J. Microbiol. Biotechnol. (1999), **9**(1), 119–121

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Sesquicillin, an Extracellular Matrix Adhesion Inhibitor, Inhibits the Invasion of B16 Melanoma Cells *In vitro*

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Received: July 28, 1998

Abstract Tumor cell interaction with the extracellular matrix is defined as the critical event of tumor invasion that signals the initiation of a metastatic cascade. Sesquicillin has been identified as an inhibitor of melanoma cell adhesion to the components of the extracellular matrix (ECM) in cultured broth of fungal strain F60063. Sesquicillin strongly inhibited the adhesion of B16 melanoma cells to laminin, fibronectin, and typeIV collagen. It also inhibited B16 melanoma cell invasion of reconstituted basement membrane Matrigel *in vitro* in a dose-dependent manner. These results suggest that sesquicillin is a new class of nonpeptidic ECM adhesion inhibitor having anti-invasive activity.

Key words: Extracellular matrix (ECM), adhesion, invasion, sesquicillin

The interaction between extracellular matrix (ECM) components, such as laminin, fibronectin, and typeIV collagen, and their cell surface receptors may play a key role in the mechanism of tumor invasion and metastasis [7]. The critical event of tumor invasion that signals the initiation of the metastatic cascade is thought to be the adhesion of tumor cells to the ECM [4]. These studies lead to the anti-adhesive concept and furthermore offer a promising strategy in the search for new types of agents effective against tumor invasion and metastasis. In the course of screening microbial metabolites for inhibitors of cell adhesion to ECM components as a whole cell assay, strong inhibitory activity was detected in the fermentation extract of isolated fungal strain F60063. Bioassay-guided fractionation led to a pure compound, identified by spectroscopies as sesquicillin (Fig. 1).

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Sesquicillin has previously been reported in patents, and is described to have antihypertensive, bronchospasmolytic, anti-inflammatory, and laxative activities [8]. Recently, it has also been reported as an inhibitor of glucocorticoid mediated signal transduction [2]. However, these effects have not been attributed to effects on adhesion to ECM proteins and the invasion of tumor cells. In this report, the identification of sesquicillin and its inhibitory activities on melanoma cell adhesion to laminin, fibronectin, and typeIV collagen, and on *in vitro* invasion are described.

The producing organism, fungal strain F60063, was isolated from soil samples in Korea and the identification of this strain is under investigation. The seed culture was prepared by transferring a loopful of surface growth from potato-dextrose agar (PDA) plate culture of strain

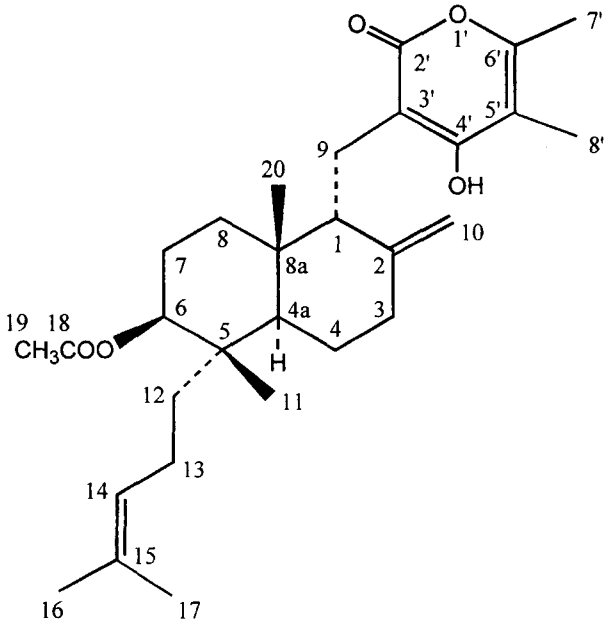

Fig. 1. Structure of sesquicillin.

F60063 into a 250-ml Erlenmeyer flask containing 50 ml of sterilized medium. The inoculated flask was shaken on a rotary shaker at 25°C for 3 days. This seed culture (50 ml) was transferred to a 5-liter fermentor containing 3 liters of medium. The seed and production medium consisted of 1.0% glucose, 0.5% tryptone, 0.3% yeast extract, and 0.3% malt extract. The pH of the medium was adjusted to 6.5 before sterilization. The fermentation was carried out at 25°C with agitation at a rate of 150 rpm and aeration of 1.0 vvm. After 6 days of fermentation, the culture broth was filtered to remove the mycelium and the filtrate was applied on a column of Diaion HP-20. After washing with water and 50% MeOH, the active principles were eluted with 90% MeOH. The active fractions were evaporated, dissolved in water, and extracted with an equal volume of EtOAc. The separated organic layer was evaporated to dryness *in vacuo* and the remaining residue was applied to a column of silica gel with hexane-EtOAc (3:2). The active eluate was concentrated and subjected to a Sephadex LH-20 column chromatography with MeOH and further purified by HPLC (YMC pack ODS-AM) with 80% CH_3CN containing 0.05% TFA. HPLC fractionation yielded the pure active compound as a white powder (16 mg).

Sesquicillin showed UV absorption maxima at 210 and 290 nm, and a molecular ion peak at m/z 470 in the EI-MS. The molecular formula, $C_{29}H_{42}O_5$, was established by HREI-MS. The structure of sesquicillin was determined by NMR experiments. The assignment of ^{13}C and ^{1}H NMR signals was facilitated by COSY and HMQC experiments (Table 1), while the carbon skeleton and relative stereochemistry of sesquicillin were determined by HMBC and NOESY experiments.

The adhesion assays were performed with B16F10 melanoma cells by a partially modified method reported by Graf *et al.* [3]. B16 melanoma cells possess multiple integrin receptors for the ECM proteins, laminin, typeIV collagen, and fibronectin [5]. B16F10 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). The cells grown up to 80% confluence in culture plates were detached with 2 mM EDTA, after washing, and were resuspended in serum-free DMEM containing 0.1% BSA. Sesquicillin at different concentrations and 10^5 cells in 100 µl of medium were added to ECM protein-coated wells. The plate was incubated for 1 h at 37°C in 5% CO_2 and 95% air. After incubation, the plate was washed to remove non-adherent cells. Adherent cells were fixed and stained with 0.5% crystal violet solution. The absorbance at 570 nm of individual wells was measured using a microplate reader (Bio-Rad). Each assay was performed in triplicate. As shown in Table 2, sesquicillin strongly inhibited the adhesion of B16 melanoma cells to all ECM proteins tested. IC_{50} values were between 2.9 and 6.1 µg/ml. Sesquicillin had no specificity against different ECM proteins.

In order to investigate the influence of the sesquicillin on cell invasiveness, *in vitro* tumor cell invasion assays through Matrigel coated filters were performed. The invasive activity of B16F10 cells was assayed in Transwell cell culture chambers with a membrane filter (Costar 3422) as described previously [1, 6], with some modifications. Briefly, the filters with 8.0-µm pore size were precoated with 1 µg of laminin on their lower surfaces and dried at room temperature. A reconstituted Matrigel (Collaborative Research Co.) was applied to the upper surfaces of the filters (5 µg/50 µl/filter) and dried in the same way. Exponentially growing B16F10 cells were harvested by 2 mM EDTA in PBS, washed with FCS-free DMEM and resuspended to the density of 2×10^6 cells/ml in the culture medium with or without sesquicillin. The cell suspension was added into the upper compartment of the chamber in triplicate and incubated for 6 hr at 37°C in an atmosphere of 5% CO_2 and 95% air. The cells on the upper surface of the filter were removed by wiping it with a cotton swab and the cells on the filters were fixed with MeOH. The fixed cells were stained with 0.5% crystal violet in 20% MeOH for 30 min. After gently rinsing with water, the crystal violet dye retained in the filters was extracted with 30% acetic acid and the

Table 1. ^{13}C and ^{1}H NMR spectral data for sesquicillin in CD_3OD.

Carbon No.	^{13}C	^{1}H	Carbon No.	^{13}C	^{1}H
1	55.5	2.13	14	124.9	5.02
2	149.3		15	131.4	
3	31.4	2.42, 2.03	16	25.2	1.62
4	23.1	1.34, 1.54	17	16.9	1.56
4a	39.6	1.79	18	171.9	
5	40.6		19	20.4	1.98
6	76.8	4.80	20	22.9	0.95
7	24.4	1.78, 1.66	1'		
8	34.3	1.77, 1.28	2'	167.4	
8a	38.1		3'	103.3	
9	21.9	2.77, 2.58	4'	167.0	
10	109.8	4.45, 4.18	5'	108.0	
11	18.1	0.84	6'	156.0	
12	38.3	1.24, 1.12	7'	16.6	2.15
13	22.0	1.90	8'	9.7	1.88

^{13}C, 125 MHz; ^{1}H, 500 MHz; δ in ppm.

Table 2. Inhibition of adhesion of B16F10 melanoma cells to ECM components by sesquicillin.

Proteins (quantity per well)	IC_{50} (µg/ml)*
Laminin (2 µg)	2.92
Fibronectin (1 µg)	6.10
TypeIV collagen (2 µg)	3.45

*IC_{50} values were defined as concentration (µg/ml) that caused 50% inhibition of cell adhesion to ECM proteins.

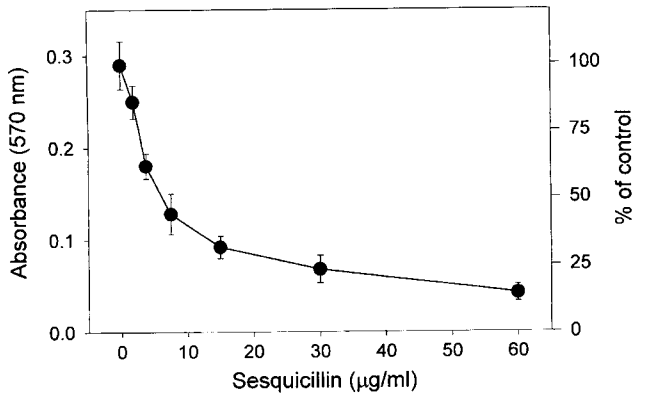

Fig. 2. Inhibition of *in vitro* invasion of B16F10 melanoma cells by sesquicillin.

The invasive activity of B16F10 cells was assayed in Transwell chambers. The data were expressed as the mean ± S.D. of triplicate cultures.

absorbance was measured colorimetrically at 570 nm. As shown in Fig. 2, sesquicillin inhibited B16F10 melanoma cell invasion of reconstituted basement membrane Matrigel *in vitro* in a dose-dependent manner. The IC_{50} value was 6.13 μg/ml. In addition, sesquicillin slightly inhibited the activity of MMP-2, one of major proteases which contributes to ECM proteolysis. The mechanism of inhibitory action of sesquicillin on tumor cell invasion may be due to the inhibitory effect of tumor cell interaction with ECM proteins.

As a result of our screening for inhibitors of cell adhesion to laminin, fibronectin, and typeIV collagen, the major components of the ECM, sesquicillin, has been isolated from fungal strain F60063. Sesquicillin has strong inhibitory activities on melanoma cell adhesion and it showed efficient anti-invasive activity of highly metastatic B16F10 melanoma cells. These results suggest that sesquicillin, a fungal diterpene, may be a promising ECM receptor antagonist and inhibitor of tumor invasion and metastasis.

Acknowledgments

We are grateful to Dr. C. J. Kim of the Korea Research Institute of Bioscience and Biotechnology for supplying the fungus used in this study.

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