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Effects of Naturally Occurring Stilbene Glucosides from Medicinal Plants and Wine, on Tumour Growth and Lung Metastasis in Lewis Lung Carcinoma-Bearing Mice

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Abstract

Stilbene glucosides are naturally occurring phytoalexins, found in a variety of medicinal plants. Among the stilbene derivatives, resveratrol 3-*O*-D-glucoside (piceid) is found in grapes and wine. We studied the effects of stilbene glucosides isolated from medicinal plants and grapes on tumour growth and lung metastasis in mice bearing highly metastastic Lewis lung carcinoma (LLC) tumours. We also studied the inhibitory effects of stilbene glucosides on differentiation of human umbilical vein endothelial cells (HUVECs) to form a capillary network.

Tumour growth in the right hind paw and lung metastasis were inhibited by oral administration of the stilbene glucosides, piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside for 33 consecutive days, in LLC-bearing mice. As the number of $CD8^+$ and $NK1.1^+$ T cells in the spleen was not affected, the inhibitory effects of these stilbene glucosides on tumour growth and lung metastasis could not be explained by natural killer or cytotoxic T lymphocyte activation. Piceid inhibited the DNA synthesis in LLC cells at a concentration of 1000 μM, but not at lower concentrations (10–100 μM). 2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside also inhibited DNA synthesis in LLC cells (IC50 81 μM). In addition, both stilbene glucosides inhibited the formation of capillary-like tube networks (angiogenesis) of HUVECs at concentrations of 100 to 1000 μM.

We suggest that the antitumour and antimetastatic activity of the stilbene glucosides, piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside, might be due to the inhibition of DNA synthesis in LLC cells and angiogenesis of HUVECs.

Stilbene glucosides are naturally occurring phytoalexins found in medicinal plants of *Polygonum* species and *Rheum* species (Polygonaceae) and *Cassia* species (Leguminosae) (Hata et al 1975, 1979; Kubo et al 1981; Kimura et al 1983a). Among the stilbene derivatives, resveratrol-3-*O*-D-glucoside (piceid) is also found in grapes and wine. We previously showed that stilbene derivatives such as piceid, resveratrol, and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside reduced the elevation of lipid levels (Arichi et al 1982), and that 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside strongly prevented liver damage induced by high lipid peroxidized diets (Kimura et al 1983b).

Tumour angiogenesis is the induction of the directional growth of blood vessels from surrounding tissue into a solid tumour. The stimuli that promote tumour angiogenesis may be provided directly by the tumour cells or indirectly by host inflammatory cells that are attracted to the tumour site. Angiogenesis is as important in the growth of secondary tumour colonies as it is in the growth of primary solid tumours. Secondary tumours often give rise to tertiary tumours in distant organs by a process termed the metastatic cascade (Blood & Zetter 1990; Ferrara 1995; Folkman 1995; Kerbel 1997), and the entry of tumour cells into the neovascular system at the secondary site is crucial for the further spread of these neoplasms. Thus, in a cyclic process, primary tumour cells require blood vessels to grow and spread to secondary organs; tumour cells in the secondary site similarly require vessels to grow and to return to the blood stream to colonize additional organ sites.

In this study, we examined the effects of stilbene glucosides isolated from medicinal plants and

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grapes on tumour growth and lung metastasis in mice bearing highly metastatic Lewis lung carcinoma (LLC) tumours. We also examined the inhibitory effects of stilbene derivatives on the differentiation of human umbilical vein endothelial cells (HUVECs) to form a capillary network.

Materials and Methods

Materials

Piceid (3,5,4′-trihydroxystilbene-3-*O*-D-glucoside) and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside were isolated from the roots of *Polygonum cuspidatum* Sieb. et Zucc. and *Polygonum multiflorium* Thunb. as previously described (Hata et al 1975; Kubo et al 1981; Kimura et al 1983a). Mouse lymphocyte separation medium (Lympholytes-Mouse) was purchased from Dainippon Pharmacy Co. Ltd (Osaka, Japan). Fluorescein isocyanate (FITC)-labelled anti-mouse CD8 and phycoerythrin (PE)-labeled anti-mouse NK1.1 were purchased from Serotec Ltd (Oxford, UK). 3′-*O*-Acetyl-2′,7′-bis (carbocyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) solution (1 mM) was purchased from Wako Pure Chemical Inc. Ltd (Osaka, Japan). Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). [Methyl-^{3}H]-thymidine (sp. act. 740 GBq mmol^{-1}) was purchased from NEN Life Science Products, Inc. (Boston, MA). Dulbecco's modified Eagle's medium (DMEM), endothelial basal medium (modified MCB 131; EBM) and CS-C medium kits were obtained from Nissui Pharmaceutical Co. Ltd (Tokyo, Japan), Clonetic (San Diego, CA) and Cell Systems Co. (Kirkland, WA), respectively, and used as culture media. Antibiotic and antimycotic solutions (100 ×) containing 10 000 U mL^{-1} penicillin, 10 mg mL^{-1} streptomycin and 25 μg mL^{-1} amphotericin B in 0·9% NaCl were purchased from Sigma Chemical Co. (Louis, MO). Foetal bovine serum (FBS) was purchased from Gibco BRL (Auckland, New Zealand). Collagen (Type I)-coated 6- and 24-well plates were purchased from Toyobo Engineering Co. Ltd (Osaka, Japan) and Sumitomo Bakelite Co. Ltd (Tokyo, Japan), respectively, and 6-, 12-, 24-, 48- and 96-well plates were purchased from Corning Glass Works (NY).

Cells

The highly metastatic, drug-resistant mouse Lewis lung carcinoma (LLC) cells were obtained from Riken Gene Bank (Tukuba, Japan) and maintained in DMEM supplemented with 10% FBS, penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and amphotericin B (0·25 μg mL^{-1}). Human umbilical vein endothelial cells (HUVECs) and bovine aorta endothelial cells (BAECs) were purchased from Clonetics (San Diego, CA) and Sannkou Junyaku Co. Ltd (Tokyo, Japan), respectively, and were seeded on collagen (Type I)-coated 6- or 24-well plates and maintained in EBM or CS-C media.

Animals

Female C57BL/6 mice (5 weeks old) were obtained from Clea Japan (Osaka, Japan). They were housed for 1 week in a room maintained at 25 ± 1°C with 60% relative humidity and had free access to food and water. The room was illuminated for 12 h each day starting at 07 00 h.

Measurement of tumour growth and lung metastasis in LLC-bearing mice

Solid-type LLC was prepared by subcutaneous transplantation of 5 × 10^5 cells (100 μL) into the right hind paw on day 0. Stilbene glucosides, piceid (300 mg kg^{-1}) or 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (150 mg kg^{-1}), were administered orally twice daily (at 0700 and 1900 h) for 32 consecutive days, starting 12 h after tumour implantation. Control mice were administered distilled water on the same schedule. The tumour volume was determined every 2 to 3 days by direct measurement with callipers and calculation of right hind paw volume minus left hind paw (non-treated) volume, where paw volume was length × width × depth/2. On day 33, the mice were killed by cervical dislocation, and the spleen, thymus and lung were immediately removed and weighed. The metastases to the lung were counted using a stereomicroscope.

Measurement of lymphocyte number and T cell population (CD8$^+$ and NK1.1$^+$ T cells) in LLC-bearing mice

The spleen tissue was gently teased to release cells using dissecting forceps in cold phosphate-buffered saline (PBS; pH 7·4) . The cell suspension (5 mL) was layered on 5 mL Lympholytes-Mouse and centrifuged at 1500 *g* for 30 min. The lymphocyte band at the interface was recovered, and the cells were rinsed three times with PBS (pH 7·4). The number of lymphocytes was measured using a Coulter Counter. The cell concentration was adjusted to 2 × 10^6 cells 100 μL^{-1}, and then 10 μL FITC-labeled anti-mouse CD8 or PE-labeled anti-

mouse NK1.1 was added to 100 μL of the cell suspension. After incubation for 30 min at 4°C, lymphocytes were rinsed three times with 1 mL PBS and centrifuged at 700 *g* for 5 min, and then $CD8^+$ and $NK1.1^+$ T cell populations were analysed by flow cytometry using a FACS Calibur (Becton & Dickinson, Mountain View, CA).

Measurement of DNA synthesis in LLC cells

LLC cells were placed in DMEM supplemented with 10% FBS at 1×10^4 cells per well in 24-well culture plates. The cells were cultured overnight and then the medium was replaced with fresh DMEM with 10% FBS. The cells were exposed to the indicated amounts of stilbenes (piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside) for 20 h, and then the medium was replaced with [^{3}H]-thymidine (18·5 KBq = 0·5 μCi per well) in DMEM with 10% FBS. After incubation for 4 h, the cells were washed twice with PBS, immersed in 1 mL 5% trichloroacetic acid (TCA) for 1 h at 4°C, washed twice with 5% TCA, and solubilized with 100 μL 0·2 M NaOH containing 0·5% Triton X-100. Thymidine incorporation into the cells was determined by liquid scintillation counting.

Measurement of DNA synthesis in BAECs and HUVECs

BAECs were placed in CS-C medium containing 10% FBS at 2×10^4 cells per well in collagen-coated 24-well culture plates. The cells were cultured overnight and then the medium was replaced with fresh CS-C medium containing 10% FBS. The cells were exposed to the indicated amounts of stilbenes for 20 h and then the medium was replaced with [^{3}H]thymidine (18·5 kBq = 0·5 μCi per well) in CS-C medium containing 10% FBS. After incubation for 4 h, cells were washed twice with ice-cold PBS, and then the thymidine incorporation was determined by liquid scintillation counting. HUVECs (1×10^4 cells per well) were seeded onto Matrigel (10 μg per well)-coated 96-well culture plates in CS-C medium containing 10% FBS. The cells were cultured overnight and then the medium was replaced with fresh medium. The cells were exposed to the indicated amounts of stilbenes for 20 h, and then thymidine incorporation was determined by liquid scintillation counting.

LLC cell adhesion to HUVECs

To load BCECF into the LLC cells, 3 μM BCECF-AM was added to the LLC cell suspension (2×10^6 cells mL^{-1}) with DMEM containing 10% FBS and 1 mM EDTA, and incubated for 30 min at 37°C with gentle agitation in a water bath. The permeable acetoxymethyl ester of BCECF is hydrolysed by cellular esterases on entering the cell and the BCECF thus formed is relatively impermeable and remains trapped in the cytoplasm. After the incubation period, the reaction mixture was centrifuged at 410 *g* to remove the medium containing BCECF-AM. The cells were then washed twice with DMEM containing 10% FBS and 1 mM EDTA and suspended in a final concentration of 1×10^6 cells mL^{-1} in CS-C medium containing 10% FBS. Confluent HUVEC monolayers (second passage) grown on collagen-coated 24-well culture plates were incubated with the indicated amounts of stilbenes for 6 h at 37°C in a humidified chamber containing 5% CO_2 in CS-C medium containing 10% FBS. After the incubation period, HUVEC monolayers were washed twice with CS-C medium containing 10% FBS, and then BCECF-labeled LLC cells (1×10^4 cells per well) were seeded onto HUVEC monolayers treated with stilbenes and incubated for 2 h. After the incubation period, HUVECs were gently washed three times with CS-C medium containing 10% FBS to remove non-adherent LLC cells. LLC cells that adhered to HUVECs were solubilized by adding 1 mL 0·5% Triton X-100, and the fluorescence of BCECF released from the LLC cells was measured by fluorimetry (JASCO, FP-777; Tokyo, Japan) with excitation at 500 nm and emission at 540 nm. LLC cell adherence to HUVEC monolayers is expressed as percent adherence, and the fluorescence released from the BCECF-labeled LLC cells seeded in each well was taken as 100% total fluorescence intensity.

Measurement of tube formation by HUVECs

Matrigel (150 μL per well) was added to a 48-well culture plate at 4°C and allowed to polymerize by incubation for 1 h at 37°C. HUVECs (second passage, 2×10^4 cells) were seeded on the Matrigel in 270 μL DMEM supplemented with 20% FBS, and incubated with the indicated amounts of stilbenes at 37°C for 24 h in a humidified 5% CO_2 atmosphere. Four different phase-contrast microscopic fields ($\times$ 40 and $\times$ 100) per well were photographed, and the photomicrograph images were stored in a computer. The total length of tube structures in each photograph ($\times$ 40) was measured using Adobe Photoshop software. The capillary length in each photograph ($\times$ 40) was measured as the average value from four fields and expressed as percentage of control value.

Statistical analysis

All values are expressed as mean ± s.e.m. Statistical analysis was performed with the Fisher's

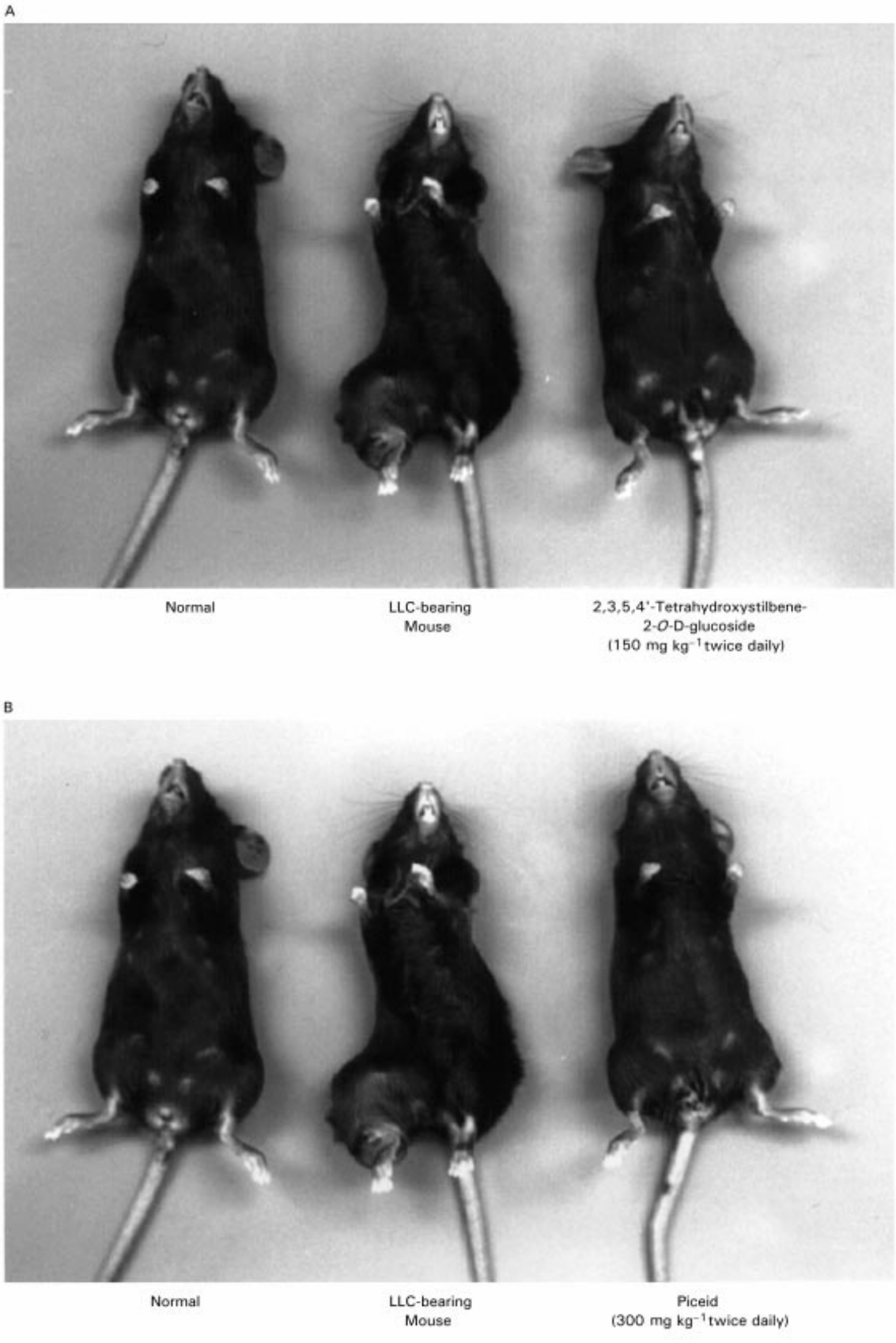

Figure 1. Antitumour effects of oral administration of 150 mg kg^{-1} 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (A) and 300 mg kg^{-1} piceid (B), twice daily, in Lewis lung carcinoma-bearing mice.

protected LSD test to determine significance using Super ANOVA software.

Results

Effects of stilbene glucosides on tumour growth and lung metastasis in LLC-bearing mice

As shown in Figures 1 and 3, 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside and piceid inhibited tumour growth time-dependently after oral administration of 150 or 300 mg kg^{-1} twice daily, respectively. As shown in Figure 2 and Table 1, 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside and piceid inhibited lung metastasis in LLC-bearing mice.

Effects of stilbene glucosides on body weight, spleen and thymus weights in LLC-bearing mice

2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside and piceid had no effect on body weight in LLC-bearing mice (data not shown). The spleen weight in LLC-bearing mice was significantly increased compared with control mice. In contrast, thymus weight in LLC-bearing mice was significantly reduced compared with normal mice (Table 1). 2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside (150 mg kg^{-1} twice daily) prevented the reduction in thymus weight in LLC-bearing mice. Piceid (300 mg kg^{-1} twice daily) prevented the increase in spleen weight and the reduction in thymus weight in LLC-bearing mice (Table 1).

Effects of stilbene glucosides on the lymphocyte numbers, CD8$^+$ and NK1.1$^+$ T cells in LLC-bearing mice

As shown in Table 2, the numbers of lymphocytes and CD8$^+$ T cells in the spleen were reduced in LLC-bearing mice compared with normal mice. Conversely, the number of NK 1.1$^+$ T cells in the spleen was increased compared with normal mice. 2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside (150 mg kg^{-1} twice daily) prevented the reduction in lymphocyte numbers in LLC-bearing mice. The reduction in lymophocyte and CD8$^+$ T cell numbers in the spleen was not prevented by the oral administration of either of the stilbene glucosides in LLC-bearing mice. The number of NK1.1$^+$ T cells in the spleen was not further increased by the oral administration of the stilbene glucosides in LLC-bearing mice.

Effects of stilbenes on DNA synthesis in LLC cells in-vitro

As shown in Figure 4, 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside inhibited thymidine incorporation

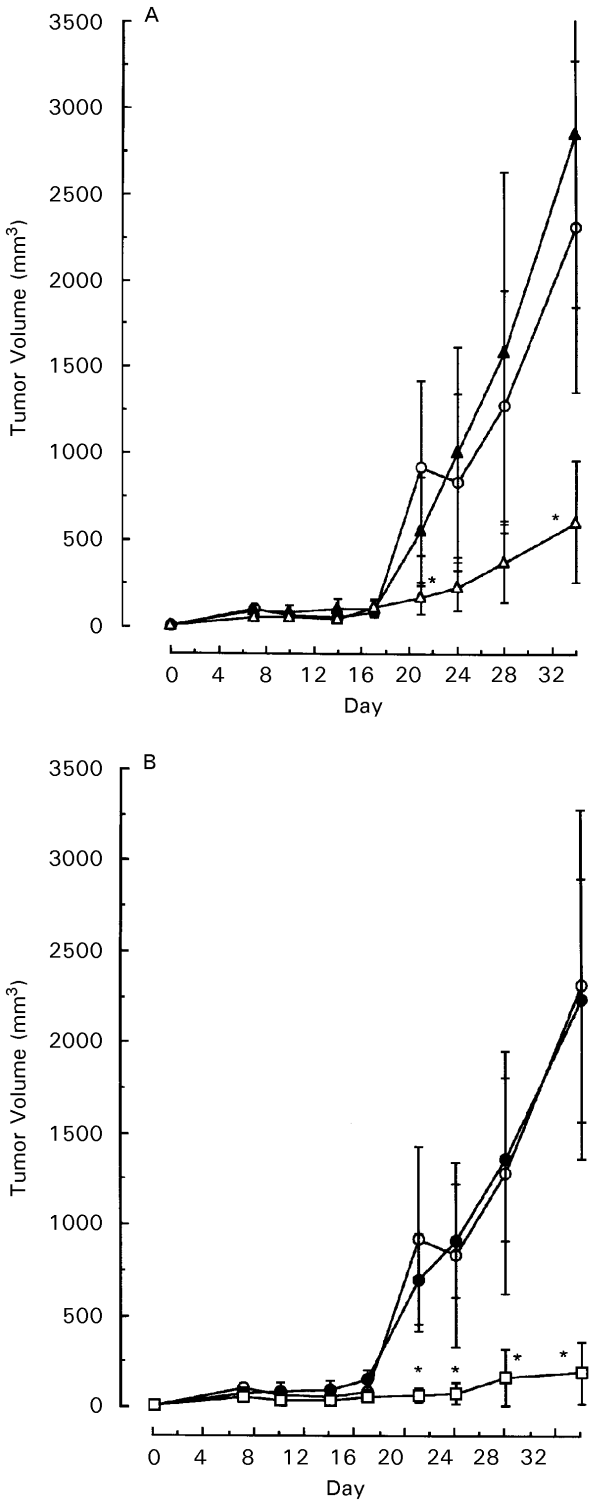

Figure 2. Effects of oral administration of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (A) and piceid (B), twice daily, on tumour growth in Lewis lung carcinoma (LLC)-bearing mice. A. LLC-bearing mice (○); 50 mg kg^{-1} 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (▲); 150 mg kg^{-1} 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (△). B. LLC-bearing mice (○); 100 mg kg^{-1} piceid (●); 300 mg kg^{-1} piceid (□). Results are expressed as means ± s.e.m. of 4–5 mice. * $P < 0.05$ significantly different compared with LLC-bearing mice.

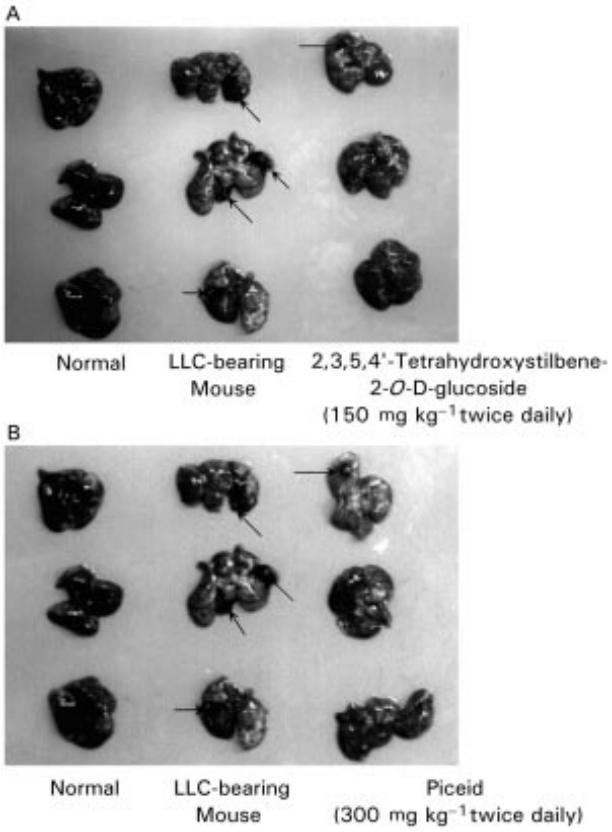

Figure 3. Effects of oral administration of 150 mg kg^{-1} 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (A) and 300 mg kg^{-1} piceid (B), twice daily, on lung metastasis in Lewis lung carcinoma-bearing mice.

into the DNA of LLC cells at concentrations of 50 to 1000 μM (IC50 81 μM). Piceid also inhibited thymidine incorporation into DNA at concentrations of 500 and 1000 μM (IC50 > 1000 μM).

Effects of stilbenes on DNA synthesis in BAECs and HUVECs in-vitro

As shown in Figure 5, 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside and piceid inhibited DNA synthesis in BAECs at the relatively high concentration of 1000 μM. As shown in Figure 6, 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside inhibited DNA synthesis in HUVECs at a concentration of 1000 μM. Piceid had no effect on DNA synthesis in HUVECs.

Effects of stilbenes on adherence of LLC cells to HUVECs in-vitro

2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside and piceid had no effect on the adherence of LLC cells to HUVECs (data not shown).

Effects of stilbenes on capillary-like network tube formation of HUVECs in-vitro

As shown in Figure 7, 2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside and piceid dose-dependently inhibited tube formation of HUVECs at concentrations of 100 to 1000 μM.

Discussion

Chemotherapeutic drugs such as 5-fluorouracil (5-FU) derivatives, cisplatin, mitomycin, adriamycin and taxisol, have been used extensively for the treatment of certain types of cancer. However, gastrointestinal toxicity, kidney damage, myelotoxicity and immunotoxicity are induced by cancer chemotherapeutic drugs as a result of the inhibition of DNA synthesis of cells in proliferating organs as well as in tumour cells. The clinical application of combinations of drugs and their modulators, has led to enhanced antitumour activity and reduced side-effects in patients with colorectal cancer, lung cancer and breast cancer. However, with these treatments, severe gastrointestinal toxicity with diarrhoea and mucosis, and haematological toxicity with leukopemia and immunosuppression appear to be dose-limiting factors. We previously reported that chitosan and carp extracts inhibited side-effects such as gastrointestinal toxicity, myelotoxicity and immunosuppression induced by 5-FU

Table 1. Effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside and piceid on cancer metastasis to lung, spleen and thymus in Lewis Lung Carcinoma (LLC)-bearing mice.

	Lung metastasis (%)	Spleen weight (mg) Mean ± s.e.m	Thymus weight (mg) Mean ± s.e.m
Normal	0 (0/4)	71·72 ± 3·10*	56·04 ± 9·21*
LLC-bearing mice	60 (3/5)	139·81 ± 42·18	27·33 ± 7·70
2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside			
50 mg kg^{-1}	100 (5/5)	145·96 ± 53·19	18·64 ± 9·10
150 mg kg^{-1}	20 (1/5)	126·47 ± 35·38	46·31 ± 8·34*
Piceid			
100 mg kg^{-1}	100 (5/5)	129·92 ± 45·55	30·79 ± 5·26
300 mg kg^{-1}	20 (1/5)	78·74 ± 11·58*	42·78 ± 7·81

2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside and picieid were administered orally twice daily. Results are expressed as means ± s.e.m. from 4–5 mice. *$P < 0.05$ significantly different compared with LLC-bearing mice.

Table 2. Effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside and piceid on the numbers of lymphocytes, $CD8^+$ and $NK1.1^+$ T cells of spleen in Lewis Lung Carcinoma (LLC)-bearing mice.

	Spleen cell number ($\times 10^6$ cells/spleen)		
	Lymphocyte	$CD8^+$ T cell	$NK1.1^+$ T cell
Normal	25·6 ± 3·00*	4·85 ± 1·15*	0·047 ± 0·008*
LLC-bearing mice	12·0 ± 4·88	1·60 ± 0·84	0·245 ± 0·086
2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside			
50 mg kg^{-1}	18·1 ± 3·74	1·60 ± 0·81	0·183 ± 0·074
150 mg kg^{-1}	24·0 ± 2·53*	2·49 ± 0·98	0·114 ± 0·096
Piceid			
100 mg kg^{-1}	20·0 ± 2·01	2·11 ± 0·75	0·152 ± 0·088
300 mg kg^{-1}	18·5 ± 3·91	2·02 ± 1·17	0·295 ± 0·114

2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside and picieid were administered orally twice daily. Results are expressed as means ± s.e.m. from 4–5 mice. *$P < 0.05$ significantly different compared with LLC-bearing mice.

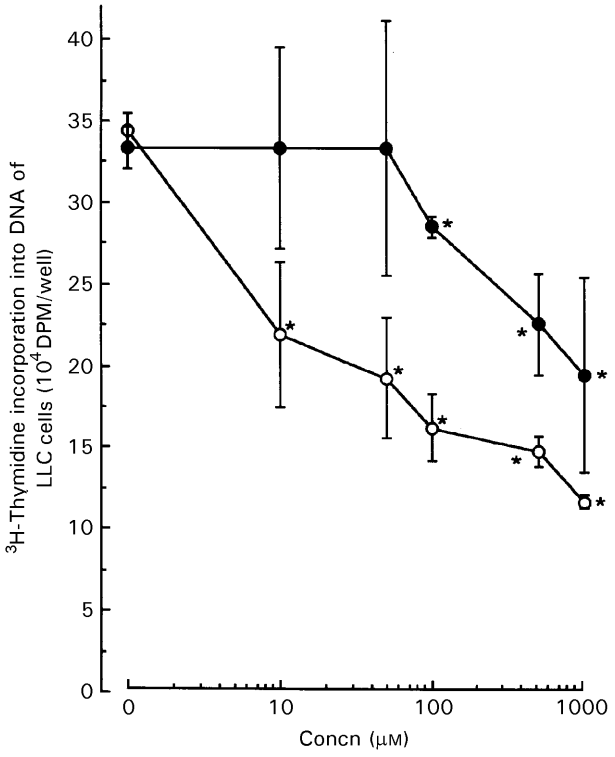

Figure 4. Effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (○) and piceid (●) on DNA synthesis in Lewis lung carcinoma (LLC) cells. Results are expressed as means ± s.e.m. of 4 experiments. * $P < 0.05$ significantly different compared with [^{3}H]-thymidine alone.

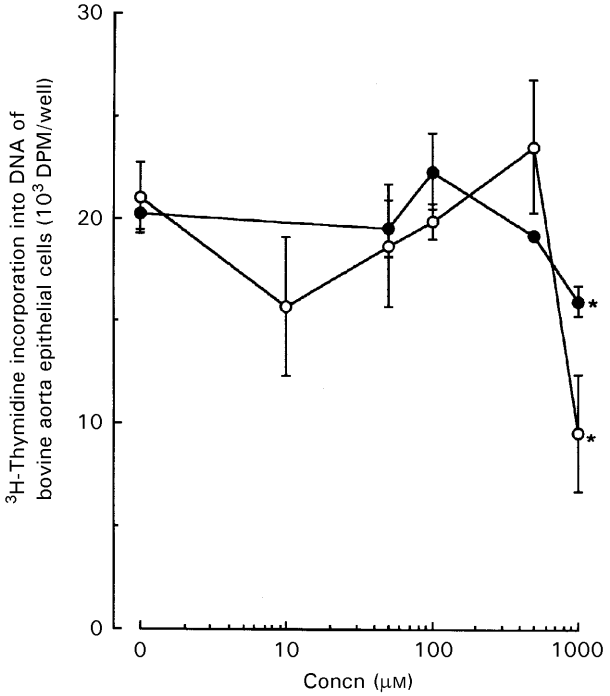

Figure 5. Effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (○) and piceid (●) on DNA synthesis in bovine aorta endothelial cells. Results are expressed as means ± s.e.m. of 4 experiments. * $P < 0.05$ significantly different compared with [^{3}H]-thymidine alone.

without loss of antitumour activity (Kimura & Okuda 1999a,b). After surgical removal of tumours, radiation therapy and adjuvant therapy with cancer chemotherapeutic drugs are used. However, the removal of certain tumours, for example, breast carcinoma, colon carcinoma and osteogic sarcoma, may be followed by the rapid growth of distant metastases to other organs such as lung and liver. Therefore, it is necessary to develop new anticancer agents with antitumour and antimetastatic activity without side-effects such as myelotoxicity, immunocompetent organ toxicity, and gastrointestinal toxicity.

In this study, we examined the effects of stilbene glucosides from medicinal plants and wine on tumour growth and lung metastasis in LLC-bearing mice. It has been reported that LLC-bearing C57 BL/6 mice had lung metastasis in addition to tumour growth (DeWys 1972; Gorelik et al 1978, 1980; O'Reilly et al 1994). In this study tumour

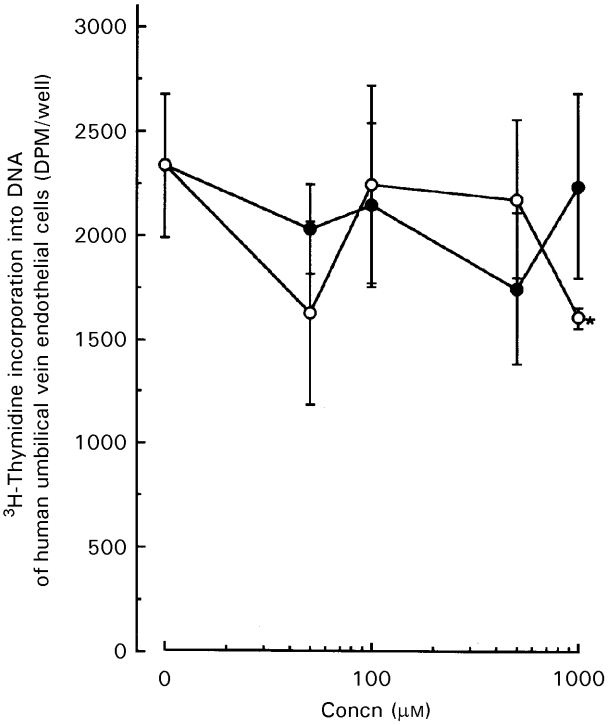

Figure 6. Effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (○) and piceid (●) on DNA synthesis in human umbilical vein endothelial cells. Values are expressed as means ± s.e.m. of 4 experiments.

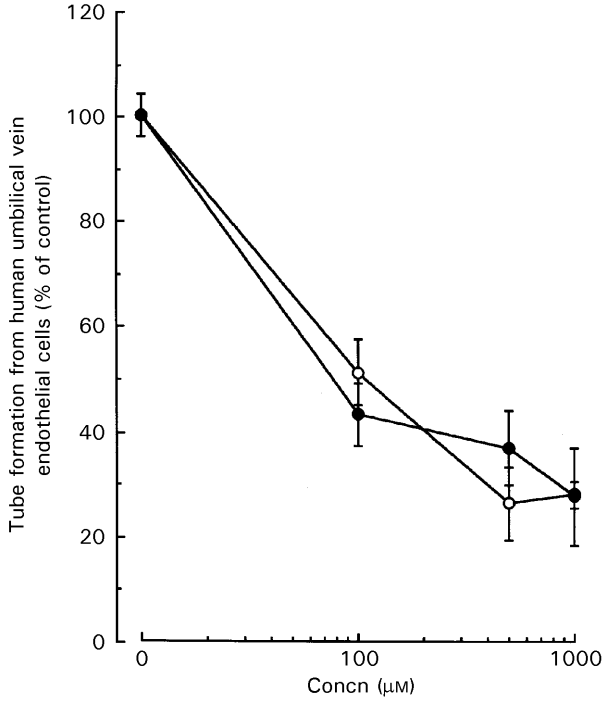

Figure 7. Effects of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (○) and piceid (●) on capillary-like network formation from human umbilical vein endothelial cells. Results are expressed as means ± s.e.m. of 3-6 experiments.

growth in the right hind paw and lung metastasis were inhibited by the oral administration of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (150 mg kg^{-1}) or piceid (300 mg kg^{-1}) twice daily for 33 consecutive days in LLC-bearing C57BL/6 mice. Metastasis to distant organs and tumour growth are reported to be prevented through natural killer cell and cytotoxic T lymphocyte activation by the administration of interleukin 12 (Brunda et al 1993; Mu et al 1995). In this study, the oral administration of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside (150 mg kg^{-1} twice daily) prevented the reduction of lymphocyte numbers in LLC-bearing mice. However, the oral administration of piceid or 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside did not affect the numbers of CD8$^+$ T cells or NK1.1$^+$ T cells in the spleens of LLC-bearing mice. Therefore, the inhibitory effects of piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside on tumour growth and lung metastasis could not be explained by NK and CTL activation. We examined the effects of piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside on the DNA synthesis in LLC cells. The results showed that piceid inhibited DNA synthesis in LLC cells at the relatively high concentration of 1000 μM, but did not at lower concentrations of 10 to 100 μM. 2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside also inhibited the DNA synthesis in LLC cells at concentrations of 50 and 100 μM (IC50 81 μM). We suggest that the inhibitory effects of piceid on tumour growth and lung metastasis might be partly caused by the inhibition of DNA synthesis. The antitumour and antimetastatic activity of 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside may also be partly mediated by the inhibition of DNA synthesis of LLC cells.

Tumour cell interactions with platelets, endothelial cells and subendothelial matrix are considered essential intermediate steps for the completion of the metastatic cascade (Liotta 1986; Weiss et al 1988; Blood & Zetter 1990; Honn & Tang 1992). To determine the antimetastatic activity of piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside, we examined their effects on the interactions of LLC cells with HUVECs. The results showed that piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside had no effect on the adherence of LLC cells to HUVECs. This indicates that the antimetastatic activity of piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside is not due to inhibition of the adherence of LLC cells to HUVECs.

Angiogenesis is the growth of new capillary blood vessels from pre-existing capillaries and postcapillary venules. Solid tumours cause neo-

vascularization, and the resultant angiogenesis from solid tumours stimulates growth and metastasis (Blood & Zetter 1990; Ferrara 1995; Folkman 1995; Koch et al 1995; Bischoff 1997; O'Reilly et al 1997; Boem et al 1997). To determine the mechanisms of the inhibitory effects of piceid and 2,3,5,4′-tetrahydroxy-stilbene-2-*O*-D-glucoside on tumour growth and lung metastasis, we examined their effects on the capillary-like network tube formation of HUVECs, as well as the inhibitory effects on DNA synthesis of LLC cells. Piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside dose-dependently inhibited the capillary-like network tube formation of HUVECs at concentrations of 100 to 1000 μM. Piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside had no effect on DNA synthesis in BAECs and HUVECs at concentrations of 100 and 500 μM, while both stilbene glucosides inhibited DNA synthesis in BAECs at a high concentration of 1000 μM. 2,3,5,4′-Tetrahydroxystilbene-2-*O*-D-glucoside inhibited DNA synthesis in HUVECs at a high concentration of 1000 μM. Piceid has no effect at this concentration. Therefore, the inhibitory effects of piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside on the tube formation of HUVECs could not be explained by the inhibition of DNA synthesis in endothelial cells. Further experiments are necessary to determine the mechanisms (e.g. regulation of vascular endothelial cell growth factor receptor Flt 1 and KDR expression) of the inhibitory effects of stilbenes on capillary-like network tube formation of HUVECs.

The results of this study suggest that the antitumour and antimetastatic activity of piceid and 2,3,5,4′-tetrahydroxystilbene-2-*O*-D-glucoside might be due to the inhibition of DNA synthesis in LLC cells, and the inhibition of tube formation of HUVECs.

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