JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Inhibitory Effect of Caffeic Acid Phenethyl Ester on Angiogenesis, Tumor Invasion, and Metastasis

HUI-FEN LIAO,[†,‡] YU-YWAN CHEN,[§] JUN-JEN LIU,[∥] MING-LING HSU,[†] HUI-JU SHIEH,[†] HUNG-JEN LIAO,[†] CHWEN-JEN SHIEH,[⊥] MING-SHI SHIAO,[‡] AND YU-JEN CHEN*[,†,∞]

Departments of Medical Research and Radiation Oncology, Mackay Memorial Hospital, Taipei 104, Taiwan; Department of Medical Research and Education, Veterans General Hospital—Taipei, Taipei 112, Taiwan; Department of Seafood Science, National Kaohsiung Institute of Marine Technology, Kaohsiung 811, Taiwan; Institute of Biochemistry, National Taiwan University, Taipei 106, Taiwan; Department of Bioindustry Technology, Dayeh University, Changhua 515, Taiwan; and Department of Martial Arts, Chinese Culture University, Taipei 111, Taiwan

Caffeic acid phenethyl ester (CAPE) derived from honeybee propolis has been used as a folk medicine and has several proven biological activities. The present study investigated the effect of CAPE on angiogenesis, tumor invasion, and metastasis. A cytotoxicity assay of CAPE in CT26 colon adenocarcinoma cells showed a dose-dependent decrease in cell viability but no significant influence on the growth of human umbilical vein epithelial cells (HUVEC). A low concentration of CAPE (1.5 $\mu$g/mL) inhibited 52.7% of capillary-like tube formation in HUVEC culture on Matrigel. CAPE (6 $\mu$g/mL)-treated CT26 cells showed not only inhibited cell invasion by 47.8% but also decreased expression of matrix metalloproteinase (MMP)-2 and -9. Vascular endothelial growth factor (VEGF) production from CT26 cells was also inhibited by treatment with CAPE (6 $\mu$g/mL). Intraperitoneal injection of CAPE (10 mg/kg/day) in BALB/c mice reduced the pulmonary metastatic capacity of CT26 cells accompanied with a decreased plasma VEGF level. CAPE treatment also prolonged the survival of mice implanted with CT26 cells. These results indicate that CAPE has potential as an antimetastatic agent.

**KEYWORDS: CAPE (caffeic acid phenethyl ester); angiogenesis; tumor invasion; metastasis; matrix metalloproteinase; human umbilical vein endothelial cell; vascular endothelial growth factor**

## INTRODUCTION

Propolis is exuded from the bark of conifer trees and carried by honeybees to their hives. Known for the variety of its beneficial effects, it has been a popular folk medicine through the ages. Caffeic acid phenethyl ester (CAPE) is a biologically active ingredient of propolis with several interesting biological properties, including antioxidant, antiinflammatory, antiviral, immunostimulatory, and carcinostatic activities (*1*−*4*). By altering oxidative processes, CAPE treatment exerts a protective effect on posterior capsule opacification (*5*), cataract formation (*6*), and corneal neovascularization (*7*) that may correlate with lower GSH level and suppressing the transformation of the lens epithelial cells. CAPE inhibits ox-LDL-mediated degradation of NF-$\kappa$B signal transduction that resulted in prevention of human coronary artery endothelial cell (*8*) and ischaemia-reperfusion injury (*9*). In chemotherapy, CAPE suppresses APC-associated intestinal carcinogenesis (*10*), and CAPE-derivative compounds are potential agents against oral cancer (*11*). In human HeLa, BEAS-2B, HL-60, and mouse ME308 cell lines assay initiated by chemical carcinogens, CAPE is chemopreventive, possibly by mitigating oxidative stress (*12*, *13*). CAPE induces apoptosis in human leukemic HL-60 cells accompanied with selective $H_2O_2$ scavenging, glutathione depletion, mitochondrial dysfunction, Bcl-2 down-regulation, Bax up-regulation, and caspase-3 activation (*14*, *15*).

Angiogenesis is a process of induction of vasculature that may contribute to the growth of tumors and the development of metastasis (*16*, *17*). The continued growth of tumors requires persistent new blood vessel formation, and inhibition of angiogenesis can result in tumor dormancy (*18*). These studies suggested that tumor cells may release several angiogenic factors, switch to an angiogenic response, resulting in tumor

* To whom correspondence should be addressed: Department of Radiation Oncology, Mackay Memorial Hospital, 92, Sec. 2, North Chung-shan Road, Taipei 10449, Taiwan. Tel: +886-2-28094661, ext 3060. Fax: +886-2-28096180. E-mail: oncoman@sinamail.com.
[†] Department of Medical Research, Mackay Memorial Hospital.
[‡] Veterans General Hospital—Taipei.
[§] National Kaohsiung Institute of Marine Technology.
[∥] National Taiwan University.
[⊥] Dayeh University.
[∞] Department of Radiation Oncology, Mackay Memorial Hospital, and Chinese Culture University.

10.1021/jf034729d CCC: $25.00 
Published on Web 11/26/2003

metastasis, disease recurrence, and reduced survival in several human malignancies (*19*−*20*).

Metastasis is the spread of cancer cells from a primary lesion to distant sites. The development of metastases is the major cause of treatment failure in cancer patients and is a principal factor in cancer mortality. The pathogenesis of metastasis is complex and consists of multiple sequential steps involving interaction of tumor cells with host factors. The process includes detachment from the primary tumor, invasion of the extracellular membrane, entry into vessels, survival in the circulation, arrest in the capillary bed, adherence to subendothelial basement membranes, entry into organ parenchyma, response to paracrine growth factors, proliferation and induction of angiogenesis, and evasion of host immune defenses (*21*, *22*).

This study was designed to examine the effect of CAPE on angiogenesis, tumor cell invasion, and metastasis using murine colon carcinoma cells for in vitro and in vivo experiments.

## MATERIALS & METHODS

**Cell and Cell Culture.** CT26 cells, *N*-nitroso-*N*-methylurethane-induced mouse undifferentiated colon carcinoma cells of BALB/c origin, purchased from the American Type Culture Collection (ATCC, Rockville, MD), were used for both in vitro and in vivo experiments. The cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT) at 37 °C in a humidified 5% $CO_2$ incubator, passaged every 2−3 days with TEG solution (0.25% trypsin, 0.1% EDTA, and 0.05% glucose in Hanks' balanced salt solution), and maintained in exponential growth. Human umbilical vein epithelial cells (HUVEC) were separated from human neonatal umbilical veins; maintained in M199 medium supplemented with 20% heat-inactivated FCS, 20 $\mu$g/mL of endothelial cell growth factor, and 50 $\mu$g/ mL of heparin; and cultured at 37 °C in a humidified 5% $CO_2$ incubator.

**Tumor Cell Viability.** Cells were cultured in a 96-well microplate ($1 \times 10^4$ cells per well in 100 $\mu$L of medium) for 12 h and then incubated with different concentrations of CAPE (0−12 $\mu$g/mL) for a further 24 h. At the end of incubation, a tetrazolium dye colorimetric test (MTT test) was used to assess cell viability, as indicated by the conversion of tetrazolium salts to a colored product, formazan, the concentration of which can be measured spectrophotometrically to calculate the $IC_{50}$ value (*23*).

**Effect of CAPE on Cell Viability of HUVEC.** HUVECs were isolated from umbilical veins after fetal delivery, cultured in 96-well microplates ($1 \times 10^4$ celle/well in 100 $\mu$L of medium) for 12 h, and then incubated with different concentrations of CAPE (0−20 $\mu$g/mL). After 24 h of incubation, MTT assay was used to evaluate cell viability.

**Matrigel Invasion Assay.** Assays of cell invasion properties were performed by using a modified Boyden chamber with poly(ethylene terephthalate) filter inserts coated with a Matrigel matrix in 24-well plates containing 8-mm pores. In brief, $10^5$ tumor cells were suspended in serum-free medium with 0.5% BSA and plated into the upper chamber followed by filling the lower chamber with the same medium with or without CAPE (0−20 $\mu$g/mL). Cells were incubated for 24 h, and then noninvading cells were gently removed and stained with the solution in a Chemicon cell invasion assay kit (ECM550, Chemicon International, Temecula, CA). Cells on the upper side of the filter were carefully removed and those cells invading the lower side were counted by microscopic examination. For quantitation, 10% acetic acid (100 $\mu$L/well) was used to dissolve stained cells, which were transferred to 96-well plates for spectrophotometric measurement at a wavelength of 560 nm.

**Tube Formation Assessment.** To determine the effect of CAPE on angiogenesis, we used an in vitro tube formation assay (*24*). HUVEC were seeded on a layer of Matrigel (Chemicon International) with or without CAPE for 24 h. The closed networks of vessel-like tubes were counted under an inverted light microscope. Incomplete networks were excluded.

**Detection of Matrix Metalloproteinases by Gelatin Zymography Analysis.** Because matrix MMP-2 and -9 proteins have gelatinase activity, their activity can be assessed by the degree of gelatin degradation shown on zymography. Analysis of gelatinolytic activity was performed by using 10% (wt/vol) polyacrylamide gel impregnated with 0.1% (wt/vol) gelatin. Total protein, 50 $\mu$g per well in conditioned media and harvested from explant cultures after 24 h of CAPE-treatment, was mixed with 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.0025% (wt/vol) bromophenol blue, and 0.5 M Tris (pH 6.8) and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Gels were washed twice in 2% (vol/vol) Triton X-100 for 30 min at room temperature to remove the SDS. They were then equilibrated with developing buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 M $CaCl_2$; pH 7.2) for 30 min at room temperature and incubated overnight with the same buffer at 37 °C. Gels were stained with 0.1% (wt/vol) Coomassie Brilliant Blue G-250 and destained in 5% acetic acid and 10% methanol in water to view zones of gelatinase activity (*25*).

**VEGF Production from Cultured CT26 Cells.** To measure concentrations of VEGF in culture medium, conditioned medium was prepared after culturing $10^6$ CT26 cells in 1 mL of serum-free RPMI 1640 medium with various concentrations of CAPE (0−6 $\mu$g/mL) for 1 and 2 days. Medium was harvested by filtration and stored at −70 °C. VEGF in the conditioned medium was quantified with an enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN).

**Pulmonary Metastasis Assay.** BALB/c male mice (6−8 weeks old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and provided water and laboratory chow ad libitum. Animals were housed in a rodent facility at 22 ± 1°C with a 12-h light−dark cycle. The mice were injected with $10^5$ CT26 tumor cells intravenously (via a tail vein) to establish pulmonary metastases. On days 1−5 after tumor challenge, the animals were treated with CAPE (10 mg/kg/day), 5-FU (10 mg/kg/day), or vehicle intraperitoneally. The numbers of mice were 7−10 per group. They were killed on day 14. Plasma was collected for VEGF assay by ELISA, and metastatic lung nodules were counted by an investigator blinded to the treatment received. For survival analysis, another group of mice was treated as described above but were not sacrificed. All experiments were performed in accordance with regulations in the NIH *Guide for the Care and Use of Laboratory Animals* (DHHS publication No. NIH 85-23, revised 1996).

**Statistical Analysis.** Results are expressed as the mean ± standard error (SE) from at least three experiments. Statistical comparisons were based on the Studant's *t*-test or analysis of variance. Differences were considered significantly at $P < 0.05$. All statistical analyses were carried out by using SPSS software (Version 8.0, SPSS Inc., Chicago, IL).

## RESULTS

**Cytotoxicity of CAPE.** The cytotoxic effect of various concentrations of CAPE (0−12 $\mu$g/mL) on tumor and normal cells is shown in **Figure 1**. The viability of CT26 colon cancer cells was decreased by CAPE in a dose-dependent manner, with an $IC_{50}$ of approximate 9 $\mu$g/mL. In contrast, the concentration of CAPE up to 12 $\mu$g/mL had no significant cytotoxic effect on normal HUVEC.

**Inhibitory Effect of CAPE against Tumor Cell Invasion.** **Figure 2a** shows dose-dependent inhibition of CT26 colon cancer cell invasion by CAPE. At a dose of 6 $\mu$g/mL, CAPE inhibited invasion by 47.8%. Moreover, CAPE treatment influenced the morphology of tumor cells, which changed from shuttle-shaped (untreated) to rounded form (treated with 6 $\mu$g/mL of CAPE) (**Figure 2b,c**).

**Inhibitory Effect of CAPE on Capillary-like Tube Formation.** Photographs in **Figure 3a** (control) and **3b** (6 $\mu$g/mL CAPE treatment) show the inhibitory effect of CAPE on the formation of capillary-like tubes by HUVEC on Matrigel culture. In **Figure 3c**, 52.7% of tubes became incomplete after treatment with low-dose (1.5 $\mu$g/mL) CAPE. At a concentration of 12 $\mu$g/mL, CAPE had little influence on HUVEC growth (**Figure 1**), but treatment resulted in 94.6% degradation of tube formation (**Figure 3c**).

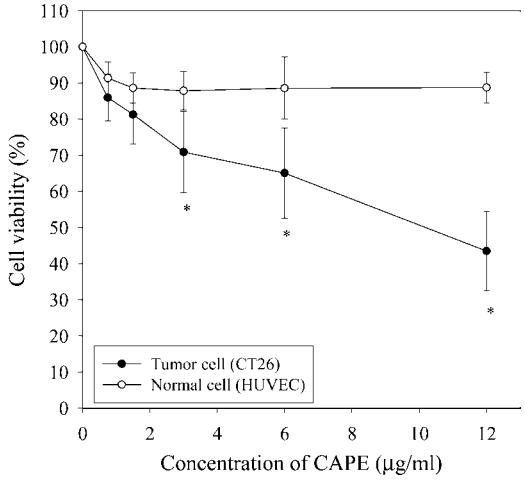
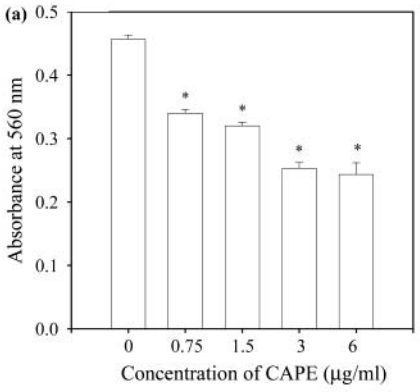



**Figure 1.** Cytotoxicity of CAPE on CT26 colon cancer cells and normal HUVECs. $10^4$ cells per well of CT26 cells (closed circle) and HUVECs (open circle) were plated in 96-well culture plates with 100 $\mu$L of culture medium. Twelve hours later, various concentrations of CAPE (0–12 $\mu$g/mL) were added to each well. After 24 h for further culture cytotoxicity was determined by MTT assay. Data from three separate experiments are expressed as mean ± SE. *Significant change compared with untreated control.



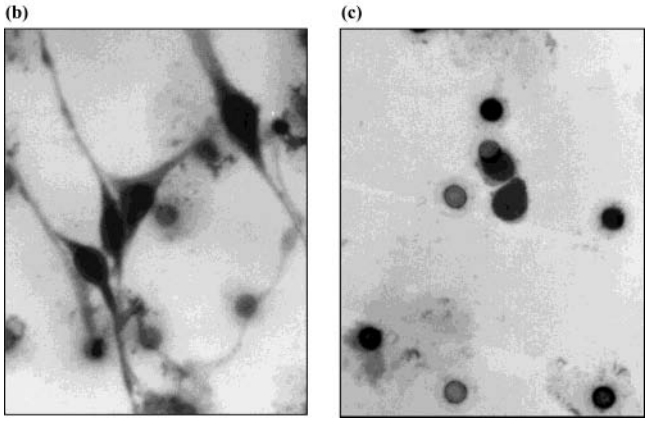




**Figure 2.** Inhibitory effect of CAPE on CT26 colon adenocarcinoma cell invasion in vitro. CT26 cells were seeded on ECM layer with various concentrations of CAPE (0 to 6 $\mu$g/mL), stained, and photographed (400×). (**a**) Absorbance by colorimetric reading at 560 nm for cell number estimation. (**b**) Morphological observation of untreated CT26 cells. (**c**) Morphological observation of CAPE (6 $\mu$g/mL)-treated CT26 cells. Data are shown as mean ± SE of at least three determinations. *Significant change compared with untreated control.

**Inhibitory Effect of CAPE on Matrix Metalloproteinase Activity.** As shown in **Figure 4**, MMP-9 and -2 are found at

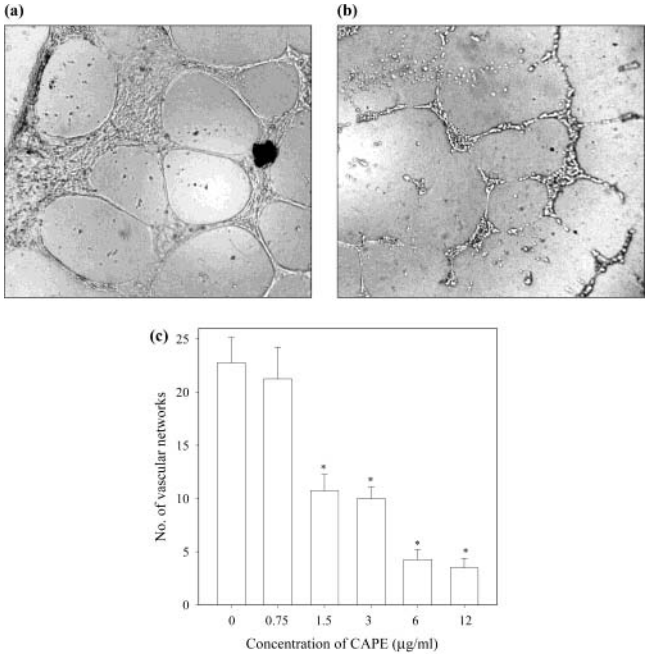



**Figure 3.** Inhibitory effect of CAPE on HUVEC tube formation in vitro. (**a**) HUVECs were seeded for 24 h to allow tube formation (control). (**b**) HUVECs were seeded with 6 $\mu$g/mL CAPE to allow tube formation. (**c**) HUVECs were seeded with various concentrations of CAPE (0–12 $\mu$g/mL), and the numbers of networks were counted under a microscope (200×). Results from four independent experiments are shown as mean ± SE. *Significant change compared with untreated controls.

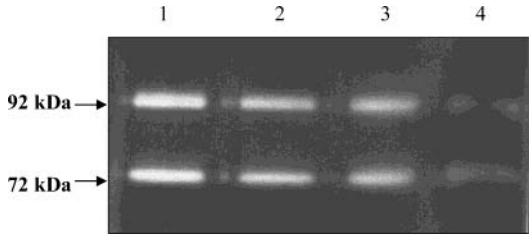



**Figure 4.** Detection of matrix metallopreteinase (MMP) activity in conditioned media from the culture of CT26 cells with or without CAPE treatment, determined by gelatin zymography. Conditioned medium was harvested from CAPE-treated CT26 cells, and the activity of MMP-2 (72 kDa) and MMP-9 (92 kDa) was assessed. Lane 1, untreated; lane 2, 1.5$\mu$g/mL of CAPE; lane 3, 3 $\mu$g/mL of CAPE; lane 4, 6 $\mu$g/mL of CAPE.

92 and 72 kDa, respectively, on SDS−PAGE. At a concentration of 6 $\mu$g/mL, CAPE effectively inhibited CT26 cells from secreting MMP-2 and -9, indicating that the inhibitory effect of CAPE occurs by down-regulating their expression in CT26 cells.

**Effect of CAPE on VEGF Secretion.** As shown in **Figure 5a**, CAPE at various concentrations (0−6 $\mu$g/mL) reduced the production of VEGF in CT26 cell culture (1 and 2 days) in a dose-dependent manner. In an animal model, plasma VEGF levels in untreated CT26 cell-bearing BALB/c mice was 257.7 ± 53.8 pg/mL (**Figure 5b**). However, treatment with 5-FU and CAPE both reduced plasma VEGF levels, 35.5% and 53.2%, respectively ($n$ = 7−10 per group).

**In Vivo Assay of CAPE.** After injection with $10^5$ murine CT26 colon adenocarcinoma cells, all untreated animals developed multiple lung metastases. As shown in **Table 1**, daily intraperitoneal injection of CAPE at a dose of 10 mg/kg/day decreased tumor colonization in the lung, and did so more effectively than 5-FU (10 mg/kg/day). Moreover, CT26-bearing

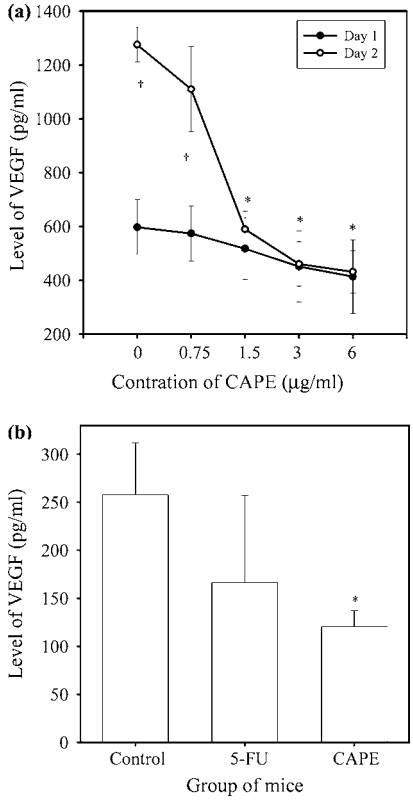



**Figure 5.** Levels of vascular endothelial growth factor (VEGF) in vitro and in vivo. (**a**) VEGF levels contained in conditioned medium from culture of CT26 colon cancer cells with the addition of various concentrations of CAPE (0–6 $\mu$g/mL) for 1 and 2 days. (**b**) VEGF levels detected in plasma of controls and 5-FU- and CAPE-treated mice using ELISA ($n = 7$–10 per group). Results are shown as mean ± SE of at least three determinations. *Significant change compared with untreated controsl. †Significant change when day 2 data were compared with day 1 data at the same concentration.

**Table 1.** Inhibitory Effect of CAPE on Pulmonary Metastasis

| group of mice[a] | no. of metastatic foci |
|---|---|
| control | 173 ± 35 |
| 5-FU (10 mg/kg/day, ip) | 78 ± 45 |
| CAPE (10 mg/kg/day, ip) | 36 ± 23*,# |

[a] BALB/c mice after iv injection of $2 \times 10^5$ CT26 colon cancer cells were treated daily with 10 mg/kg/day of CAPE, 5-FU, or vehicle for five consecutive days ($n = 7$–10 per group). *Significant change compared with control group, and #significant change compared with 5-FU group by one-way ANOVA followed by the Student–Newma–Keuls method.

mice treated with CAPE or 5-FU survived longer than untreated controls (**Figure 6**).

## DISCUSSION

We have previously reported that CAPE induced apoptosis associated with selective scavenging of hydrogen peroxide (*14*), accompanied by regulation of caspase-3, Bcl-2, and Bax (*15*). An antiinflammatory effect of CAPE has been suggested related to antiangiogenesis in chick embryo chorioallantoic membrane assay (*26*). In this study, CAPE not only eliminated secretion of VEGF in a culture of CT26 colon cancer cells but also reduced the level of VEGF in plasma of tumor-bearing mice. Tumor and normal cells secrete angiogenic growth factors (such as VEGF) that bind to specific receptors on endothelial cells and mediated angiogenesis with selective mitogenic activity (*19, 20*). The ligand−receptor interaction leads to endothelial cell proliferation, migration, invasion, and, eventually, angiogenesis (*27*). The inhibitory effect of CAPE on VEGF production may be an important way to inhibit angiogenesis and tumor invasion.

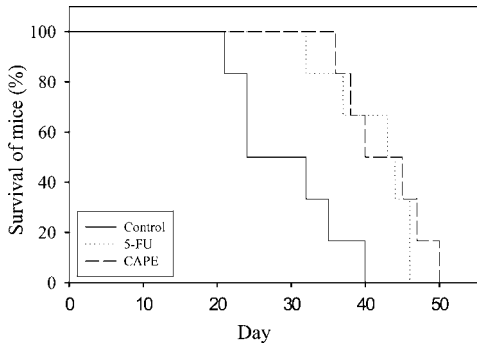



**Figure 6.** Survival of CT26 colon cancer-bearing mice intraperitoneally injected for five consecutive days with CAPE (10 mg/kg/day), 5-FU (10 mg/kg/day), or vehicle. CT26 colon adenocarcinoma cells ($10^5$) were injected via tail vein.

Interaction between tumor cells and the basement membrane is a critical event for tumor invasion that signals initiation of the metastatic cascade (*28, 29*). Basement membranes are composed of collagen, laminin, and heparin sulfate proteoglycans. Several investigators stated the three-step hypothesis of tumor cell invasion: tumor cell attachment to the basement membrane, creation of proteolytic defects in the basement membrane, and migration of tumor cells through these defects (*28, 29*). Matrix proteolytic enzymes, such as matrixins or MMPs, are considered to play an important role in tumor cell invasion and subsequent spread from the site of origin (*30*). Overexpression of MMP in tumor cells has been suggested by a correlation between MMP activity in the primary tumor and metastasis (*31, 32*). Two members of this protein family, MMP-2 and -9, have gelatinase activity, and inhibition of these proteins is a key point to stop the extracellular matrix (ECM) degradation that mediates tumor cell invasion (*33*). In this study, CAPE inhibited CT26 colon cancer cell secretion of MMP-2 and MMP-9, which may be the mechanism by which it prevents tumor cell invasion and metastasis. Besides, the morphological changes in CAPE-treated CT26 cells relate to changes in cell adhesion. Weyant et al. reported that CAPE reduced cell invasion by modulation of integrin-mediated signaling pathways and inhibition of focal adhesion kinase (FAK) (*34*). Further investigation of CAPE on adhesion molecule expression will be undertaken to clarify the relation between adhesion and antimetastatic effect.

In this study, CAPE had significant cytotoxicity on CT26 tumor cells but not on normal HUVECs, implying a differential activity of CAPE. Several researchers reported that short exposure to CAPE caused a significant drop in intracellular glutathione levels in tumorigenic cells but not in normal cells (*6, 35*), indicating that CAPE might be a pro-oxidant for cancer cells and an antioxidant for normal cells. Therefore, we postulate that CAPE might affect metastatic processes of tumor cells via redox imbalance without significant toxicity in normal tissue.

In patients with colon cancer, 5-FU is a standard chemotherapeutic agent. Our in vivo experiments show that CAPE was more effective than 5-FU in inhibiting pulmonary metastases from CT26 colon cancer cells. The exact mechanism of this antimetastatic activity of CAPE is as yet unknown. It could be due to a combination of any of the effects we have demonstrated in this study, including antiangiogenesis, selective cytotoxicity on tumor cells, inhibition of tumor cell invasion,

reduction of MMP activity in tumor cells, destruction of capillary-like tube formation, and elimination of VEGF level in vitro and in vivo. There are only few effective antimetastatic chemotherapeutic agents currently available for clinical use, and most have life-threatening adverse effects. It is concluded that CAPE might be a novel antimetastatic and antiangiogenic agent with low toxicity for normal cells.

## SAFETY CONSIDERATION

There is no significant toxicity of CAPE reported in the literature (*36*). The only safety consideration involves the flammable tendency of ethanol, the solvent we used for CAPE.

## ABBREVIATIONS USED

BSA, bovine serum albumine; CAPE, caffeic acid phenethyl ester; ECM, extracellular matrix; FCS, fetal calf serum; 5-FU, 5-fluorouracil; HUVEC, human umbilical vein epithelial cells; MMP, matrix metalloproteinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor; SDS, sodium dodecyl sulfate; SE, standard error.

## ACKNOWLEDGMENT

The authors wish to thank Dr. Mary Jeanne Buttrey for critical reading and correction of the manuscript.

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**Received for review July 6, 2003. Revised manuscript received October 22, 2003. Accepted October 27, 2003. This study was supported by grant MMH-9336 from Mackay Memorial Hospital and NSC 92-2311-B-195-002 from the National Science Council in Republic of China.**

JF034729D