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

Chen-Yu Lee,¹ Hui-Fang Sher,² Huei-Wen Chen,⁴ Chun-Chi Liu,² Ching-Hsien Chen,² Choun-Sea Lin,⁶ Pan-Chyr Yang,⁶ Hsin-Sheng Tsay,³ Chun-Chi Liu,² Ching-Hsien Chen,² Choun-Sea Lin,⁶ Chen-Yu Lee,¹ Hui-Fang Sher,² Huei-Wen Chen,⁴ and Jeremy J.W. Chen²

¹Department of Agronomy and ²Institutes of Biomedical Sciences and Molecular Biology, National Chung-Hsing University; ³Institute of Biotechnology, Chao-yang University of Technology, Taichung, Taiwan, Republic of China and ⁴Department and Institute of Pharmacology, School of Medicine, National Yang-Ming University; ⁵Agricultural Biotechnology Research Center, Academia Sinica; ⁶Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan, Republic of China

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 *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 *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 Na₂HCO₃, 4.5 g/L glucose, and 10% fetal bovine serum (Invitrogen). Both cell lines were incubated at 37°C in 20% O₂ and 5% CO₂. Before the experiments, THP-1 cells were pretreated with 3.2 × 10⁻⁷ 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 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 μg/mL dexamethasone, 10 μg/mL tanshinone I, 10 μg/mL tanshinone IIA, or 5 μg/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 ×200 magnification. For in vitro invasion assay, 1 × 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 ≥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 Cytoomics 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 vascular endothelial growth factor (VEGF) promoter, a gift from Dr. Ann (32), are used for luciferase reporter assay. Briefly, 5 × 10^5 CL1-5 cells were cotransfected with the individual IL-8 promoter or VEGF promoter construct and a pSV-β-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 harvested and the genes were identified by luciferase activity using the Dual-Light Luciferase Reporter Gene Assay System (Invitrogen). All experiments, including those on nontransfected and vector-treated cells (as negative controls), were done in triplicate.

Double-stranded oligonucleotides containing two sequences coding the IL-8-specific AP-1 (5'-AAAAATTGATGAGTCAGGGTTGCTAGACTCGATTGT-3') annealed with 5'-AACAAAAACTCTGATCATACTCACCCTGAGTCACTACACT-3' and NF-κB (5'-AAAAAAAATCTCGAGATTTCCTCCCCCCTCCCCGA-3') annealed with 5'-AATCCGAGGAAATTTTCAGATT-CGGGGGAAAAATTTCAAGATT-3') binding sites (italicized) were designed for electrophoretic mobility shift assay. 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).

**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 triplicates. The relative expression level of interest against that of TATA box binding protein was calculated as ΔCT = [CT interest - CT TATA box binding protein].

**Tumorigenesis and Metastasis In vivo**
The CL1-5 cells or CL1-5 cells with enhanced green fluorescent protein-expressing construct (CL1-5/EGFP; 5 × 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 percentage 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 (×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...
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 (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 LD₅₀ 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 LD₅₀ (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 $72 \pm 9.6\%$ for dexamethasone-treated cells, to $73 \pm 8.9\%$ for tanshinone I-treated cells, to $49 \pm 4.9\%$ for tanshinone IIA-treated cells, and to $50 \pm 6.6\%$ for cryptotanshinone-treated cells ($P < 0.01$ for each). Cell proliferation continued to decrease at 72 h, to

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 $10 \mu$g/mL tanshinone I; c, treated with $10 \mu$g/mL tanshinone IIA; d, treated with $5 \mu$g/mL cryptotanshinone. Original magnification, x400. 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; ** and #, $P < 0.01$ for tanshinone IIA-treated cells at 48 and 72 h, compared with treatment with 0.1% DMSO, respectively. T1 (10), $10 \mu$g/mL tanshinone I; T2A (10), $10 \mu$g/mL tanshinone IIA; Cry (5), $5 \mu$g/mL cryptotanshinone. Mix (3, 3, 1.7), mixture of $3 \mu$g/mL tanshinone I, $3 \mu$g/mL tanshinone IIA, and $1.7 \mu$g/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](https://example.com/figure4.png)

**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-IL-6 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

CL1-5 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 CL1-5 cell morphology compared with control cells (Fig. 3B, a).

To study the effects of tanshinones on cell cycle regulation in CL1-5 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 CL1-5 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 CL1-5 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 CL1-5 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 antiangiogenic effects in CL1-5-bearing SCID mice (data not show). 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 CL1-5/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 CL1-5-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, $\times$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 ($n = 5$). 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 genes 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”).

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Putative gene name</th>
<th>Function</th>
<th>Decrease (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Microarray</td>
<td>RTQ-PCR</td>
</tr>
<tr>
<td><strong>Induction of apoptosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_003842</td>
<td>Tumor necrosis factor receptor superfamily, member 10b</td>
<td>Signal transducers</td>
<td>32.03</td>
</tr>
<tr>
<td>NM_003804</td>
<td>Receptor (tumor necrosis factor-RSF)-interacting serine/threonine kinase 1</td>
<td>Signal transducers</td>
<td>37.37</td>
</tr>
<tr>
<td><strong>Extracellular matrix-receptor interaction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_000610</td>
<td>CD44 antigen</td>
<td>Cell–cell interactions, cell adhesion and migration</td>
<td>61.32</td>
</tr>
<tr>
<td>NM_001846</td>
<td>Collagen, type IV, α2</td>
<td>Component of basement membranes</td>
<td>78.3</td>
</tr>
<tr>
<td><strong>Rac1 signaling pathway</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_003028</td>
<td>SHB adaptor protein</td>
<td>Signal transducers</td>
<td>66.97</td>
</tr>
<tr>
<td>NM_002646</td>
<td>Phosphatidylinositol 3-kinase, class 2, β polypeptide</td>
<td>Signal transducers</td>
<td>47.58</td>
</tr>
<tr>
<td>NM_198829</td>
<td>Ras-related C3 botulinum toxin substrate 1 (Rho family, small GTP-binding protein Rac1)</td>
<td>Cytoskeletal reorganization</td>
<td>17.63</td>
</tr>
<tr>
<td><strong>Ras-MAPK signaling pathway</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_005343</td>
<td>v-Ha-ras Harvey rat sarcoma viral oncogene homologue</td>
<td>Signal transducers</td>
<td>15.39</td>
</tr>
<tr>
<td>BC029822</td>
<td>PDGF-β polypeptide</td>
<td>Growth factor</td>
<td>71.46</td>
</tr>
<tr>
<td>NM_145110</td>
<td>MAPK kinase 3</td>
<td>Signal transducers</td>
<td>66.11</td>
</tr>
<tr>
<td>NM_002524</td>
<td>Neuroblastoma RAS viral (v-ras) oncogene homologue</td>
<td>Signal transducers</td>
<td>59.38</td>
</tr>
<tr>
<td>NM_002467</td>
<td>V-myc avian myelocytomatosis viral oncogene homologue</td>
<td>Transcription factor</td>
<td>44.74</td>
</tr>
<tr>
<td><strong>Cell cycle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_057118</td>
<td>Cyclin E1</td>
<td>Promoting cell cycle</td>
<td>48.11</td>
</tr>
<tr>
<td>NM_005901</td>
<td>MAD homologue 2</td>
<td>Signal transducers and transcriptional modulators</td>
<td>65.46</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_022377</td>
<td>Intercellular adhesion molecule 4</td>
<td>Cell adhesion and migration</td>
<td>53.11</td>
</tr>
<tr>
<td>NM_002087</td>
<td>Granulin</td>
<td>Growth factor</td>
<td>56.27</td>
</tr>
<tr>
<td>NM_002074</td>
<td>Guanine nucleotide binding protein, β polypeptide 1</td>
<td>Signal transducers</td>
<td>50.79</td>
</tr>
<tr>
<td>NM_002865</td>
<td>RAB2, member RAS oncogene family-like</td>
<td>Protein transport</td>
<td>47.50</td>
</tr>
<tr>
<td>BC007922</td>
<td>IFN-stimulated gene (20 kDa)</td>
<td>Immune response</td>
<td>75.73</td>
</tr>
<tr>
<td>NM_003015</td>
<td>Fas-activated serine/threonine kinase</td>
<td>Signal transducers</td>
<td>76.09</td>
</tr>
</tbody>
</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.

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

Discussion

Inflammation in the tumorigenic microenvironment plays a critical role in cancer progression (3). Some nonsteroidal anti-inflammatory drugs, such as aspirin, can reduce the risk of developing colorectal and breast cancers (16), and selective cyclooxygenase-2 inhibitors can inhibit angiogenesis in vitro (36). Our previous studies have shown that several anti-inflammatory agents can effectively suppress the expression of the angiogenic factor IL-8 (11, 31). In the
in CL1-5 cells and that tanshinones strongly inhibited that macrophage-CM significantly induced IL-8 expression angiogenesis, and metastasis of NSCLC (28, 31). We found (10, 11). IL-8 plays a crucial role in tumorigenesis, IL-8 expression in both cancer cells and stromal cells, such as fibroblasts and macrophages, can also increase (33). The interaction of NSCLC cells and stromal cells, such as fibroblasts and macrophages, can also increase.

Expression of IL-8 mRNA in tumor specimens of NSCLC growth, tumor progression, and metastasis (28, 37). 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 co-injected with CM but not with PBS. Co-injected 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.](image-url)
role of CM remains unclear and needs further study. Our study confirms that tanshinone I exhibits antitumorigenic and antimitastatic effects in vivo.

We used cDNA microarray and RTQ-PCR analyses to investigate the possible functional mechanism responsible for the antitumorigenic and antimitastatic 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 antimitastatic 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 mitogenic 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

Mol Cancer Ther 2008;7(11). November 2008

Downloaded from mct.aacrjournals.org on April 13, 2017. © 2008 American Association for Cancer Research.
tanshinone I isolated from
Kim SY, Moon TC, Chang HW, Son KH, Kang SS, Kim HP. Effects of
Salvia miltiorrhiza
from interleukin-12 and interferon-
Kang BY, Chung SW, Kim SH, Ryu SY, Kim TS. Inhibition of

23. Kim SY, Moon TC, Chang HW, Son KH, Kang SS, Kim HP. Effects of
tanshinone I isolated from Salvia miltiorrhiza Bunge on arachidonic acid
metabolism and in vivo inflammatory responses. Phytother Res 2002;16:
616 – 20.

Pharmacologic Function of Tanshinone I in Lung Cancer
Salvia miltiorrhiza

25. Wu WL, Chang WL, Chen CF. Cytotoxic activities of tanshinones


27. Yuan D, Pan YM, Fu WW, Makino T, Kano Y. Quantitative analysis of
the marker compounds in Salvia miltiorrhiza root and its phytochemical

28. Chen JJ, Lin YC, Yao PL et al. Tumor-associated macrophages:
the double-edged sword in cancer progression. J Clin Oncol 2005;23:
953 – 64.

lethal sepsis by selectively attenuating a late-acting proinflam-
atory mediator, high mobility group box 1. J Immunol 2007;178:
3856 – 64.

30. Chen HW, Chien CT, Yu SL, Lee YT, Chen WJ. Cyclosporine A
regulate oxidative stress-induced apoptosis in cardiomyocytes: mecha-
nisms via ROS generation, iNOS and Hsp70. Br J Pharmacol 2002;137:
771 – 81.

interleukin-8 expression in lung cancer cells. Am J Respir Cell Mol Biol

32. Chau CH, Chen KY, Deng HT, et al. Coordinating Etk/Bmx activation
and VEGF upregulation to promote cell survival and proliferation.

acid expression correlates with tumor progression, tumor angiogenesis,
patient survival, and timing of relapse in non-small cell lung cancer. Am J

34. Hong TM, Yang PC, Peck K, et al. Profiling the downstream genes of
tumor suppressor PTEN in lung cancer cells by complementary DNA

for composite regulatory signature discovery. Nucleic Acids Res 2006;34:
W571 – 7.

nonsteroidal anti-inflammatory drugs: insight into mechanisms and impli-

37. Pollard JW. Tumour-educated macrophages promote tumour progres-

38. Mukaida N, Mahe Y, Matsushima K. Cooperative interaction of
nuclear factor-κB- and cis-regulatory enhancer binding protein-like factor
binding elements in activating the interleukin-8 gene by pro-inflammatory

1162 – 72.

40. Yao PL, Tsai MF, Lin YC, et al. Global expression profiling of
theophylline response genes in macrophages: evidence of airway anti-

41. Karin M, Greten FR. NF-κB: linking inflammation and immunity to

42. Ostman A. PDGF receptors – mediators of autocrine tumor growth
and regulators of tumor vasculature and stroma. Cytokine Growth Factor

43. Claesson-Welsh L. Mechanism of action of platelet-derived growth

44. Wang XT, Liu PY, Tang JB. PDGF gene therapy enhances expression
of VEGF and bFGF genes and activates the NF-κB gene in signal pathways
138 – 9.

45. Hall A. Rho GTPases and the actin cytoskeleton. Science 1998;279:
509 – 14.

46. Raftopoulou M, Hall A. Cell migration: Rho GTPases lead the way.

47. Marhaba R, Zoller M. CD44 in cancer progression: adhesion,

48. Nagano O, Saya H. Mechanism and biological significance of CD44

factor-κB: linking inflammation and immunity to

50. Mishra JP, Mishra S, Gee K, Kumar A. Differential involvement of
calmodulin-dependent protein kinase II-activated AP-1 and c-Jun N-
terminal kinase-activated EGR-1 signaling pathways in tumor necrosis
factor-α and lipopolysaccharide-induced CD44 expression in human
Molecular Cancer Therapeutics

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


Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/7/11/3527

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2008/11/04/7.11.3527.DC1

Cited articles
This article cites 50 articles, 13 of which you can access for free at:
http://mct.aacrjournals.org/content/7/11/3527.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/7/11/3527.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.