Effect of Caffeic Acid Phenethyl Ester, an Antioxidant from Propolis, on Inducing Apoptosis in Human Leukemic HL-60 Cells

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Caffeic acid phenethyl ester (CAPE) is an active component isolated from propolis. The aim of this study was to investigate the mechanism of CAPE-induced apoptosis in human leukemic HL-60 cells. It was found that CAPE entered HL-60 cells very quickly and then inhibited their survival in a concentration- and time-dependent manner. CAPE induced characteristic DNA fragmentation and morphological changes typical of apoptosis in these cells. Estimation of the apoptotic percentage showed a time-dependent increase after CAPE (6 μg/mL) treatment (up to 66.7 ± 2.0% at 72 h). Treatment with CAPE caused rapid activation of caspase-3 after 4 h, down-regulation of Bcl-2 expression after 6 h, and up-regulation of Bax expression after 16 h. These results suggest that CAPE is a potent apoptosis-inducing agent; its action is accompanied by activation of caspase-3, down-regulation of Bcl-2, and up-regulation of Bax in human leukemic HL-60 cells.

Keywords: *Caffeic acid phenethyl ester (CAPE); apoptosis; caspase-3; Bcl-2; Bax*

INTRODUCTION

Honeybee propolis has been widely used as a healthy food and folk medicine in many countries. Caffeic acid phenethyl ester (CAPE), a phenolic compound isolated from propolis (*1*), possesses a broad spectrum of biological activities including antioxidant (*2*−*4*), anti-inflammatory (*5*−*7*), antiviral (*8*), reperfusion injury preventive (*9*), and anticancer effects (*1*, *10*, *11*). Although propolis samples from different sources have similar qualitative compositions, the contents of CAPE vary greatly (*12*).

Apoptosis of tumor cells can be triggered via different mechanisms by various treatments such as irradiation (*13*), Fas/APO-1 (a cell surface molecule mediating apoptosis) ligand (*14*), and cytotoxic agents (*15*, *16*). The regulation and execution of apoptosis require two major protein families, namely, Bcl-2-related gene products and caspases (*17*, *18*). The growing Bcl-2 protein family contains several homologous proteins including anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, etc.) and proapoptotic proteins (Bax, Bad, Bak, Bik, Bcl-xS, etc.) (*17*). The caspase family comprises a group of cysteine proteases possessing homology with each other and acting in a cascade-like fashion. Caspases are present in cytosol as inactive procaspases until activation via cleavage by other caspases. For example, activated caspase-1 cleaves procaspase-3 to produce the active caspase-3 (*18*). Some caspase substrates have been shown to be proteolyzed by caspases during apoptosis. For example, caspase-2, -3, -7, and -9 may cleave poly(ADP-ribose) polymerase (PARP) (*19*), and caspase-6 cleaves nuclear lamins (*20*).

CAPE has been observed to induce apoptosis in transformed cells (*21*) and exhibit cytotoxicity in oral cancer cells (*10*), but the detailed molecular mechanism remains unclear. Differential cytotoxicity toward tumor cells has been demonstrated through modulation of the cellular redox state (*21*). Hence, CAPE is a potent antioxidant as well as an apoptosis-inducing agent, but these two activities seem to be independent. To elucidate the anticancer mechanism of CAPE, we investigated the changes of the Bcl-2 protein family and caspases in CAPE-induced apoptosis in human HL-60 leukemic cells.

MATERIALS AND METHODS

Cell Culture and Treatments. The human myeloid leukemic cell line, HL-60, was obtained from Dr. M. A. S. Moore (Memorial Sloan-Kettering Cancer Center, New York, NY). The cells were cultured in RPMI1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), passaged (change medium) every 3 days, and maintained in an exponential growth status. For induction of apoptosis, the cells were incubated in 35-mm or 100-mm Petri dishes at an initial concentration of 1×10^5/mL and grown at 37 °C in a humidified 5% CO_2 incubator. CAPE was synthesized by chemical reaction of caffeic acid and phenethyl alcohol according to the procedure of Grunberger et al. (*1*) with modification. Purified CAPE (≥99%) was dissolved in absolute ethanol (stock solution). CAPE was added to the cell cultures (1−10 μg/mL) with a final ethanol concentration of 0.1% v/v. In previous studies, we found that this concentration of ethanol had no significant effect on the growth of HL-60 cells.

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Determination of Intracellular CAPE Amount by HPLC. After the addition of 6 μg/mL CAPE into cell culture, HL-60 cells were collected every 10 min and washed twice in phosphate-buffered saline (PBS) by centrifugation at 400*g* for 10 min. To extract intracellular CAPE, the extraction solution containing Triton X-100 and dimethyl sulfoxide (DMSO) at a ratio of 0.1:100 (v/v) was added to the cell pellets and continuously shaken for 16 h. The resulting solution was then mixed with an equal volume of acetonitrile for deproteination. The denatured protein precipitate was separated by centrifugation at 8000*g* for 3 min, and a 10 μL aliquot of the supernatant was directly injected into the HPLC. Liquid chromatographic grade solvents and reagents were obtained from E. Merck (Darmstadt, Germany). Triply deionized water (Millipore, Bedford, MA) was used for all preparations. The HPLC system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN), an on-line injector (CMA 160, Stockholm, Sweden) equipped with a 10 μL sample loop, and an ultraviolet detector (Dynamax, Walnut Creek, CA). CAPE was separated using a reversed phase C18 microbore column (150 × 1 mm i.d.; particle size = 5 μm) maintained at ambient temperature. The mobile phase was composed of acetonitrile/20 mM monosodium phosphate (pH 4.0) (55:45, v/v) and 0.1 mM 1-octanesulfonic acid, and the flow rate of the mobile phase was 0.05 mL/min. The buffer was filtered through a Millipore 0.22 μm filter and degassed prior to use. The optimal UV absorbance wavelength at 330 nm of CAPE was measured by spectrometry and referred to our previous study for caffeic acid (*22*). Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA). The calibration of CAPE concentration was made prior to the experiments, achieving correlation coefficient values of at least 0.995. The relative standard deviation (RSD) was calculated from the observed concentrations as follows: precision (% RSD) = [standard deviation (SD)/C_{obs}] × 100. Precision (% RSD) values within ±15% covering the actual range of experimental concentrations were considered to be acceptable. The recovery of CAPE was performed by the effective protein precipitation method. The main goal is to remove proteins that can precipitate when it contacts with the mobile phase and thereby block tubing, causing increases in back pressure or deterioration of HPLC column performance. The method has been validated to have an overall mean precision, defined by the RSD, of 0.8−13%.

Cell Growth Kinetics and Micrographs. After treatment with various concentrations of CAPE on days 1, 2, and 3, HL-60 cells were harvested by gently rubbing the dishes with a rubber policeman (Bellco Glass, Vineland, NJ). The numbers of viable cells were counted using a trypan blue dye exclusion test. The growth inhibition was calculated using the equation as (cell number in control group − cell number in treated group)/cell number in control group. After treatment with 6 μg/mL CAPE for 24 h, the cells were collected and cytocentrifuged onto a microscope slide using a Cytospin2R (Shandon Southern Instrument Inc.) and then stained with Wright's stain. Light micrographs were taken under an Olympus microscope at a magnification of 1000×.

DNA Extraction and Gel Electrophoresis. Both untreated and CAPE-treated HL-60 cells (10^6/mL) were lysed with 0.5 mL of lysis buffer containing 5 mM Tris−borate at pH 8.0, 0.25 mL of Nonidet P-40, and 1 mM EDTA, followed by the addition of RNase (Sigma, St. Louis, MO) at a final concentration of 20 μg/mL and incubation at 37 °C for 1 h. Cells were further treated with proteinase K (300 μg/mL) for another 1 h, and DNA was then isolated from the cultured cells as described (*23*). Electrophoresis was carried out on 1.5% agarose gel in 5 mM Tris−borate buffer (pH 8.0) containing 1 mM EDTA. The DNA on the gel was stained with ethidium bromide.

Quantitation of Apoptosis by Flow Cytometer. Hypodiploid DNA was analyzed using propidium iodide (PI) staining and flow cytometry as in our previous study (*23*). Both treated and untreated leukemic cells were harvested, washed with PBS, and resuspended (10^6/mL) in 1.5 mL of hypotonic fluorochromic solution (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100) (Sigma) for 60 min at 4 °C in the dark. The PI fluorescence of individual nuclei was analyzed by a FACScaliber flow cytometer (Becton Dickinson, Lincoln Park, NJ). As an estimate of the proportion of apoptotic cells, the percentage of hypodiploid cells in a population of 10000 cells was calculated using ModFIT cell cycle analysis software version 2.01.2 (Becton Dickinson).

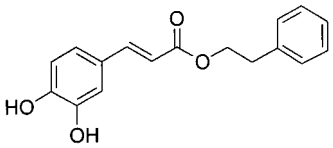

Figure 1. Chemical structure of caffeic acid phenethyl ester (CAPE).

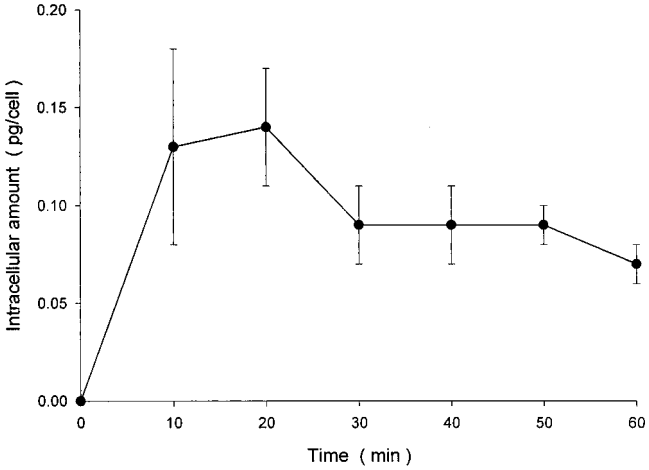

Figure 2. Time-dependent changes in intracellular amount of CAPE after 6 μg/mL CAPE treatment. Data from three separate experiments are expressed as mean ± SEM.

Western Blotting. The cellular proteins were isolated from human leukemic HL-60 cells after treatment with 6 μg/mL CAPE for 0, 2, 4, 6, and 16 h. The following reagents were added to extract total protein for 30 min on ice: 200 μL of lysis buffer containing 50 mM Tris, pH 7.4, 1 mM phenylmethanesulfonyl fluoride, 1% Nonidet P-40, and 10 μg/mL leupeptin. The cell lysates were centrifuged at 10000*g* for 30 min, and the protein concentration was determined by a bicinchoninic acid assay (*24*) kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg in each lane) were electrophoresed in 20% SDS−polyacrylamide gel at a constant current of 20 mA and transferred onto a blotting membrane. The membrane was blocked with 5% defatted milk and then immunoblotted with primary antibodies including anti-Bcl-2 (Santa Cruz Biotech., Santa Cruz, CA), anti-Bax (Santa Cruz Biotech.), and anti-caspase-3 (Upstate Biotech., Lake Placid, NY) at room temperature for 3 h. This was followed by the addition of horseradish peroxidase-labeled second antibodies (Transduction Laboratories, Lexington, KY) and development using the enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, NJ).

RESULTS

Time-Dependent Changes in Intracellular Amount of CAPE in HL-60 Cells. The structure of CAPE is illustrated in Figure 1. CAPE entered HL-60 cells very quickly. The maximal amount (0.14 ± 0.05 pg/cell) was measured at 20 min after 6 μg/mL CAPE treatment and declined slowly thereafter (Figure 2).

Inhibition of Proliferation in HL-60 Cells by CAPE. Proliferation of HL-60 cells was significantly inhibited by CAPE in a dose- and time-dependent manner (Figure 3). A concentration of 1 μg/mL CAPE had a

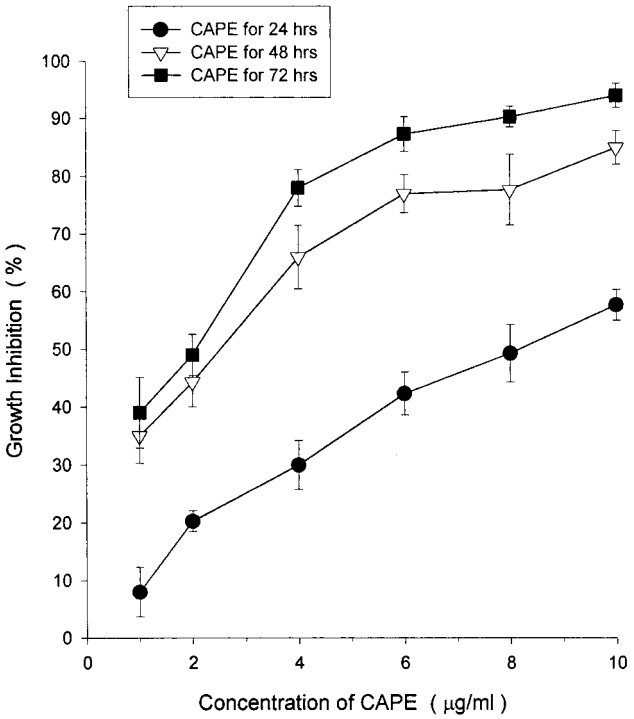

Figure 3. Time- and dose-dependent growth inhibition of HL-60 cells by CAPE (6 μg/mL). Data from three separate experiments are expressed as mean ± SEM.

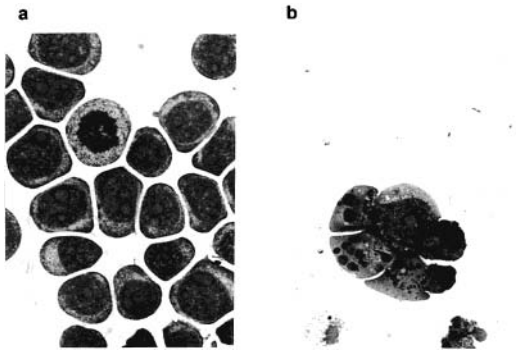

Figure 4. Morphologic features of HL-60 cells cultured with or without CAPE (6 μg/mL) for 24 h, illustrating typical apoptotic changes: (a) untreated HL-60 cells of slightly varying size show a rounded contour and no changes of cell death (one mitotic cell is noted in the upper third of the figure); (b) cells undergoing apoptosis show a condensed and fragmented nucleus. Magnification ×1000 (figure is reproduced here at 50% of its original size).

minimal effect on cell viability compared with untreated cells. However, concentrations up to 6 μg/mL of CAPE caused marked growth inhibition (77.3 ± 3.3% at 48 h and 87.3 ± 3.0% at 72 h).

Morphological Changes in CAPE-Treated HL-60 Cells Undergoing Apoptosis. Untreated HL-60 cells are morphologically myeloblast-like cells, with a round cell contour, scanty cytoplasm containing some granules, and an ovoid-shaped nucleus with some nucleoli (Figure 4a). After 24 h of incubation with 6 μg/mL CAPE, many cells exhibited characteristic apoptotic changes such as cytoplasmic membrane blebs, condensed chromatin, and apoptotic bodies containing dense micronuclear spheres (Figure 4b).

Effect of CAPE on Inducing Apoptosis. Similar to growth inhibition, nuclear fragmentation in HL-60

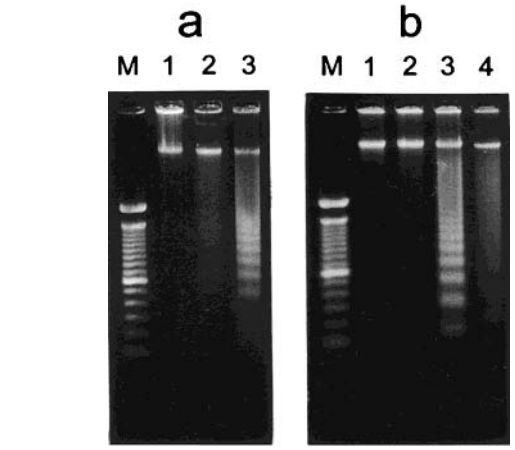

Figure 5. Induction of DNA fragmentation in HL-60 cells by CAPE: (a) HL-60 cells treated with increasing doses of CAPE for 6 h (lane M, molecular weight marker; lane 1, untreated cells; lanes 2 and 3, cells treated with 2 and 6 μg/mL CAPE, respectively); (b) time-dependent increase of DNA fragmentation induced by 6 μg/mL CAPE (lane M, molecular weight marker; lane 1, untreated cells; lane 2, 2 h; lane 3, 6 h; lane 4, 24 h).

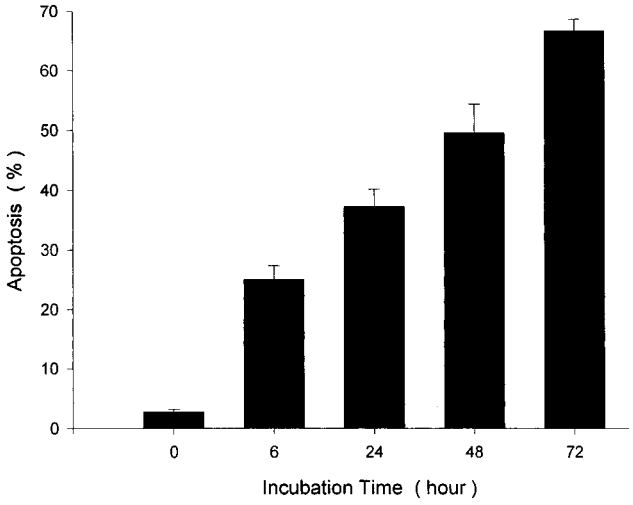

Figure 6. Flow cytometric detection of time-dependent induction of apoptosis by CAPE (6 μg/mL) in HL-60 cells. Data from three separate experiments are expressed as mean ± SEM.

cells induced by CAPE was dose- and time-dependent. The oligonucleosome-length DNA ladder shown by agarose gel electrophoresis after treatment with 6 μg/mL CAPE was more evident than at a lower concentration of 2 μg/mL for the same incubation time (6 h) (Figure 5a). After 2 h of treatment with 6 μg/mL CAPE, the DNA ladder was not clearly noted but became obvious after 6 h of incubation (Figure 5b). The less marked DNA fragmentation after 24 h treatment may due to the lower number of cells as a result of substantial growth inhibition.

The percentage of apoptotic cells in the untreated HL-60 population was 2.8 ± 0.4%. Cells treated with CAPE (6 μg/mL) had a significantly higher percentage (25.1 ± 2.3%) after 6 h of treatment. Moreover, the percentage of apoptotic cells increased to 66.7 ± 2.0% after 72 h of treatment (Figure 6).

Time-Dependent Effect of CAPE on Expression of Bcl-2 and Bax and Activity of Caspase-3. The expression of Bcl-2 was inhibited after treatment with 6 μg/mL CAPE for 6 h. In contrast, the expression of

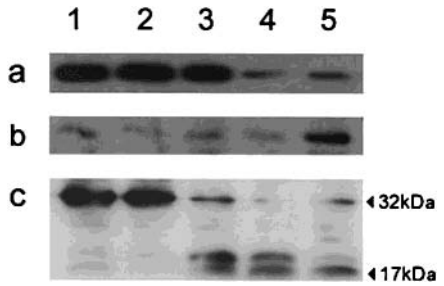

Figure 7. Western blot analysis for expression of Bcl-2 protein family and activity of caspase-3: (a) Bcl-2; (b) Bax; (c) caspase-3; lane 1, untreated cells; lane 2, 2 h; lane 3, 4 h; lane 4, 6 h; lane 5, 16 h after 6 μg/mL CAPE treatment.

Bax, a pro-apoptotic protein, was stimulated after 16 h of incubation with the same dose of CAPE (Figure 7a,b). Caspase-3 was activated after treatment with 6 μg/mL CAPE for 4 h, as assessed by cleavage of caspase-3 (32-kD) into a 17-kDa active form (Figure 7c).

DISCUSSION

Our findings indicate that CAPE is a potent apoptosis-inducing agent; its action is accompanied by activation of caspase-3, down-regulation of Bcl-2, and up-regulation of Bax in human leukemic HL-60 cells. In Figure 5a, we showed that 2 μg/mL (7 μM) CAPE induced DNA fragmentation within 6 h of treatment, which suggests that CAPE is more potent than garcinol and curcumin because they need a larger dose and a longer time to induce DNA fragmentation (*25*).

CAPE-induced apoptosis occurred within several hours in HL-60 cells, as did changes in caspase-3, Bcl-2, and Bax. This is compatible with the view that apoptosis is induced by activating pre-existing apoptotic machinery. However, the time sequence of these events bears consideration. CAPE entered HL-60 cells rapidly and reached a maximal intracellular amount after 20 min of treatment. We found that CAPE treatment caused caspase-3 activation by cleaving procaspase-3 prior to the onset of apoptosis, down-regulation of Bcl-2, and up-regulation of Bax. Our previous work demonstrated that CAPE rapidly depleted intracellular glutathione to less than half after 1 h in HL-60 cells (*26*). CAPE also depletes ~40% of glutathione in transformed rat fibroblasts within 4 h (*21*). A recent study has shown that thiol depletion can effectively activate caspase-3 and subsequently induce cancer cell apoptosis (*27*). From these observations, it appears that the early activation of caspase-3 may be due to a rapid depletion of intracellular glutathione after CAPE treatment. This glutathione-related activation of caspase-3 may occur prior to Bcl-2-family mediated apoptosis.

The Bcl-2 protein family has been mainly localized to the cytosol. Overexpression of Bcl-2 and Bcl-xL has been found to cause resistance to chemotherapeutic drugs by inhibiting apoptosis (*28*). Bax, a pro-apoptotic protein, may act as a tumor suppressor and is mutated in human gastrointestinal cancer and some leukemias (*29*−*31*). The differential effect of Bcl-2 and Bax was confirmed in our experiments showing a decrease of Bcl-2 expression and an increase of Bax expression during CAPE-induced apoptosis.

In conclusion, CAPE entered HL-60 cells rapidly and exhibited a growth inhibitory effect by inducing apoptosis, which was associated with activation of caspase-3, down-regulation of Bcl-2, and up-regulation of Bax.

SAFETY CONSIDERATION

There is no significant toxicity of CAPE reported in the literature (*9*). The only safety consideration is due to the flammable tendency of ethanol, the solvent we used for CAPE.

ABBREVIATIONS USED

CAPE, caffeic acid phenethyl ester; HPLC, high-performance liquid chromatography.

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LITERATURE CITED

(1) Grunberger, D.; Banerjee, R.; Eisinger, K.; Oltz, E. M.; Efros, L.; Caldwell, M.; Estevez, V.; Nakanishi, K. Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis. *Experientia* **1988**, *44*, 230−232.

(2) Sud'ina, G. F.; Mirzoeva, O. K.; Pushkareva, G. A.; Korshunova, G. A.; Sumbatyan, N. V.; Varfolomeev, S. D. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. *FEBS Lett.* **1993**, *329*, 21−24.

(3) Bhimani, R.; Troll, W.; Grunberger, D.; Frenkel, K. Inhibition of oxidative stress in HeLa cells by chemopreventive agents. *Cancer Res.* **1993**, *53*, 4528−4533.

(4) Jaiswal, A. K.; Venugopal, R.; Mucha, J.; Carothers, A. M.; Grunberger, D. Caffeic acid phenethyl ester stimulates human antioxidant response element-mediated expression of the NAD(P)H:quinone oxidoreductase (NQO1) gene. *Cancer Res.* **1997**, *57*, 440−446.

(5) Michaluart, P.; Masferrer, J. L.; Carothers, A. M.; Subbaramaiah, K.; Zweifel, B. S.; Koboldt, C.; Mestre, J. R.; Grunberger, D.; Sacks, P. G.; Tanabe, T.; Dannenberg, A. J. Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. *Cancer Res.* **1999**, *59*, 2347−2352.

(6) Mirzoeva, O. K.; Calder, P. C. The effect of propolis and its components on eicosanoid production during the inflammatory response. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **1996**, *55*, 441−449.

(7) Frenkel, K.; Wei, H.; Bhimani, R.; Ye, J.; Zadunaisky, J. A.; Huang, M. T.; Ferraro, T.; Conney, A. H.; Grunberger, D. Inhibition of tumor promoter-mediated processes in mouse skin and bovine lens by caffeic acid phenethyl ester. *Cancer Res.* **1993**, *53*, 1255−1261.

(8) Fesen, M. R.; Pommier, Y.; Leteurtre, F.; Hirogushi, S.; Yung, J.; Kohn, K. W. Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. *Biochem. Pharmacol.* **1994**, *48*, 595−608.

(9) Koltuksuz, U.; Ozen, S.; Uz, E.; Aydinc, M.; Karaman, A.; Gultek, A.; Akyol, O.; Gursoy, M. H.; Aydin, E. Caffeic acid phenethyl ester prevents intestinal reperfusion injury in rats. *J. Pediatr. Surg.* **1999**, *34*, 1458−1462.

(10) Lee, Y. J.; Liao, P. H.; Chen, W. K.; Yang, C. Y. Preferential cytotoxicity of caffeic acid phenthyl ester analogues on oral cancer cells. *Cancer Lett.* **2000**, *153*, 51−56.

(11) Chen, J. H.; Shao, Y.; Huang, M. T.; Chin, C. K.; Ho, C. T. Inhibitory effect of caffeic acid phenethyl ester on human leukemic HL-60 cells. *Cancer Lett.* **1996**, *108*, 211−214.

(12) Hegazi, A. G.; Abd E1 Hady, F. K.; Abd Allah, F. A. Chemical composition and antimicrobial activity of European propolis. *Z. Naturforsch. C* **2000**, *55*, 70−75.

(13) McGowan, A. J.; Fernandes, R. S.; Verhaegen, S.; Cotter, T. G. Zinc inhibits UV radiation-induced apoptosis but fails to prevent subsequent cell death. *Int. J. Raidiat. Biol.* **1994**, *66*, 343−349.
(14) Strand, S.; Hofmann, W. J.; Hug, H.; Muller, M.; Otto, G.; Strand, D.; Mariani, S. M.; Stremmel, W.; Krammer, P. H.; Galle, P. R. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells−a mechanism of immune evasion? *Nat. Med.* **1996**, *2*, 1361−1366.
(15) Gunji, H.; Kharbanda, S.; Kufe, D. Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. *Cancer Res.* **1991**, *51*, 741−743.
(16) Cotter, T. G.; Glynn, J. M.; Echeverri, F.; Green, D. R. The induction of apoptosis by chemotherapeutic agents occurs in all phases of the cell cycle. *Anticancer Res.* **1992**, *12*, 773−779.
(17) Adams, J. M.; Cory, S. The Bcl-2 protein family: arbiters of cell survival. *Science* **1998**, *281*, 1322−1326.
(18) Cohen, G. M. Caspases: the executioners of apoptosis. *Biochem. J.* **1997**, *326*, 1−16.
(19) Tewari, M.; Quan, L. T.; O'Rourke, K.; Desnoyers, S.; Zeng, Z.; Beidler, D. R.; Poirier, G. G.; Salvesen, G. S.; Dixit, V. M. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **1995**, *81*, 801−809.
(20) Takahashi, A.; Alnemri, E. S.; Lazebnik, Y. A.; Fernandes-Alnemri, T.; Litwack, G.; Moir, R. D.; Goldman, R. D.; Poirier, G. G.; Kaufmann, S. H.; Earnshaw, W. C. Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin 1 beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8395−8400.
(21) Chiao, C.; Carothers, A. M.; Grunberger, D.; Solomon, G.; Preston, G. A.; Barrett, J. C. Apoptosis and altered redox state induced by caffeic acid phenethyl ester (CAPE) in transformed rat fibroblast cells. *Cancer Res.* **1995**, *55*, 3576−3583.
(22) Tsai, T. H.; Chen, Y. F.; Chen, I. F.; Chen, C. F. Measurement of unbound caffeic acid in rat blood by on-line microdialysis coupled with liquid chromatography and its application to pharmacokinetic studies. *J. Chromatogr. B* **1999**, *729*, 119−125.
(23) Wang, S. Y.; Hsu, M. L.; Hsu, H. C.; Tzeng, C. H.; Lee, S. S.; Shiao, M. S.; Ho, C. K. The anti-tumor effect of Ganoderma lucidum is mediated by cytokines released from activated macrophages and T lymphocytes. *Int. J. Cancer* **1997**, *70*, 699−705.
(24) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76−85.
(25) Pan, M. H.; Chang, W. L.; Lin-Shiau S. Y.; Ho, C. T.; Lin, J. K. Induction of apoptosis by garcinol and curcumin through cytochrome *c* release and activation of caspases in human leukemia HL-60 cells. *J. Agric. Food Chem.* **2001**, *49*, 1464−1474.
(26) Chen, Y. J.; Shiao, M. S.; Wang, S. Y. The antioxidant caffeic acid phenethyl ester induces apoptosis associated with selective scavenging of hydrogen peroxide in human eukemic HL-60 cells. *Anti-Cancer Drugs* **2001**, *12*, 143−149.
(27) Coffey, R. N.; Watson, R. W.; Hegarty, N. J.; O'Neill, A.; Gibbons, N.; Brady, H. R.; Fitzpatrick, J. M. Thiol-mediated apoptosis in prostate carcinoma cells. *Cancer* **2000**, *88*, 2092−2104.
(28) Nuessler, V.; Stotzer, O.; Gullis, E.; Pelka-Fleischer, R.; Pogrebniak, A.; Gieseler, F.; Wilmanns, W. Bcl-2, Bax and Bcl-xL expression in human sensitive and resistant leukemia cell lines. *Leukemia* **1999**, *13*, 1864−1872.
(29) Rampino, N.; Yamamoto, H.; Ionov, Y.; Li, Y.; Sawai, H.; Reed, J. C.; Perucho, M. Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* **1997**, *275*, 967−969.
(30) Yamamoto, H.; Sawai, H.; Perucho, M. Frameshift somatic mutations in gastrointestinal cancer of the microsatellite mutator phenotype. *Cancer Res.* **1997**, *57*, 4420−4426
(31) Meijerink, J. P.; Mensink, E. J.; Wang, K.; Sedlak, T. W.; Sloetjes, A. W.; de Witte, T.; Waksman, G.; Korsmeyer, S. J. Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. *Blood* **1998**, *91*, 2991−2997.

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