Antitumor and Antimetastatic Activity of Synthetic Hydroxystilbenes Through Inhibition of Lymphangiogenesis and M2 Macrophage Differentiation of Tumor-associated Macrophages

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Abstract. *An increase in tumor-associated macrophages (TAMs) around the tumor microenvironment has been closely associated with a poor prognosis in patients with cancer, and M2 TAMs promote tumor growth and tumor metastasis by stimulating angiogenesis or lymphangiogenesis in tumors. We herein examined the effects of nine synthetic hydroxystilbenes on M2 macrophage activation and differentiation, and three selected dihydroxystilbenes on vascular endothelial cell growth factor (VEGF)-C-induced tube formation in human lymphatic endothelial cells (HLECs) (in vitro). We also investigated the antitumor and antimetastatic effects of three synthetic dihydroxystilbenes in LM8-bearing mice in vivo. The three selected synthetic stilbenes (at concentrations of 5, 10, 25, and 50 μM) inhibited the production of interleukin-10 and monocyte chemoattractant protein-1 in M2 macrophages, but promoted that of transforming growth factor-β1. The three dihydroxystilbenes (at concentrations of 10-50 μM) inhibited the phosphorylation of signal transducer and activator of transcript 3 without affecting its expression in the differentiation of M2 macrophages. Furthermore, the 2,3- and 4,4'-dihydroxystilbene inhibited VEGF-C-induced lymphangiogenesis in HLECs. Both 2,3- and 4,4'-dihydroxystilbene (at 10 and 25 mg/kg, twice daily) inhibited tumor growth and metastasis to the lung in mice. These results suggested that the antitumor and antimetastatic effects of 2,3- and 4,4'-dihydroxystilbene were partly due to anti-lymphangiogenesis, and the regulation of M2 macrophage activation and differentiation.*

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Tumor-associated macrophages (TAMs) derived from circulating monocytes have been identified as the main components of the tumor microenvironment and have been shown to stimulate tumor growth and metastasis (1-3). An increase in TAMs around the tumor microenvironment has been closely associated with a poor prognosis in patients with cancer (4-7). Different macrophage phenotypes reflect the expression of different receptors and different cytokines, and TAMs represent a subset of alternatively activated (M2) macrophages induced by Th2 cytokines such as interleukin (IL)-4 and IL-13 (4-10). Consequently, M2 TAMs accelerate tumor growth, invasion, and metastasis.

Stilbenes are natural polyphenolic phytoalexines found in medicinal plants of the *Polygonum* (Polygonaceae) and *Cassia* species (Leguminosae) (11-13). Among stilbene derivatives, resveratrol (3,4',5-trihydroxystilbene) has been found to exhibit potent preventive effects on lifestyle-related disorders such as hyperlipidemia, obesity, coronary heart disease and cancer, as well as on aging (14-18). Savio *et al.* (19) and Maccario *et al.* (20) reported that the resveratrol analog 4,4'-dihydroxystilbene inhibited the proliferation of LF1 cells (human lung fibroblasts) and MCF-7 cells (human breast adenocarcinoma). Resveratrol and its related 4,4'-dihydroxystilbene alone exhibit anti-proliferative effects against human fibroblast or breast cancer cell lines as chemopreventive agents.

In the present study, we examined the effects of nine hydroxystilbene derivatives on M2 macrophage activation (using MCP-1 production as a marker) using human THP-1 monocytes *in vitro* in order to evaluate the tumor

microenvironment. We selected three out of the nine dihydroxystilbenes based on the results of M2 macrophage activation and investigated their effects on lymphangiogenesis (*in vitro*) as well as on tumors and metastasis using highly metastatic osteosarcoma LM8-bearing C3H/He male mice *in vivo*. We also examined the effects of these three dihydroxystilbenes on the differentiation and activation of M2 macrophages (*in vitro*).

Materials and Methods

Synthetic stilbenes. Nine stilbenes were supplied by Professor K. Baba (Department of Pharmacognosy, Osaka University of Pharmaceutical Sciences, Osaka, Japan) (21) (Figure 1). These stilbenes were dissolved in ethanol, without exceeding a final concentration of 0.25% v/v for ethanol in the culture medium.

Materials. Culture medium, Dulbecco's modified Eagle's medium (DMEM), and RPMI-1640 medium were obtained from Nissui Pharm. Co. (Tokyo, Japan). Endothelial base medium (EBM) (EBM™-2) was purchased from Lonza Japan (Tokyo, Japan). Antibiotic and antimycotic solutions (100×) containing 10,000 units of penicillin, 10 mg/ml of streptomycin, and 25 μg/ml of amphotericin B in 0.9% NaCl were obtained from Sigma-Aldrich (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Gibco BRL (Auckland, New Zealand). The 100-mm and 60-mm culture dishes, and 96-well plates were purchased from Corning Inc. (NY, USA). Human interleukin (IL)-10, transforming growth factor (TGF-)-β_1, and monocyte chemoattractant protein (MCP)-1, enzyme-linked immunosorbent assay (ELISA) kits as well as human recombinant IL-13 and IL-4 were purchased from R & D Systems Inc. (Minneapolis, MN, USA). Rabbit monoclonal anti-signal transducer and activator of transcript 3 (STAT3) and rabbit monoclonal anti-phospho STAT3 (Tyr 705) were purchased from Cell Signaling Technology Japan (Tokyo, Japan). The mouse monoclonal antibody to β-actin was purchased from Sigma-Aldrich Japan. All chemicals used in this study were of reagent grade and obtained from Wako Pure Chemical Co. (Osaka, Japan).

Cells. Human monocyte THP-1 cells and highly metastatic osteosarcoma LM8 cells were obtained from the Institute of Development, Aging, and Cancer, Tohoku University, Japan. THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (0.25 μg/ml), while LM8 cells were maintained in DMEM containing the above FBS and antibiotic solutions. Human lymphatic endothelial cells (HLECs, third passage) were purchased from Lonza Japan, seeded on 100-mm culture dishes and maintained in EBM supplemented with growth factor (EGM).

Animals. Male C3H/He mice (5 weeks old) were obtained from Japan SLC Co. (Shizuoka, Japan), housed for 1 week in a room with controlled temperature and humidity, and given free access to food and water during the experiments. Animal experiments were performed according to the ethical guidelines for Animal Experimentation, Ehime University and Japanese Pharmacological Society, and the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The Animal Studies Committee of Ehime University approved the experimental protocol (approval no. 05-YA-1-1).

IL-10, MCP-1, and TGF-β1 production in M2-polarized THP-1 macrophages (in vitro). THP-1 monocytes (5×10^6 cells/well) were cultured with 200 nM phorbol 12-myristate 13 acetate (PMA) for 72 h in a 60-mm culture dish to induce macrophage differentiation. After the incubation period, differentiated cells adhered to the culture dish and displayed morphological similarities to macrophages. M2-polarized THP-1 macrophages were obtained by the methods of Tjiu *et al*. (22). THP-1 macrophages (5×10^6 cells/well) were cultured with IL-4 (25 ng/ml) and IL-13 (25 ng/ml) for 24 h in a 60-mm culture dish, and the differentiated M2 macrophages were then washed twice with Dulbecco's phosphate-buffered saline (PBS) (pH 7.4). The differentiated M2 macrophages were cultured with the nine different mono- and di-hydroxystilbenes at 50 μM for 24 h. After the incubation period, IL-10, MCP-1, and TGF-β_1 levels were measured in the medium using the respective ELISA kits.

STAT3 expression and phosphorylation in the differentiation of M2-polarized THP-1 macrophages (in vitro). PMA-treated THP-1 macrophages were cultured with IL-4 (25 ng/ml) and IL-13 (25 ng/ml) in order to differentiate M2 macrophages in the presence of three of the dihydroxystilbenes, namely 2,3-, 3,4-, and 4,4'-dihydroxystilbene, for 24 h in a 60-mm culture dish. The cells were washed with ice-cold PBS (pH 7.0) and then lysed and centrifuged using the same methods described above. After centrifuging, the supernatant was used to measure the phosphorylation of STAT3 as well as its protein levels. Samples (80 μg of protein) were subjected to electrophoresis on a 7.5% polyacrylamide gel, and the separated proteins were then transferred to blotting sheets of the polyvinylidene difluoride (PVDF) membrane (Bio-Rad Lab. Hercules, CA, USA). The blot was incubated with 5% skimmed milk to bind on the PVDF membrane. The solution of the anti-STAT3 rabbit, anti-phosphoSTAT3 rabbit monoclonal, or anti-β-actin mouse monoclonal antibody was added to bind to their respective specific proteins (primary antibody reaction), and alkaline phosphatase-conjugated anti-rabbit IgG goat antibody (MP Biomedicals, Tokyo, Japan) or alkaline phosphatase-conjugated anti-mouse IgG goat antibody (Sigma-Aldrich) was then added to detect these proteins (secondary antibody reaction). The locations of the respective antibodies were detected by 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Sigma-Aldrich).

Lymphangiogenesis (tube-like network formation) in HLECs (in vitro). Lymphangiogenesis induced in HLECs by vascular endothelial growth factor (VEGF)-C was assessed by a slightly modified version of previously described methods (23, 24). Briefly, Matrigel (reduced growth factor) (50 μl) was placed into each well of a 96-well culture plate at 4˚C and allowed to polymerize during incubation for 1 h at 37˚C. HLECs (third passage, 2×10^4 cells) were seeded onto the Matrigel (reduced growth factor) and cultured with each of 2,3-, 3,4-, and 4,4'-dihydroxystilbene and VEGF-C (100 ng/ml) in a total volume of 200 μl for 12 h. Two different microscopic fields (×50 and ×100 magnification) per well were photographed and light micrographic images were stored in a computer. The total length of the tube structures in each photograph (×50 magnification) was measured using a Coordinating Area and Curvimeter Machine (X-PLAN 360 dII; Ushitaka Co, Tokyo, Japan).

Tumor growth and metastasis in LM8-bearing mice (in vivo). Solid-type LM8 was prepared by the subcutaneous transplantation of 1×10^5 cells into the backs of mice (n=59) on day 0. Three dihydroxystilbenes (2,3-, 3,4-, and 4,4'-dihydroxystilbene) were suspended in 5% γ-cyclodextrin. The three dihydroxystilbenes (10

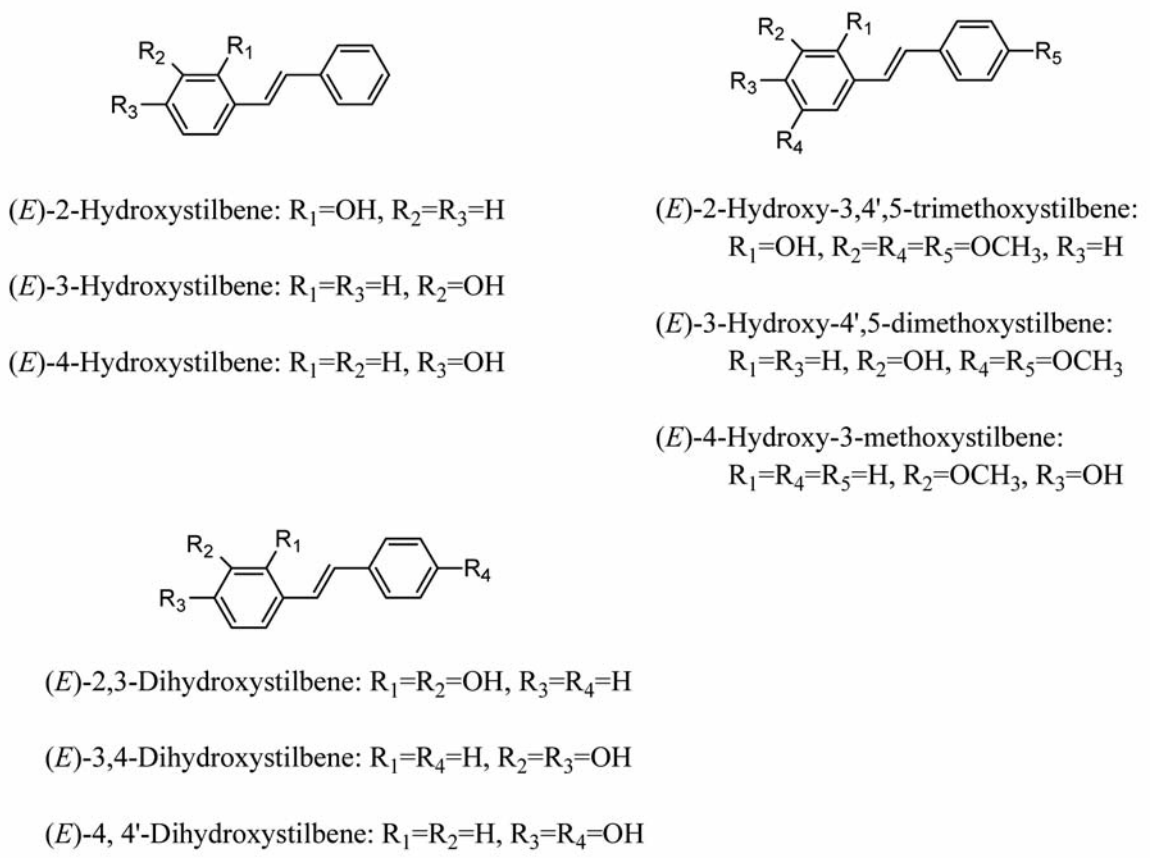

Figure 1. *Structures of the studied hydroxystilbenes.*

or 25 mg/kg body weight, twice daily) were administered orally for 30 days, starting 12 h after the implantation of tumor cells. Non-LM8-bearing mice (normal) and LM8-bearing mice (control) were orally administered 5% γ-cyclodextrin solution alone according to the same schedule. Tumor volumes were determined every 2-3 days by direct measurements with calipers; volumes were calculated as the length × width^2/2. On day 31, mice were killed with an overdose of diethylether, and the liver, lung, spleen, thymus, and tumors were quickly removed and weighed. Metastatic nodules in the liver or lung were counted using a stereoscopic microscope: tumor metastases of the liver and lung were photographed.

Statistical analysis. All values are expressed as means±SE. When data followed a normal distribution, they were analyzed by a one-way ANOVA or repeated-measures ANOVA. When the F-test was significant, means were compared using the Turkey-Kramer or Dunnett's test with Stat View (SAS Institute Inc., Tokyo, Japan). Differences were considered significant at $p<0.05$.

Results

IL-10, MCP-1 and TGF-β_1 production in M2-polarized THP-1 macrophages. Among the nine stilbenes examined, 2,3-, 3,4-, and 4,4'-dihydroxystilbene inhibited the production of MCP-1 in M2-polarized THP-1 macrophages at a concentration of 50 μM. The inhibition ratios for MCP-1 production of 2,3-, 3,4-, and 4,4'-dihydroxystilbene were 31.8, 41.3, and 56.4%, respectively (Table I). On the other hand, monohydroxystilbenes (2-, 3-, and 4-hydroxystilbene, and 4-hydroxy-3-methoxy-, 3-hydroxy-4',5-dimethoxy-, and 2-hydroxy-3,4',5-trimethoxystilbene) had no effect on the production of MCP-1 at 50 μM (Table I).

2,3-, 3,4-, and 4,4'-dihydroxystilbenes were further examined for their effects on the production of IL-10 and TGF-β_1, as well as MCP-1, by M2-poralized THP-1 macrophages at concentrations of 5, 10, 25, and 50 μM. 2,3-Dihydroxystilbene at 25 and 50 μM, 3,4-dihydroxystilbene at 50 μM, and 4,4'-dihydoxystilbene at 10, 25 and 50 μM significantly inhibited the production of IL-10 by M2 THP-1 macrophages (Figure 2A). The production of MCP-1 by M2 THP-1 macrophages was also inhibited by 2,3-, 3,4-, and 4,4'-dihydroxystilbenes at concentrations of 5 to 50 μM (Figure 2B). The production of TGF-β_1 was greater in M2 THP-1 macrophages than in THP-1 macrophages. The production of TGF-β_1 in M2 THP-1 macrophages was further enhanced by adding 2,3-dihydroxystilbene at 25 and

Table I. *Effects of nine synthetic stilbenes on monocyte chemoattractant protein-1 (MCP-1) production in M2-polarized THP-1 macrophages.*

Stilbene (50 μM)	MCP-1 production (pg/mg protein) (%)
None (medium alone)	241.3±4.2 (100%)
2-Hydroxystilbene	251.2±5.1 (104.1%)
3-Hydroxystilbene	265.3±7.3 (109.9%)
4-Hydroxystilbene	227.3±3.9 (94.2%)
2-Hydroxy-3,4',5-trimethoxystilbene	233.2 ±10.4 (96.6%)
3-Hydroxy-4',5-dimethoxystilbene	216.9±11.5(89.9%)
4-Hydroxy-3-methoxystilbene	244.1±4.8 (101.2%)
2,3-Dihydroxystilbene	164.6±2.5 (68.2%)
3,4-Dihydroxystilbene	141.6±3.6 (58.7%)
4,4'-Dihydroxystilbene	105.2±3.1 (43.6%)

Values are the mean±SE of three experiments.

50 μM, 3,4-dihydroxystilbene at 10 to 50 μM, or 4,4'-dihydroxystilbene at 5 to 50 μM (Figure 2C).

Expression of STAT3 protein and STAT3 phosphorylation (pSTAT3) in the differentiation of M2-polarized THP-1 macrophages. p-STAT3 was increased in the process of M2-polarized THP-1 macrophage differentiation stimulated by IL-4 plus IL-13, whereas the expression of STAT3 protein was not affected by M2 THP-1 macrophage differentiation. The three dihydroxystilbenes, 2,3-, 3,4-, and 4,4'-dihydroxystilbenes, at concentrations of 10-50 μM inhibited p-STAT3 increase during M2 THP-1 macrophage differentiation induced by IL-4 plus IL-13 (Figure 3).

VEGF-C-induced capillary-like tube formation of HLECs. 2,3-Dihydroxystilbene inhibited VEGF-C-induced capillary-like-tube formation in HLECs at concentrations of 10 to 50 μM (Figure 4A). Both 3,4- and 4,4'-dihydroxystilbenes also inhibited VEGF-C-induced lymphangiogenesis at concentrations of 5 to 50 μM (Figure 4B and C).

Tumor growth and tumor metastasis in C3H/He mice bearing LM8 highly metastatic osteosarcoma. 2,3-Dihydroxystilbene inhibited tumor growth on days 12 to 40 (Figure 5A) and final tumor weights at doses of 10 and 25 mg/kg (Figure 5D) in LM8-bearing mice. 3,4-Dihydroxystilbene inhibited tumor growth on days 12 to 30 and on days 36 and 40 at a dose of 25 mg/kg (Figure 5B), but had no effect on final tumor weights (Figure 5E). 4,4'-Dihydroxystilbene (10, 25 and 50 mg/kg, twice daily) also inhibited tumor growth on day 10 and on days 13 to 36 (Figure 5C), as well as final tumor weights (Figure 5F).

2,3-Dihydroxystilbene (10 and 25 mg/kg, twice daily) and 4,4'-dihydroxystilbene (10 and 25 mg/kg, twice daily) both inhibited tumor metastasis to the lung and increases in lung weights in LM8-bearing mice (Figure 6A and D, C and F, and Table II), whereas 3,4-dihydroxystilbene had no effect on lung metastasis (Figure 6B and E). Liver metastasis and kidney metastasis were inhibited by 2,3-, 3,4-, and 4,4'-dihydroxystilbenes (Table II).

No significant differences were observed in final body, liver, spleen, or thymus weights among normal, LM8-bearing mice (control) and 2,3-, 3,4-, and 4,4'-dihydroxystilbene-treated LM8-bearing mice (Table III). On the other hand, 2,3- and 4,4'-dihydroxystilbenes (25 mg/kg, twice daily) inhibited increases in lung weights induced by lung metastasis (Table III).

Discussion

The number of patients with cancer is increasing worldwide. Cancer therapy involves the surgical resection of malignant tumors in the body, followed by radiation with/without adjuvant chemotherapy; however, severe adverse reactions are associated with such therapies. Furthermore, metastasis to the lung, liver, and kidney through tumor-induced angiogenesis and to regional lymph nodes through the lymphatic system has been reported in patients with resected cancer such as breast carcinoma, colon carcinoma, and osteogenic sarcoma (25-29). Using a model of osteosarcoma, LM8-bearing mice, we previously demonstrated that lymphangiogenesis and TAMs in tumors were enhanced, and, as a consequence, tumor growth and lung metastasis were more prominent (30, 31). Macrophages have been divided into classically activated macrophages (M1-polarized macrophages) and alternative activated macrophages (M2-polarized macrophages) based on their abilities to produce T -helper type 1 (Th1) and Th2 cytokines, respectively (10). Previous studies reported that the differentiation of M2 macrophages was closely associated with the activation of TAMs (8-10). STAT3 has also been associated with the tumor microenvironment, as well as tumor growth and metastasis, and its signaling in macrophages has been shown to play a role in the regulation of immunosuppression and angiogenesis (32-34). Sica and Bronte found that the activation of STAT3 (pSTAT3) was essential for the differentiation of M2 macrophages (35).

In the present study, we examined the inhibitory effects of nine synthetic mono- or dihydroxystilbenes on the production of MCP-1 from M2-polarized macrophages as a marker of activation of M2-polarized macrophages. Among the nine stilbenes tested, three dihydroxystilbenes: 2,3-, 3,4- and 4,4'-dihydroxystilbene, inhibited the production of MCP-1 from M2 macrophages. These results demonstrate that the inhibitory effects of stilbenes with dihydroxy groups on the production of MCP-1 were greater than those with mono-hydroxyl groups. Furthermore, among the three dihydroxystilbenes examined, the inhibitory effects of 4,4'-

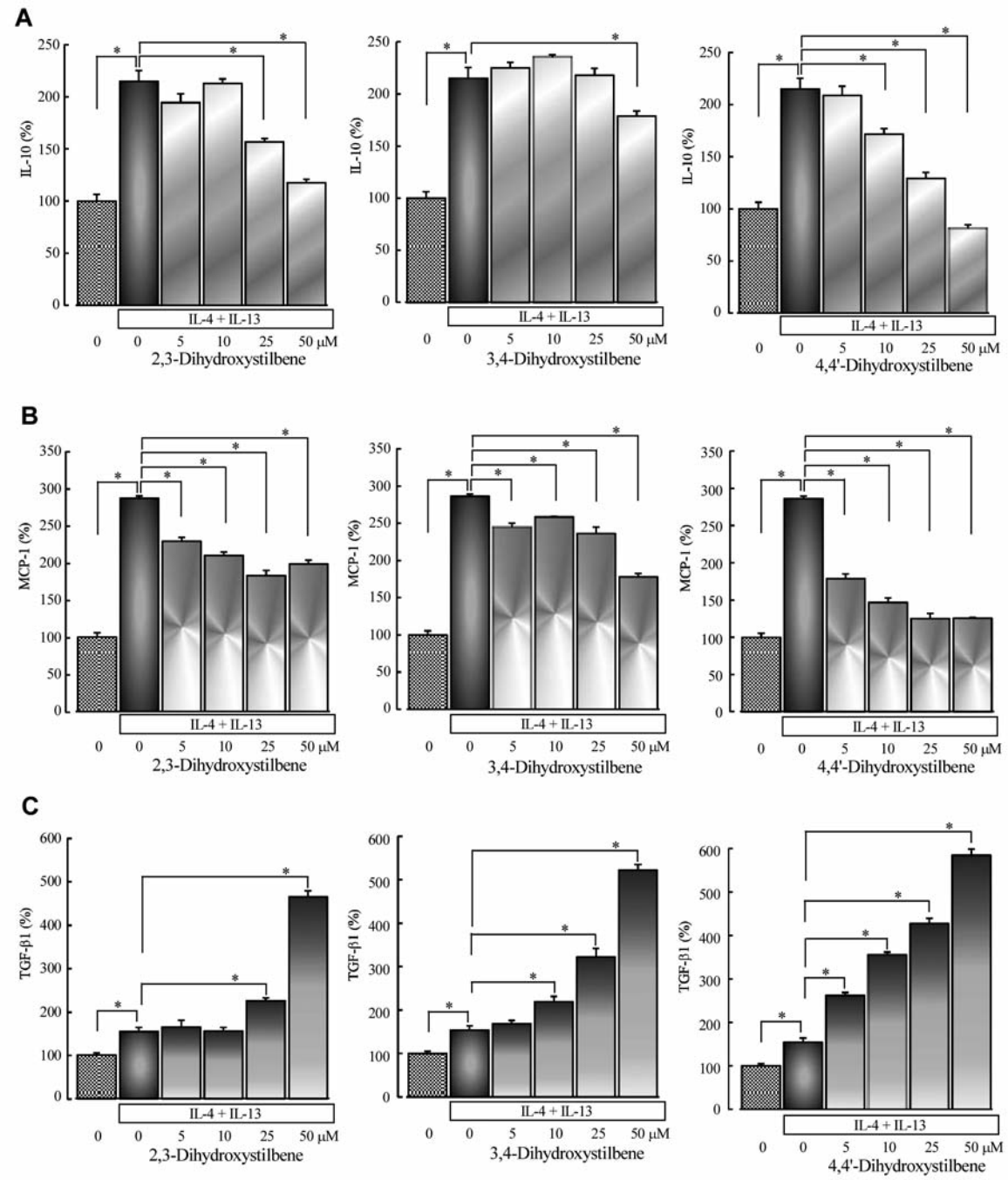

Figure 2. *Effects of 2,3-, 3,4- and 4,4'-dihydroxystilbenes on the production of interleukin-10 (IL-10) (A), monocyte chemoattractant protein-1 (MCP-1) (B), and transforming growth factor-β1 (TGF-β1) (C) by M2-polarized THP-1 macrophages. Values are expressed as the mean±S.E. of four experiments. *Significantly different at p<0.05 from cells cultured in medium containing IL-4 plus IL-13.*

dihydroxystilbene on M2 macrophage differentiation (STAT3 phosphorylation) and activation (MCP-1 and IL-10 production) were stronger than those of 2,3- and 3,4-dihydroxystilbenes. The extent of the antitumor effects of the three dihydroxystilbenes was in the order of 4,4'-dihydroxystilbene=2,3-dihydroxystilbene>3,4-dihydroxystilbene at a dose of 10 mg/kg. These physiological effects of various stilbenes may be related to the combined positions of the hydroxyl groups or the number of phenolic hydroxyl groups in the stilbene skeleton. Further studies are needed in

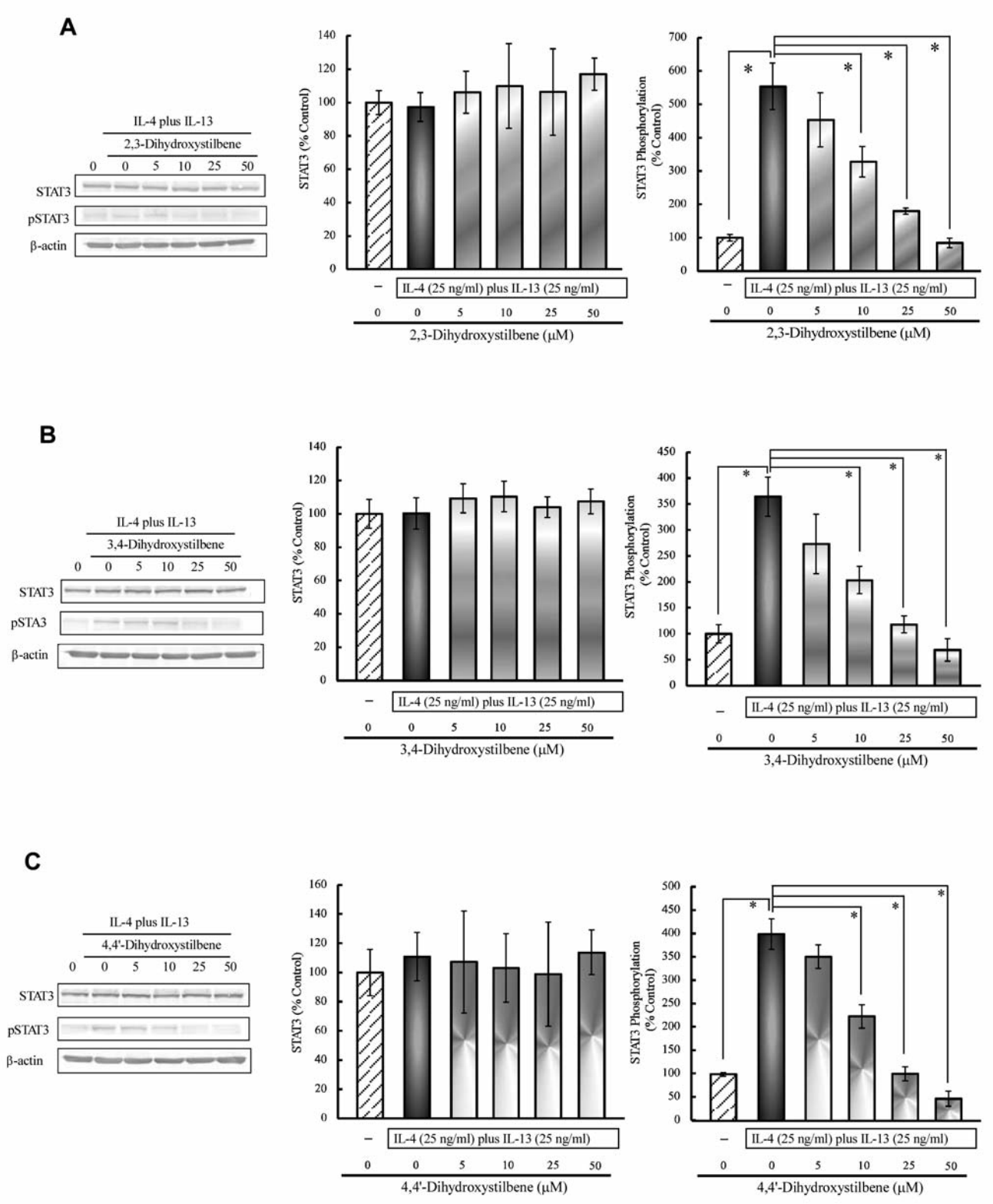

Figure 3. *Effects of 2,3-dihydroxystilbene (A), 3,4-dihydroxystilbene (B) and 4,4'-dihydroxystilbene (C) on the expression of the signal transducer and activator of transcript 3 (STAT3) protein and phosphorylation in the differentiation of M2-polarized THP-1 macrophages treated with interleukin-4 (IL-4) plus IL-13. Values are expressed as the mean±S.E. of four experiments. *Significantly different at p<0.05 from cells cultured in medium containing IL-4 plus IL-13.*

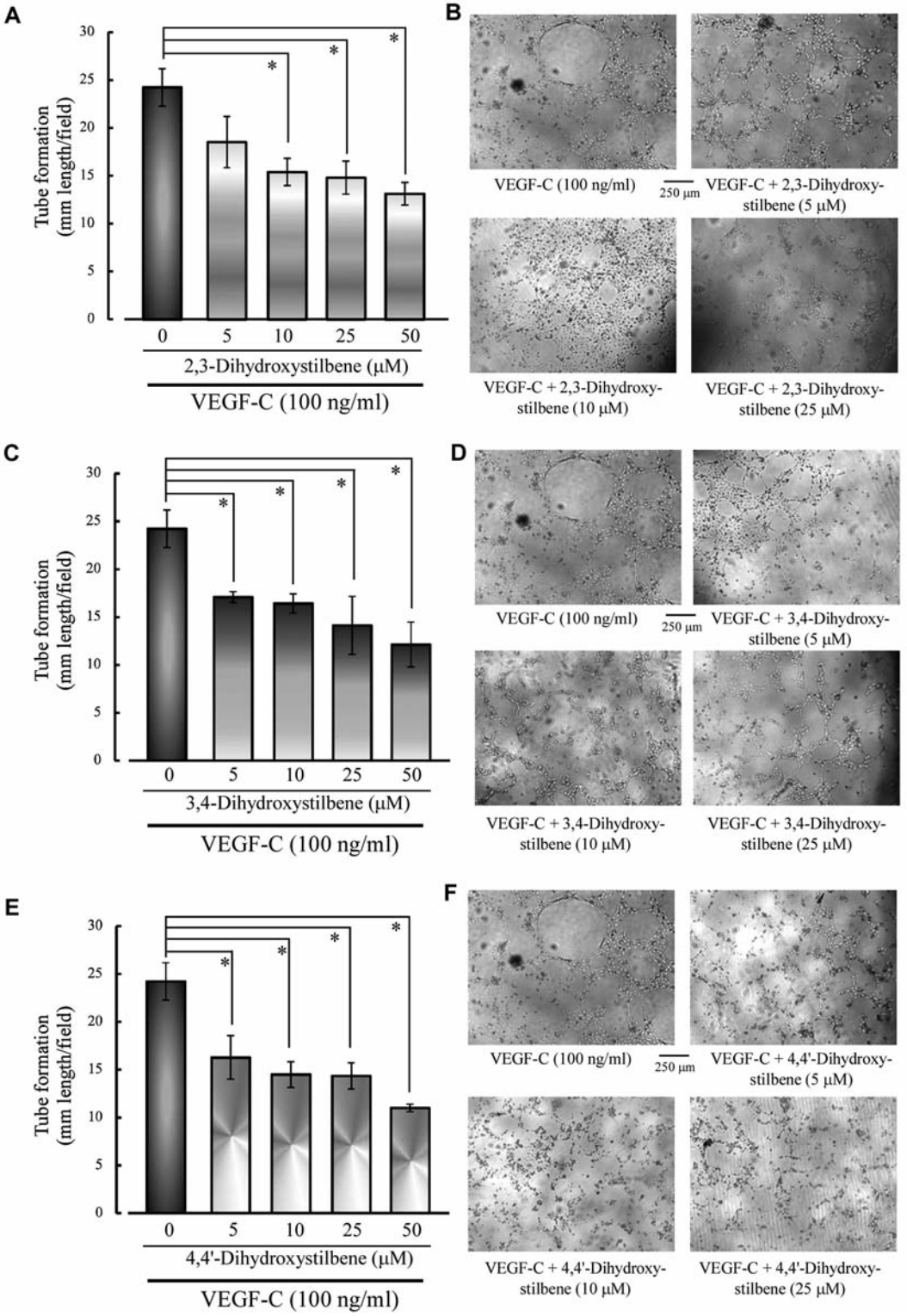

Figure 4. *Effects of 2,3-dihydroxystilbene (A and B), 3,4-dihydroxystilbene (C and D) and 4,4'-dihydroxystilbene (E and F) on vascular endothelial growth factor-C (VEGF-C)-induced capillary-like tube formation in human lymphatic endothelial cells (HLECs). A, C and E: Values are expressed as the mean±S.E. of four experiments. *Significantly different at p<0.05 from cells cultured in medium alone. B, D and F: Light micrographs showing the VEGF-C-induced formation of capillary-like tubes by HLECs in the presence of 2,3-dihydroxystilobene, 3,4-dihydroxystilbene and 4,4'-dihydroxystilbene (5, 10, 25 and 50 μM).*

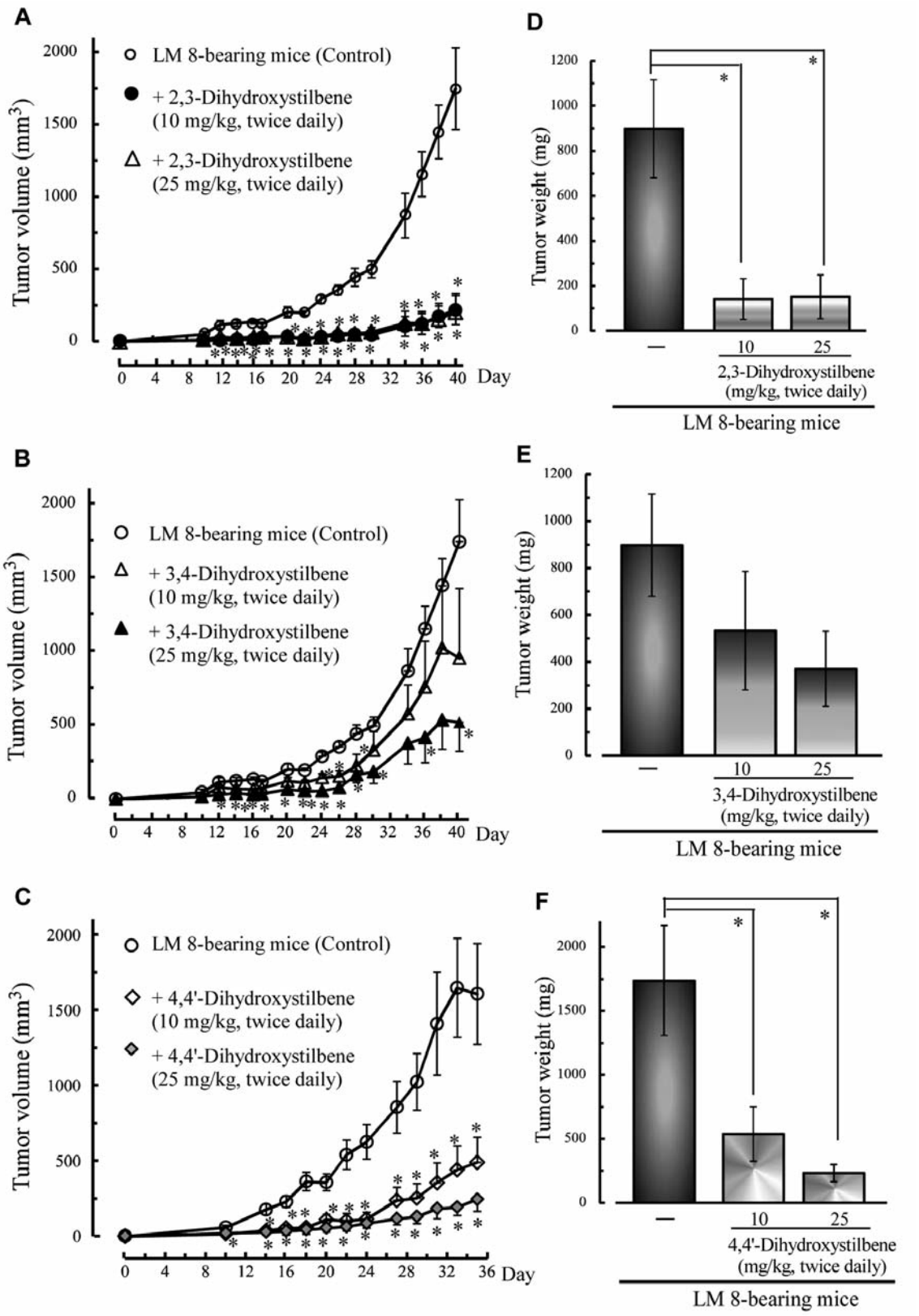

Figure 5. *Effects of 2,3-, 3,4-, and 4,4'-dihydroxystilbenes on tumor growth (A, B, and C) and final tumor weight (D, E and F) in LM8-bearing C3H/He male mice. Values are expressed as the mean±S.E. of 7-8 mice. *Significantly different at p<0.05 from untreated LM8-bearing mice (control).*

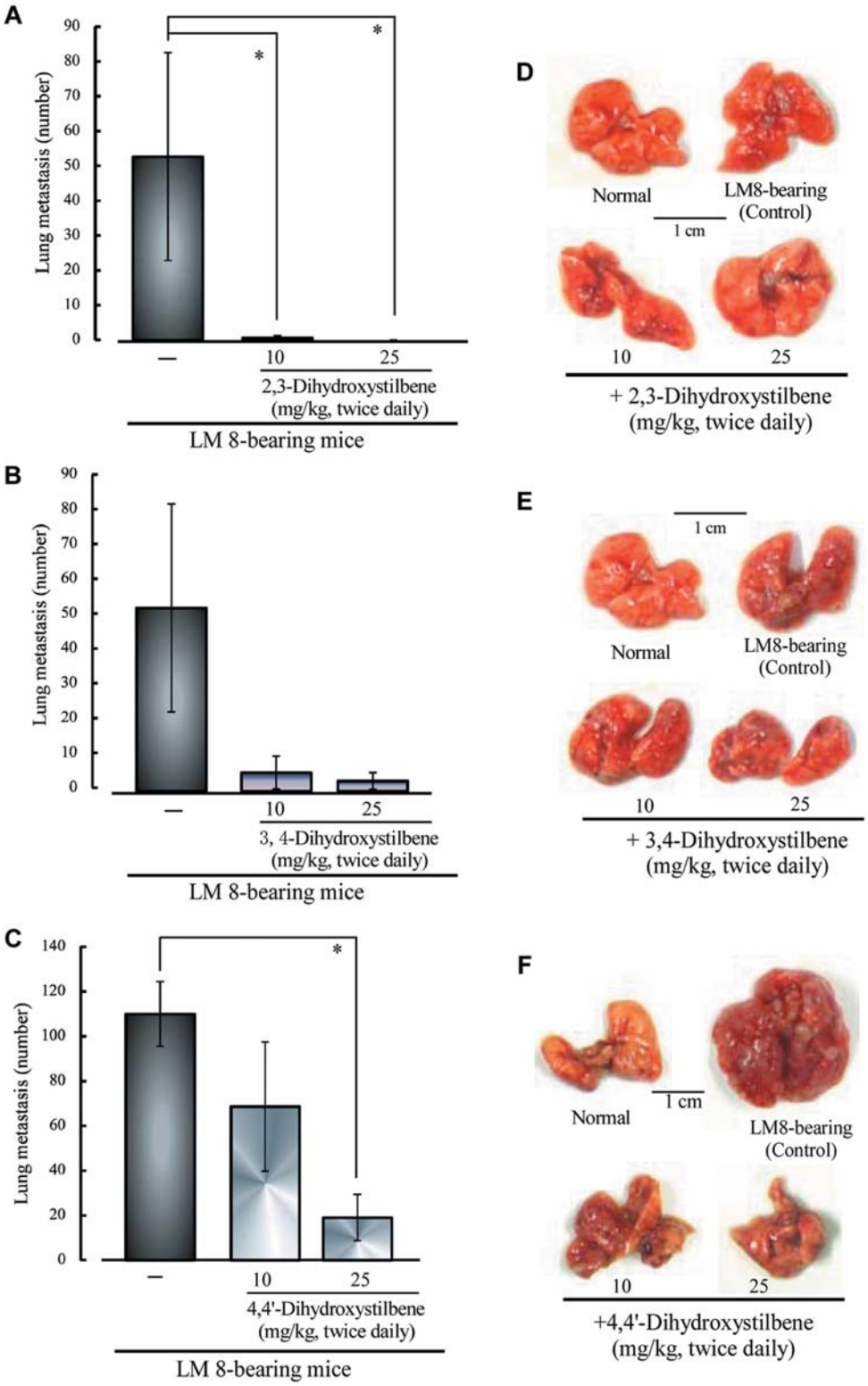

Figure 6. *Effects of 2,3-, 3,4-, and 4,4'-dihydroxystilbenes on metastasis to the lung in LM8-bearing mice. A, B and C: Frequency of metastasis to the lung. Values are expressed as the mean±S.E. of 7-8 mice. *Significantly different at p<0.05 from untreated LM8-bearing mice (control). D, E, and F: Photographs showing metastasis to the lung in LM8-bearing mice and 2,3-dihydroxystilbene-, 3,4-dihydroxystilbene-, and 4,4'-dihydroxystilbene-treated LM8-bearing mice.*

Table II. *Effects of 2,3-, 3,4-, and 4,4'-dihydroxystilbenes on metastasis to the liver, lung, and kidney in LM8-bearing mice. Data are expressed as the number of mice with metastases as a percentage of mice.*

	Liver metastasis	Lung metastasis	Kidney metastasis
Experiment 1			
No treatment (Control)	6/8 (75.0%)	6/8 (75.0%)	4/8 (50.0%)
2,3-Dihydroxystilbene 10 mg/kg, twice daily	0/7 (0%)	1/7 (14.3%)	0/7 (0%)
25 mg/kg, twice daily	0/7 (0%)	0/7 (0%)	0/7 (0%)
3,4-Dihydroxystilbene 10 mg/kg, twice daily	2/7 (28.6%)	2/7 (28.6%)	0/7 (0%)
25 mg/kg, twice daily	1/7 (14.3%)	2/7 (28.6%)	1/7 (14.3%)
Experiment 2			
No treatment (Control)	5/7 (71.4%)	7/7 (100%)	6/7 (85.7%)
4,4'-Dihydroxystilbene 10 mg/kg, twice daily	3/8 (37.5%)	4/8 (50.0%)	0/8 (0%)
25 mg/kg, twice daily	1/8 (12.5%)	3/8 (37.5%)	0/8 (0%)

Table III. *Effects of 2,3-. 3,4-, and 4,4'-dihydroxystilbenes on body and various tissue weights in LM8-bearing mice.*

	Final body weight (g)	Liver (mg)	Lung (mg)	Spleen (mg)	Thymus (mg)
Experiment 1					
Normal (no LM-8)	23.3±0.44	1,062.6±46.0	139.6±3.71*	58.0±2.70	23.4±1.21
No treatment (Control)	22.6±0.51	1,049.0±79.6	245.5±29.8	63.6±4.92	19.9±1.47
2,3-Dihydroxystilbene					
10 mg/kg, Twice daily	23.5±0.60	1,090.6±48.6	205.9±6.48	64.1±2.60	19.9±1.18
25 mg/kg, Twice daily	23.5±0.36	1,043.6±76.3	157.6±6.62*	66.4±6.01	22.1±1.44
3,4-Dihydroxystilbene					
10 mg/kg, Twice daily	24.1±0.44	1,091.9±71.8	214.1±15.7	67.4±4.05	19.9±1.79
25 mg/kg, Twice daily	22.7±0.52	1,055.6±35.8	207.6±9.74	65.6±3.69	19.7±1.66
Experiment 2					
Normal (no LM8)	24.1±0.27	1,191.7±22.3	135.4±2.96*	63.4±1.17	25.1±0.99
No treatment (Control)	22.2±0.37	973.3±122.7	361.0±87.1	68.0±7.83	19.4±2.67
4,4'-Dihydroxystilbene					
10 mg/kg, Twice daily	23.1±0.47	1,156.4±31.1	243.9±45.1	78.5±4.84	18.3±1.70
25 mg/kg, Twice daily	23.9±0.62	1,400.5±149.2	178.8±27.8*	76.1±4.48	18.9±1.98

Values are the mean±S.E. of 7-8 mice.

order to clarify the relationship between the structure and physiological effects of more stilbenes.

In the present study, we demonstrated that 2,3-, 3,4-, and 4,4'-dihydroxystilbene inhibited the differentiation of M2 macrophages by inhibiting the phosphorylation of STAT3 without affecting its expression in the differentiation of M2 macrophages induced by IL-4 plus IL-13. Previous studies reported that chemokines such as MCP-1 and C-C chemokine ligand-21 (secondary lymphoid tissue chemokines) played important roles in lymphangiogenesis through cytokine-induced macrophage activation (36-39). Maeng *et al*. showed that the inhibition of TGF-β_1 expression reduced lymphangiogenesis and tumor metastasis, and suggested that TGF-β_1 was a potential target for the treatment of tumor metastasis (40). On the other hand, Lee *et al*. reported that TGF-β_1 inhibited VEGF-C-induced migration, invasion, and capillary-like tube formation in HLECs (41). On the other hand, we found that the production of TGF-β_1 was increased by 2,3-, 3,4-, and 4,4'-dihydroxystilbene in M2 macrophages. In the present study, 2,3-, 3,4-, and 4,4'-dihydroxystilbene inhibited VEGF-C-induced capillary-like tube formation in HLECs. In the *in vivo* study, 2,3- and 4,4'-dihydroxystilbene inhibited tumor growth and tumor metastasis to the lung and liver. We previously showed an increase in TAMs and the lymphangiogenesis of tumors in mice bearing highly metastatic LM8 osteosarcoma (30, 31). These findings suggest that the antitumor and antimetastatic

actions of 2,3- and 4,4'-dihydroxystilbenes were due to the inhibition of VEGF-C-induced lymphangiogenesis in HLECs through the regulation (reduction in IL-10 and MCP-1, and increase in TGF-β_1) of M2 macrophages, and the inhibition of M2 macrophage differentiation by inhibition of STAT3 phosphorylation without affecting STAT3 protein expression.

As far as we are aware, this is the first study to show that synthetic dihydroxystilbenes prevented tumor growth and metastasis to the lung and liver by inhibiting lymphangiogenesis induced by the activation and differentiation of M2 TAMs.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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This article is dedicated to Dr. M, Sumiyoshi, 42 years old, who passed away on December 11th, 2014. We wish to express our posthumous gratitude to Dr. M. Sumiyoshi for her great commitment to carrying out the tests, the discussion of the results, and the writing of the text, and to the overall process of preparing this study.

All Authors read and approved the final manuscript.

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