# Inhibitory Effects of Caffeic Acid Phenethyl Ester Analogues on Experimental Lung Metastasis of Murine Colon 26-L5 Carcinoma Cells

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**We have previously examined the antiproliferative activity of caffeic acid phenethyl ester (CAPE) and its 20 analogues against six tumor cell lines, and found that CAPE analogues possess selective antiproliferative activity toward the murine colon 26-L5 carcinoma cell line. To extend our study, the effects of CAPE analogues on the metastatic development of murine colon 26-L5 carcinoma cells in the lung were examined. The oral administration of CAPE (5 mg/mice/d) for 7 d after tumor inoculation decreased the tumor weight and the number of tumor nodules in the lung by 50% and 50%, respectively, compared to the control, while CAPE (5 mg/mice/d) administered for 7 d before tumor inoculation showed no significant effect. Besides CAPE, 4-phenylbutyl caffeate, 8-phenyl-7-octenyl caffeate, 2-*cyclo*hexylethyl caffeate and *n*-octyl caffeate at an oral dose of 2 mg/mice/d caused a 55%, 43%, 55% and 35% reduction of the tumor nodules in their lung metastasis formation, respectively. These results further elaborate the possibility of CAPE and its analogues to become a new class of chemopreventive agents for the treatment of colon cancer metastasis.**

**Key words** caffeic acid phenethyl ester; lung metastasis; colon 26-L5 carcinoma

Tumor metastasis is a major cause of death in cancer patients. Tumor invasion is an important step in the sequential process of metastasis, and is subdivided into 3 steps including tumor cell adhesion, migration and enzymatic degradation of the extracellular matrix and basement membrane. Therefore, many attempts have been made to discover promising agents with anti-metastatic activity.

Caffeic acid phenethyl ester (CAPE; **1**, Fig. 1) is a well known constituent of European propolis, which possesses various biological properties including antimicrobial,[1] anti-inflammatory,[2] antioxidant[3] and antitumor activities.[4—10] Besides these activities, CAPE (**1**) also possesses enzyme inhibitory activities against HIV-1 integrase,[11,12] ornithine decarboxylase,[13] cyclooxygenase[14] and lipoxygenase.[15] In our recent work, we have isolated CAPE (**1**), together with benzyl caffeate and cinnamyl caffeate, from Netherlands propolis, as potent antiproliferative agents.[16] Moreover, we synthesized 21 analogues of caffeic acid esters and then tested their antiproliferative activity against six tumor cell lines.[17] They showed selective and strong antiproliferative activity toward the murine colon 26-L5 carcinoma cell line. The CAPE analogues are mainly divided into four different groups according to the nature of their alcoholic part: (1) esters having an alkyl group with a phenyl group at the end of the alkyl chain, (2) esters having an alkyl group with a styryl group at the end of the alkyl chain, (3) an ester having an alkyl group with a cyclohexyl group at the end of the alkyl chain, and (4) esters having a straight alkyl chain. To extend our study, we further examined the effect of CAPE analogues on the metastatic development of murine colon 26-L5 carcinoma cells in the lung. For this purpose, we selected compounds **2**—**5**, having the strongest antiproliferative activity in each group ($EC_{50}$ values, 0.02—0.22 $\mu$M), and compared their activities with that of CAPE (**1**). In this paper, we report the anti-metastatic activities of these CAPE analogues.

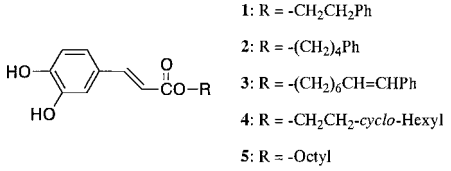


Fig. 1. Structure of CAPE Analogues

## MATERIALS AND METHODS

**Materials** CDDP (*cis*-diamine-dichloroplatinum; Randa®, 0.5 mg/ml) was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). RPMI-1640 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Heat-inactivated fetal calf serum (FCS) and trypsin were from Gibco BRL Products (Gaitherburg, MD, U.S.A.). A cell culture flask (150, 75 $cm^2$) coated with polystyrene (Corning Incorporated, Corning, NY, U.S.A.) was used to culture the cells. EDTA and Tween-20 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), glutamine, sodium bicarbonate and corn oil were from Wako Pure Chemical Industries (Osaka, Japan). Caffeic acid esters (**1**—**5**) were synthesized by esterification of caffeic acid chloride with corresponding alcohol, as describe in our previous report, and were pure in TLC and $^1$H-NMR spectral observation.[17]

**Cell Lines and Cell Culture** The murine colon 26-L5 carcinoma cell line was established by one of the authors (I. Saiki).[18] Murine colon 26-L5 carcinoma cells were cultured in RPMI-1640 medium, supplemented with 0.1% sodium bicarbonate and 2 mM glutamine and 10% fetal calf serum. The cells were propagated at 37 °C in a humidified atmosphere containing 5% $CO_2$.

**Animals** Inbred 6-week-old female Balb/c mice were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. All mice were housed in a controlled environment with a 12 h light/dark cycle, a temperature of 24±2 °C and a humidity of 55±10%, and they were given a laboratory pellet chow (Labo MR, Nosan, Kanagawa, Japan) and tap water *ad libitum*. This study was conducted in accordance

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with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

**Experimental Lung Metastasis Assay** Log-phase cell cultures of colon 26-L5 cells were harvested with 1 mM EDTA in phosphate-buffered saline (PBS), washed three times with serum-free RPMI-1640, and resuspended at $2\times10^4$ cells/200 $\mu$l concentration in serum-free medium, then 200 $\mu$l of the cell suspension was injected intravenously into the mice. The mice were then divided randomly into different groups of 6 mice per group. The CAPE analogues **1**—**5** were suspended in corn oil/Tween 20/water (6/1/13, w/w) and administered orally by gastric tube to mice at the appropriate dose for 7 d before or after tumor inoculation. CDDP was injected intravenously at the dose of 80 $\mu$g/mice on the 1st and 8th days after tumor inoculation. At the 15th day after tumor inoculation, the mice were sacrificed under an anesthetic condition and their lungs were removed. The weight of the lung was taken immediately after washing with distilled water and drying on tissue paper. The tumor nodules in the lungs were counted under a dissection microscope. The tumor weight was calculated by subtracting the lung weight of the normal mice group (200 $\mu$l serum free medium inoculated group) from that of colon 26-L5 cell treated group.

**Statistical Analysis** All data are expressed as the mean±S.D. Student's *t*-test for unpaired observations between control and tested samples was carried out to identify statistical differences; *p* values of less than 0.05 were considered significantly different.

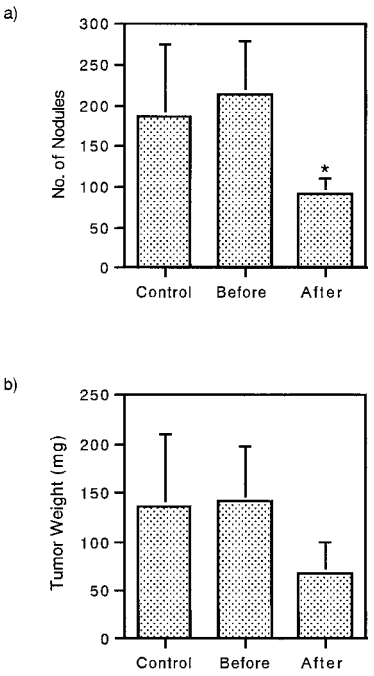


Fig. 2. Effect of Before or After Administration of CAPE (**1**) on Colon 26-L5 Carcinoma Cell Inoculation in Experimental Lung Metastasis Formation

CAPE (**1**) was administered orally daily for 7 d before or after tumor inoculation into mice. At the 15th day after tumor inoculation, the number of nodules on the surface of the lungs was counted (a), and tumor weight calculated (each group average lung weight−normal group average lung weight) (b). The data are expressed as mean±S.D. of 6 mice. $*p<0.05$.

## RESULTS

**Effect of CAPE Before or After Cell Inoculation on Lung Metastasis Formation** Initially, we investigated the effect of CAPE (**1**, 5 mg/mice/d), by oral administration before or after inoculation of colon 26-L5 carcinoma cells, on the experimental lung metastasis model. Mice administered CAPE (**1**) before cell inoculation did not show any suppression of tumor formation. In contrast, mice administered CAPE (**1**) after cell inoculation showed a decrease in their lung tumor formation; the tumor weight was reduced by 50% and the number of nodules by 50% (Fig. 2). Thus, the anti-metastatic effect of CAPE (**1**) might be due to cytotoxicity or growth inhibition against tumor cells, or due to blocking of the invasion process, an initial step of lung metastasis.

**Effect of CAPE at Various Doses** We further examined the effect of CAPE (**1**) on the same experimental model at different doses, or of CDDP, a well-known anticancer drug having strong anti-metastatic properties toward lung metastatsis.[19] Oral administration of CAPE (**1**) at 5 and 2 mg/mice/d did not produce any significant difference in suppressive effect on the formation of lung tumor (Figs. 3a, b). The tumor weight, as well as the number of nodules, was reduced by 30—40% at both doses, while CDDP suppressed tumor weight by 89% and the number of nodules by 72%. However, CDDP injection caused a significant reduction in body weight, *i.e.*, a toxic effect, but CAPE (**1**) at 5 mg/mice/d had little effect on body weight (Fig. 3c). These results suggest that CAPE (**1**) could suppress tumor metastasis without side effects. On the other hand, there was no significant anti-metastatic effect of CAPE (**1**, 5 mg/mice/d) on lung metastasis by murine B16-BL6 melanoma cells[20] (data not shown), which led to the conclusion that CAPE (**1**) selectively inhibits the metastasis of colon 26-L5 carcinoma cells.

**Effect of CAPE Analogues** In our previous *in vitro* study, we observed that 4-phenylbutyl caffeate (**2**), 8-phenyl-7-octenyl caffeate (**3**), 2-*cyclo*hexylethyl caffeate (**4**) and *n*-octyl caffeate (**5**) (Fig. 1) had potent antiproliferative activity toward the murine colon 26-L5 carcinoma cell line.[17] Thus, these compounds **2**—**5** were subjected to an *in vivo* experiment at a dose of 2 mg/mice/d. Ester **2** showed the highest degree of suppressive effect on the reduction of the number of nodules by 55% (Fig. 4), but there was no significant reduction in tumor weight, even it was reduced by 58%. Similarly, ester **4** possessed stronger inhibitory activity than CAPE (**1**) at 2 mg/mice/d. On the other hand, ester **3** and **5** possessed inhibitory activities a little weak but identical with CAPE (**1**).

## DISCUSSION

The results indicate that CAPE and its analogues possess anti-metastatic properties. CAPE was previously reported to be a strong antioxidant, to inhibit soybean 15-lipoxygenase, and to completely block the production of reactive oxygen species (ROS) in human neutrophils and in the cell-free xanthine/xanthine oxidase (XOD) system.[21] These antioxidative properties of CAPE are considered important factors in the protection of cancer formation in mice bearing an *Apc* gene.[8] CAPE at a dietary level of 0.15% decreased tumor formation in C57BL/6J-Min/+ mice bearing germline mutation in the *Apc* gene.[8] The author concluded that the tumor

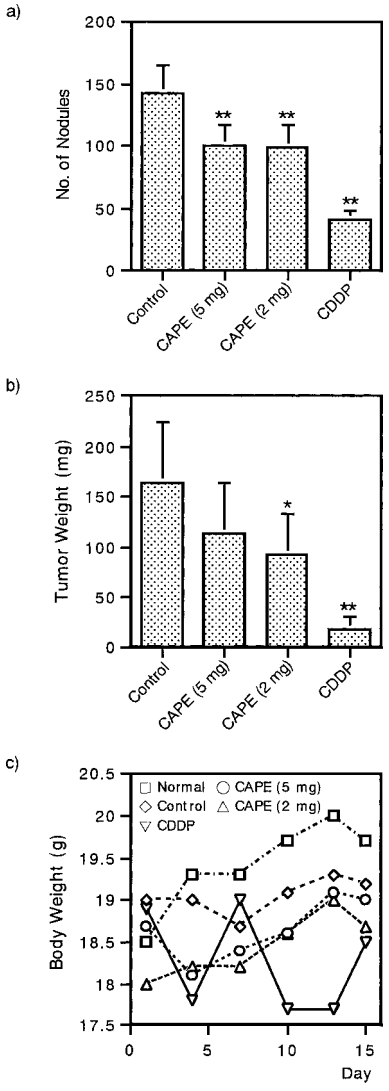


Fig. 3. Effect of CAPE (**1**) at Various Doses

CAPE (**1**) was administered orally daily for 7 d after tumor inoculation into mice. CDDP was injected intravenously into mice on day 1 and day 8. At the 15th day after tumor inoculation, the number of nodules on the surface of the lungs was counted (a), tumor weight calculated (each group average lung weight−normal group average lung weight) (b), and mean body weight of the mice measured during the experiment (c). The data are expressed as mean±S.D. of 6 mice. $*p<0.05$, $**p<0.01$.

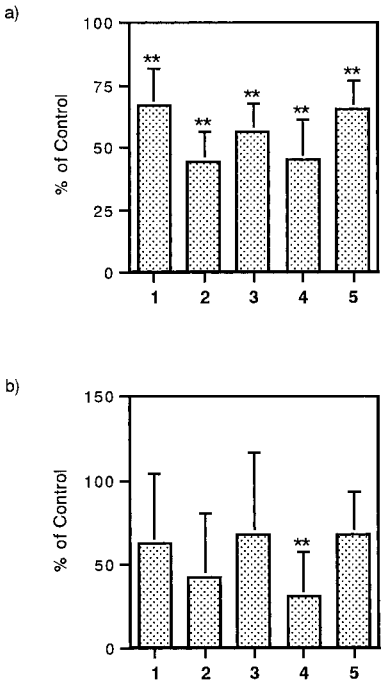


Fig. 4. Effect of CAPE (**1**) Analogues **2**—**5**

CAPE (**1**) and its analogues **2**—**5** were administered orally daily for 7 d after tumor inoculation into mice. At the 15th day after tumor inoculation, the number of nodules on the surface of the lungs was counted and expressed as a % of the control (a), then tumor weight was calculated (each group average lung weight−normal group average lung weight) and expressed as a % of the control (b). The data are expressed as mean±S.D. of 6 mice. $**p<0.01$.

prevention by CAPE was associated with increased enterocyte apoptosis and proliferation closely associated with ROS.[8] In our previous report, we also observed that CAPE inhibits the proliferation of the colon 26-L5 carcinoma cell line *via* apoptosis.[17] Moreover, it has been well documented that ROS are associated with tumor promotion. ROS are thought to act as second messengers for signal transduction pathways that regulate cell proliferation,[22] and by reducing intracellular peroxides, antioxidants may expect to inhibit carcinogenesis. We further tested the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of CAPE and its analogues. All these compounds possessed an equal strength of DPPH free radical scavenging activity ($EC_{50}$, 5.20—6.99 $\mu$M) to that of caffeic acid ($EC_{50}$, 4.83 $\mu$M). Thus, it may be partly due to its antioxidative property that CAPE and its analogues show anti-metastatic activity.

Besides the antioxidative action of CAPE, CAPE also showed inhibition of the tumor promoter 12-*O*-tetradecanoylphorbol-13-*O*-acetate (TPA)-induced carcinogenesis associated with the initiation of cancer.[7] We, for the first time, have demonstrated that CAPE and its analogues show anti-metastatic activity against lung metastasis of murine colon 26-L5 carcinoma cells in mice. CAPE was previously reported to inhibit the invasion of human colon carcinoma cells through affecting focal adhesion kinase (FAK) expression or tyrosine phosphorylation.[10] Moreover, CAPE was reported to have the ability to restore a deficiency in gap junctional intercellular communication (GJIC), which is shown to be a characteristic of cancer cells.[23] Though the present study alone is unable to explain the exact mechanism, considering previous reports, CAPE and its analogues may suppress tumor formation in colon 26-L5 inoculated lung metastasis in mice partly by inhibiting the invasion of the colon 26-L5 cells or by restoring GJIC.

In conclusion, oral administration of CAPE (5 mg/mice) for 1 week after tumor inoculation significantly decreased the tumor weight and the number of nodules in the lung. Besides CAPE, 4-phenylbutyl caffeate, 8-phenyl-7-octenyl caffeate, 2-cyclohexylethyl caffeate and *n*-octyl caffeate also significantly reduced the tumor nodules in their lung metastasis formation. These results further elaborate the possibility of CAPE and its analogues as a new class of chemopreventive agents for the treatment of colon cancer metastasis.

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