Growth inhibition of human breast and prostate cancer cells by cinnamic acid derivatives and their mechanism of action

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SUMMARY

Cancer is the leading cause of death and there is a particularly pressing need to develop effective treatments for breast and prostate cancer. In the current study, we show the inhibitory effects of cinnamic acid derivatives, including caffeic acid phenethyl ester (CAPE, 1), on the growth of breast and prostate cancer cells. Among the compounds examined, 3,4,5-trihydroxycinnamic acid decyl ester (6) showed the most potent inhibition of cancer cell growth by the induction of apoptosis. Compound 6 could be a new anti-cancer agent for use against breast and prostate cancer.

Key words: cinnamic acid; caffeic acid phenethyl ester; breast cancer; prostate cancer; apoptosis
INTRODUCTION

Caffeic acid serves as a key intermediate in lignin biosynthesis. Caffeic acid phenethyl ester (CAPE) (1, Fig. 1), is a naturally occurring lipophilic derivative of caffeic acid, which is found in many plants, particularly in propolis of the honeybee hive. Compound 1 has been known to exhibit numerous biological activities, including anti-oxidant,1) anti-inflammatory,2) anti-viral,3) and immunostimulatory4) properties. Compound 1 can display antioxidant effects by blocking production of reactive oxygen species.5) It also exhibits anti-inflammatory properties through the down-regulation of prostaglandin and leukotriene synthesis by inhibiting cyclooxygenase.6)

We have previously examined the effects of the cinnamic acid derivatives 3,4-dihydroxycinnamic acid 6-phenylhexyl ester (3), 3,4-dihydroxycinnamic acid decyl ester (5) and 3,4,5-trihydroxycinnamic acid decyl ester (6) (Fig. 1) on pancreatic lipase activity, lipid absorption and lipid accumulation during 3T3-L1 cell differentiation.7) We found that 1, 3, 5, and 6 inhibit pancreatic lipase activity in vitro, and that 5 and 6 suppress increases in blood triglyceride (TG) levels in mice administered corn oil orally. We attribute these latter effects to inhibition of pancreatic lipase activity. In addition, 3, 5, and 6 prevent 3T3-L1 differentiation (lipid accumulation) at low concentrations. Compound 6, which represents a new agent, was the most potent compound, inhibiting lipid accumulation to a much greater extent than the parent compound 1.

Breast and prostate cancer are estimated to lead the incidence of new cases of female and male cancer in United States of America.8) In addition, breast and prostate cancer are the second leading cause of cancer deaths in the United States.8) There is a pressing need to develop new effective treatments. In this study, we have investigated the influence of CAPE and cinnamic acid derivatives on the proliferation of breast and prostate cancer cells.
MATERIALS AND METHODS

**Chemicals**  Ethylenediaminetetraacetic acid (EDTA), and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO). All other chemicals were of reagent grade. The synthesis of esters 1, 4-hydroxycinnamic acid 2-phenylethyl ester (2), 3, 4-hydroxycinnamic acid 6-phenylhexyl ester (4), 5, 6, 3-hydroxycinnamic acid decyl ester (7), and 4-hydroxycinnamic acid decyl ester (8) were as described previously.7)

**Cell lines and culture conditions** MCF-7 and PC-3 cells were grown in RPMI 1640 medium containing 10% FBS and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Invitrogen, Carlsbad, CA, USA). Attached cells were removed with trypsin-EDTA (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

**Cell growth** Cells were trypsinized and suspended in appropriate medium containing 10% FBS. Cells (4 x 10⁴ cells/mL) were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. After 1 d, various concentrations of chemicals were added to the cultures. Controls were prepared by treating cells with culture medium containing 0.1% DMSO without other chemicals. Cells were incubated for 72 h, and then viable cell number was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.9) Values for percent net cell growth were calculated with the following formula: [(absorbance of experimental cell concentration) – (absorbance of initial cell concentration)/(absorbance of control cell concentration) – (absorbance of initial cell concentration)] x 100.

**Flow Cytometry** MCF-7 and PC-3 cells (4×10⁴ cells/mL) were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. After 24 h, cells were treated with various concentrations of 6 or DMSO for 48 h. Cells were harvested and fixed in 70% ethanol at 4 °C overnight. Before the analysis, cells were washed with PBS twice, treated with 100 μg/mL
RNase A at 37 °C for 30 min, and then stained with 10 µg/mL propidium iodide (PI). Cell cycle analysis was performed using a FACSVerse flow cytometer (Becton Dickinson, San Jose, CA, USA).

Caspase-3 assay For measurement of caspase-3 activity in cellular lysates, the Caspase-3/CPP32 Colorimetric Assay Kit (MBL, Nagoya, Japan) was used according to the manufacturer’s instructions.

RNA isolation and RT-PCR Total RNA was extracted from cells treated with compounds using an ISOGEN RNA extraction kit (Nippon Gene, Toyama, Japan). cDNA was synthesized from 0.5 µg of RNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer’s instructions. RT-PCR was done according to the instructions of the manufacturer using Blend Taq® (TOYOBO; Osaka, Japan). The reactions were amplified with specific primers for human bax (166 bp: sense 5’-ATG CGT CCA CCA AGA AGC TGA G-3’, antisense: 5’- CCC CAG TTG AAG TTG CCA TCA G-3’), human bcl-2 (293 bp: sense 5’-TGC ACC TGA CGC CCT TCA C-3’, antisense 5’-AGA CAG CCA GGA GAA ATC AAA CAG-3’), and β-actin (202 bp: sense 5’-CCT TCC TGG GCA TGG AGT CCT G-3’, antisense: 5’-GGA GCA ATG ATC TTG ATC TTC-3’). The PCR products were separated by electrophoresis in 2% agarose gel and visualized.

Statistical analysis and presentation of results The data are expressed as the mean ± standard deviation (SD). Data were analyzed using Prism version 6. Statistical significance was assessed using one-way ANOVA followed by Dunnett’s multiple comparisons test or Student’s t test. *p<0.05, **p<0.01, and ***p<0.001 vs. control.
RESULTS AND DISCUSSION

Inhibitory effects of cinnamic acid derivatives on cancer cell growth

The effects of compounds 1-8 on the growth of cancer cells were examined using a MTT assay. The growth of breast cancer MCF-7 cells was inhibited approximately 14%, 12%, 41%, and 12%, by 1, 2, 3, and 4, at 4 µM, respectively (Fig. 2A). At 4 µM concentration, compounds 6, 5, 7, and 8 inhibited the growth of MCF-7 cells approximately 98%, 58%, 6%, and 9%, respectively (Fig. 2B). Furthermore, at 4 µM concentration, the growth of PC-3 prostate cancer cells was inhibited approximately 62%, 9%, 80%, and 20%, by 1, 2, 3, and 4, respectively (Fig. 2C). Compounds 6, 5, 7, and 8 inhibited cell growth approximately 94%, 88%, 18%, and 28%, respectively (Fig. 2D). The newly synthesized compounds 3, 5, and 6 were more effective than 1 against both MCF-7 and PC-3 cells. These results suggest that 3, 5, and 6 inhibit the growth of breast and prostate cancer cells to a greater extent than the parent compound 1 without showing cytotoxicity.

Inhibitory effects of cinnamic acid phenethyl ester and phenylhexyl ester derivatives on cancer cell growth

Next, we examined the dose dependency on cell growth inhibition by cinnamic acid derivatives. In MCF-7 cells, 1 significantly inhibited cell growth in dose-dependent manner; 1 µM: 8.9%, 2 µM: 11.6%, 4 µM: 21.3%, 8 µM: 42.7%, and 10 µM: 52.3% (Fig. 3A). In contrast, 2, which has one less hydroxyl group than 1, inhibited cell growth to a lesser extent (1 µM: 2.7%, 2 µM: 10.4%, 4 µM: 10.2%, 8 µM: 10.2%, and 10 µM: -0.9%) (Fig. 3B). Compound 3 also inhibited cell growth in a dose-dependent manner; 1 µM: 12.4%, 2 µM: 24.7%, 4 µM: 57.0%, 8 µM: 82.2%, and 10 µM: 86.0% (Fig. 3C), while compound 4, which
has one less hydroxyl group than 3, inhibited cell growth only slightly (1 µM: 10.7%, 2 µM: 12.4%, 4 µM: 22.9%, 8 µM: 26.9%, and 10 µM: 20.5%) (Fig. 3D).

Against PC-3 cells, 1 significantly inhibited cell growth in a dose-dependent manner (1 µM: 37.5%, 2 µM: 50.7%, 4 µM: 65.4%, 8 µM: 81.7%, and 10 µM: 81.8%) (Fig. 4A), while 2 inhibited cell growth only slightly (1 µM: 12.7%, 2 µM: 11.5%, 4 µM: 13.5%, 8 µM: 11.9%, and 10 µM: 12.8%) (Fig. 4B). In contrast, 3 showed dose-dependent inhibition of cell growth (1 µM: 36.1%, 2 µM: 59.3%, 4 µM: 85.3%, 8 µM: 92.7%, and 10 µM: 91.4%) (Fig. 4C), while 4 inhibited cell growth slightly (1 µM: 19.2%, 2 µM: 20.3%, 4 µM: 31.1%, 8 µM: 31.9%, and 10 µM: 27.8%) (Fig. 4D). These results suggested that caffeic acid derivatives (1 and 3) having two hydroxyl groups were more effective in MCF-7 and PC-3 cells than coumaric acid derivatives (2 and 4) having one hydroxyl group.

Inhibitory effects of cinnamic acid decyl ester derivatives on cancer cell growth

Next, we examined the effects of cinnamic acid decyl ester derivatives having two (5), three (6), and one (7, 8) aromatic hydroxyl groups. In MCF-7 cells, 5 inhibited cell growth in dose-dependent manner (0.4 µM: 9.3%, 0.8 µM: 13.6%, 1 µM: 19.0%, 2 µM: 41.3%, and 4 µM: 70.6%) (Fig. 5A). Compound 6, which has one more hydroxyl group than 5, inhibited cell growth more than 5 in a dose-dependent fashion (0.4 µM: 18.8%, 0.8 µM: 35.8%, 1 µM: 44.9%, 2 µM: 77.7%, and 4 µM: 98.7%) (Fig. 5B). In contrast, compound 7, which has one less hydroxyl group than 5, at the R₁ position, inhibited cell growth only slightly (1 µM: 9.4%, 2 µM: 10.0%, 4 µM: 15.5%, 8 µM: 18.8%, and 10 µM: 13.6%) (Fig. 5C). In addition, 8, which has one less hydroxyl group than 5, at the R₂ position, also inhibits cell growth only slightly (1 µM: 9.4%, 2 µM: 12.1%, 4 µM: 14.1%, 8 µM: 26.0%, and 10 µM: 27.1%) (Fig. 5D). The inhibitory effects on cell growth by compounds 7 and 8 were less than by 5 and 6.

Against PC-3 cells, 5 significantly inhibited cell growth in dose-dependent manner
Compound 6 inhibited cell growth more potently than 5 in a dose-dependent fashion (0.4 µM: 29.1%, 0.8 µM: 48.3%, 1 µM: 54.9%, 2 µM: 76.5%, and 4 µM: 91.9%) (Fig. 6A). Both compounds 7 (1 µM: 12.9%, 2 µM: 16.2%, 4 µM: 23.7%, 8 µM: 27.2%, and 10 µM: 26.1%) (Fig. 6C) and 8 (1 µM: 23.2%, 2 µM: 24.3%, 4 µM: 28.4%, 8 µM: 35.3%, and 10 µM: 33.6%) (Fig. 6D) inhibited cell growth only slightly. These results suggest that tri-hydroxycinnamic acid derivatives (6) are more effective than mono-hydroxycinnamic acid (7 and 8) and di-hydroxycinnamic acid (5) derivatives in both MCF-7 and PC-3 cells.

**Summary of the IC₅₀ values of cinnamic acid derivatives for cancer cell growth inhibition**

Table 1 shows the IC₅₀ values of mono-hydroxycinnamic acid derivatives (2, 4, 7, and 8), di-hydroxycinnamic acid derivatives (1, 3, and 5), and tri-hydroxycinnamic acid derivative (6). The IC₅₀ values of CAPE (1) were 8 µM in MCF-7 cells and 1.2 µM in PC-3 cells. Compounds 3, 5, and 6 showed lower IC₅₀ values than 1 against both MCF-7 and PC-3 cells. Compounds 2, 4, 7, and 8 had higher IC₅₀ values than 1 against both cell lines. Growth inhibitory effects were enhanced by converting the phenylethyl moiety (1) to a phenylhexyl moiety (3) or to an alkyl chain (5).

Our previous studies have shown that the inhibitory effects on lipid absorption and accumulation are stronger than 1 for cinnamic acid derivatives depending on the ester moieties (3, 5, and 6) and that 6 (tri-hydroxycinnamic acid) is more potent than 5 (di-hydroxycinnamic acid). These results indicate that inhibitory effects become stronger with increasing number of hydroxyl groups on the cinnamic acid aryl ring. Our current study shows that the anti-cancer effects are also greater for 6 than 5 against MCF-7 cells. This suggests that increasing the number of hydroxyl groups on cinnamic acid aryl ring enhances
anti-proliferative potencies. Therefore, cinnamic acid derivatives (3, 5, and 6, particularly 6) may have potential value as anti-cancer drugs against breast and prostate cancer.

**Mechanisms of cancer cell growth inhibition by tri-hydroxycinnamic acid decyl ester**

Compound 6 was the most potent analog among the cinnamic acid derivatives examined. Since apoptosis is one possible mechanism of action for 6, we examined cell cycle distribution and caspase-3 activity as well as mRNA expression of Bax and Bcl-2 in MCF-7 and PC-3 cells treated without and with 6. First, we analyzed the cell cycle by flow-cytometry using propidium iodide (PI), which binds to DNA. Increases in cell populations in sub-G1 phase were significant in MCF-7 cells treated with 6 as compared with control and in dose-dependent fashion (Fig. 7, left panel). Sub-G1 populations in the cell cycle profiles for MCF-7 cells treated with 4 µM, 10 µM and 20 µM for 48 h markedly increased, approximately 3-fold, 7.2-fold, and 11.8-fold as compared with control. The increase in sub-G1 populations indicated that cycle-arrested cells were undergoing apoptosis. In contrast, sub-G1 populations in the cell cycle profiles in PC-3 cells treated with 6 appeared in a dose-dependent manner (Fig. 7, right panel). Compound 6 at concentrations of 4 µM, 10 µM and 20 µM increased the population of sub-G1 cells to 4.3% (2.3-fold), 7.4% (4-fold) and 15.6% (8.4-fold) when compared with control 1.8%, respectively. Increases of cell populations in sub-G1 phase were significant in PC-3 cells treated with 6 at the concentrations of 4~20 µM. These results indicate that 6 is an inducer of apoptosis in MCF-7 and PC-3 cells.

Next, we examined caspase-3 activity and the expression of Bax and Bcl-2 mRNAs quantified by RT-PCR. As shown in Fig. 8, 6 increased caspase-3 activity approximately 2.1-fold and 2.3-fold in MCF-7 and PC-3 cells, respectively. In addition, the expression of Bax mRNA was not significantly affected by treatment with 6 in MCF-7 and PC-3 cells (Fig. 9A and Fig. 9B). In contrast, treatment with 6 significantly decreased the expression of Bcl-2 mRNA in both MCF-7 and PC-3 cells (approximately 73% and 63%, respectively) (Fig. 9C and Fig. 9D). These results suggest that 6 induced apoptosis in MCF-7 and PC-3 cells.
Previous studies have shown that 1 and its derivatives affect the growth of a number of cancer cell lines. Recently, it was reported that a derivative of 1 having a 4-nitro group in the phenethyl ester moiety (1-\textit{p}NO2 derivative) exhibits anti-cervical and anti-colon cancer activities and induces cellular apoptosis.\textsuperscript{10,11) While experimental conditions and cell lines were different among these reports, the inhibitory potencies of the derivatives were weaker than for the cinnamic acid derivatives 3, 5, and 6, when compared with 1 as a standard.\textsuperscript{10-13) In the current study, we have shown that 6 is the most potent compound among the cinnamic acid derivatives tested and that it induces apoptosis in breast and prostate cancer cells. Currently, a detail mechanism study of apoptosis induction is under investigation. As shown in Table 1, compound 6, having three hydroxyl groups (IC50: 0.8 \(\mu\)M), exhibits cell growth inhibitory action greater than compound 5, having two hydroxyl groups (IC50: 1.7 \(\mu\)M), and compound 7 or 8, having one hydroxyl group exhibit little effect (IC50: > 10 \(\mu\)M). While it is extremely difficult in a short timeframe to find and analyze the target proteins, it is assumed that target proteins might be enzymes, receptors, transcription factors etc. involved in cell proliferation and apoptosis. The three hydroxyl-bearing compound could potentially bind to these target proteins in a stronger and more stable fashion than the compounds with two hydroxyl groups. Once bound, it might affect (promote/inhibit) the functions of these target proteins, and induce apoptosis, resulting in inhibition of proliferating of cancer cells. It would be interesting to explore the proteins that bind to the compound with high affinity. It should be noted that previously, Kikuzaki et al. have reported that caffeic acid and ferulic acid exhibit antioxidant activity.\textsuperscript{14) The authors showed that lipid peroxidation was inhibited by caffeic acid and ferulic acid to a greater extent than \textit{p}-coumaric acid or alkyl ferulate. In the current study, we have not examined antioxidant activities.
CONCLUSION

In conclusion, we have shown that certain cinnamic acid derivatives suppress cancer cell growth more strongly than 1 and that against MCF-7 and PC-3 cells, compound 6 induces growth inhibition through apoptosis. We conclude that cinnamic acid derivatives, in particular 6, may have potential for the treatment of patients with breast and prostate cancer.
Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.
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Fig. 1. Chemical structure of cinnamic acid derivatives

1; \( R^1 = \text{OH}, R^2 = \text{OH} \)
2; \( R^1 = \text{H}, R^2 = \text{OH} \)
3; \( R^1 = \text{OH}, R^2 = \text{OH} \)
4; \( R^1 = \text{H}, R^2 = \text{OH} \)
5; \( R^1 = \text{OH}, R^2 = \text{OH}, R^3 = \text{H} \)
6; \( R^1 = \text{OH}, R^2 = \text{OH}, R^3 = \text{OH} \)
7; \( R^1 = \text{OH}, R^2 = \text{H}, R^3 = \text{H} \)
8; \( R^1 = \text{H}, R^2 = \text{OH}, R^3 = \text{H} \)
Fig. 2. Growth inhibition with cinnamic acid derivatives.

MCF-7 (A and B) and PC-3 (C and D) cells were treated with 4 μM of cinnamic acid derivatives for 72 h. Cell growth was determined by MTT method. Data shown are mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 vs Control (C) (Dunnett's multiple comparisons test).
Fig. 3. Growth inhibition with cinnamic acid phenethyl and phenylhexyl ester derivatives in MCF-7 cells.

MCF-7 cells were treated with various concentrations of cinnamic acid derivatives for 72 h. Cell growth was determined by MTT method. Data shown are mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 vs Control (Dunnett's multiple comparisons test).
Fig. 4. Growth inhibition with cinnamic acid phenethyl and phenylhexyl ester derivatives in PC-3 cells.

PC-3 cells were treated with various concentrations of cinnamic acid derivatives for 72 h. Cell growth was determined by MTT method. Data shown are mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 vs Control (Dunnett's multiple comparisons test).
Fig. 5. Growth inhibition with cinnamic acid decyl ester derivatives in MCF-7 cells.

MCF-7 cells were treated with various concentrations of cinnamic acid derivatives for 72 h. Cell growth was determined by MTT method. Data shown are mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 vs Control (Dunnett's multiple comparisons test).
Fig. 6. Growth inhibition with cinnamic acid decyl ester derivatives in PC-3 cells.

PC-3 cells were treated with various concentrations of cinnamic acid derivatives for 72 h. Cell growth was determined by MTT method. Data shown are mean ± SD (n=4). *p<0.05, **p<0.01, ***p<0.001 vs Control (Dunnett's multiple comparisons test).
Fig. 7. Effect of compound 6 on cell cycle distribution in MCF-7 and PC-3 cells.

MCF-7 and PC-3 cells (4 x 10^4 cells/ml) were treated with various concentrations of 6 and DMSO for 48 h. Both floating and attached cells were collected and processed for analysis of cell cycle distribution. The sizes of subpopulations are given as percentage of total populations of cell cycle histograms of MCF-7 and PC-3 cells obtained cell cycle analysis by flow cytometry after PI staining. Results represent the mean ± SD of each group. **p<0.01, and ***p<0.001 versus control.
Fig. 8. Levels of Caspase-3 activity in MCF-7 and PC-3 cells.

Cells were treated with 4 μM of 6 for 24 h. The cells were then lysed and caspase-3 activity was measured colorimetrically. The activity of control (C) was defined as 1.0. Results are expressed as ratios against control value ± S.D. (n=3).

**p<0.01, ***p<0.001 vs Control (C) (Student's t test).
Fig. 9. Levels of Bax and Bcl-2 mRNA in MCF-7 (A and C) and PC-3 (B and D) cells. Cells were treated with 4 μM of 6 for 24 h. The expression levels of Bax (A and B) and Bcl-2 (C and D) mRNAs were analyzed by RT-PCR, followed by an agarose gel (2%) electrophoresis. The intensity of control (C) was defined as 1.0. Data shown are mean ± SD (n=3). **p<0.01, ***p<0.001 vs Control (Student’s t test).
Table 1 IC₅₀ values of cinnamic acid derivatives against MCF-7 and PC-3 cells.

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