW480 cells at 24, 48, 72, 96 h after CAPE treatment were 20.27, 10.38, 6.15, and 5.44 μg/ml, respectively.

Effect of caffeic acid phenethyl ester on apoptosis induction of human colon cancer cells

We explored whether induction of apoptosis by CAPE was a mechanism for the inhibitory effect of CAPE. To determine the occurrence of apoptosis in cells treated with CAPE, we stained the cells with both Annexin-V–FITC

Fig. 1



Dose-dependent and time-dependent growth inhibition of human colon cancer cells by caffeic acid phenethyl ester (CAPE). HCT116 (a) and SW480 (b) cells were treated with CAPE at various concentrations (0, 2.5, 5, 10, 20, 40 and 80 μg/ml) and cell viability was determined by 3-(4, 5-dimethylthiazol-2-y[1])-2, 5-diphenyltetrazolium bromide assay at the indicated time period. The values are expressed as the mean ± standard deviation from six independent experiments.

and PI and performed flow cytometry. After treatment with CAPE for 24 h, the Annexin-positive and PI-negative cell population (apoptotic cell population) clearly increased in HCT116 and SW480 cells. Moreover, CAPE induced apoptosis in a dose-dependent manner in HCT116 and SW480 cells (Fig. 2).

Caffeic acid phenethyl ester arrests human colon cancer cells in G_0/G_1 cell cycle phase

To study the effect of CAPE treatment on cell populations in different phases of the cell cycle, we treated exponentially growing cells with 2.5, 5 or 10 μg/ml of CAPE for 24 h. Using conventional DNA flow cytometry, we determined the effect of CAPE on the distribution of cells in various phases of the cell cycle. In untreated HCT116 and SW480 cells, 40–46% of cells were in the G_0/G_1 phase, 45–49% were in S phase and the remaining cells were in the G_2/M phase. In HCT116 and SW480 cells, CAPE treatment, however, resulted in an accumulation of cells in the G_0/G_1 phase, and these results were dose-dependent and were paralleled by a decrease of cells in S phase (Fig. 3). Interestingly, CAPE treatment resulted in an increase of the percentage of G_2/M phase in SW480 cells, especially with 5 μg/ml of CAPE (Fig. 3).

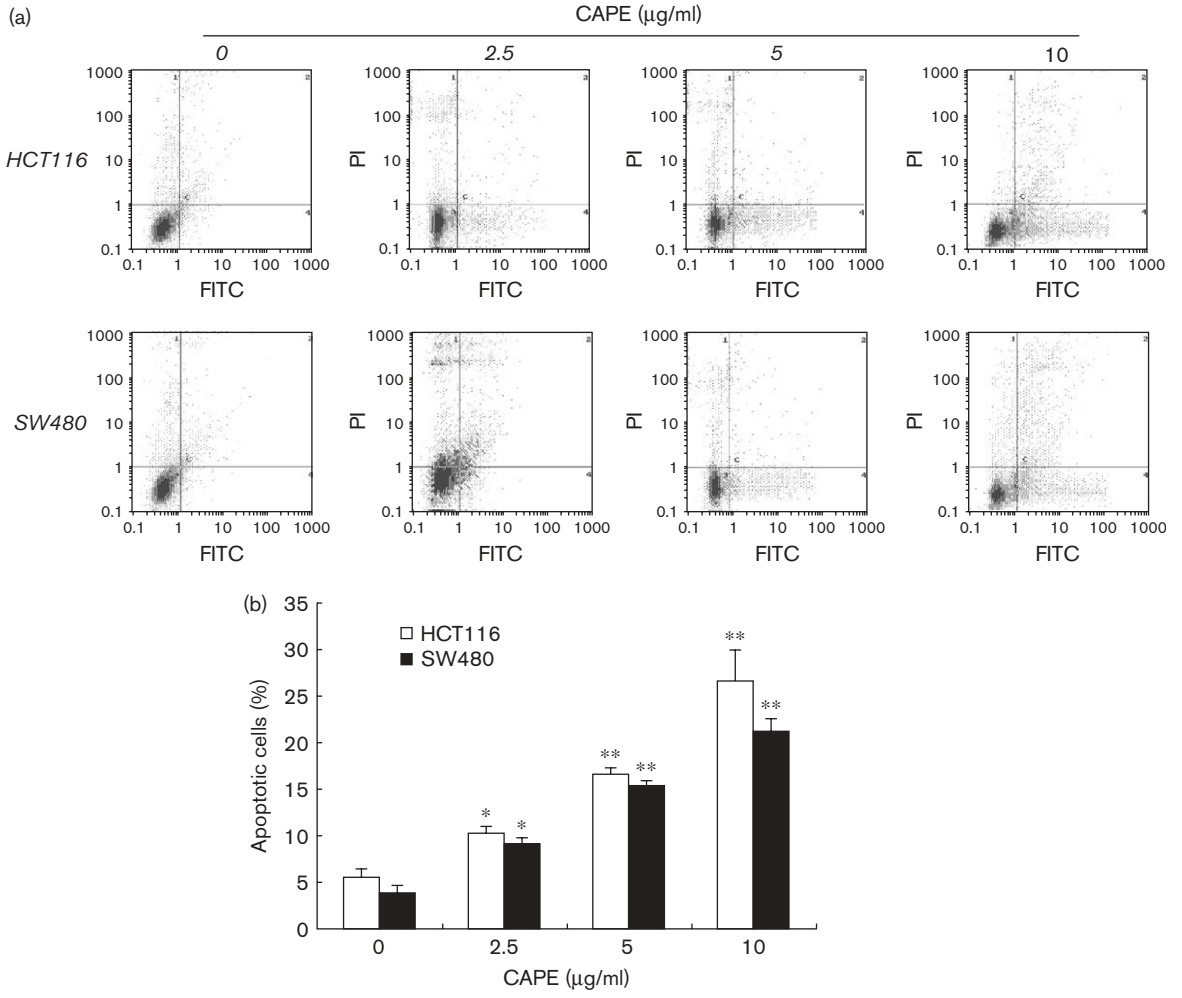
β-Catenin, cyclin D1 and c-*myc* expression during caffeic acid phenethyl ester treatment

β-Catenin is involved in both colon carcinogenesis and target genes (cyclin D1 and c-*myc*) transcriptional regulation. Therefore, we investigated whether CAPE-induced G_1 arrest and apoptosis were associated with changes in protein and mRNA expression of β-catenin, c-*myc* and cyclin D1 by Western blot and RT-PCR analysis. HCT116 and SW480 cells were treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml). After 24 and 48 h, Western blot analysis showed that CAPE treatment was associated with a dose-dependent and time-dependent decrease in β-catenin protein expression in both HCT116 and SW480 cells (Fig. 4a). After 24 h, CAPE treatment was not associated with a decrease in β-catenin mRNA expression in both HCT116 and SW480 cells. After 48 h, CAPE treatment was associated with a slight decrease in β-catenin mRNA expression in both HCT116 and SW480 cells (Fig. 4b), which was not paralleled by a decrease in the β-catenin protein expression. After 24 and 48 h, CAPE treatment was associated with a dose-dependent and time-dependent decrease in cyclin D1 and c-*myc* protein (Fig. 4a) and mRNA (Fig. 4b) expression in both HCT116 and SW480 cells.

Effect of caffeic acid phenethyl ester on β-catenin localization in human colon cancer cells

Translocation of β-catenin into the nucleus is required for its transcriptional activity as a coactivator [3,32]. As β-catenin exists both in the cytosol and in the nucleus, we explored the possibility that CAPE may alter its distribution between the nucleus and the cytosol, thereby inhibiting target genes (cyclin D1 and c-*myc*) transcriptional activity. HCT116 and SW480 cells were treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml) for 24 or 48 h, and β-catenin was detected by immunofluorescence. Figure 5 shows strong nuclear expression of β-catenin in control HCT116 and SW480 cells. CAPE

Fig. 2



Caffeic acid phenethyl ester (CAPE) promotes apoptosis in the human colon cancer cells. HCT116 and SW480 cells were treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml) for 24 h, and the cells were stained with Annexin-V (*x*-axis) and propidium iodide (PI) (*y*-axis) to quantify apoptosis and necrosis, respectively. Early apoptotic cells were localized in the lower right quadrant of a dot-plot graph using Annexin-V–fluorescein isothiocyanate versus PI (a). (b) Bar graphs represent the mean values of triplicate determinations ± standard deviation. **P* < 0.05 versus vehicle; ***P* < 0.01 versus vehicle.

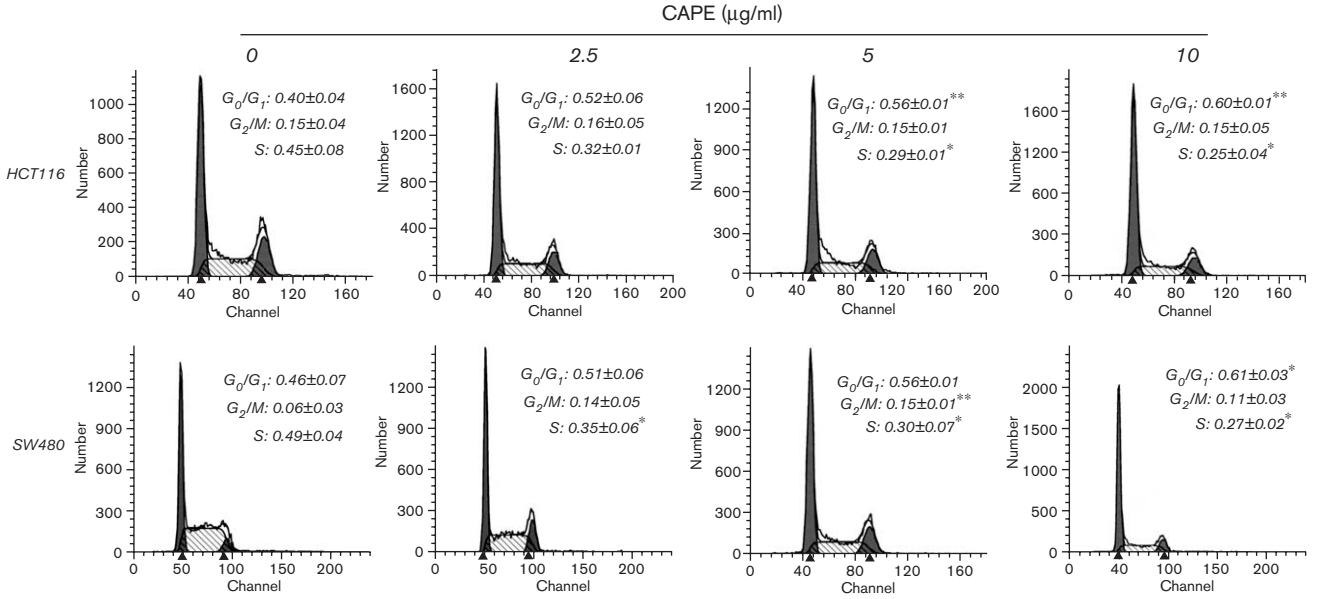
treatment was associated with decreased nuclear β-catenin expression and a concurrent increase in β-catenin protein expression at cell–cell contacts, especially after HCT116 cells were treated with higher concentrations of CAPE (10 μg/ml) for 48 h (Fig. 5).

Caffeic acid phenethyl ester downregulates β-catenin/T-cell factor transcriptional activity

We were then interested to determine the effect of CAPE on β-catenin/Tcf transcriptional activity in human colon cancer cells. HCT116 and SW480 cell lines have a constitutively active transcriptional activity of β-catenin/Tcf. Cells were transiently transfected with a synthetic Tcf reporter plasmid TOPflash (which consists of three Tcf-binding sites upstream of a minimal thymidine kinase promoter and the luciferase open reading frame), prior to treatment with CAPE.

In order to exclude nonspecific effects of CAPE on gene expression, TOPflash activity in CAPE-treated cells was corrected for any drug-induced effects on FOPflash (which is identical to TOPflash except that it contains mutant inactive Tcf-binding sites). Figure 6 shows that CAPE suppressed the Tcf transcriptional activity in HCT116 and SW480 cell lines in a dose-dependent and time-dependent manner. Within 48 h of treatment, 10 μg/ml concentration of CAPE reduced β-catenin/Tcf transcriptional activity of HCT116 and SW480 cells by 63 ± 7 and 57 ± 6% compared with the corresponding control, respectively, whereas CAPE treatment was associated

Fig. 3



Caffeic acid phenethyl ester (CAPE) induces cell cycle arrest in the G_0/G_1 cell cycle phase in human colon cancer cells. HCT116 and SW480 cells were treated with a range of CAPE (0, 2.5, 5 and 10 μg/ml) for 24 h. Cell cycle analysis was performed after propidium iodide staining of ethanol-permeabilized cells. The percentages of cells within the G_0/G_1 phase, the S phase and the G_2/M phase are indicated for each cell in the figure. Values represent mean percentages ± standard deviation of three separate experiments. $*P<0.05$ versus vehicle; $**P<0.01$ versus vehicle.

with numerically small, variable effects on FOPflash activity.

Discussion

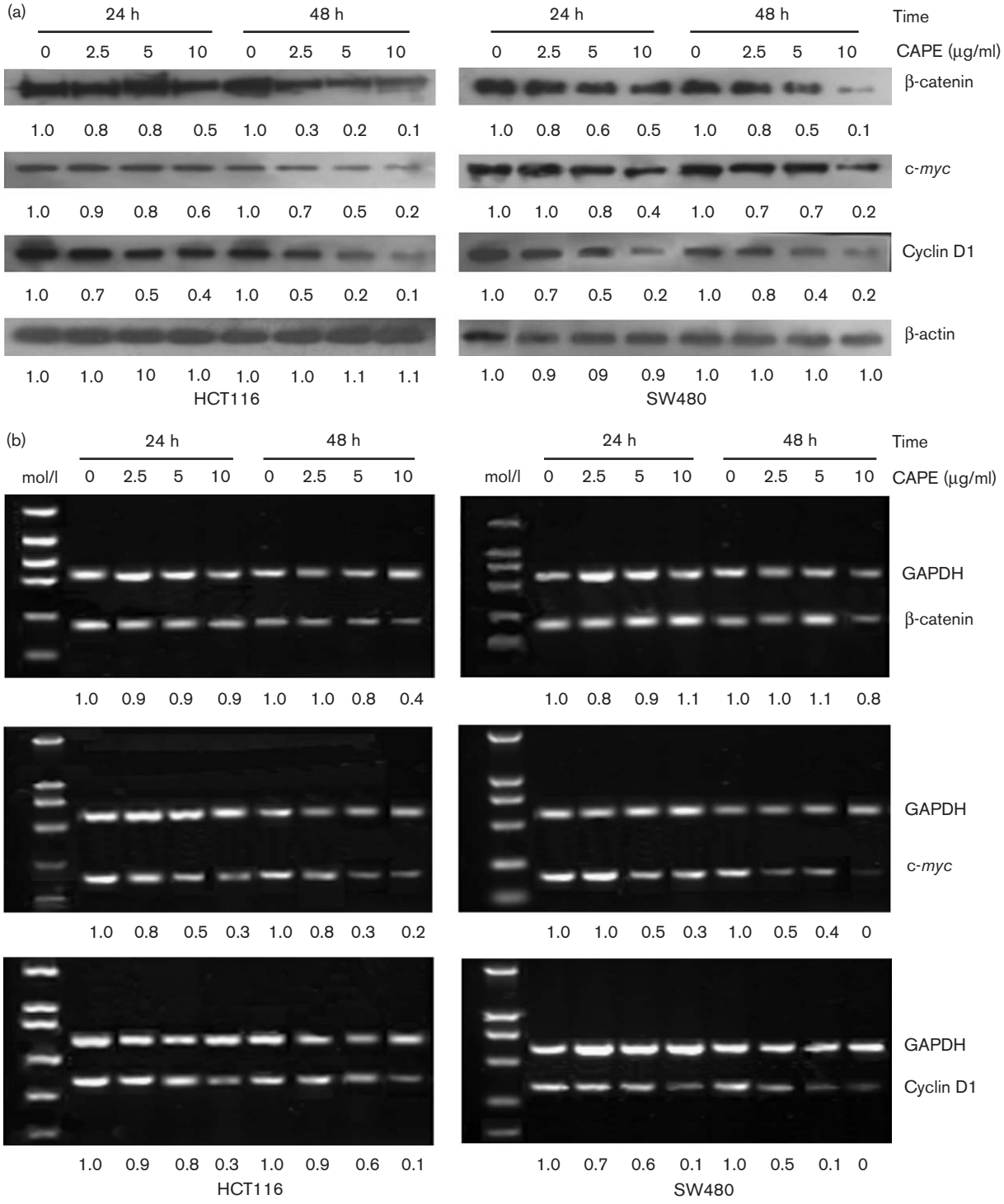
Epidemiological studies of CRC incidence suggest that the development of this disease can be modulated by dietary factors, with a high intake of fruits and vegetables providing a protective effect. Many diet-derived substances, such as plant polyphenolic compounds, calcium, antioxidant vitamins and ω-3 fatty acids have demonstrated significant efficacy in tumor prevention. CAPE is also a phenolic compound and an active component of honeybee propolis [33,34]. It has previously been shown to have anticancer activities in cell culture of both hematological and solid tumors [20–25]. CAPE has also demonstrated chemopreventive activity in a variety of laboratory animal models, including azoxymethane-induced colonic tumorigenesis and diethylnitrosamine-treated hepatocarcinogenesis in rats [29,35].

In the present study, we investigated the effect of CAPE on the growth, cell cycle and apoptosis of colon cancer cells. Our data demonstrated that CAPE treatment was associated with a strong inhibition of growth in a dose-dependent and time-dependent manner, along with induction of G_0/G_1 arrest and apoptosis in both HCT116 and SW480 colon cancer cells. Similar to previous findings, CAPE entered HL-60 cells very quickly, and then inhibited their survival in a dose-dependent and time-dependent manner, and induced characteristic DNA fragmentation and morphological changes typical of apoptosis in these cells [20,21].

The elevated β-catenin/Tcf signaling is an important event in the genesis of a number of malignancies, such as colon cancer. Mutations in the regulatory region of β-catenin or loss of APC function have been identified in human colon cancers [9,36]. Activation of an abnormal APC/β-catenin/Tcf signaling pathway and alterations in cellular adhesion mediated through changes in β-catenin homeostasis within the colonic epithelium are initiating factors in the development of the majority of colorectal cancers. Moreover, β-catenin signaling plays an important role in the growth and apoptosis of colon cancer cells [19,37].

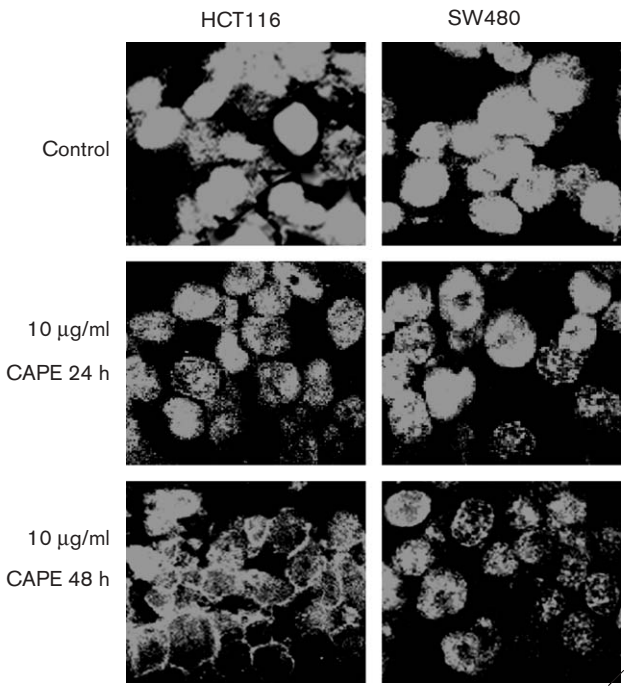
We hypothesized that the antitumor effects of CAPE in colon cancer are mediated by its ability to downregulate the β-catenin/Tcf signaling. Studies on the inhibitory agent against β-catenin/Tcf signaling in cancer cell lines have been performed. Dihlmann *et al.* [38] and Nath *et al.* [39] reported that nonsteroidal anti-inflammatory drug and nitric oxide-donating aspirin, respectively, were good inhibitors of β-catenin/Tcf signaling in colon cancer cell lines. Dashwood *et al.* [40] also reported that epigallocatchin gallate (EGCG) inhibited β-catenin/Tcf activity in HEK293 cells transiently transfected with constitutively active mutant β-catenin gene. In addition, it was

Fig. 4



Effects of caffeic acid phenethyl ester (CAPE) on β-catenin, c-*myc* and cyclin D1 expression in human colon cancer cells. HCT116 and SW480 cells were treated with vehicle or various concentrations of CAPE (2.5–10 μg/ml) for 24 or 48 h. (a) Western blotting of cell lysates for the protein expression of β-catenin, c-*myc*, cyclin D1 and β-actin. Fold induction of protein level was based on densitometric measurements and is shown below each immunoreactive band. β-Actin was used as a standard for each sample and protein levels in untreated cells were defined as 1.0. Data are representative of three independent experiments. (b) Reverse transcriptase-polymerase chain reaction of cell lysates for the mRNA expression of β-catenin, c-*myc*, cyclin D1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH served as internal loading controls and mRNA levels in untreated cells were defined as 1.0. Data are representative of three independent experiments.

Fig. 5



Caffeic acid phenethyl ester (CAPE) inhibits expression of nuclear β-catenin in HCT116 and SW480 cells. Indirect immunofluorescence for β-catenin was performed on adherent cells following 24 or 48 h treatment with CAPE. Confocal microscopy was performed at the same magnification for all cell preparations (× 400).

revealed by Jaiswal *et al.* [41] that curcumin inhibited the transcriptional activity of β-catenin/Tcf so as to induce growth arrest and apoptosis in HCT116 colon cancer cells. As the importance of β-catenin as a cause of tumorigenesis increases, many more studies on the β-catenin inhibitor and its inhibitory mechanism are being conducted.

Our data showed that CAPE treatment was associated with a dose-dependent decrease in β-catenin protein expression in both HCT116 and SW480 colon cancer cells. β-Catenin mRNA levels, however, were not significantly different from untreated controls. From these data, we conclude that the reduced β-catenin protein expression is not related to β-catenin transcription, but related to β-catenin ubiquitination and proteasomal degradation.

Cyclin D1 and c-*myc* are known β-catenin target gene, therefore, we also investigated the protein and mRNA expression of cyclin D1 and c-*myc* by Western blot and RT-PCR analysis. Figure 4 shows that CAPE treatment was associated with a dose-dependent and time-dependent decrease in cyclin D1 and c-*myc* protein and mRNA expression in both HCT116 and SW480 cells, which was paralleled by a decrease in the β-catenin protein expression.

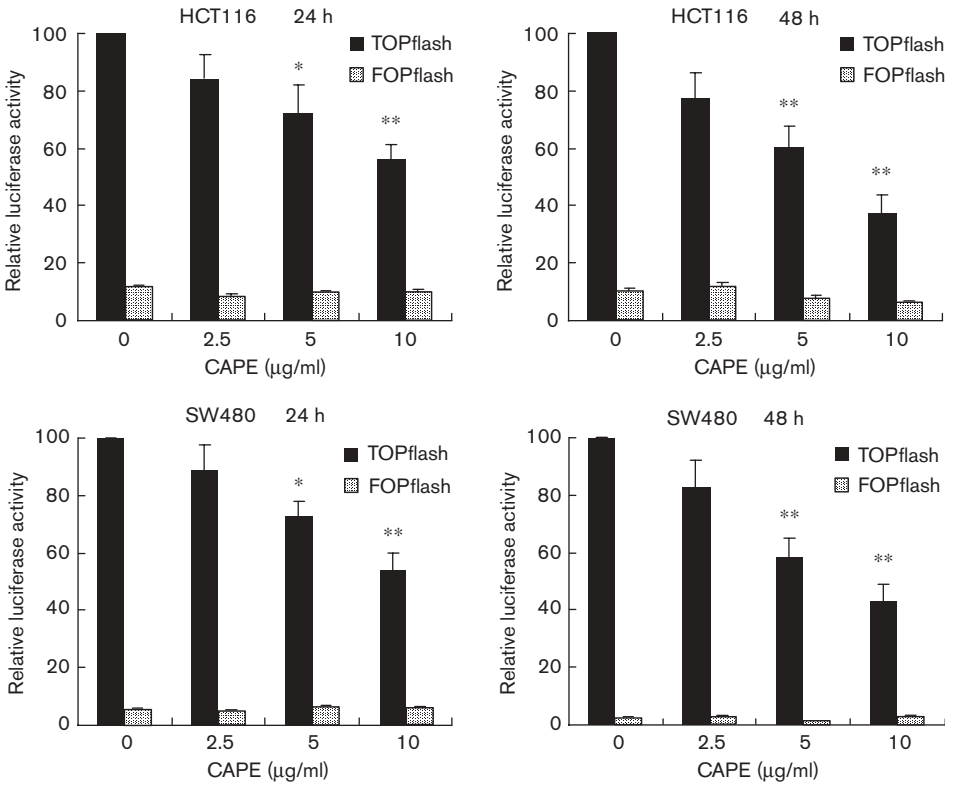
As we know, β-catenin is ubiquitous to and moves freely in a cell. It contributes to the cell–cell adhesion in the membrane and functions as a transcriptional activator in the nucleus [1]. β-Catenin is transcriptionally active in most CRC cell lines and after forming a complex with Tcf/Lef transcription factor in the nucleus, it regulates the expression of β-catenin/Tcf target genes. To determine β-catenin subcellular localization, immunofluorescence staining was performed. The results indicate that CAPE treatment was associated with decreased nuclear β-catenin and a concurrent increase in β-catenin protein expression at cell–cell junctions.

The changes in the subcellular localization of β-catenin might result in the downregulation of β-catenin/Tcf signaling. To prove this assumption, β-catenin/Tcf transcriptional activity was determined by transient transfection and reporter gene assay. Our data of luciferase activity showed that β-catenin/Tcf-driven transcription (measured by TOPflash activity) was suppressed strongly by CAPE in both HCT116 and SW480 human colon cancer cells dose dependently (Fig. 6), which showed that CAPE was a good inhibitor of β-catenin/Tcf signaling.

Multiple molecular mechanisms seem to be involved in the tumor-suppressive effects of CAPE. Recently, it is reported that CAPE induced apoptosis via Fas signal activation in human breast cancer MCF-7 cells [42]. Moreover, tumor-suppressor protein p53 and p38 mitogen-activated protein kinase play a prominent role in the CAPE-induced apoptotic cell death, which might contribute to the antitumor effects of CAPE in C6 glioma cells [43]. The present study for the first time provides evidence that the β-catenin/Tcf signaling pathway is a target of CAPE in colon cancer cells.

Eventually, we could conclude that the reduction of β-catenin in the nucleus is the root of the suppressed β-catenin/Tcf signaling. Now, we can postulate several possibilities to account for the decreased nuclear β-catenin product due to CAPE. The first possibility is that β-catenin is translocated to the cytosol from the nucleus by CAPE, then degraded in the cytosol via GSK-3β phosphorylation. Our data demonstrated that CAPE treatment was resulted in a dose-dependent and time-dependent loss of total and nuclear β-catenin protein. It is already well known that β-catenin is exported to the cytosol from the nucleus by an APC-dependent or APC-independent pathway, and that Lef-1 blocks the APC-mediated nuclear export of β-catenin [44–46]. CAPE may enhance the β-catenin exporting system. The second possibility is that β-catenin is degraded in the nucleus, not in the cytosol via GSK3β phosphorylation, by CAPE

Fig. 6



Caffeic acid phenethyl ester (CAPE) inhibits the transcriptional activity of β-catenin/T-cell factor (Tcf) in HCT116 cells and SW480 cells. Cells were cotransfected with reporter genes harboring Tcf-4-binding sites (TOPflash) or a mutant Tcf-binding site (FOPflash), respectively, and pRL-TK. Three hours post-transfection, increasing amounts of CAPE as indicated were added to the cells. Luciferase activity was determined 24 or 48 h post-treatment, normalized against values for the corresponding pRL-TK activity. Values represent means ± standard devition of three independent experiments. *$P<0.05$ versus vehicle; **$P<0.01$ versus vehicle.

through an unknown mechanism. The HCT116 human colon cancer cell line has no APC mutations, but harbors a somatic β-catenin mutation coding for a 3-bp deletion, which results in the removal of one amino acid (serine 45) in the coded protein [9]. Ser45 is one site for APC/GSK-3β-dependent phosphorylation, a key event in targeting the β-catenin protein for ubiquitination and proteasomal degradation. If phosphorylation of Ser 45 is essential for proteasomal degradation of β-catenin induced by CAPE, the HCT116 cell line would be expected to be resistant to these effects. Like the SW480 cell line, treatment of HCT116 cells with CAPE resulted in a dose-dependent and time-dependent loss of β-catenin protein. These results indicate that GSK-3β-dependent phosphorylation at Ser 45 is not required for CAPE-induced down-regulation of β-catenin. Curcumin, also plant-derived phenolic compound, induced growth arrest and apoptosis in colon cancer cells, which were associated with caspase-3-mediated cleavage of β-catenin, decreased transactivation of β-catenin/Tcf and the damaged β-catenin/Tcf signaling is not recovered by caspase-3 inhibitor, Z-DEVD-fmk, which blocks the cleavage of β-catenin by caspase-3 [41].

In summary, we have identified that CAPE could inhibit human colon cancer cell growth, and induce cell cycle arrest and apoptosis. This paper reveals the molecular mechanism underlying the antitumor effect of CAPE by suppressing β-catenin/Tcf signaling via decreasing the nuclear β-catenin proteins. In further studies, we will focus on the way in which nuclear β-catenin protein was decreased and the effects of CAPE on β-catenin/Tcf signaling in CRC cells *in vivo*.

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References

1 Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004; **303**:1483–1487.
2 Willert K, Nusse R. Beta-catenin: a key mediator of Wnt signaling. *Curr Opin Genet Dev* 1998; **8**:95–102.
3 Polakis P. Wnt signaling and cancer. *Genes Dev* 2000; **14**:1837–1851.
4 He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, *et al.* Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281**:1509–1512.
5 Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* 1999; **398**:422–426.

6 Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, *et al.* The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* 1999; **96**:5522–5527.

7 Shimokawa T, Furukawa Y, Sakai M, Li M, Miwa N, Lin YM, *et al.* Involvement of the FGF18 gene in colorectal carcinogenesis, as a novel downstream target of the beta-catenin/T-cell factor complex. *Cancer Res* 2003; **63**:6116–6120.

8 Tan X, Apte U, Micsenyi A, Kotsagrelos E, Luo JH, Ranganathan S, *et al.* Epidermal growth factor receptor: a novel target of the Wnt/beta-catenin pathway in liver. *Gastroenterology* 2005; **129**:285–302.

9 Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, *et al.* Activation of β-catenin–Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* 1997; **275**:1787–1790.

10 Fujie H, Moriya K, Shintani Y, Tsutsumi T, Takayama T, Makuuchi M, *et al.* Frequent β-catenin aberration in human hepatocellular carcinoma. *Hepatol Res* 2001; **20**:39–51.

11 Woo DK, Kim HS, Lee HS, Kang YH, Yang HK, Kim WH. Altered expression and mutation of β-catenin gene in gastric carcinomas and cell lines. *Int J Cancer* 2001; **95**:108–113.

12 Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, *et al.* APC mutations occur early during colorectal tumorigenesis. *Nature* 1992; **359**:235–237.

13 Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. *Cancer Res* 1998; **58**:1130–1134.

14 Bright-Thomas RM, Hargest R. APC, β-catenin and hTCF-4; an unholy trinity in the genesis of colorectal cancer. *Eur J Surg Oncol* 2003; **29**:107–117.

15 Baldus SE, Monig SP, Huxel S, Landsberg S, Hanisch FG, Engelmann K, *et al.* MUC1 and nuclear beta-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. *Clin Cancer Res* 2004; **10**:2790–2796.

16 Wong SC, Lo ES, Lee KC, Chan JK, Hsiao WL. Prognostic and diagnostic significance of beta-catenin nuclear immunostaining in colorectal cancer. *Clin Cancer Res* 2004; **10**:1401–1408.

17 Dihlmann S, von Knebel Doeberitz M. Wnt/beta-catenin-pathway as a molecular target for future anti-cancer therapeutics. *Int J Cancer* 2005; **113**:515–524.

18 Luu HH, Zhang R, Haydon RC, Rayburn E, Kang Q, Si W, *et al.* Wnt/beta-catenin signaling pathway as a novel cancer drug target. *Curr Cancer Drug Targets* 2004; **4**:653–671.

19 Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against beta-catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. *Clin Cancer Res* 2003; **9**:1291–1300.

20 Chen YJ, Shiao MS, Wang SY. The antioxidant caffeic acid phenethyl ester induces apoptosis associated with selective scavenging of hydrogen peroxide in human leukemic HL-60 cells. *Anticancer Drugs* 2001; **12**: 143–149.

21 Chen YJ, Shiao MS, Hsu ML, Tsai TH, Wang SY. Effect of caffeic acid phenethyl ester, an antioxidant from propolis, on inducing apoptosis in human leukemic HL-60 cells. *J Agric Food Chem* 2001; **49**:5615–5619.

22 Lee YJ, Liao PH, Chen WK, Yang CY. Preferential cytotoxicity of caffeic acid phenethyl ester analogues on oral cancer cells. *Cancer Lett* 2000; **153**:51–56.

23 Usia T, Banskota AH, Tezuka Y, Midorikawa K, Matsushige K, Kadota S. Constituents of Chinese propolis and their antiproliferative activities. *J Nat Prod* 2002; **65**:673–676.

24 Nomura M, Kaji A, Ma W, Miyamoto K, Dong Z. Suppression of cell transformation and induction of apoptosis by caffeic acid phenethyl ester. *Mol Carcinog* 2001; **31**:83–89.

25 Nagaoka T, Banskota AH, Tezuka Y, Saiki I, Kadota S. Selective antiproliferative activity of caffeic acid phenethyl ester analogues on highly liver-metastatic murine colon 26-L5 carcinoma cell line. *Bioorg Med Chem* 2002; **10**:3351–3359.

26 Weyant MJ, Carothers AM, Bertagnolli ME, Bertagnolli MM. Colon cancer chemopreventive drugs modulate integrin-mediated signaling pathways. *Clin Cancer Res* 2000; **6**:949–956.

27 Liao HF, Chen YY, Liu JJ, Hsu ML, Shieh HJ, Liao HJ, *et al.* Inhibitory effect of caffeic acid phenethyl ester on angiogenesis, tumor invasion, and metastasis. *J Agric Food Chem* 2003; **51**:7907–7912.

28 Nagaoka T, Banskota AH, Tezuka Y, Harimaya Y, Koizumi K, Saiki I, *et al.* Inhibitory effects of caffeic acid phenethyl ester analogues on experimental lung metastasis of murine colon 26-L5 carcinoma cells. *Biol Pharm Bull* 2003; **26**:638–641.

29 Borrelli F, Izzo AA, Di Carlo G, Maffia P, Russo A, Maiello FM, *et al.* Effect of a propolis extract and caffeic acid phenethyl ester on formation of aberrant crypt foci and tumors in the rat colon. *Fitoterapia* 2002; **73**:S38–S43.

30 Mahmoud NN, Carothers AM, Grunberger D, Bilinski RT, Churchill MR, Martucci C, *et al.* Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis. *Carcinogenesis* 2000; **21**:921–927.

31 Yang DC, Jiang XP, Elliott RL, Head JF. Inhibition of growth of human breast carcinoma cells by an antisense oligonucleotide targeted to the transferrin receptor gene. *Anticancer Res* 2001; **21**:1777–1787.

32 Tolwinski NS, Wieschaus E. A nuclear function for armadillo/beta-catenin. *PLoS Biol* 2004; **2**:e95.

33 Banskota AH, Tezuka Y, Kadota S. Recent progress in pharmacological research of propolis. *Phytother Res* 2001; **15**:561–571.

34 Murad JM, Calvi SA, Soares AM, Bankova V, Sforcin JM. Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*. *J Ethnopharmacol* 2002; **79**:331–334.

35 Carrasco-Legleu CE, Marquez-Rosado L, Fattel-Fazenda S, Arce-Popoca E, Perez-Carreon JI, Villa-Trevino S. Chemoprotective effect of caffeic acid phenethyl ester on promotion in a medium-term rat hepatocarcinogenesis assay. *Int J Cancer* 2004; **108**:488–492.

36 Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, *et al.* Constitutive transcriptional activation by a β-catenin–Tcf complex in $APC^{-/-}$ colon carcinoma. *Science* 1997; **275**:1784–1787.

37 Wang H, MacNaughton WK. Overexpressed beta-catenin blocks nitric oxide-induced apoptosis in colonic cancer cells. *Cancer Res* 2005; **65**:8604–8607.

38 Dihlmann S, Siermann A, von Knebel Doeberitz M. The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate β-catenin/Tcf-4 signaling. *Oncogene* 2001; **20**:645–653.

39 Nath N, Kashfi K, Chen J, Rigas B. Nitric oxide-donating aspirin inhibits beta-catenin/T cell factor (TCF) signaling in SW480 colon cancer cells by disrupting the nuclear beta-catenin–TCF association. *Proc Natl Acad Sci U S A* 2003; **100**:12584–12589.

40 Dashwood WM, Orner GA, Dashwood RH. Inhibition of beta-catenin/Tcf activity by white tea, green tea, and epigallocatechin-3-gallate (EGCG): minor contribution of H_2O_2 at physiologically relevant EGCG concentrations. *Biochem Biophys Res Commun* 2002; **296**:584–588.

41 Jaiswal AS, Marlow BP, Gupta N, Narayan S. Beta-catenin-mediated transactivation and cell–cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells. *Oncogene* 2002; **21**:8414–8427.

42 Watabe M, Hishikawa K, Takayanagi A, Shimizu N, Nakaki T. Caffeic acid phenethyl ester induces apoptosis by inhibition of NFkappaB and activation of Fas in human breast cancer MCF-7 cells. *J Biol Chem* 2004; **279**: 6017–6026.

43 Lee YJ, Kuo HC, Chu CY, Wang CJ, Lin WC, Tseng TH. Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells. *Biochem Pharmacol* 2003; **66**:2281–2289.

44 Henderson BR. Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat Cell Biol* 2000; **2**:653–660.

45 Eleftheriou A, Yoshida M, Henderson BR. Nuclear export of human beta-catenin can occur independent of CRM1 and the adenomatous polyposis coli tumor suppressor. *J Biol Chem* 2001; **276**:25883–25888.

46 Henderson BR, Galea M, Schuechner S, Leung L. Lymphoid enhancer factor-1 blocks adenomatous polyposis coli-mediated nuclear export and degradation of beta-catenin. Regulation by histone deacetylase 1. *J Biol Chem* 2002; **277**:24258–24264.