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Ginseng Intestinal Bacterial Metabolite IH901 as a New Anti-metastatic Agent

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Anti-metastatic activities of IH901, an intestinal bacterial metabolic derivative formed from Ginseng protopanaxadiol saponins, was determined *in vitro* and *in vivo*. Under *in vitro* conditions, IH901 inhibited the migration of bovine aortic endothelial cells 25 times stronger than suramin and suppressed the invasion of HT1080 human fibrosarcoma cells into reconstituted basement membrane components of Matrigel 1000 times stronger than RGDS peptide. IH901 also showed inhibitory effect on type-IV collagenase secretion from HT1080 cells and platelet aggregation. When the anti-metastatic activity of IH901 was evaluated in comparison with that of 5-FU using a spontaneous lung metastatic model of Lewis lung carcinoma, the administration of IH901 (10 mg/kg p. o.) to tumor-bearing mice led to a significant decrease in lung metastasis (43% of untreated control), which was slightly more effective than that obtained with 5-FU (56% of control). Thus, IH901 seems to exhibit its anti-metastatic activity partly through the inhibition of tumor invasion which results from the blockade of type IV collagenase secretion and also through anti-platelet and anti-angiogenic activities.

Key words : Ginseng saponin, Intestinal bacterial metabolite IH901, Angiogenesis, Metastasis, Invasion, Type IV-collagenase, Platelet aggregation

INTRODUCTION

Currently there is great interest in means for prevention of cancer metastasis, in order to improve the prognosis of cancer metastasis. Cancer metastasis develops by multiple and sequential steps: step 1, release of tumor cells from the primary site; step 2, tumor cell invasion of surrounding tissues and vascular or lymphatic circulation; step 3, transit in the circulation; step 4, arrest of the circulating tumor cells in the microvasucular of target organs; step 5, extravasation from circulation; step 6, growth at apparently selective sites which are distant from the original tumor site. Few cells in the primary tumor can complete all these steps necessary to achieve metastasis. During the sequential steps of metastasis, metastasizing tumor cells encounter various host cells (platelet, lymphocytes or endothelial cells) and/or extracellular matrix and basement membrane components (fibronectin and laminin) (Fidler, 1984; Nicolson, 1987; Hart, 1983; Liotta *et al.*, 1983). Among these steps, the invasion is the most characteristic process, control of which is likely to provide a new strategy for metastasis prevention, and some compounds such as RGDS peptide (Saiki *et al.*, 1989a), bestatin (Talmadge *et al.*, 1986) and batimastat (Wang *et al.*, 1994) have been so far examined for anti-metastatic activity. It is desirable that the type of agents used for long term anti-metastasis therapy avoid their possible adverse side effects. From this view point, our attention was focused on the components of medicinal plants having a long history of their use in folklore medicine.

IH901 [20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol] is a major intestinal bacterial metabolite formed from Ginseng (the root of *Panax ginseng* C. A. Meyer, Araliaceae) protopanaxadiol saponins (Hasegawa *et al.*, 1996) and has been shown to possess some pharmacological activities such as the inhibition of glucose uptake by tumor cells and the reversal of multidrug-resistance in bacteria and tumor cells (Hasegawa *et al.*, 1994; 1995a; 1995b). In the course of searching for new anti-cancer agents recently performed by the Screening Committee in Japan, IH901 has been found to exhibit anti-angiogenic and metastatic activities *in vitro*.

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The present paper deals with its anti-metastatic potential *in vivo* as well as *in vitro*.

MATERIALS AND METHODS

Chemicals

Ginseng saponin intestinal bacterial metabolite IH 901 was prepared in the same manner as described in our preceding paper (Hasegawa *et al.*, 1996). Its structure is shown in Fig. 1. 5-FU was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cells

Metastatic human fibrosarcoma HT1080 cell line was obtained from ATCC (Rockville, MD) and maintained as monolayer cultures in Eagles's minimum essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine. Lewis lung carcinoma (LLC) cell line was given by RIKEN Cell Bank (Tukuba, Ibaraki) and maintained as monolayer cultures in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% FBS, 100 μg/ml streptomycin. Bovine aortic endothelial (BAE) cells were isolated from bovine aortic artery and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 60 μg/ml kanamycin as reported previously (Sato *et al.*, 1988).

Mice

Specific pathogen free C57BL/6 mice at 6 weeks of age were purchased from Japan SLC, Inc. (Hamamatsu). The mice were maintained under laminar air flow conditions.

Assay for BAE cell migration

This assay for BAE cell migration was carried out by the method described previously (Sato *et al.*, 1988), with slight modifications. BAE cells grown to confluency in 6-well-microtiter plates were washed three times in PBS and stripped from plate by razor and then exposed to 10% FBS-BMEM medium. After 1 hr of incubation, test sample solutions were added to the cultures to make final concentrations of IC_{10} and IC_{50} of BAE cell growth, and further incubated for 24 hr. After incubation, the cells were fixed with MeOH, stained with Giemsa and the cells that had migrated from the place stripped were counted under a microscope.

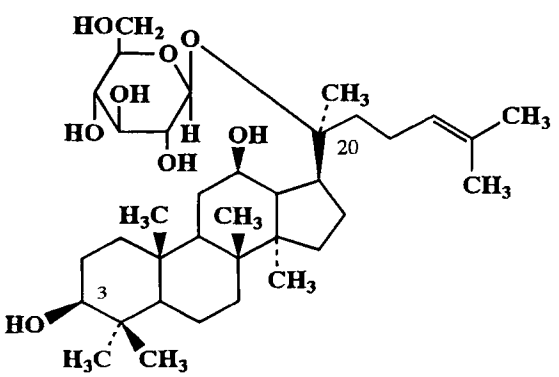

Fig. 1. Chemical structure of IH901.

Tumor invasion assay

The invasion activity of tumor cells was assayed using Transwell cell-culture chambers (Costar 3422, Cambridge, MA) according to the method previously reported (Saiki *et al.*, 1989b). Briefly, the lower surface of polyvinylpyrrolidone-free polycarbonate filters with an 8.0 μm pore size (Nucleopore, Pleasanton, CA) was pre-coated with 5 μg fibronectin (Iwaki Glass, Tokyo) in a volume of 50 μl and dried at room temperature. A reconstituted basement membrane Matrigel (containing laminin, type IV collagen, heparan sulfate proteoglycan and entactin: Collaborative Research Inc. MA), diluted to 100 μg/ml with cold PBS, applied to the upper surfaces of the filters (10 μg/filter) and dried at room temperature under a hood. These prepared filters were designated as Matrigel/fibronectin-coated filters. The coated filters were washed extensively with PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 1 mM EDTA in PBS, washed three times with serum-free EMEM, and re-suspended to a final concentration of 2×10^6 cells/ml in EMEM with 0.1% bovine serum albumin. Cell suspensions (100 μl) were added to the upper compartment, and incubated in the presence or absence of test compounds for the appropriate hours at 37°C in a 5% CO_2 atmosphere. The filters were fixed with methanol, and stained with hematoxylin and eosin. The cells on the upper surfaces of the filter were removed by careful wiping with cotton swabs. The cells that had invaded to various areas of the lower surface were manually counted under a microscope at ×400 magnification.

Gelatin zymography

The gelatinolytic activity of conditioned medium of tumor cells was performed by electrophoresis in a gelatin-embedded polyacrylamide gel followed by incubation and Coomassie Blue staining, based on the methods described (Moll *et al.*, 1990). HT1080 cells grown to confluency in 12-wells microtiter plate were washed three times in PBS and changed to serum free MEM (1.6 ml) with non-cytotoxic concentrations (1.56~12.5 μM) of IH901 and then incubated for 24 hr at 37°C. Aliquots (10 μl) of conditioned medium

generated for 24 h in the absence or presence of IH 901 were analyzed on gelatin zymograms using SDS-PAGE gels with copolymerized gelatin at a final concentration of 1 mg/ml. Enzyme-containing samples were dissolved in SDS sample buffer in the absence of reducing agents and boiling and were electrophoresed on 7.5% or 5~15% gradient gels. After electrophoresis, the gels were washed 2×30 min in 2.5% Triton X-100 to remove SDS and incubated overnight at 37°C in 40 mM Tris-200 mM NaCl-10 mM $CaCl_2$, pH 7.5, and then stained in Coomassie Brilliant Blue (0.1%). Clear zones of gelatin lysis against a blue background stain indicated the presence of enzyme.

Measurement of platelet aggregation

Platelet-rich plasma (PRP) was obtained from blood collected from the retro-orbital sinus under light ether anesthesia, anti-coagulated with sodium citrate (3.2%, 1:9) and centrifuged for 10 min at 900 rpm at room temperature. The blood sample from which PRP was removed was re-centrifuged for 10 min at 3000 rpm to obtain platelet-poor plasma (PPP). PRP (100 μl) was pre-incubated with IH901 at 37°C for 1 min, and ADP (3 μM) or collagen (5 μg/ml) was added. Aggregation was measured by the light transmission method (O'Brien, 1962), using an aggregometer (NBS Hema Tracer 801, M. C. Medical, Tokyo), in which the absorbance of PRP was taken as 0% aggregation and that of PPP as 100% aggregation.

Assay for spontaneous lung metastases

Spontaneous lung metastasis was assayed following the reported method (Kobayashi *et al.*, 1995). Six C 57BL/6 male mice were given oral administration with IH901 or 5-FU at 10 mg/kg/d for 2 weeks after a s. c. injection (0.2 ml/mouse) of LLC cells (1×10^6) in the abdominal wall. Other six mice were given PBS only for control. The mice were killed 21 d after tumor inoculation. The lungs were fixed in Bousin's solution and the lung tumor colonies were counted under a dissecting microscope.

Statistical Analysis

The significance of difference between groups was calculated by applying the Student's two-tailed t-test.

RESULTS

Effect of IH901 on cell growth

The cytotoxicity of test compounds against normal or tumor cells was examined and the results are listed in Table I. LLC cells were resistant to 5-FU.

Table I. Cytotoxicity of test samples against normal or tumor cells

Cell line	Test compound	IC_{10}	IC_{50} (μM)
BAE	Suramin	70	700
	IH901	10	28
HT 1080	IH901	16	55
LLC	5-FU	5	13
	IH901	15	39

BAE (5×10^3), HT1080 (2×10^5) and LLC (5×10^4) were incubated with or without test compounds at 37°C. After 2 days of incubation the cell viability was measured using the MTT dye assay. Each assay was performed in duplicate. The IC_{10} and IC_{50} values were calculated by using Hill plots.

Effects of IH901 on aortic endothelial cell migration

The effect of IH901 on BAE cell migration was examined using suramin (Gagliardi *et al.*, 1992) as a positive control for anti-angiogenic activity. As shown in Table II, the inhibition of BAE cell migration was caused by 10~28 μM IH901 was almost same as that observed with 70~700 μM suramin.

Effect of IH901 on tumor cell invasion

The effects of IH901 on tumor cell invasion was examined using RGDS peptide (Saiki *et al.*, 1989a) as a positive control for anti-metastatic activity. As shown in Table III, IH901 induced a marked inhibitory effect on the invasion of HT1080 human fibrosarcoma cells into reconstituted basement membrane components of Matrigel at a concentration-dependent manner and it was a 1000-fold stronger inhibitor than RGDS peptide (IC_{50}=3.2 μM of IH901 v. s. 4 mM of RGDS peptide).

Effect of IH901 on the degradation of gelatin in zymograms

Fig. 2 shows the effect of IH901 on the degradation

Table II. Effect of IH901 on the migration of BAE cells

	Con. (μM)	No. of Migrated cell	% of control
Suramin	0	480±34	
	70	498±28	103.8
	700	302±24*	62.9
IH901	0	433±42	
	10	413±28	95.4
	20	246±30*	56.8

BAE cells grown to confluency were washed three times with PBS and stripped from plate by razor and then changed to 10% FBS-BMEM medium. After 1 hr of incubation, test sample solutions were added to the cultures to make final concentrations of IC_{10} and IC_{50} of BAE cell growth. After further incubation for 24 hr, the cells were fixed with MeOH, stained with Giemsa and the cells that had migrated from the place stripped were counted. Each value shows the mean±S. D. of duplicate determinations. Differences concerning the respective control group were evaluated by Student's two-tailed t-test (*$p<0.05$).

Table III. Effect of IH901 on the invasion of HT1080 human fibrosarcoma cell line into Matrigel/fibronectin-coated filters

Compound	Con. (μM)	No. of invaded cells/field	Inhibition of invasion (%)
Control		118±8	
RGDS peptide	4000	61±9	48
IH901	1	73±4	38
	IC_{50}=3.2	45±9	50
	10	0	62
	20		100

HT1080 cells (1×10^5) in 0.1% BSA-MEM medium were seeded with or without IH901 onto the Matrigel/fibronectin-coated filters. After 4-hr incubation, the cells which had invaded to the lower surface were counted. Each value shows the mean±S.D. of quintuplet determinations. The IC_{50} values were calculated by using Hill plots.

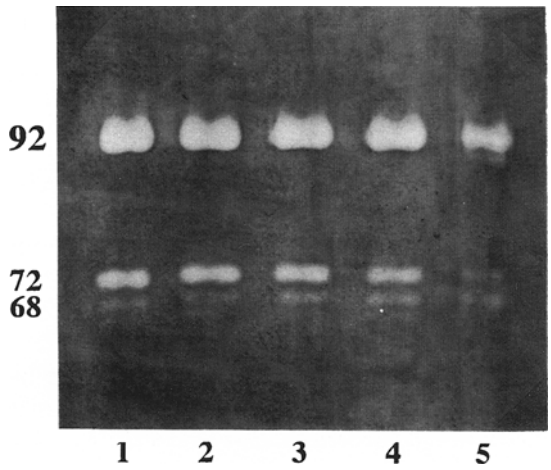

Fig. 2. Type IV collagenolytic activity by conditionated medium of IH901-treated HT1080 cells. HT1080 cells grown to confluency were washed three times in PBS and changed to serum free MEM (1.6 ml) containing IH901 at non-cytotoxic concentrations (1.56~12.5 μM) and then incubated for 24 hr at 37°C. Aliquots of conditioned medium generated for 24 h in the absence or presence of IH901 were analyzed on gelatin zymograms using SDS-PAGE gel (Control, Lane 1; 1.56 μM, Lane 2; 3.13 μM, Lane 3; 6.25 μM, Lane 4; 12.5 μM, Lane 5). Numbers indicate the molecular weight of the proteinase bands estimated in relation to the migrations of molecular weight standards.

in zymograms of gelatin substrates by conditioned medium of fibrosarcoma cell line HT1080. The zymography of type-IV collagenase from HT1080 revealed three bonds of gelatinolytic activity with molecular weights of 92, 72 and 68 (Lane 1). It is well known that collagenases exist in both latent (92 kDa and 72 kDa) and active (68 kDa) forms on the gelatin zymograms using SDS-PAGE gels. Although the treatment of cells with the concentration ranging from 1.56 μM to 6.25 μM of IH901 failed to induce any significant inhibition of enzyme level (Lanes 2-4), the treatment of cells with 12.5 μM of IH901 resulted in the remarkable disappearance of the 72 kDa enzyme level and slight reduction of the 92 kDa enzyme level (Lane 5). While no significant differences were observed in gelatinolytic activities between the conditioned media of HT1080 incubated in the presence and absence of IH901 (data not shown). Therefore, IH901 is supposed to induce the inhibitory effect on cell-mediated type-IV collagenolytic activity, in other words, the type-IV collagenase secretion from tumor cells, but not on the type-IV collagenolytic activity in the conditioned media.

Effect of IH901 on platelet aggregation

Since tumor interactions with host cells or components in the processes of metastatic progression are associated with platelet aggregation-factor, the effect of IH901 on platelet aggregation was examined. As shown in Table IV, platelet aggregation induced by ADP and collagen was inhibited by IH901 dose-dependently (IC_{50}, 15 μM for ADP; 8 μM for collagen).

Effect of IH901 on spontaneous lung metastasis

The anti-metastatic activity of IH901 was examined in a spontaneous metastatic model in comparison with that of 5-FU. When IH901 and 5-FU were administered orally to mice at the dose of 10 mg/kg for 2 weeks after the s. c. tumor injection, the lung metastasis was significantly decreased by IH901 (43% of control) and its efficacy was slightly superior to that of 5-FU as shown in Fig. 3.

DISCUSSION

Tumor interactions with host cells or components in the processes of metastatic progression are associated with several functions (growth-, adhesion-, migration-, platelet aggregation-, blood coagulation- and angiogenesis-factors or receptors, and matrix-degrading enzymes). Initial *in vitro* examinations using BAE migration and HT1080 invasion assay systems revealed the remarkable inhibitory potential of IH901 (Tables II and III). To elucidate the mechanism of inhibition of HT

Table IV. Effect of IH901 on platelet aggregation

Con. (μM)	Inhibition of aggregation (%)	
	ADP	Collagen
5	NE	4±2
8*		50
10	9±2	76±6
15*	50	
20	82±9	87±19

PRP was pre-incubated with IH901 at 37°C for 1 min, then ADP (3 μM) or collagen (5 μg/ml) was added. Each value shows the mean±S.D. of quadruplicate determinations. NE, No effect.
*The IC_{50} values were calculated by using Hill plots.

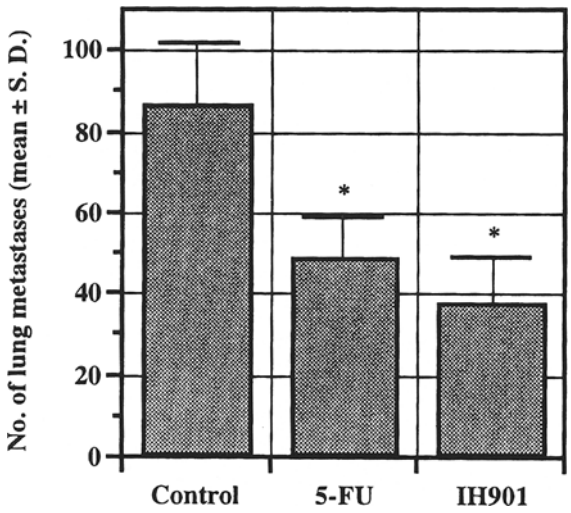

Fig. 3. Effect of p. o. administration of IH901 and 5-FU on lung metastasis produced by LLC cells in C57BL/6 mice. Mice were given oral administration of 10 mg/kg IH901 or 5-FU from day 1 to day 14 after the s. c. injection of LLC cells (1×10^6). Mice were killed 21 days after tumor inoculation and the lungs were examined. *$p<0.01$ (compared with untreated control by Student's two-tailed t-test).

1080 invasion by IH901, its effects on migration of the tumor cells and type IV collagenase secretion were investigated. Although the effect of IH901 on the migration was hardly observed (data not shown), IH901 decreased the secretion of type IV collagenase from the tumor at the non-toxic concentration of 12.5 μM (Table I and Fig. 2). In addition, IH901 reduced platelet aggregation (Table IV).

The p. o. treatment of tumor-bearing mice with IH 901 induced a significant decrease in the number of metastasizing tumors into lung (Fig. 3). The fact that the efficacy of IH901 on *in vivo* anti-metastasis was slightly superior to that of 5-FU seems related to the resistance of LLC cells to 5-FU (Table I). Moreover, the toxicity of IH901 is found to be very low ($LD_{50}>5$ g/kg). Therefore IH901 has a promising potential as an anti-metastatic oral agent without adverse side effects. Further studies on its therapeutic properties are now in progress.

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REFERENCES CITED

Fidler, I. J., The Ernst W. Bernet memorial award lecture: the evolution of biological heterogeneity in metastatic neoplasms. In Nicolson G. L. and Milas, L. (Eds.). *Cancer invasion and metastases: Biologic and therapeutic aspects*. Raven Press New York, pp.5-26 (1984).

Gagliardi, A., Hadd, H. and Collins, D. C., Inhibition of angiogenesis by suramin. *Cancer Res.*, 52, 5073-5075 (1992).

Hasegawa, H., Matsumiya, S., Uchiyama, M., Kurokawa, T., Inouye, Y., Kasai, R., Ishibashi, S. and Yamasaki, K., Inhibitory effect of some triterpenoid saponins on glucose transport in tumor cells and its application to *in vitro* cytotoxic and antiviral activities. *Planta Med.*, 60, 240-243 (1994).

Hasegawa, H., Matsumiya, S., Uchiyama, M., Inouye, Y., Kasai, R. and Yamasaki, K., Reversal of efflux-mediated tetracycline resistance in *Staphylococcus aureus* clinical isolates by Ginseng prosapogenins. *Phytother. Res.*, 9, 260-263 (1995a).

Hasegawa, H., Sung, J. H., Matsumiya, S., Uchiyama, M., Inouye, Y., Kasai, R. and Yamasaki, K., Reversal of daunomycin and vinblastine resistance in multidrug-resistant P388 leukemia *in vitro* through enhanced cytotoxicity by triterpenoids. *Planta Med.*, 61, 409-413 (1995b).

Hasegawa H, Sung J, H., Matsumiya, S. and Uchiyama, M., Main Ginseng saponin metabolites formed by intestinal bacteria. *Planta Med.*, 62, 453-457 (1996).

Hart, I. R., "Seed and soil" revisited: mechanisms of site specific metastasis. *Cancer Metastasis Rev.*, 1, 5-16 (1982).

Kobayashi, H., Shinohara, H., Fujie, M., Gotoh, J., Itoh, M., Takeuchi, K. and Terao, T., Inhibition of metastasis of Lewis lung carcinoma by urinary trypsin inhibitor in experimental and spontaneous metastasis model. *Int. J. Cancer,* 63, 455-462 (1995).

Liotta, L. A., Rao, C. V. and Barsky, S. H., Tumor invasion and the extracellular matrix. *Lab. Invest.*, 49, 636-649 (1983).

Moll, U. M., Youngeleib, G. L., Rosinski, K. B. and Quigley, J. P., Tumor promotor-stimulated Mr 92, 000 gelatinase secreted by normal and malignant human cells: Isolation and characterization of the enzyme from HT1080 tumor cells. *Cancer Res.*, 50, 6162-6170 (1990).

Nicolson, G. L., Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogene to oncofetal expression. *Cancer Res.*, 47, 1473-1487 (1987).

O'Brien, J. R., Platelet aggregation. *J. Clin. Pathol.*, 15, 446-455 (1962).

Saiki, I., Iida, J., Murata, J., Ogawa, R., Nishi, N., Sugimura, K., Tokura, S. and Azuma, I, Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequences of cell-adhesive molecules. *Cancer Res.*, 49, 3815-3822 (1989a).

Saiki, I., Murata, J., Watanabe, K., Fujii, H., Abe, F. and Azuma, I., Inhibition of tumor-cell invasion by ubenimex (bestatin) *in vitro*. *Jpn. J. Cancer Res.*, 80, 873-878 (1989b).

Sato, Y. and Rifkin, D. B., Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *J. Cell Biol.*, 107, 1199-1205 (1988).

Talmadge, J. E., Lenz, B. F., Pennington, R., Long, C., Phillips, H., Schneider, M. and Tribble, H., Immunomodulatory and therapeutic properties of bestatin in mice. *Cancer Res.*, 46, 4505-4510 (1986).

Wang, X., Fu, X., Brown, P. D. Crimmin, M. J. and Hoffman, R. M., Matrix metalloprotease inhibitor BB-94 (Batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. *Cancer Res.*, 54, 4726-4728 (1994).