EFFECT ON SPONTANEOUS METASTASIS OF MOUSE LEWIS LUNG CARCINOMA BY A TRIFLUOROACETAMIDE ANALOGUE OF SIASTATIN B

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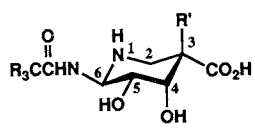
(Received for publication March 24, 1994)

As reported in our previous papers[1,2], (3*R*,4*R*,5*R*,6*R*)-6-(trifluoroacetamido)-3,4,5-trihydroxypiperidine-3-carboxylic acid (tentatively named SF1) and (3*S*,4*S*,5*R*,6*R*)-6-(trifluoroacetamido)-4,5-dihydroxypiperidine-3-carboxylic acid (SF4) which were synthesized as trifluoroacetamide analogues of siastatin B[3] (Fig. 1), were highly inhibitory against β-glucuronidase (EC 3.2.1.31) from bovine liver (IC_{50} 0.073 and 0.029 μM, respectively) and significantly inhibited experimental pulmonary metastasis of the highly metastatic variants of B16 melanoma (B16 variant) by pretreatment and iv injection into the tail veins of mice at 180 μM by 80 and 91%, respectively. These results prompted us to investigate the effect of the more active SF4 on tumor cell invasion and spontaneous metastasis. Here we demonstrate that SF4 inhibits the highly metastatic B16 variant and Lewis lung carcinoma (3LL) cell invasion through reconstituted basement membranes and suppresses spontaneous lung metastases of 3LL cells in mice.

Fig. 1. Structures of siastatin B and its trifluoroacetamide analogues.



Siastatin B	R=H	R'=H
SF1	R=F	R'=OH
SF4	R=F	R'=H

Materials and Methods

Chemicals

SF-4 was synthesized from L-ribose[2] and also prepared by chemical modification of siastatin B in the Institute of Microbial Chemistry. A large scale preparation of siastatin B from the culture of *Streptomyces*[3] was carried out by Meiji Seika Kaisha, Ltd. Polyvinylpyrrolidone-free polycarbonate filters (8-μm pore size) were purchased from Nuclepore (California); Matrigel and laminin from Collaborative Research (Bedford, MA); DULBECCO's modified minimum essential medium (DMEM) supplemented with 0.1% bovine serum albumin fraction V (BSA) and DULBECCO's modified EAGLE's medium supplemented with 10% fetal bovine serum from Nissui Seiyaku (Tokyo, Japan); lentinan from Morishita-Roussel (Osaka, Japan).

Mice and Tumor Cells

Five-week-old female C57BL/6 mice were obtained from Charles River Japan (Kanagawa, Japan) and kept under pathogen-free conditions. The highly metastatic B16 variant cells were obtained by FIDLER's modified method[4,5], and 3LL cells were supplied by the National Cancer Center Research Institute, Japan. B16 variant and 3LL cells were maintained in DULBECCO's modified EAGLE's medium supplemented with 10% fetal bovine serum under atmosphere of 5% CO_2 and 95% air at 37°C.

Invasion Assay

The invasion assay was performed by the method described by ALBINI *et al.*[6] and SAIKI *et al.*[7] with some modifications, using Transwell cell culture chambers (Costar No. 3422, Cambridge, MA) equipped with 8.0 μm pore size polyvinylpyrrolidone-free polycarbonate membranes. The upper surface of the membrane was coated with Matrigel (5 μg/filter), and its lower surface was coated with laminin (10 μg/filter) as cell attractants. The membrane was dried at room temperature under a hood. The bottom chamber was filled with 0.6 ml of a solution containing DMEM supplemented with 0.1% BSA. Cells (5.0×10^5) grown with (or without) SF4 for 72 hours (B16 variant) or 15 hours (3LL) were harvested by a 20-minute treatment with 0.08% sodium citrate, washed twice with a solution of DMEM supplemented with 0.1% BSA, and suspended in 1 ml of DMEM containing 0.1% BSA. The cell suspension (0.1 ml) was added to the upper Transwell chamber and incubated at 37°C in a

Table 1. Inhibitory effect of SF4 on tumor cell invasion.

Treatment	Concentration (μM)	Incubation (hours)		No. of invaded cells		Average percent inhibition of invasion	
		B16	3LL	B16	3LL	B16	3LL
None	—	3	4	10.3±6.1	35.3±25.4	0	0
SF4	370	3	4	7.3±3.7	9.3±2.3	29.1	73.7
	740	—	4	—	3.0±1.0	—	91.5
	1,100	3	4	3.7±3.8	7.3±2.1	64.1	79.3
None	—	5	6	54.3±16.3	38.7±20.8	0	0
SF4	370	5	6	51.3±8.1	10.7±2.1	5.5	72.4
	740	—	6	—	7.7±0.6	—	80.1
	1,100	5	6	37.7±31.6	7.7±2.5	30.6	80.1

B16 variant or 3LL carcinoma cells were cultured with or without SF4 for 72 hours (B16 variant) or 15 hours (3LL), and then B16 variant or 3LL cells (5.0×10^4/well) were seeded on the Matrigel/laminin-coated filters in the upper Transwell chamber. After incubation, the invaded cells on the lower surface (the mean±SD/0.3 mm^2 of 3 determinations) were counted.

Table 2. Inhibitory effect of SF4 on the spontaneous lung metastasis.

Sample	Administered dose (mg/kg)	No. of lung metastasis		Inhibition of metastasis (%)
		(Mean±SD)	(Range)	
Saline (0.9%)	×5	43.4±16.3	35~52	0
SF4	10×5	41.2±9.3	27~53	5.1
	50×5	33.2±10.4	19~41	23.5
	100×5	18.6±6.2	9~26	57.1
Lentinan	2×5	17.4±10.4	9~35	59.9

Five female C57BL/6 mice per group inoculated with 3LL cells (1×10^6) by intrafootpad injection were dosed iv with SF4 for 5 days starting on the day of the surgical excision of primary tumors on day 9. Mice were sacrificed 10 days after tumor excision.

humid 5% CO_2 atmosphere. After 3-, 4-, 5- and 6-hour incubations, the cells on the upper surface of the filters were completely removed by wiping with a cotton swab, and filters were fixed with methanol and stained with hematoxylin. Cells from various areas (0.3 mm^2) of the lower surface were counted under a microscope (×200) and each assay was performed in triplicate.

Assay for Spontaneous Lung Metastasis

Spontaneous pulmonary colonization was assayed essentially as described by SHIINO *et al.*[8] and SAIKI *et al.*[9] using lentinan as a reference agent. Mice were inoculated with 3LL cells (1×10^6) by sc injections into the right hind footpad. SF4 was administered iv for five days starting on the day of the surgical excision of primary tumors on day 9. Mice were killed 10 days after surgery, and the lung tumor colonies were counted under dissecting microscope.

Results and Discussion

As shown in Table 1, the invasion of B16 variant and 3LL cells into the Matrigel/laminin-coated filters was inhibited by SF4 in a dose-dependent fashion in Transwell chamber analysis. SF4 at 370, 740 and 1,100 μM inhibited the 3LL cell invasion by >70, >80 and >80%, respectively. SF4 also inhibited B16 variant cell invasion at 370 and 1,100 μM by about 30 and 64%, respectively. SF4 at 1,100 μM had no effect on the growth and viability of B16 variant and 3LL cells, respectively.

As shown in Table 2, spontaneous lung metastasis by sc inoculation of 3LL cells into the footpad was significantly inhibited by iv injections of SF4 for five days starting on the day of the excision of primary tumors by amputation of the tumor-bearing leg from an articulation. SF4 dose-dependently inhibited metastasis at administration doses of 10, 50 and 100 mg/kg by about 5, 24 and 57%, respectively. No toxicity of SF4 was observed at iv injection of 100 mg/kg in mice.

As already mentioned[2], we found that SF4 inhibited strongly β-glucuronidase activity and experimental pulmonary metastasis of B16 variant. In further evaluations, SF4 inhibited the invasion

of B16 variant and 3LL cells through reconstituted basement membrane (Matrigel/laminin-coated filter) in a dose-dependent fashion (Table 1). Moreover, SF4 significantly suppressed spontaneous lung metastasis of 3LL cell by sc inoculation with iv administration for five days starting on the day of the excision of primary tumor (Table 2). SF4 had no significant effects on cell growth at the concentrations used in this study. These results suggest that the antimetastatic effect of SF4 may be due to its antiinvasive rather than antiproliferative activities. Other derivatives related to SF4 may contribute to the study of metastatic processes through better understanding of the mechanism of action of proteolytic enzymes secreted by tumors, and they may be of pharmaceutical interest in the treatment of cancer.

References

1) NISHIMURA, Y.; T. KUDO, S. KONDO & T. TAKEUCHI: Totally synthetic analogues of siastatin B. II. Optically active piperidine derivatives having trifluoroacetamide and hydroxyacetamide groups at C-2. J. Antibiotics 45: 963~970, 1992
2) NISHIMURA, Y.; T. KUDO, S. KONDO, T. TAKEUCHI, T. TSURUOKA, H. FUKUYASU & S. SHIBAHARA: Totally synthetic analogues of siastatin B. III. Trifluoroacetamide analogues having inhibitory activity for tumor metastasis. J. Antibiotics 47: 101~107, 1994
3) UMEZAWA, H.; T. AOYAGI, T. KOMIYAMA, H. MORISHIMA, M. HAMADA & T. TAKEUCHI: Purification and characterization of a sialidase inhibitor, siastatin, produced by *Streptomyces*. J. Antibiotics 27: 963~969, 1974
4) TSURUOKA, T.; H. FUKUYASU, M. AZETAKA, Y. IIZUKA, K. KAWAHARAJO, S. INOUYE, M. HOSOKAWA & H. KOBAYASHI: Inhibition of pulmonary metastases in experimental tumor by D-glucaro-δ-lactam sodium salt. Proceedings of the Second Joint Meeting of the American Association of Cancer Research and the Japanese Cancer Association, B-3, Hawaii, February 10~14, 1992
5) FIDLER, I. J.: Biological behavior of malignant melanoma cells correlated to their survival *in vivo*. Cancer Res. 35: 218~224, 1975
6) ALBINI, A.; Y. IWAMOTO, H. K. KLEINMAN, G. R. MARTIN, S. A. AARONSON, J. M. KOZLOWSKI & R. N. MCEWAN: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res. 47: 3239~3245, 1987
7) SAIKI, I.; J. MURATA, K. WATANABE, H. FUJII, F. ABE & I. AZUMA: Inhibition of tumor cell invasion by Ubenimex (Bestatin) *in vitro*. Jpn. J. Cancer Research 80: 873~878, 1989
8) SHIINO, T.; K. OHISHI, I. NITTSU, H. HAYASHIBARA, Y. TSUCHIYA, T. YOSHIHAMA & H. MORIYUKI: Combination use of lentinan with X-ray therapy in mouse experimental tumor system (Part 3). Combination effect on the metastatic tumors. Jpn. J. Cancer Chemother. 15: 481~485, 1988
9) SAIKI, I.; J. YONEDA, Y. IGARASHI, M. AOKI, N. KUSUNOSE, K. ONO & I. AZUMA: Antimetastatic activity of polymeric RGDT peptides conjugated with poly(ethylene glycol). Jpn. J. Cancer Research 84: 558~565, 1993