Anticancer effects of tanshinone I in human non-small cell lung cancer

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Abstract

Tanshinones are the major bioactive compounds of Salvia miltiorrhiza Bunge (Danshen) roots, which are used in many therapeutic remedies in Chinese traditional medicine. We investigated the anticancer effects of tanshinones on the highly invasive human lung adenocarcinoma cell line, CL1-5. Tanshinone I significantly inhibited migration, invasion, and gelatinase activity in macrophage-conditioned medium-stimulated CL1-5 cells in vitro and also reduced the tumorigenesis and metastasis in CL1-5-bearing severe combined immunodeficient mice. Unlike tanshinone IIA, which induces cell apoptosis, tanshinone I did not have direct cytotoxicity. Real-time quantitative PCR, luciferase reporter assay, and electrophoretic mobility shift assay revealed that tanshinone I reduces the transcriptional activity of interleukin-8, the angiogenic factor involved in cancer metastasis, by attenuating the DNA-binding activity of activator protein-1 and nuclear factor-κB in conditioned medium-stimulated CL1-5 cells. Microarray and pathway analysis of tumor-related genes identified the differentially expressed genes responding to tanshinone I, which may be associated with the Ras-mitogen-activated protein kinase and Rac1 signaling pathways. These results suggest that tanshinone I exhibits anticancer effects both in vitro and in vivo and that these effects are mediated at least partly through the interleukin-8, Ras-mitogen-activated protein kinase, and Rac1 signaling pathways. Although tanshinone I has a remarkable anticancer action, its potential anticoagulant effect should be noted and evaluated. [Mol Cancer Ther 2008;7(11):3527–38]

Introduction

Lung cancer is the most common and most lethal disease in the world, and most patients in whom therapy fails have distant metastases (1). Metastasis is a complicated multi-step process that involves interactions between cancer cells and their surrounding microenvironment (2). It is now becoming clear that inflammatory cells that exist in the tumor microenvironment play an indispensable role in cancer progression. This may explain why different types of cancer arise from sites of chronic irritation and inflammation (3).

Angiogenesis is a critical step in tumor growth and metastasis (4, 5), which are regulated closely by the local increase of the activity of a variety of angiogenic factors, such as interleukin-8 (IL-8) and others (6, 7). Substantial evidence also suggests that stroma cells adjacent to the cancer cells, including fibroblasts and inflammatory cells such as macrophages, neutrophils, and lymphocytes, can interact with cancer cells and express angiogenic factors (7–9). The interaction of non-small cell lung cancer (NSCLC) cells and stroma fibroblasts can promote the expression of angiogenic factor IL-8 in both cancer cells and fibroblasts (10). IL-8 expression increases in both NSCLC cells and macrophages when cocultured together (11, 12).

The macrophage is the pivotal inflammatory cell within the tumor stroma. On activation, the tumor-associated macrophages release a vast diversity of growth factors, proteolytic enzymes, cytokines, and inflammatory mediators. Many of these factors are key agents in angiogenesis. Recent reports indicate that tumor-infiltrating macrophages are associated with vessel density in ovarian (13), breast (14), and other (15) malignancies. Our previous report also indicated that tumor-infiltrating macrophage density is positively correlated with tumor IL-8 mRNA expression and intratumoral microvessel counts and negatively correlated with NSCLC patient survival (11).

The chemopreventive effects of anti-inflammatory drugs on carcinogenesis have attracted much recent attention, especially to the possible role of nonsteroidal anti-inflammatory drugs in reducing the risk of colorectal, breast,
lung, esophageal, and stomach cancers (16, 17). Herbal medications are now used in cancer therapy (18, 19), and several plant-derived compounds are used successfully in clinical practice; these include vinblastine (Velban), vincristine (Oncovin), etoposide (VP-16; VePesid), teniposide (VM-26; Vumon), paclitaxel (Taxol), vinorelbine (Navelbine), docetaxel (Taxotere), topotecan (Hycamtin), and irinotecan (Camptosar). The roots of \textit{Salvia miltiorrhiza} Bunge, generally known as Danshen, have been used in Chinese traditional medicine to treat cardiovascular disorders and hepatitis in Asia for thousands of years. The major bioactive compounds of Danshen, tanshinone I, tanshinone IIA, and cryptotanshinone, exhibit diverse biological effects such as antibacterial activity (20), antioxidative activity (21), anti-inflammatory activity (22, 23), cytotoxicity (24, 25), and act as inhibitors of platelet aggregation (26).

The objective of this study was to investigate the effects of tanshinones on inflammation, tumorigenesis, and metastasis in NSCLC and to identify the mechanism responsible for the pharmacologic function of tanshinones, especially tanshinone I.

**Materials and Methods**

**Reagents**

Authentic, high-performance liquid chromatography-grade samples of tanshinone I, tanshinone IIA, and cryptotanshinone (purity, NLT 99%) were obtained from Formosa Kingstone Bioproducts International. The structures of tanshinones and their yield properties in root of \textit{S. miltiorrhiza} Bunge are shown in Fig. 1 (27). Dexamethasone was purchased from the Sigma.

**Cell Lines**

The human monocyte cell line THP-1 (ATCC TIB 202; American Type Culture Collection) and the highly invasive human lung adenocarcinoma cell line CL1-5 (2) were grown in RPMI 1640 (Invitrogen) supplemented with 1.5 g/L Na$_2$HCO$_3$, 4.5 g/L glucose, and 10% fetal bovine serum (Invitrogen). Both cell lines were incubated at 37°C in 20% O$_2$ and 5% CO$_2$. Before the experiments, THP-1 cells were pretreated with 3.2 \times 10^{-7} \text{ mol/L} phorbol myristate acetate (Sigma) for 24 h. The preparation of conditioned medium (CM) derived from phorbol myristate acetate-pretreated THP-1 cells has been described previously (28).

**Cell Viability and Proliferation Assays**

CL1-5 cells were treated with a range of concentrations of tanshinone I, tanshinone IIA, and cryptotanshinone (0, 1, 10, 20, 40, 80, or 160 \text{ \mu g/mL}) for 24 h. The cell numbers and viability were assessed by trypan blue exclusion under an inverse light microscope (Carl Zeiss). For the cell proliferation assay, CL1-5 cells were seeded in 96-well microplates (Corning Glass Works) at 1 \times 10^4 per well and incubated for 24 h in 100 \text{ \mu L} culture medium. Cells were then treated with dexamethasone (5 \text{ \mu g/mL}), tanshinone I (10 \text{ \mu g/mL}), tanshinone IIA (10 \text{ \mu g/mL}), or cryptotanshinone (5 \text{ \mu g/mL}) for 24, 48, or 72 h. Because tanshinones are structurally related to steroids (29), the glucocorticoid analogue dexamethasone is employed to compare with those tanshinone compounds. The viable cells were identified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) assay, and the percentage of growth inhibition was determined. The detailed procedures have been described in the previous report (30).

**Wound-Healing and \textit{In vitro} Invasion Assays**

CL1-5 cells were seeded into 24-well plates and grown in medium containing 10% fetal bovine serum to nearly confluent cell monolayers and then scratched carefully using a sterile pipette tip. After wounding and washing, cells were cultured in CM with different concentrations of drugs (5 \text{ \mu g/mL} dexamethasone, 10 \text{ \mu g/mL} tanshinone I, 10 \text{ \mu g/mL} tanshinone IIA, or 5 \text{ \mu g/mL} cryptotanshinone) and incubated for 24 h. The movement distance of cells...
migrating into the wound track was evaluated under a phase-contrast microscope and photographed at \( \times 200 \) magnification. For \textit{in vitro} invasion assay, \( 1 \times 10^5 \) CL1-5 cells resuspended in CM containing different concentrations of drugs were seeded onto the Matrigel and incubated overnight at 37°C by using Transwell apparatus. The detailed procedures have been described previously (28). The experiments were done at least three times.

**Propidium Iodide Staining and Flow Cytometry**

Cells were trypsinized, harvested, and washed once with cold PBS. The cell pellets were then fixed with cold 70% ethanol for \( \geq 30 \) min and incubated with 100 μg/mL RNase A and 50 μg/mL propidium iodide in PBS. The fixed cells were incubated at 4°C in the dark for at least 15 min and analyzed using Cytomix FC 500 Series Flow Cytometry Systems (Beckman Coulter).

**Zymographic Analysis**

CL1-5 cells were cultured for 24 h in CM containing the designated agents, and the culture medium was collected and centrifuged to remove cellular debris. Without heating, each culture medium was mixed with sample buffer and subjected to electrophoresis in 10% polyacrylamide gels containing 0.1% gelatin (Sigma) in the presence of SDS under nonreducing conditions. The subsequent detailed procedures have been described previously (28). The clear bands on the blue background, representing areas of gelatinolysis, were quantified by ImageJ software (NIH). The gelatinase activity of CM-treated CL1-5 cells is defined as 100% to relatively compare with the other treatments.

**Luciferase Reporter and Electrophoretic Mobility Shift Assay**

The constructions of the IL-8 proximal promoter, the site-directed mutagenesis of the IL-8 activator protein-1 (AP-1), nuclear factor (NF)-IL-6, and NF-κB sites (31), as well as putative gene names related to cell adhesion, motility, and vascular endothelial growth factor (VEGF) promoter, a gift from Dr. Ann (32), are used for luciferase reporter assay. Briefly, 5 \( \times 10^5 \) CL1-5 cells were cotransfected with the individual IL-8 promoter or VEGF promoter construct and a pSV-b-galactosidase plasmid (Promega) with LipofectAMINE (Invitrogen), and the cells were treated with tanshinone I, tanshinone IIA, or cryptotanshinone at the designated concentrations for 24 h. The cell lysate was subjected to electrophoresis in 10% polyacrylamide gels under nonreducing conditions. The subsequent detailed procedures have been described previously (28). The measurements of drug effects were seeded onto the Matrigel and incubated overnight at 37°C by using Transwell apparatus. The experiments were done in triplicate.

**Real-time Quantitative PCR**

Total RNA was extracted from CL1-5 cells treated with or without agent using RNAzol B. Briefly, each amplification mixture (50 μL) containing 10 ng cDNA and 25 μL SYBR Green PCR master mix (Applied Biosystems) was subjected to 40 cycles of PCR. The TATA box binding protein was quantified as an internal control using the primers described in a previous report (11). The primer sets used are listed in Supplementary Table S1. All experiments were done in triplicate. The relative expression level of interest against that of TATA box binding protein was defined as \( \Delta CT = [CT_{interest} - CT_{TATA\ box\ binding\ protein}] \). The measured mRNA/TATA box binding protein mRNA ratio was calculated as \( 2^{\Delta CT} \times K \), where \( K \) is a constant (11, 33).

**Tumorigenesis and Metastasis In vivo**

The CL1-5 cells or CL1-5 cells with enhanced green fluorescent protein-expressing construct (CL1-5/EGFP; 5 \( \times 10^5 \) cells per injection site) were injected s.c. into 5-week-old female severe combined immunodeficient (SCID) mice and allowed to grow for 3 days. The animals were maintained under sterile conditions in laminar flow rooms and provided with sterilized food and water. Thereafter, mice were divided into five groups (six mice per group) and treated as follows: (a) PBS alone, (b) CM alone, (c) tanshinone I (0.3 mg/kg/d) suspended in CM, (d) tanshinone IIA (0.3 mg/kg/d) suspended in CM, and (e) tanshinone I (0.3 mg/kg/d) plus tanshinone IIA (0.3 mg/kg/d) suspended in CM. Vehicle or tanshinones were injected i.p. on a daily basis. After 12 days, the mice were sacrificed and the tumor xenografts were removed, photographed, and weighed. All lung tissues were fixed in 10% buffered formalin for at least 24 h, progressively dehydrated in solutions containing an increasing percent- age of ethanol (70%, 80%, 95%, and 100%), cleared in xylene, embedded in paraffin under vacuum, sectioned at 3 μm thickness, deparaffinized, and stained with H&E. Metastatic tumor colonies in the lung were photographed and counted manually. Mouse experiments were approved by The Institutional Animal Care and Use Committee of National Chung Hsing University.

**Immunohistochemical Staining for Microvessels**

Microvessels were stained using rabbit anti-CD31 polyclonal antibody (1:50 dilution; Abcam) as the primary antibody. Immunohistochemical staining was carried out using a modified avidin-biotin peroxidase complex method (Zymed). Microvessels were counted in 20 high-power fields (\( \times 200 \)) from five tumors of each treatment group.

**Microarray Analysis**

We obtained 1,152 human expressed sequence tag clones with putative gene names related to cell adhesion, motility, and vascular endothelial growth factor. Oligonucleotides were labeled with digoxigenin-11-dUTP (Roche Molecular Biochemicals) using Klenow polymerase (Invitrogen) and purified by MicroSpin G-50 columns (Amersham Pharmacia). The details of nuclear extract preparation, electrophoresis, and detection have been described previously (11, 31).
angiogenesis, signal transduction, tumorigenesis, metastasis, etc. (2), from the IMAGE consortium libraries through its distributor (Research Genetics). Three copies of 1,152 PCR-amplified cDNA fragments were spotted per microarray membrane (measuring 18 × 27 mm). Four concentrations of tanshinone I in CM were used (0, 0.1, 1, and 10 μg/mL) in addition to a control condition without CM. Total RNA (30 μg) derived from CL1-5 cells from each treatment described above was labeled with digoxigenin during reverse transcription as described in previous reports (2, 34). All experiments were done three times. The details of target preparation, hybridization, color development, image analysis, and spot quantification have been described previously (2, 28). The data discussed in this publication have been deposited in National Center for Biotechnology Information Gene Expression Omnibus and are accessible through Gene Expression Omnibus series accession no. GSE9315.

Statistical Analysis

Data collected from at least three independent experiments were analyzed by ANOVA (Excel; Microsoft). P < 0.05 was considered significant. Wherever applicable, the data are presented as mean ± SD. Gene expression data obtained from the microarray experiments were processed and normalized using the procedure described in our previous report (28). Genes were clustered into groups based on expression profiles by the self-organizing maps algorithm using the Acuity 3.0 program (Axon Instruments).

Results

Effects of Tanshinone I, Tanshinone IIA, and Cryptotanshinone on CL1-5 Cell Viability and Proliferation

Exposure of CL1-5 cells to tanshinone I, tanshinone IIA, and cryptotanshinone reduced the cell viability in a concentration-dependent manner (Fig. 2A). The LD_{50} was 80 μg/mL for tanshinone I and tanshinone IIA and 30 μg/mL for cryptotanshinone. Based on these results, the cancer cells were treated with the concentration equivalent to the LD_{10} (10 μg/mL for tanshinone I or tanshinone IIA and 5 μg/mL for cryptotanshinone) for
24, 48, and 72 h. As shown in Fig. 2B, a significant inhibitory effect of CL1-5 cell proliferation was observed at 48 h; compared with control values in non-CM-treated cells, proliferation decreased to 72 ± 9.6% for dexamethasone-treated cells, to 73 ± 8.9% for tanshinone I-treated cells, to 49 ± 4.9% for tanshinone IIA-treated cells, and to 50 ± 6.6% for cryptotanshinone-treated cells (P < 0.01 for each). Cell proliferation continued to decrease at 72 h, to

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![Zymographic analysis, morphologic change, and apoptosis.](image)

**Figure 3.** Zymographic analysis, morphologic change, and apoptosis. **A,** gelatinase activity of CL1-5 cell culture medium obtained after different treatments. The zymography below the bar chart is a representative experiment of at least three experiments. **B,** morphologic characteristics of CL1-5 cells observed with light microscopy. **a,** no treatment; **b,** treated with 10 μg/mL tanshinone I; **c,** treated with 10 μg/mL tanshinone IIA; **d,** treated with 5 μg/mL cryptotanshinone. Original magnification, ×400. **C,** apoptotic analysis of CL1-5 cells after treatment with various agents. After treatment for 24, 48, and 72 h with different agents, the cells were harvested and stained with propidium iodide, and the number of cells in the sub-G₁ phase, representing an apoptotic state, was calculated. *, P < 0.05 for tanshinone IIA-treated cells at 24 h; **, P < 0.01 for tanshinone IIA-treated cells at 48 and 72 h, compared with treatment with 0.1% DMSO, respectively. T1 (10), 10 μg/mL tanshinone I; T2A (10), 10 μg/mL tanshinone IIA; Cry (5), 5 μg/mL cryptotanshinone. Mix (3, 3, 1.7), mixture of 3 μg/mL tanshinone I, 3 μg/mL tanshinone IIA, and 1.7 μg/mL cryptotanshinone.
55 ± 7% of control values for dexamethasone-treated cells, to 71 ± 3% for tanshinone I-treated cells, to 58 ± 7% for tanshinone IIA-treated cells, and to 41 ± 5% for cryptotanshinone-treated cells (P < 0.01 for each).

Inhibition of Cell Migration and Invasion In vitro by Tanshinone I

As shown in Fig. 2C, migration activity increased significantly in CM-treated CL1-5 cells and decreased in the presence of each of the four reagents, especially in cells treated with tanshinone I (10 μg/mL), whose migration activity decreased to 36.7 ± 3.59% of the value from non-CM-treated cells (P = 0.014). The invasive potential of CL1-5 cells stimulated with CM increased by 27.9 ± 3.2%, and only tanshinone I (1 or 10 μg/mL) significantly inhibited cancer cell invasion; compared with non-CM-treated cells, invasive activity decreased to 44.5 ± 10.47% at the lower dose and to 12.8 ± 6.89% at the higher dose (P < 0.01 for each; Fig. 2D).

Zymographic analysis revealed that CM markedly increased the gelatinase activity in CL1-5 cells and that treatment with tanshinone I, tanshinone IIA, and cryptotanshinone significantly inhibited gelatinase activity (Fig. 3A). Compared with the CM-treated cells, pro-matrix metalloproteinase-2 activity decreased to 73.1% in tanshinone

Figure 4. Transcriptional inhibition of IL-8 expression by tanshinones. CL1-5 cells were stimulated with CM at different concentrations of tanshinones for an additional 24 h and then subjected to the following assays. A, IL-8 mRNA expression detected by RTQ-PCR analysis. *, P < 0.05; **, P < 0.05, compared with CM. B, luciferase assay of the wild-type IL-8 promoter construction. *, P < 0.05, compared with CM. C, luciferase assay of site-directed mutagenesis of the IL-8 promoter. Left, schema of the IL-8 promoter constructs. -133IL-8, wild-type IL-8 promoter; mAP-1, AP-1 mutation; mNF-IL6, NF-IL6 mutation; mNF-κB, NF-κB mutation. Right, luciferase assay. *, P < 0.01; **, P < 0.01, compared with wild-type. D, electrophoretic mobility shift assay was used to detect NF-κB-binding capability (left) and AP-1-binding capability (right). Mean ± SD. Representative of at least three experiments, each done in triplicate. T1, 10 μg/mL tanshinone I; T2A, 10 μg/mL tanshinone IIA; Cry, 5 μg/mL cryptotanshinone.
I-treated cells, to 47.6% in tanshinone II-treated cells, and to 52.1% in cryptotanshinone-treated cells. The respective values for matrix metalloproteinase-2 activity were 70.7%, 38%, and 37.4%.

**Effects of Tanshinones on Cell Morphology and Apoptosis in NSCLC**

CL-1-5 cells treated with tanshinone IIA (10 µg/mL) showed condensed and vacuolated nuclei and cell shrinkage (Fig. 3B, c). In contrast, tanshinone I (10 µg/mL; Fig. 3B, b) and cryptotanshinone (5 µg/mL; Fig. 3B, d) had no obvious influence on CL-1-5 cell morphology compared with control cells (Fig. 3B, a).

To study the effects of tanshinones on cell cycle regulation in CL-1-5 cells, the cells treated with tanshinones for 24, 48, and 72 h were analyzed by flow cytometry. In cells treated with 10 µg/mL tanshinone IIA, the percentage of cells in the sub-G1 phase increased in a time-dependent manner. The apoptotic cell percentage was 3.35 ± 0.41% at 24 h (P = 0.013), 6.94 ± 2.21% at 48 h (P = 0.028), and 43.4 ± 4.7% at 72 h (P < 0.01). Tanshinone I and cryptotanshinone had no effects on cell apoptosis and had a similar apoptotic cell percentage as control cells treated with 0.1% DMSO alone (0.52 ± 0.09%) at 72 h (Fig. 3C).

**Tanshinone I and Tanshinone IIA Suppress IL-8 mRNA Expression in CL-1-5 Cells Stimulated with CM**

The expression of IL-8 mRNA correlates with the metastasis and angiogenesis of cancer (11). We measured IL-8 mRNA expression in cells cultured in CM and treated with different concentrations of tanshinones (tanshinone I or tanshinone IIA, 1 and 10 µg/mL; cryptotanshinone, 0.5 and 5 µg/mL). As shown in Fig. 4A, compared with the CM-treated control, the expression of IL-8 mRNA increased 15 times in CL-1-5 cells after stimulation with CM, and this effect was suppressed significantly to 49.4% after the addition of 1 µg/mL tanshinone I (P = 0.074) and to 10.9% after the addition of 10 µg/mL tanshinone I (P < 0.01). Tanshinone IIA had a similar effect, decreasing IL-8 mRNA expression to 29.3% of the control value when added at 1 µg/mL (P = 0.026) and to 27.2% at 10 µg/mL (P = 0.042). The luciferase reporter assay of the wild-type IL-8 promoter also showed that treatment with CM increased the transcriptional activity of the IL-8 promoter by 10 times compared with no CM treatment. Compared with the values in CM-treated cells, IL-8 promoter transcriptional activity was suppressed significantly by tanshinone I to 50.8% of the CM-treated level at 1 µg/mL (P = 0.018) and to 41.6% at 10 µg/mL (P = 0.011). In contrast, tanshinone IIA at 10 µg/mL did not significantly alter IL-8 promoter activity (P = 0.092) compared with CM-treated cells (Fig. 4B). No luciferase activity was observed in the mock-transfected and non-transfected control cells.

**Tanshinone I Inhibits the Transcriptional Activity of the IL-8 Promoter through NF-κB and AP-1 Pathways**

To determine which cis-element in the IL-8 promoter region is affected by CM, three mutant constructs were subjected to the reporter assay, including AP-1, NF-IL-6, and NF-κB (Fig. 4C, left). The site-directed mutagenesis indicated that the mutation of the AP-1 and NF-κB binding sites reduced the promoter activities to 29% for AP-1 binding and to 8% for NF-κB (P < 0.01 for each) compared with the wild-type construct (Fig. 4C, right). There was no significant difference between the NF-IL-6 mutant and the wild type (P = 0.549).

The effects of tanshinone I on CM-mediated activation of NF-κB and AP-1 in the IL-8 promoter was studied with electrophoretic mobility shift assay. As shown in Fig. 4D (left), nuclear extracts from CL-1-5 control cells showed weak binding to the NF-κB probe (lane 2). In contrast, the binding capacity was significantly higher in cells stimulated with CM (lane 3); this effect could be competed away with a 200-fold excess of the unlabeled NF-κB probe (lane 4). The addition of tanshinone I (10 µg/mL) caused significant decrease in the binding capability of NF-κB compared with CM treatment (Fig. 4D, left, lane 5). Moreover, no obvious change in binding activity was observed in cells treated with 10 µg/mL tanshinone IIA or 5 µg/mL cryptotanshinone (lanes 6 and 7). Similar results were obtained in the experiments using the AP-1 probe (Fig. 4D, right).

**Inhibitory Effects of Tanshinone I on Tumorigenesis, Angiogenesis, and Metastasis in SCID mice**

Tanshinone I and tanshinone IIA (diluted with PBS) had no significant antitumorigenic or antimetastatic effects in CL-1-5-bearing SCID mice (data not shown). Interestingly, treatment with tanshinone I diluted with CM significantly decreased tumor size by 85% compared with CM alone (P < 0.01; Fig. 5A). Treatment with tanshinone I and tanshinone IIA administered together also decreased tumor size by 85% (P < 0.01) compared with CM alone. Tanshinone IIA by itself had no effect on tumor size.

Fluorescent microscopy showed numerous metastatic nodules on the surface of the lungs of CL-1-5/EGFP-bearing mice treated with CM only (Fig. 5B, a), but these nodules did not appear in the lungs of normal mice (Fig. 5B, b). Representative H&E-stained sections are shown in Fig. 5B (c). Only a few nodules, none of them metastatic, were observed in the lungs of mice treated with tanshinone I diluted with CM (Fig. 5B, d). Compared with CM-treated mice, mice treated with tanshinone I diluted with CM had significantly fewer tumor metastases in the lung (tanshinone I with CM, P = 0.0296; tanshinone I + tanshinone IIA with CM, P = 0.034; Fig. 5C). Tanshinone IIA alone had no significant effect on the number of tumor metastases in the lung (data not shown).

To further investigate the effect of tanshinone I on antiangiogenesis, microvessel density was quantified from tumors of untreated and tanshinone-treated CL-1-5-bearing SCID mice. The immunohistochemistry showed that the intratumoral microvessels were stained brown by anti-CD31 antibodies (Fig. 5C, left). Treatment with tanshinone I significantly inhibited tumor vascularity induced by CM (P < 0.01), whereas tanshinone IIA by itself did not suppress (Fig. 5C, right).
Identification of the Genes Responding to Tanshinone I by Microarray and Real-time Quantitative PCR Validation

The coefficient of variation for the three replicates of each gene, averaged over the 1,152 genes and all treatment groups, was 10.9%. After self-organizing maps clustering, the differentially expressed genes were grouped into four clusters. One cluster of expression profile was selected according to the descending trend, and this cluster

Figure 5. Inhibition of tumor growth, angiogenesis, and metastasis in SCID mice by tanshinone I. A, tanshinone I reduces tumorigenicity in SCID mice. After treatment for 12 d, mice were sacrificed and the tumors were removed. Left, representative tumors derived from CL1-5 cells, including those treated and untreated with tanshinone I; right, tumors were weighed. *, P < 0.01, compared with CM (n = 6). B, metastatic nodule image and histochemical staining. Left, arrows, metastatic nodules in the lung of the CL1-5/EGFP-bearing mice (a), compared with the lung of normal mice (b), under fluorescence microscopy. c and d, H&E-stained sections of the lungs of untreated and tanshinone I-treated CL1-5-bearing mice, respectively. Original magnification, ×100. Right, numbers of metastatic nodules in the lung of CL1-5/EGFP-bearing mice treated with different agents were counted. *, P < 0.05, compared with CM (n = 6). C, microvessel counts in SCID mice. Left, immunohistochemistry of tumoral microvessel of untreated (a) and tanshinone I-treated (b) CL1-5-bearing mice. Right, intratumoral microvessels of each treatment were counted. *, P < 0.01, compared with CM (n = 6). Mean ± SD. T1, tanshinone I; T2A, tanshinone IIA; T1 + T2A, tanshinone I and tanshinone IIA together.
contained 291 genes. We focused on the expression of the suppressed genes of interest in this cluster, choosing 38 gene expressions that showed a return to near-baseline or lower than baseline levels (CL1-5 alone without CM treatment) after tanshinone I treatment and applied SYBR Green Real-time Quantitative PCR (RTQ-PCR) analysis. These clones were sequenced retrospectively after differential expressions were found to confirm that they represented the true transcript. As shown in Table 1, only 20 genes were available in RTQ-PCR, and their expression trends were consistent with those from the microarray studies. These clones were grouped into six categories according to their involved pathways based on the literature. These categories included genes involved in the Ras-mitogen-activated protein kinase (MAPK) signaling pathway, extracellular matrix-receptor interaction, Rac1 signaling pathway, apoptosis pathway, cell cycle, and anonymous genes that correlated positively with invasiveness (marked as “others”). Genes with multiple roles were included in more than one category. Our in-house data mining tool revealed that the Ras-MAPK, Rac1 signaling, and regulation of actin cytoskeleton pathways were suppressed by tanshinone I (Fig. 6). A full list of genes and data relating to treatment with tanshinone I are deposited in Gene Expression Omnibus (series accession no. GSE9315).

### Table 1. Genes suppressed by tanshinone I in CL1-5 cells following stimulation with CM

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<td>v-Ha-ras Harvey rat sarcoma viral oncogene homologue</td>
<td>Signal transducers</td>
<td>15.39 58.68</td>
</tr>
<tr>
<td>BC029822</td>
<td>PDGF-β polypeptide</td>
<td>Growth factor</td>
<td>71.46 79.37</td>
</tr>
<tr>
<td>NM_145110</td>
<td>MAPK kinase 3</td>
<td>Signal transducers</td>
<td>66.11 67.13</td>
</tr>
<tr>
<td>NM_002524</td>
<td>Neuroblastoma RAS viral (v-ras) oncogene homologue</td>
<td>Signal transducers</td>
<td>59.38 51.49</td>
</tr>
<tr>
<td>NM_002467</td>
<td>v-myc avian myelocytomatosis viral oncogene homologue</td>
<td>Transcription factor</td>
<td>44.74 35.7</td>
</tr>
<tr>
<td><strong>Cell cycle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_057182</td>
<td>Cyclin E1</td>
<td>Promoting cell cycle</td>
<td>48.11 67.95</td>
</tr>
<tr>
<td>NM_005901</td>
<td>MAD homologue 2</td>
<td>Signal transducers and transcriptional modulators</td>
<td>65.46 53.74</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_002377</td>
<td>Intercellular adhesion molecule 4</td>
<td>Cell adhesion and migration</td>
<td>53.11 61.4</td>
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<tr>
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<td>Granulin</td>
<td>Growth factor</td>
<td>56.27 37.6</td>
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<tr>
<td>NM_002074</td>
<td>Guanine nucleotide binding protein, β polypeptide 1</td>
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<td>50.79 44.84</td>
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<tr>
<td>NM_002865</td>
<td>RAB2, member RAS oncogene family-like</td>
<td>Protein transport</td>
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<td>BC007922</td>
<td>IFN-stimulated gene (20 kDa)</td>
<td>Immune response</td>
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<tr>
<td>NM_033015</td>
<td>Fas-activated serine/threonine kinase</td>
<td>Signal transducers</td>
<td>76.09 49.61</td>
</tr>
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</table>

NOTE: Differentially expressed genes related to cell cycle, growth, invasion, and adhesion were identified by an in-house cDNA microarray and were further validated by SYBR Green real-time PCR. CL1-5, human lung adenocarcinoma cells with highly invasive capability.

*Percentage decrease in the level of gene expression by tanshinone I compared with the untreated control.

8 http://biochip.nchu.edu.tw/crsd1
present study, we investigated the anti-inflammatory, antitumorigenic, and antimetastatic effects of three major bioactive compounds contained in Danshen, including tanshinone I, tanshinone IIA, and cryptotanshinone in NSCLC. The specific actions of each compound were summarized in Supplementary Table S2.7 Among these compounds, tanshinone I significantly inhibits lung cancer cell migration, invasion, and gelatinase activity in vitro and reduces metastasis, angiogenesis, and tumorigenesis in vivo. Several previous studies have shown that tumor-infiltrating macrophages have protumorigenic activity and that their activity correlates with tumor microvessel growth, tumor progression, and metastasis (28, 37). Expression of IL-8 mRNA in tumor specimens of NSCLC patients correlates with tumor progression, tumor angiogenesis, survival, and the occurrence of tumor metastasis (33). The interaction of NSCLC cells and stromal cells, such as fibroblasts and macrophages, can also increase IL-8 expression in both cancer cells and stromal cells (10, 11). IL-8 plays a crucial role in tumorigenesis, angiogenesis, and metastasis of NSCLC (28, 31). We found that macrophage-CM significantly induced IL-8 expression in CL1-5 cells and that tanshinones strongly inhibited this effect. In addition to IL-8, tanshinone I could also significantly down-regulate VEGF promoter activity and mRNA expression induced by CM in CL1-5 cells (Supplementary Fig. S1).7 These results indicate that tanshinones have the potential to modulate the chronic inflammatory response in the microenvironment between inflammatory cells (macrophages) and cancer cells (CL1-5) by attenuating IL-8 and VEGF expression.

Previous reports have shown that AP-1, NF-IL-6, and NF-κB binding elements located in IL-8 promoter region are essential for the transcriptional regulation of IL-8 gene expression in most cells (38). The results of our reporter assay and electrophoretic mobility shift assay indicated that the transcription factors AP-1 and NF-κB are involved in CM-induced IL-8 expression in CL1-5 cells and that tanshinone I significantly suppresses this induction. We also showed that tanshinone I prevents NF-κB and AP-1 from binding to the IL-8 promoter and further suppresses IL-8 transcriptional regulation. Further studies to examine the role of tanshinone I on NF-κB and AP-1 are ongoing. Interestingly, tanshinone I had significant antitumorigenic and antimetastatic effects in CL1-5-bearing SCID mice when coinjected with CM but not with PBS. Coinjected mice had significantly smaller tumor mass and metastatic nodule formation after treatment of tanshinone I, whereas tanshinone IIA had no effect. These results suggest that tanshinone I has more potent effects than does tanshinone IIA on inhibiting the growth and metastasis of tumor xenografts. However, these results raise a critical question about the role of CM in the antitumorigenic and antimetastatic effects of tanshinone I. The CM is a serum-free medium with 24 h incubation with phorbol myristate acetate-treated THP-1 cells. Our previous studies and the other reports showed that the CM might contain proinflammatory cytokines such as tumor necrosis factor-α, IL-1, IL-8, and IL-12; anti-inflammatory cytokines such as transforming growth factor-β, IL-4, IL-10, and IL-13; and IL-6 with both proinflammatory and anti-inflammatory properties (3, 11, 27, 39, 40). Previous reports have shown that the imbalance between proinflammatory and anti-inflammatory cytokines can influence neoplastic outcome (3). These proinflammatory cytokines might be regulated by NF-κB signaling pathway (41), which could be inhibited by tanshinone I. The treatment of tanshinone I alone might attenuate these certain cytokines and chemokines to result in limited inflammation and restricted tumor growth. The CM treatment alone would invoke inflammation and neovascularization and promote tumor growth due to the abundant proinflammatory cytokines. On the contrary, the treatment of tanshinone I with CM might alter the balance of proinflammatory and anti-inflammatory cytokines due to tanshinone I suppressing the proinflammatory cytokines (e.g., IL-8 and VEGF-A), which might mimic the previous model of inflammation and cancer (3) to facilitate a switch from a Th1-type to a Th2-type inflammatory response and result in excessive inflammation and then repress tumor growth. However, the exact mechanism responsible for the

Figure 6. Scheme of the tanshinone I regulatory pathways. The effects of tanshinone I on tumorigenesis and metastasis are hypothesized to occur through the Ras-MAPK and Rac1 signaling pathways. This diagram shows that tanshinone I might inhibit the mRNA expression of the target genes, including PDGF-β, Ras, MAP2K3, CD44, Shb, phosphatidylinositol 3-kinase, Rac1, and IL-8.
role of CM remains unclear and needs further study. Our study confirms that tanshinone I exhibits antitumorigenic and antimetastatic effects in vivo.

We used cDNA microarray and RTQ-PCR analyses to investigate the possible functional mechanism responsible for the antitumorigenic and antimetastatic effects of tanshinone I. This analysis identified a panel of invasion-related genes, including the genes associated with angiogenesis, cell adhesion, cell motility, signal transduction, and cell proliferation [e.g., platelet-derived growth factor (PDGF)-β, v-myc, Shb, H-ras, N-Ras, MAPK kinase 3, phosphatidylinositol 3-kinase, CD44, Rac1, and collagen type IV], which were down-regulated by tanshinone I in CL1-5 cells in a concentration-dependent manner. Some of these candidate genes may be responsible for the antimetastatic effects of tanshinone I.

Metastasis is associated with the movement of cells. Those genes whose expression is involved in the cellular cytoskeleton and motility may play an important role in metastasis. Human PDGF-β is a potent mitogen, a mediator of actin cytoskeletal rearrangements, and a chemoattractant for cells that express functional PDGF receptors of mesenchymal origin (42). In PDGF-mediated mitogen signaling, Shc is activated by the PDGF receptor to regulate the activity of the small GTP-binding protein Ras, which then initiates the MAPK pathway (43). This initiation might activate the NF-κB pathway and promote the expression of IL-8 and other angiogenic factors (VEGF, basic-fibroblast-like growth factor, and PDGF itself; ref. 44). Shb contains a COOH-terminal SH2 domain that interacts with the PDGF-β receptor, activates phosphatidylinositol 3-kinase, and affects the small GTP-binding protein Rac1-mediated pathways (45). Furthermore, members of the Rho superfamily of low molecular weight GTPases are important in many signaling pathways, including the regulation of cell shape, adhesion, movement, and growth (46). Our results lead us to hypothesize that tanshinone I limits cancer metastasis by inhibiting the PDGF-β signaling pathway and related gene expression (Fig. 6).

CD44, an adhesion molecule with binding domains for hyaluronic acid, glycosaminoglycans, collagen, laminin, and fibronectin (47), is expressed in most human cell types. The interaction of CD44 with its ligand activates Rac1 signaling, and this has been implicated in a wide variety of physiologic and pathologic processes, including lymphocyte homing, activation, and cell migration, as well as tumor cell growth and metastasis (48). Our results show that tanshinone I inhibits the Rac1 signaling pathway (Fig. 6). Previous studies have shown that PDGF-β (49) and CD44 (50) can be regulated by NF-κB or AP-1. Our results are consistent because we found that tanshinone I down-regulates these proteins at least partly by inhibiting AP-1 and NF-κB. Further characterization of the genes identified in this study is in progress.

In conclusion, our study shows that tanshinone I can significantly reduce metastasis and tumorigenesis. These actions may occur through PDGF-β and its downstream pathways. This is the first report to show that tanshinone I suppresses the expression of the angiogenic factor IL-8 through the NF-κB and AP-1 pathways. Our data suggest that tanshinone I has potential as an agent to inhibit tumor progression. The potential clinical use of this agent should be evaluated in future. Further studies to clarify the mechanisms responsible for the inhibitory effect of tanshinone I on tumorigenesis, angiogenesis, and metastasis will be undertaken.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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