Inhibitory Effect of Methyl Caffeate on Fos-Jun-DNA Complex Formation and Suppression of Cancer Cell Growth

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The fos and jun proteins form a heterodimeric complex that interacts with the DNA regulatory element known as the AP-1 binding site. To search for inhibitors of the fos-jun-DNA complex formation, several natural plants extracts were screened. One of them, the methanol extract of *Perilla frutescens* showed inhibitory effect against the complex formation between the fos-jun dimer and the AP-1 binding site (TGAG/CTCA). Methyl caffeate and dibutyl phthalate (DBP) were isolated as the active constituents from the extract of *P. frutescens* by repeated column chromatography and HPLC. And their IC_{50} values for fos-jun-DNA complex formation were estimated as 0.29 mM and 0.55 mM, respectively. The more potent inhibitor, methyl caffeate showed cytotoxic effect against human cancer cell line (A549 and K562).

Keywords : AP-1 binding site, Fos-Jun heterodimer, *P. frutescens*, Methyl caffeate, Dibutyl phthalate (DBP).

Introduction

Activator protein-1 (AP-1) was first identified as a transcription factor that binds to an essential cis-element of the human metallothionein 2a(hMT2a) promotor.[1] AP-1 is multiprotein complex composed of the products of the *fos* and *jun* proto-oncogene families.[2,3] The protein encoded by the *jun* oncogene contains a region of 60 amino acids adjacent to its c-terminus that is 44% identical to the DNA-binding domain of the yeast transcription factor GCN4. The ability of fos to interact with jun raised the possibility that fos participate directly in regulating gene expression by using the jun protein as an anchor to bind to DNA binding site within the regulatory regions of specific target genes.[4-6]

The amino acid sequences of jun and fos reveal domains with sequence similarity to the DNA binding domain of GCN4. These domains are composed of two elements : the amino-terminal is rich in basic amino acid while the carboxy-terminal, referred to as the 'leucine zipper', has five leucines separated from each other by six amino acids. Dimerization of jun and fos proteins occurs *via* hydrophobic interactions between their 'leucine zipper' regions. The fos protein does not dimerize with itself because of the electrostatic repulsions between negatively-charged side chains that are abundant in its 'leucine zipper' region.[7,8] The increased DNA-binding acivity of the jun-fos heterodimer is due to its increased thermostability and a number of salt bridges between the two 'leucine zipper' region.[9] The activated oncogene products produce continuous signals for cell proliferation, which could lead to neoplastic transformation.[10,11]

Curcumin, a dietary pigment responsible for the yellow color of curry, is known to inhibit the mRNA expression of c-jun in mouse fibroblast cell that is stimulated by phorbol ester, and also inhibit c-jun/AP-1 binding to its cognate motif.[12,13] In the previous papers, we found that momordin and dihydroguaiaretic acid isolated from the natural plants inhibit the AP-1 activity in TPA-induced tumor cells and suppress the tumor cell growth.[14,15]

The leaf of *Perilla frutescens* is one of the most popular garnishes in Japan and is also a folk medicine as a detoxicant, antitussive and orders in Japan and China.[16,17]

We isolated dibutyl phthalate and methyl caffeate from the extract of *P. frutescens.* They reduced the formation of fos-jun-DNA complex. The methyl caffeate is a derivative of caffeic acid. Thus, the inhibitory activity of caffeic acid was compared with that of methyl caffeate. It is also found that methyl caffeate and caffeic acid showed the cytotoxic effects against A-549 and K-562 human cancer cell lines.

Experimental Section

Expression of c-jun and c-fos proteins in *E. coli* BL21 (DE3). c-Jun and c-fos cloned into pLMI were kindly provided by Mark Glover, Harvard University. These vectors were over-expressed in *E. coli* strain BL21 (DE3) using IPTG induction. Cells were pelleted. The pelleted cells were then resuspended in lysis buffer (250 mM NaCl, 50 mM K_3PO_4, 1 mM EDTA, 0.1% β-mercaptoethanol, pH 7.5) containing protease inhibitors (1 μg/mL leupeptin, 0.1 mg/mL PMSF, and 1 μg/mL pepstatin) at 1/40th volume of the original culture volume. The cells were lysed using sonication.

Protein dimerization. The c-jun and c-fos proteins were expressed individually and then dimerized. The cell extracts containing equivalent amount of c-jun and c-fos were mixed at room temperature for 30 minutes.

Preparation of ^{32}P-labeled probe DNA. ^{32}P-labeled probe DNA was prepared by the standard 5'-terminal labeling method. 1.75 pmol of AP-1 consensus oligonucleotide (purchased from Promega) was mixed with 10 μCi (γ-^{32}P)ATP and T4 polynucleotide kinase. The mixture was incubated at 37 °C for 30 minutes and the reaction was

quenched with 0.5 M EDTA.

Electrophoresis mobility shift assay (EMSA). The binding of the jun-fos dimer with the DNA probe was investigated using electrophoresis mobility shift assay. The dimer and DNA were mixed in gel shift buffer (20% glycerol, 5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 mg/mL poly (dI-dC)). After 30-minute incubation at room temperature, the samples were resolved on a 6% nondenatured gel. The gel was autoradiographed. To quantify the shifted band, each band corresponding to the jun-fos-DNA complex was excised from the gel and the radioactivity was measured using liquid scintillation counter.

Isolation of inhibitors. The dry leaves of *P. frutescens* were extracted with methanol under reflux condition for about 2 hours. The extracts were concentrated *in vacuo*, and then, partitioned between ethyl acetate and water. The ethyl acetate-soluble fraction was then evaporated. The resides were separated into nine fractions by silica gel column chromatography, eluted with hexane-ethyl acetate. The inhibitory activity of each fraction was examined by the electrophoresis mobility shift assay. The active fractions were further separated by repeated silica gel column chromatography. Then, the final fraction was applied to the HPLC with a C_{18} reverse-phase column and eluted with acetonitrile-water, to give compounds. The structure of these compounds were determined using GC-MS, ^{1}H-NMR (400 MHz) and ^{13}C-NMR (500 MHz).

Cell culture. A549 (human lung cancer), K562 (human leukemia) cell lines were purchased from Korean Cell Line Bank. Each of tumor cells was cultured in RPMI 1640 medium containing 10% fetal bovine serum and antibiotic antimicotic solution (Gibco BRL) and periodically subculture in trypsin-EDTA solution containing 0.05% trypsin and 0.53 mM EDTA. Each of the cell lines (1×10^4 cells/100 μL) was plated on 96-well microplate and cultured for 24h at 37 in a 5% CO_2 incubator.

Cell cytotoxicity test. The sample was dissolved in DMSO, sterilized with a 0.22 μm PVDF filter, and serially 5-fold diluted from 400 μg/mL to 0.0256 μg/mL. It was then added into a 96-well microplate containing the human tumor cells. The concentration of DMSO was below 0.5%. The cells were cultured for 48 h at 37 ºC in a CO_2 incubator. MTT (3-(4,5-Dimethyl-thiazole-2-yl)-2,5-diphenyltetrazoliumbromide) was dissolved in physiological saline (2 mg/mL) and filtered through a 0.22 μm PTFE filter. After the MTT solution was added into the each well of the 96-well microplate, the plate was incubated for an additional 4 hours at 37 ºC in an incubator. Microplates were centrifuged at 1000 rpm for 10 min, and supernatants were removed. The formazan formed from MTT was dissolved in 100 μL of DMSO. The absorbance was measured at 540 nm. The IC_{50} (50% Inhibition Concentration) value was calculated by the linear regression method.

Results and Discussion

Expression of c-jun and c-fos proteins. Cloning vectors (pLM1) that contained the B-Zip region of c-*fos* and c-*jun* gene sequences were expressed in *E. coli.* The B-Zip proteins were dimerized and used to investigate the formation of the dimer and DNA complex at an electrophoresis mobility shift assay. To verify that the band of interest was mostly attributed to c-jun/c-fos, the anti-c-jun/AP-1 antibody was used. When the cell extract was pre-incubated with the antibody, the band of interest in EMSA was weekened (Figure 1).

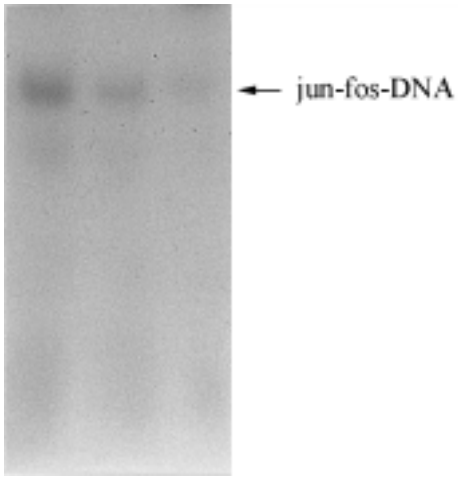


Figure 1. Autoradiograph showing the result from the electrophoresis mobility shift assay, with antibodies against c-jun/AP-1. Anti-c-jun/AP-1 polyclonal antibody was purchased from the Santa Cruz Biochemistry. In lane 1, no antibodies were added; lane 2, antibody 0.2 μg; lane 3, 0.5 μg.

Purification and identification of inhibitors. The methanol extracts of *P. frutescens* showed the inhibitory effect against the fos-jun-DNA complex formation in concentration of 10 mg/mL. The methanol extract was further purified by repeated silica gel chromatography and HPLC to give two active compounds. The purification efficiency of methyl caffeate was about 0.0015%. The structures of these compounds were identified to be dibutyl pthalate (DBP) and methyl caffeate by the instrumental analysis data (Figure 2A and B). The ^{13}C-NMR and the ^{1}H-NMR spectra of DBP are shown in Table 1. The weight of the molecular ion was determined by mass-spectrum analysis and found to be 278.4 ($C_{16}H_{22}O_4$: dibutyl phthalate) and 194.1 ($C_{10}H_{10}O_4$: methyl caffeate).

Inhibitory effect on jun-fos-DNA complex formation. The activation of fos-jun is a crucial factor in transmitting the tumor promoting signal from the extracellular environment to nuclear transcription machinery. The final steps in signal transduction is the binding of fos-jun to the AP-1 DNA site in order to express genes. It is expected that the inhibitory drugs for the fos-jun dimer function will play an important role in reversing the tumor promotion. The inhibitory effect of the isolated compounds were evaluated by the EMSA method. Shown in Figures 3 and 4, the IC_{50} values of DBP and MC are 0.55 mM and 0.29 mM, respectively.

DBP is used as a plasticizer in films covering tablets,

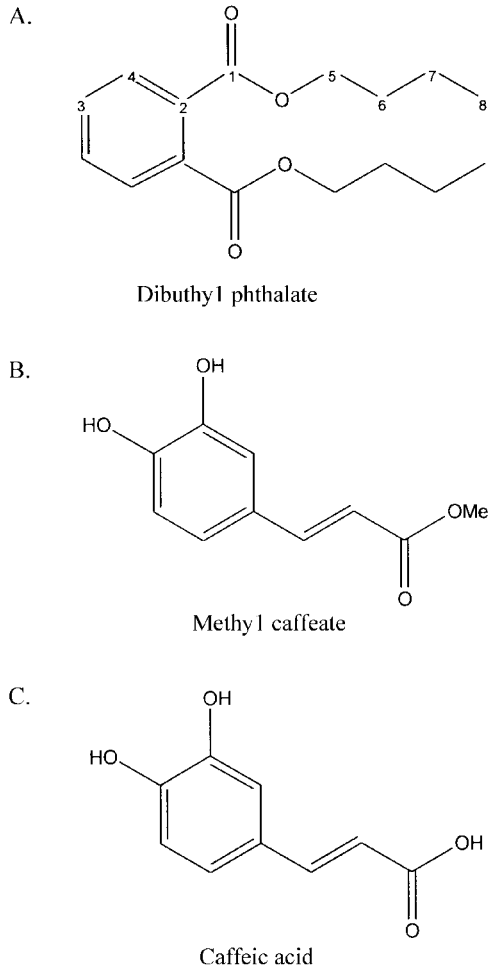


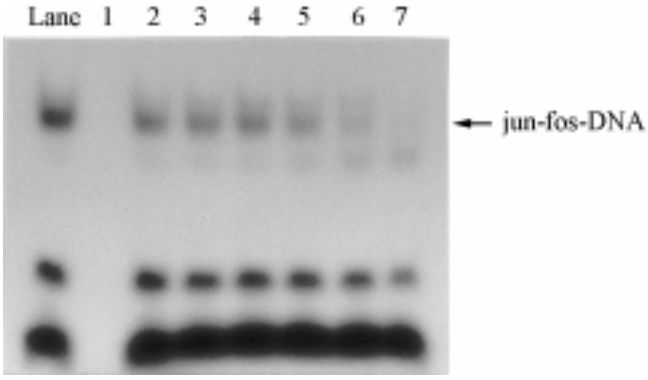
Figure 2. The structures of inhibitors.

Table 1. ^{13}C-NMR and ^{1}H-NMR chemical shift of DBP

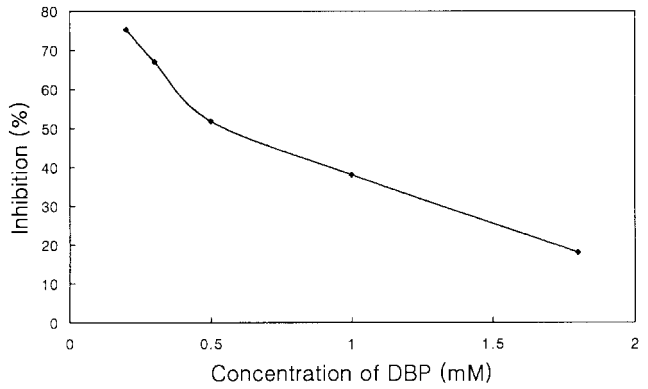
No. of C	δ (ppm)	No. of H	δ (ppm)
C-1	167.68		
C-2	132.33		
C-3	130.89	C3-H	7.46
C-4	128.82	C4-H	7.64
C-5	65.88	C5-H	4.23
C-6	36.57	C6-H	1.65
C-7	22.98	C7-H	1.35
C-8	14.16	C8-H	0.80

dragees and capsules. Testicular toxicity and male reproductive tract effects of DBP have been reported. Besides, it was known as a triggering agent of anaphylactic reactions to drugs.[19,20] We regarded that DBP is not adequate as an anticancer drug.

Methyl caffeate was known as antioxidants and its inhibitory effects on cytokine-induced proliferation of murine cultured mesangial cells were investigated.[21] In addition, methyl caffeate is known to be a potent inhibitor of human colon tumor cell growth.[21,22] Methyl caffeate is one of derivatives of caffeic acid contained in *P. frutescens.* Because of



(A) Autoradiography showing the decreasing band of jun-fos-DNA by DBP



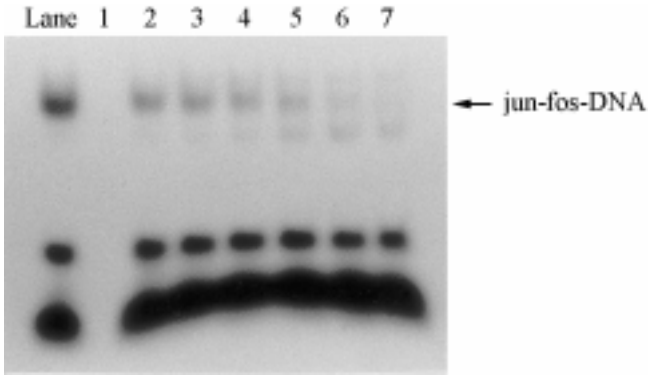
(B) Inhibitory activity profile of DBP

Figure 3. Inhibitory effect of DBP on fos-jun-DNA complex formation. The entire reaction mixture with DBP solubilized in DMSO was incubated at room temperature for 30 minutes, and then electrophoresis was performed. After autoradiography, each band corresponding to jun-fos-DNA complex was excised from the gel and radioactivity was measured by liquid scintillation counting. The value of DBP-treated samples was compared with that of DMSO-treated controls. Lane 1: control (w/o drug), lane 2: 0.2 mM of DBP, lane 3: 0.3 mM, lane 4: 0.5 mM, lane 5: 1 mM, lane 6: 1.8 mM, lane 7: 3 mM.

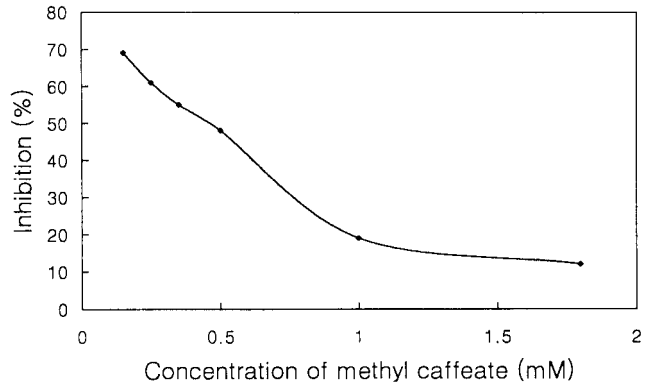
the potential usefulness of caffeic acid ester derivatives in tumor inhibition, we investigated the activity of caffeic acid. Caffeic acid showed relatively lower inhibitory activity than methyl caffeate. (IC_{50} of caffeic acid, 0.58 mM)

To specify whether the inhibitor binds to dimer or to DNA, the EMSA was used. The retardation band which was decreased by methyl caffeate was not restored, in spite of increasing dimer concentrations (data not shown), but the decreased band was restored to the original state with increased DNA concentrations (Figure 6). And then, the change rate of radioactivies with increasing dimer was equivalent to that of control radioactivity without an inhibitor. It is assumed that the inhibitory action of DBP and methyl caffeate may not come from dimer-binding, but rather from inhibitory interaction with DNA.

Cytotoxicity of inhibitors. The cytotoxicity was investigated by the MTT assay. As shown in Table 2, the IC_{50} value of methyl caffeate for A549 was 0.07 mM, and for K562 was 0.2 mM.



(A) Autoradiography showing the decreasing band of jun-fos-DNA by methyl caffeate



(B) Inhibitory activity profile of methyl caffeate

Figure 4. Inhibitory effect of methyl caffeate on fos-jun-DNA complex formation. The entire reaction mixture with methyl caffeate solubilized in DMSO was incubated at room temperature for 30 minutes, and then electrophoresis was performed. After autoradiography, each band corresponding to jun-fos-DNA complex was excised from the gel and radioactivity was measured by liquid scintillation counting. The value of inhibitor-treated samples was compared with that of DMSO-treated controls. Lane 1: control (w/o drug), lane 2: 0.2 mM of methyl caffeate, lane 3: 0.25 mM, lane 4: 0.3 mM, lane 5: 0.5 mM, lane 6: 1 mM, lane 7: 1.8 mM.

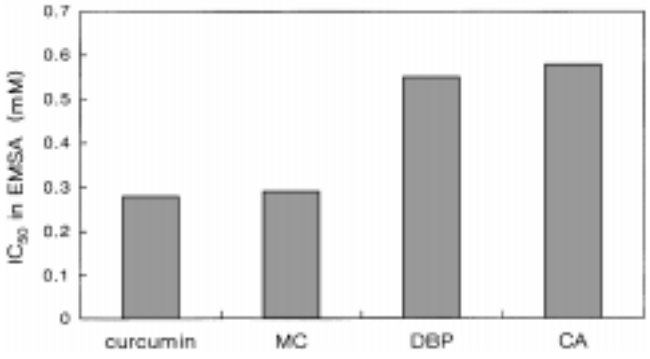


Figure 5. IC_{50} values in EMSA of curcumin, methyl caffeate, DBP and caffeic acid

Conclusion

Few studies have been done and little is known about the molecular mechnism of *P. frutescens* as Chinease drug. In this study, we concluded that methyl caffeate block DNA binding onto transcription factor, jun-fos dimer. This natural product may facilitate the design of other improved agents which reverse the cancer promotion by interrupting the jun-fos transcription factor.

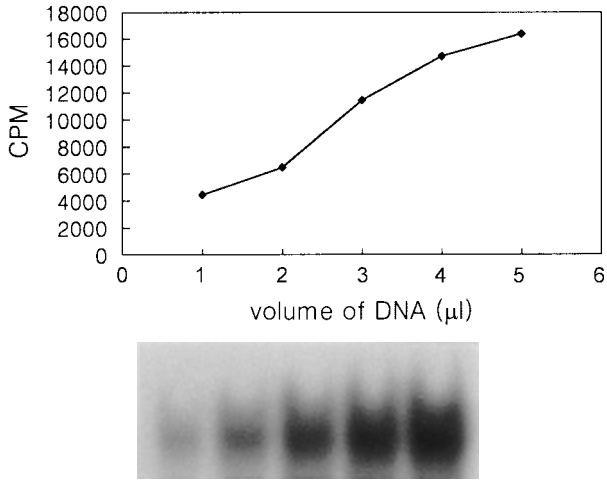


Figure 6. Radioactivity with increasing DNA concentration. fos-lun dimer: 5 μL, methyl caffeate: 0.24 mM, lane 1-5: 1, 2, 3, 4, 5 μL of DNA.

Table 2. IC_{50} values of inhibitors in the MTT cytotoxicity test (mM). Data was averaged from the triplet of the two independent experiments

	A549	K562
curcumin	0.0038	0.0019
methyl caffeate	0.07	0.2
caffeic acid	> 1	N. A

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References

1. Lee, W.; Haslinger, A.; Karin, M.; Tjian, R. *Nature* **1987**, *325*, 368.
2. Angel, P.; Imagawa, M.; Chiu, R.; Stein, B.; Imbra, R. J.; Rahmsdorf, H. J.; Jonat, C.; Herrlich, P.; Karin, M. *Cell* **1987**, *49*, 729.
3. Lee, W.; Mitchell, P. J.; Tjian, R. *Cell* **1987**, *49*, 741.
4. Vogt, P. K.; Bos, T. J.; Doolittle, R. F. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 3316.
5. Hope, I. A.; Struhl, K. *Cell* **1985**, *43*, 177.
6. Curran, T.; Teich, N. M. *Virology* **1982**, *116*, 221.
7. Thanos, D. H.; Katia, G.; Michael, E. G.; Philip, L. *Cell* **1988**, *55*, 917.
8. Smeal, T.; Angel, P.; Meek, J.; Karin, M. *Genes Dev* **1989**, *3*, 2091.
9. Abate, C.; Baker, S. J.; Lees-Miller, S. P.; Anderson, C. W.; Marshak, D. R.; Curran, T. *Proc. Natl. Acad. Sci. USA*

1990, *90*, 6766.
10. Auwerx, J.; Sassone-Corsi, P. *Oncogene* **1992**, *7*, 2271.
11. Abate, C.; Patel, L.; Rauscher, F. J.; Curran, T. *Science* **1990**, *209*, 1157.
12. Lu, Y.-P.; Chang, R. L.; Lou, Y.-R.; Huang, M.-T.; Newmark, H. L.; Reuhl, K. R.; Conney, A. H. *Carcinogenesis* **1994**, *15*, 2363.
13. Huang, T.-S.; Lee, S.-C.; Lin, J.-K. *Proc. Natl. Acac. Sci. USA* **1991**, *88*, 5292.
14. Lee, D. *Anticancer Research* **1998**, *18*, 119.
15. Park, S.; Lee, D.; Yang, C.-H. *Cancer Letters* **1998**, *127*, 23.
16. Tada, M.; Matsumoto, R.; Yamaguchi, H.; Chiba, K. *Biosci. Biotech. Biochem*. **1996**, *60*, 1093.
17. Makino, T.; Ono, T.; Muso, E.; Honda, G. *Planta Med.* **1998**, *54*, 541.
18. Park, S.; Song, J.-S.; Lee, D.; Yang, C.-H. *Bull. Korean Chem. Soc.* **1999**, *20*, 925.
19. Gall, H.; Kohler, A.; Peter, R. U. *Dermatology* **1999**, *199*, 168.
20. Wilkinson, C. F.; Lamb, J. C. *Regulatory Toxicology and Pharmacology* **1999**, *30*, 140.