Suppression of AP-1 Activity by Tanshinone and Cancer Cell Growth Inhibition

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The process of transcription is the major point at which gene expression is regulated. The jun and fos families of eukaryotic transcription factor heterodimerize to form complexes capable of binding 5'-TGAGTCA-3' DNA elements (AP-1 binding site). To search for the inhibitors of the jun-fos-DNA complex formation, several natural products extracts were screened and methanol extract of tanshen (the dried roots of *Salvia miltiorrhiza Bunge*) showed remarkable inhibitory activity. The active compounds of the extracts were purified using repeated column chromatography and recrystallization. Their structures were identified as tanshinone I and tanshinone II_A. Through the electrophoresis mobility shift assay and cell cytotoxicity test, tanshinone I and tanshinone II_A were identified as inhibitors that suppress not only AP-1 function but also the cell proliferation. Tanshinone I also suppressed the jun-fos-DNA complex formation in TPA-induced NIH 3T3 cells.

Introduction

The process of transcription, whereby a RNA product is produced from the DNA, is an essential element in gene expression. The central role of transcription in the process of gene expression also renders it an attractive control point for regulating the expression of genes in particular cell types or in response to particular signal.[1,2]

The *jun* and *fos* oncogene products play a critical role in the regulation of specific cellular genes in normal cells, to produce the controlled activity of their genes necessary for normal controlled growth.[3-5]

The jun and fos proteins form a heterodimeric complex that interact with a DNA regulatory element known as the AP-1 binding site (5′-TGAGTCA-3′). Following *in vitro* translation, jun binds as a homodimer to the AP-1 binding site, while fos fails to dimerize and displays no apparent affinity for the AP-1 site. Cotranslated jun and fos proteins bind 25 times more does the jun homodimer.[6,7] Dimerization occurs *via* interaction within leucine zipper domains at their carboxyl terminals, and the actual contact with the DNA occurs *via* basic region, which is located immediately upstream of the leucine zipper.[8,9] Jun-fos dimers regulate the transcription of jun gene.[10,11]

Their ability to transduce afferent growth signals into specific genetic responses means that they represent a critical nexus between normal and aberrant cell growth. It has ever been reported that abnormal regulation and/or expression of these proteins are implicated in the transformed cells.[12-15]

Tanshen, the dried roots of *Salvia miltiorrhiza Bunge*, has been used in Chinese traditional medicine for the treatment of hematological abnormalities, heart diseases and hepatitis. The extract of tanshen is known to be rich in diterpene pigments, so called tanshinones, which have ortho- or para-naphthoquinone chromophores.[16] They are unique components exclusively found in the Salvia genus and have attracted particular attention of many chemists and clinicians because many of them exhibit diverse biological activities such as antibacterial, antifungal, antioxidant, antimutagenic, anti-inflammatory, and antiplatelet aggregation activities.[16] For these reasons, extensive studies on the chemical composition of tanshen have been conducted over the last 50 years and resulted in the isolation of more than 40 tanshinones from these species. However there was no report so far which is related to jun-fos transcription factor. So, tanshen was selected as a target for AP-1 function inhibitor and the active compounds were isolated successively through the screening system and various purification methods. Among the tanshinones, tanshinone I and tanshinone II_A showed inhibitory effect on the AP-1 function. And these compounds also exhibited a significant cytotoxicity against cultured human cancer cell lines. Out of these results, it may be suggested that the anticancer mechanism of tanshinone I and tanshinone II_A come from their inhibitory effect on the jun-fos-DNA complex formation.

Experimental Section

Extraction and Purification. Tanshen, the dried roots of *Salvia miltiorrhiza Bunge*, was purchased from a Chinese drug store in Seoul, Korea. The roots of *Salvia miltiorrhiza Bunge* (1.2 kg) were finely cut and extracted with methanol (2 L × 4) under reflux for 10 hours. The extract was freed of solvents in a rotary evaporator to be prepared for silica gel column chromatography. The residue was dissolved in water and extracted with ethyl acetate. The residue of the ethyl acetate layer was dissolved in a small amount of acetone, and subjected to the column chromatography.

A glass column of a diameter 50 mm and length 300 mm was packed with silica gel (Silica gel 60, particle size 0.040-0.063 mm, Merck) in hexane slurries. The column was developed first with n-hexane: ethyl acetate/10 : 1, stepwise increasing portions of ethyl acetate in hexane, stepwise increasing portions of methanol in ethyl acetate, and finally pure methanol. The distinct colorful bands were collected separately. The first and the third major fraction inhibited

DNA-dimer complex formation. So, this fraction was recrystallized with methanol and acetone as solvents. Repeated operations were needed to afford pure crystals. These compounds were identified as tanshinone I and tanshinone II_A, by comparison with the spectroscopic data.

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 g/mL leupeptin, 0.1 mg/mL PMSF, and 1 g/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 therefore should be dimerized. Protein dimerization was carried out *in vitro*. 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, loading buffer was added. Samples were resolved on a 6% nondenatured gel. The gel was autoradiographed.[17] To quantitate the shifted band, each band corresponding to the jun-fos-DNA complex was excised from the gel and radioactivity was measured by liquid scintillation counting.

Cell culture. A549 (human lung cancer), Colo205 (human colon cancer), K562 (human leukemia) cell lines were purchased from Korean Cell Line Bank. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 10% calf serum and antibiotic antimicotic solution (Gibco BRL). 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.

Cell cytotoxicity test. The sample was dissolved in DMSO, sterilized with a 0.22 μm PVDF filter, and serially 5-fold diluted from 400 g/mL to 0.0256 g/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 °C in a CO_2 incubator. MTT (3-(4,5-Dimethyl-thiazole-2-yl)-e, 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 °C 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.

TPA treatment and preparation of cell extracts. NIH 3T3 confluent cells were serum-starved for 40 hours and then treated with both TPA and tanshinone I for 60 minutes. TPA-treated NIH 3T3 cells were scraped and resuspended in an NP40 lysis buffer (10 mM Tris-HCl / pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40). The nuclear pellet was resuspended in an NE buffer (20 mM Tris-HCl pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol) with a glass homogenizer.[18,19] After centrifugation, the pellet was resuspended in 0.6 × NE buffer and mixed with a ^{32}P-labeled AP-1 oligonucleotide

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. The band of interest was mostly attributed to the c-jun/AP-1 protein was verified using anti-c-jun/AP-1 antibody in our previous published data.[20] Combining the jun and fos protein in different ratio showed the discrimination between the jun-jun and the jun-fos band (Figure 1).

Isolation and Identification of the Inhibitors. When many kinds of natural plants extracts were screened using EMSA, some of these were expected to have compounds

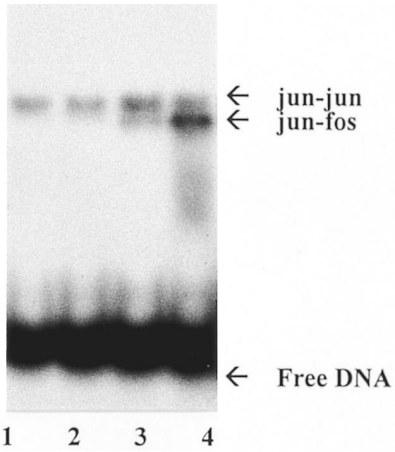
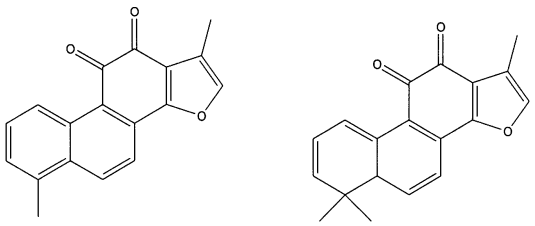


Figure 1. Autoradigraph of jun-jun and jun-fos expressed in *E. coli*. The lysed cell extracts were combined in different volume ratio, jun (lane 1), jun/fos, 1/1 (lane 2), 1/2 (lane 3), 1/3 (lane 4). Electrophoresis mobility shift assay was performed as indicated in Experimental.



tanshinone I tanshinone II_A

Figure 2. The structures of tanshinone I and tanshinone II_A.

which could inhibit jun-fos-DNA complex formation. One of these natural plants, the tanshen (the dried roots of *Salvia miltiorrhiza Bunge*) showed inhibitory activity. Tanshen is an important herb used frequently in traditional Chinese medicine for its tranquilizing, sedative and circulation-promoting effects.[21] The active principles of the thanshen were isolated and purified by repeated column chromatography and recrystallization. Mass spectra were obtained by EI method at the Korean Basic Science Center in Seoul National University. NMR spectra were obtained with a 500 MHz NMR instrument at the Korean Basic Science Center in Seoul National University. Their structures were identified as tanshinone derivatives by comparison with the reported spectral data. Their structures are shown in Figure 2 and their chemical properties are determined as below.

Tanshinone I: $C_{18}H_{12}O_3$ Brown red needles. Mp 230-236 °C.
λ_{max} (EtOH) 241, 268, 281, 290, 340, 440 nm.

Tanshinone II_A: $C_{19}H_{18}O_3$ Bright red needles. Mp 198-214 °C.
λ_{max} (EtOH) 250, 270, 345, 460 nm.

Inhibitory effect of tanshinone on jun-fos-DNA complex formation. To investigate the jun-fos-DNA complex formation, EMSA was used. Tanshinone I and tanshinone II_A reduced the interaction of jun-fos and DNA (Figure 3). And their IC_{50} values were 0.15 mM and 0.22 mM. (Figure 4).

Cell Cytotoxicity of Tanshinone. The inhibitors (tanshinone I and tanshinone II_A) showed cell cytotoxicity against human cancer cell lines. The IC_{50} values of tanshinone I against cancer cells was from 8 μM to 90 μM depending on the cell lines (A549, K562, Colo205). As correlated with the result of the EMSA, tanshinone II_A showed less strong cytotoxicity than tanshinone I. Its IC_{50} values was from 64 μM to 230 μM depending on the cell lines (A549, K562, Colo205). The values were shown in Table 1.

Doxorubicin, a well-known antitumor agent, was used as a

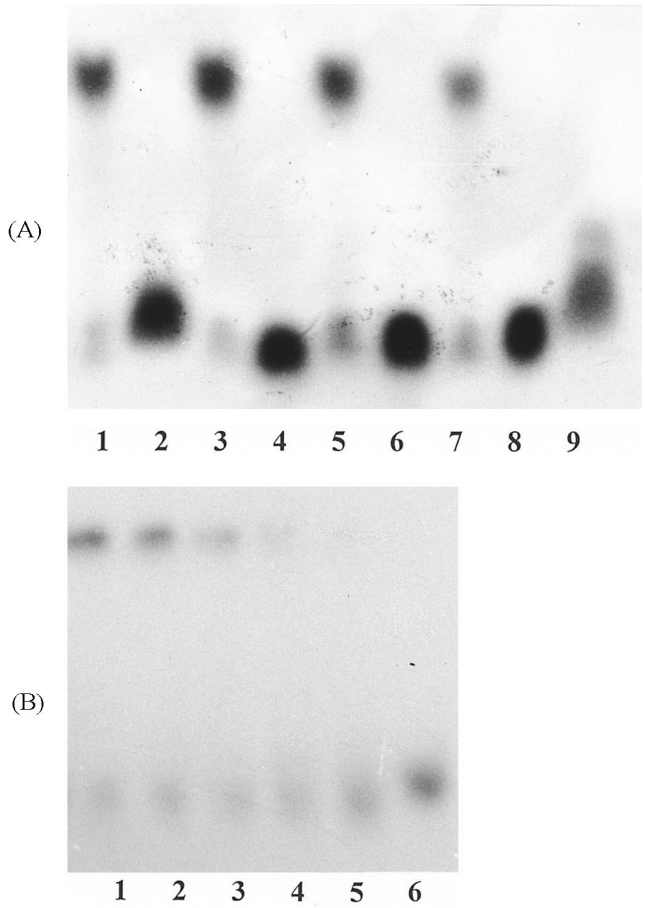


Figure 3. Autoradiograph showing the inhibitory action of tanshinone I on jun-fos-DNA complex formation. A. Total extracts and each fraction of column chromatography of tanshen was applied to the electrophoresis mobility shift assay. Lane 1: positive control, lane 2: AP-1oligonucleotide, lane 3: solvent control (DMSO), lane 4: total extract of tanshen, lane 5: none-active fraction, lane 6: active fraction, lane 7: none-active fraction, lane 8: purified tanshinone I, lane 9: tanshinone II_A B. Tanshinone I was applied to the electrophoresis mobility shift assay in concentration dependent manner. Lane 1: positive control, lane 2: 0.03 mM, lane 3: 0.1 mM, lane 4: 0.15 mM, lane 5: 0.2 mM, lane 6: 0.25 mM of tanshinone I.

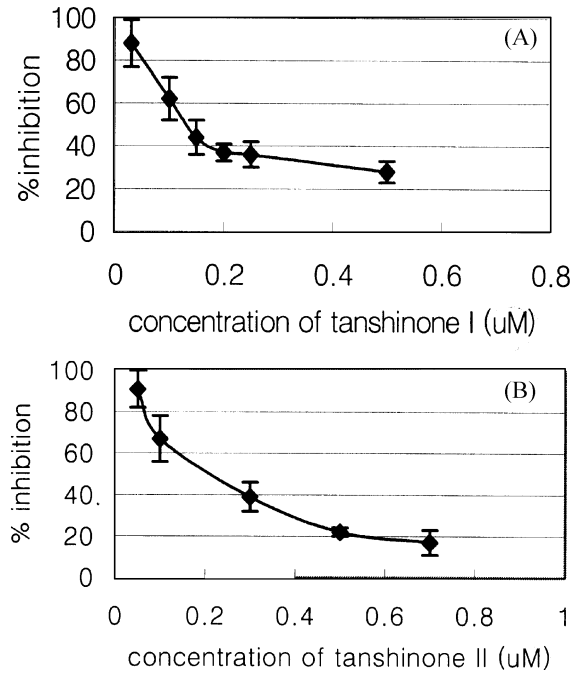


Figure 4. Inhibitory effect of tanshinone. The entire reaction mixture with tanshinone I solubilized in DMSO was incubated at room temperature for 30 minutes, and then electrophoresis was performed. After autoradiography, each band corresponding to jun-fos-DNA complex was excised from the gel and radioactivity was measured by liquid scintillation counting. The value of tanshinone I-treated samples was compared with that of DMSO-treated controls.

Table 1. IC_{50} values of inhibitors in the MTT cytotoxicity test (μM)

	A549	K562	Colo205
Tanshinone I	10	8	90
Tanshinone II_A	64	70	230
Doxorubicin	2.2	0.77	0.24

Data was averaged from the triplet of the two independent experiments

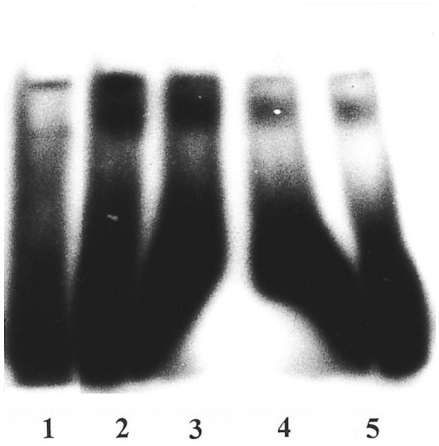


Figure 5. Autoradiograph of c-jun/AP-1 binding with DNA in TPA-induced NIH3T3 cells. Nuclear extracts were obtained from the cells treated with 70 ng/mL of TPA with or without tanshinone I at 1 mM. Lane 1: untreated NIH 3T3 cells, lane 2: cells treated with TPA at 50 ng/mL, lane 3: 0.7 μg of poly (dI-dC) was added to the cell extracts of lane 2, lane 4: cells treated with TPA at 50 ng/mL and tanshinone I at 1 mM, lane 5: 0.7 μg of poly (dI-dC) was added to the cell extracts of lane 4.

reference drug for the cytotoxicity evaluation of tanshinones, because the planar anthraquinone feature of doxorubicin was similar in some extent with common naphthoquinone or phenanthroquinone moiety of tanshinones. One of the major problems of doxorubicin in clinical application is that many malignant tumor cells in patients had rapidly developed resistance to this drug, which caused a serious decrease of the therapeutic effect of the drug.[22, 23]

Inhibition of jun-fos complex formation in TPA-treated NIH3T3 cells. After NIH3T3 cells were treated with TPA with or without tanshinone I for 60 min., the nuclear extracts were examined using EMSA. TPA increased the level of c-jun/AP-1 binding with DNA as previously known.[19] Treatment of tanshinone I decreased the level of c-jun/AP-1 binding with DNA (Figure 5). These results show that the tanshinone I penetrate into cells and effectively block the c-jun/AP-1 binding with DNA.

It has been shown that the inhibitors of jun-fos-DNA complex formation is correlated with their cytotoxicity in our previous studies.[20,24] The results of present studies revealed that tanshinone I and tanshinone II_A, as inhibitors of the jun-fos-DNA complex formation, induce cancer cell death. These results provide one of evidences that the inhibition of transcription activator could be the control point to reduce the cancer cell growth.

Proposed mechanism of tanshinone I and tanshinone II_A. Tanshinones (components of *Salvia miltiorrhiza*) were discovered to inhibit the fos-jun-DNA complex formation. Tanshinone I appears to be more potent inhibitor on fos-jun-DNA complex formation than tanshinone II_A and two inhibitors show selectivity on fos-jun-DNA complex formation versus another transcription activator, myc-max-DNA complex formation (data not shown). Although tanshinone I and tanshinone II_A differ only in that the latter has an extra methyl group and torsion at the flat conformation, the inhibitory action is different. Their biological action, cell cytotoxicity is more different than the inhibitory action. Considering the relationship between structural difference and the biological activity, it is suggested that the extended aromatic system exert inhibitory activity of tanshinones. Although the experimental evidences are not yet provided, like other aromatic compounds, tanshinones that are hydrophobic and relatively flat can slip between the stacked layers of base pairs in the DNA helix.

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