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Anti-invasive and anti-angiogenic activities of naturally occurring dibenzodiazepine BU-4664L and its derivatives

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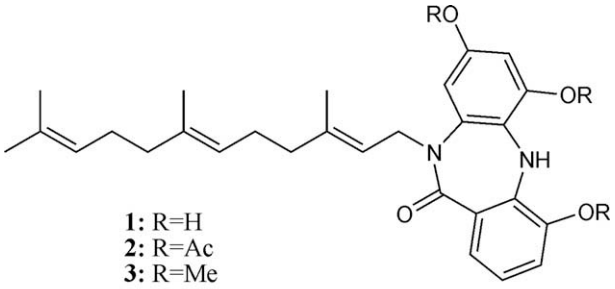
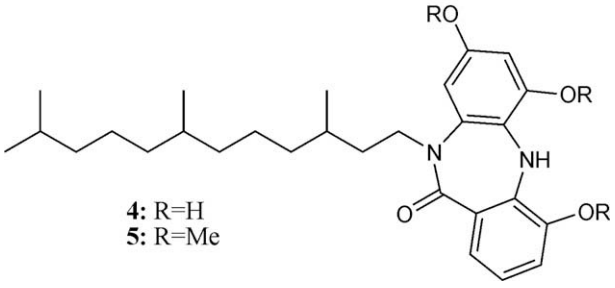
ABSTRACT

In the screening for antitumor leads from microbial secondary metabolites, BU-4664L (**1**), a naturally occurring dibenzodiazepine, was found to inhibit tumor invasion and angiogenesis in vitro. Compound **1** inhibited the gelatinase activities of MMP-2 and MMP-9 and the cellular motility. Four derivatives (**2**–**5**) were synthesized from **1** and their antitumor activities were evaluated. Compounds **3** and **4** exhibited potent anti-angiogenic effects on HUVEC, together with remarkable inhibition of cell migration at nanomolar concentrations, and showed much lower cytotoxicity.

Tumor metastasis is the process by which a tumor cell leaves the primary tumor, disseminates to a distant site via the circulatory system, and establishes a secondary tumor.[1] In the metastatic process, translocation of tumor cells across extracellular barriers, namely invasion, is a critical step to accomplish the metastasis. The process of invasion includes tumor cell adhesion, local proteolysis of extracellular matrix proteins, and migration. Angiogenesis is another crucial step in metastasis. A high degree of tumor vascularization increases the chance for tumor cells to enter the circulatory system and metastasize.[2] Angiogenesis is also necessary for delivery of oxygen and nutrition to support tumor growth. The process of angiogenesis requires steps similar to invasion: (1) proliferation of endothelial cells, (2) degradation of the extracellular matrix, and (3) migration of endothelial cells. These processes required for metastasis are common to tumor cells of different genetic origins. Therefore, the suppression of invasiveness and angiogenesis is a promising therapeutic approach to various types of solid tumors.[3]



1: R=H
2: R=Ac
3: R=Me



4: R=H
5: R=Me

In our continued screening for anti-invasive and/or anti-angiogenic compounds from microbial secondary metabolites,[4] BU-4664L (**1**)[5] was found to exhibit modest inhibitory effects on tumor cell invasion and angiogenesis. Compound **1** was initially discovered from a soil-derived actinomycete of the genus *Micromonopora*

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in the screening for 5-lipoxygenase inhibitors, aiming at anti-inflammatory drug discovery, by researchers at Bristol-Myers Squibb.[5a] They also demonstrated the antitumor effect of **1** against B16 melanoma and P388 leukemia in a mouse zenograft model. Recently, researchers at Thallion Pharmaceutical reported potent therapeutic effects of **1** in human breast cancer MDA-MB-231 and prostate cancer PC-3 xenograft experiments.[6] In spite of these in vivo antitumor efficacy, action mechanism of **1** still remains unclear. In this Letter, we describe the anti-invasive and anti-angiogenic activities of **1** and the results from preliminary structure–activity relationship and mode of action studies.

Invasion of murine colon 26-L5 carcinoma cells through Matrigel/fibronectin-coated filters[7] was inhibited by BU-4664L (**1**) with an IC_{50} value of 1.0 μg/mL (=2.2 μM) in a concentration dependent manner (Table 1). Compound **1** showed no effects on cell proliferation at the same concentration, but 50% growth inhibition was observed at 7.8 μg/mL (Table 2). Tumor cell invasion consists of mainly three steps: cell adhesion, degradation of extracellular matrix, and migration. To gain insights into the mode of action, effects of **1** on these steps were examined. Compound **1** did not inhibit the attachment of colon 26-L5 cells to extracellular matrix proteins such as fibronectin, laminin, and Matrigel (data not shown). On the other hand, **1** suppressed the migration of the same cell line at similar concentrations (IC_{50} = 0.63 μg/mL). MMP-2 and MMP-9, gelatinases responsible for degradation of basement membrane, are the matrix metalloproteinases (MMPs) most frequently overexpressed in tumor cells.[8] Compound **1** inhibited the proteolytic activities of MMP-2 and MMP-9 with an IC_{50} value of 0.46 μg/mL and 0.60 μg/mL, respectively (Fig. 1). These observations indicated that **1** acts on both steps of extracellular matrix degradation and migration.

Angiogenesis is the process of forming blood vessels from pre-existing vessels. In response to angiogenic factors such as vascular endothelial growth factor (VEGF), endothelial cells migrate into the surrounding extracellular matrix where they form a capillary blood vessel.[2] Therefore, cell migration is the crucial step common to angiogenesis and invasion. Anti-angiogenic effect of **1** was evaluated by the in vitro vascular organization model using HUVECs (human umbilical vein endothelial cells).[9] Compound **1** inhibited vascular formation of HUVECs with an IC_{50} value of 0.72 μg/mL. Furthermore, effect of **1** on migration of endothelial cells was evaluated by the wound healing assay.[9] Confluently grown HUVECs were wounded by a micropipette tip and the migrated cells were counted after 8 h. Compound **1** suppressed the cell migration in a similar concentration range (IC_{50} = 0.43 μg/mL), leading to the suggestion that **1** inhibits vascular formation by interrupting cellular motility.

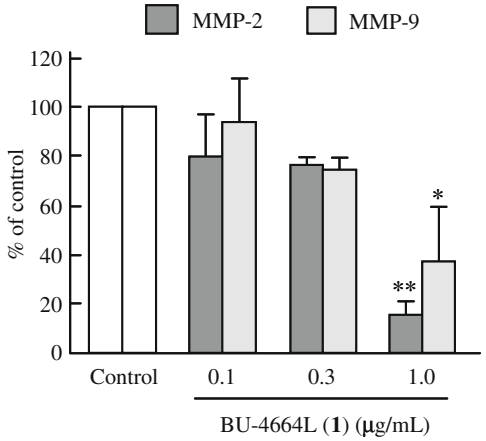

Figure 1. Inhibition of MMP-2 and MMP-9 by BU-4664L (**1**). The data were expressed as the mean ± SD. $^{*}p$ <0.01, $^{**}p$ <0.025 as compared with the control by student's two tailed *t*-test.

BU-4664L (**1**) comprises of two distinct substructures: the dibenzodiazepine core and the aliphatic farnesyl side chain. In order to examine which part is responsible to exert bioactivities, four derivatives were prepared from **1**.[5b] Compounds **2**[10] and **3**[11] were obtained by acetylation and methylation of **1**, respectively. Compound **4**[12] was obtained by catalytic hydrogenation of the farnesyl moiety from **1**. Compound **5**[13] was prepared by hydrogenation of **3**.

Effects of compounds **2**–**5** on proliferation of three cell lines were examined in comparison with BU-4664L (**1**) (Table 2). Compound **1** displayed moderate toxicity toward colon 26-L5 cells and Renca (murine renal cell carcinoma) cells and higher toxicity toward HUVECs. On the other hand, most of the derivatives showed lower or no cytotoxicity. Especially, it is interesting to note a considerable reduction in toxicity of the derivatives **2**–**5** toward HUVECs.

Next we examined effects of compounds **2**–**5** on invasion (Table 1). Triacetate **2** was not active or weaker but O-methylated analogue **3** maintained potency equivalent to the parent compound **1**. Hydrogenation of the unsaturated side chain led to the loss of anti-invasive activity as demonstrated by compound **4**. However, compound **5** that has a saturated side-chain and an O-methylated core still retained good activity. These findings suggested that the dibenzodiazepinone core has a larger contribution to the anti-invasiveness than the side-chain moiety.

SAR for anti-angiogenesis is partially not consistent with that for anti-invasiveness (Table 3). Triacetate **2** was inactive again, but the side-chain saturated analogue **4** displayed more potent inhibition than **1**. The O-methylated analogue **3** was the most potent inhibitor with an IC_{50} value of 0.11 μg/mL. Contrary to our expectation, compound **5** which has the side chain of **4** and the aromatic core of **3** showed lower activity than **1**.

Table 1
Anti-invasive activity of compounds **1**–**5**

Compound	IC_{50}[a] (μg/mL)	
	Colon 26-L5	Renca
1	1.0	0.78
2	>1.0 (0%)	>1.0 (40%)
3	1.0	0.78
4	>1.0 (30%)	>1.0 (40%)
5	1.0	>1.0 (44%)

[a] Inhibition percentage at the indicated concentrations is shown in parentheses.

Table 2
Cytotoxicity of compounds **1**–**5**

Compound	IC_{50}[a] (μg/mL)		
	Colon 26-L5	Renca	HUVEC
1	7.8	8.3	2.0
2	7.6	>10 (0%)	>10 (0%)
3	>10 (30%)	>10 (40%)	>10 (0%)
4	10	>10 (0%)	>10 (0%)
5	>10 (8%)	>10 (31%)	>10 (32%)

[a] Inhibition percentage at the indicated concentrations is shown in parentheses.

Table 3
Anti-angiogenic activity of compounds **1**–**5** and fumagillin

Compound	IC_{50}[a] (μg/mL)
1	0.72
2	>1.0 (0%)
3	0.11
4	0.23
5	>1.0 (30%)
Fumagillin	0.034

[a] Inhibition percentage at the indicated concentrations is shown in parentheses.

Table 4
Inhibition of cellular motility by compounds **1**, **3**, **4**, and fumagillin

Compound	IC_{50}[a] (μg/mL)	
	Colon 26-L5	HUVEC
1	0.63	0.43
3	0.67	0.0076
4	>1.0 (22%)	0.0024
Fumagillin	NT[b]	0.023

[a] Inhibition percentage at the indicated concentrations is shown in parentheses.
[b] NT: not tested.

Furthermore, the effects of **3** and **4** that showed higher anti-angiogenic activity were examined toward cellular motility of colon 26-L5 cells and HUVECs (Table 4). Compounds **3** and **4** displayed a remarkable improvement of inhibition against the cell migration of HUVECs with IC_{50} values of 7.6 ng/mL (=15 nM) and 2.4 ng/mL (=5.2 nM), respectively, while the potency toward colon 26-L5 cells was similar to or lower than **1**. Meanwhile, **3** lost the inhibitory activities against MMP-2 and MMP-9, suggesting that the phenolic hydroxyl groups are involved in gelatinase inhibition but not important for anti-invasive and anti-angiogenic properties. Although further SAR study is necessary, these results indicate that BU-4664L has a large capacity of structural modification to improve toxicity and anti-angiogenic activity.

We demonstrated that BU-4664L (**1**) suppresses invasion and angiogenesis by inhibiting cellular motility. Compound **1** has been shown to prolong the survival time[5a] and reduce the tumor volume size[6] in mouse zenograft experiments. These antitumor effects of **1** may be attributed not only to its anti-proliferative property but also to anti-invasive and anti-angiogenic properties. It is noteworthy that the inhibitory potency of **3** toward vascular formation and motility of HUVECs is comparable to that of fumagillin[14] which used to be under clinical development as an antitumor drug.

Because invasion and angiogenesis are complex, multistep processes, a large number of potential target molecules exist. Farnesyltransferase (FTase)[15] and VEGF receptor tyrosine kinase[16] are the examples of attractive targets for anti-metastatic cancer chemotherapy. Inhibition of these enzymes results in the suppression of invasiveness and angiogenesis, but BU-4664L did not show any inhibitory effects on them. Further experiments directing to target identification is in progress.

To the best of our knowledge, BU-4664L (**1**) is the only example of dibenzodiazepine found from nature,[17] while synthetic dibenzodiazepines are well known as ligands for receptors in central and peripheral nervous systems.[18] Compound **1** has been isolated from *Micromonospora* sp. collected from various niches such as sediment, marine invertebrate, and seawater.[5,17,19] In fact, we encountered this compound from soil- and marine-derived *Micromonopora* strains in our culture collection, but analogous metabolites have not been identified so far. This uniqueness renders BU-4664L interesting to be studied as a lead scaffold in the design and synthesis of anti-angiogenic agents. Evaluation of in vivo antitumor efficacy of **3** and further chemical modification based on the dibenzodiazepine pharmacophore are under investigation.

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10. To a stirred solution of **1** (10 mg, 0.022 mmol) in dry pyridine (1 mL) was added acetic anhydride (1 mL) at room temperature. After stirring at the same temperature overnight, the mixture was poured into ice-water and extracted with EtOAc. The organic layer was washed with saturated $CuSO_4$ solution and brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. The residue was purified on a silica gel column (hexane/EtOAc = 1:0–1:1) to give **2** (3.2 mg, 25% yield): IR (neat) ν_{max} 1768, 1615 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.58 (3H, s), 1.59 (3H, s), 1.67 (3H, s), 1.70 (3H, s), 1.95 (2H, m), 2.05 (6H, m), 2.25 (3H, s), 2.41 (6H, s), 4.56 (1H, d, J = 7.9 Hz), 5.07 (2H, m), 5.40 (1H, t, J = 7.4 Hz), 6.09 (1H, s), 6.83 (1H, d, J = 3.0 Hz), 6.93 (1H, d, J = 3.0 Hz), 7.00 (1H, t, J = 10 Hz), 7.15 (1H, dd, J = 10, 1.8 Hz), 7.73 (1H, dd, J = 10, 1.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.0, 16.5, 17.7, 21.0, 21.1, 21.2, 25.7, 26.4, 26.8, 39.6, 39.7, 49.3, 112.9, 114.3, 120.5, 122.2, 123.8, 124.3, 125.3, 126.7, 130.3, 131.3, 133.1, 135.3, 136.5, 138.9, 139.1, 140.8, 141.6, 145.8, 167.1, 167.7, 168.1, 168.9; HR-ESITOFMS m/z $[M+Na]^+$ 611.2738 (calcd for $C_{34}H_{40}N_2O_7Na$, 611.2728).
11. To a stirred solution of **1** (16 mg, 0.035 mmol) in MeOH–benzene solution (0.5–1 mL) was added 2 M trimethylsilyldiazomethane in Et_2O (0.40 ml, 1.1 mmol) at room temperature. After stirring at the same temperature overnight, the mixture was concentrated in vacuo and the residue was purified on the column of LH-20 (CH_2Cl_2) to give **3** (11 mg, 60% yield): IR (neat) ν_{max} 1618 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.58 (6H, s), 1.67 (3H, s), 1.72 (3H, s), 1.95 (2H, m), 2.02–2.15 (6H, m), 3.67 (3H, s), 3.88 (3H, s), 3.92 (3H, s), 4.57 (2H, d, J = 7.5 Hz), 5.08 (2H, br s), 5.47 (1H, t, J = 7.0 Hz), 6.28 (1H, d, J = 3.5 Hz), 6.44 (1H, d, J = 3.5 Hz), 6.86 (1H, dd, J = 9.5, 3.0 Hz), 6.89 (1H, t, J = 9.5 Hz), 6.93 (1H, br s), 7.45 (1H, d, J = 9.0, 3.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.0, 16.5, 17.7, 25.7, 26.6, 26.7, 39.6, 39.7, 48.9, 55.5, 56.3, 56.5, 96.1, 99.5, 113.1, 121.0, 121.8, 123.9, 124.3, 124.4, 125.3, 127.6, 131.3, 135.2, 135.5, 137.8, 142.1, 148.1, 150.4, 155.8, 168.5; HR-ESITOFMS m/z $[M+Na]^+$ 527.2888 (calcd for $C_{31}H_{40}N_2O_4Na$, 527.2880).
12. A mixture of **1** (10 mg, 0.022 mmol) and 10% Pd/C (5 mg) in MeOH (2.5 mL) was stirred under H_2 at room temperature. After 3 h, the mixture was filtered through Celite, and the filtrate was concentrated in vacuo. The residue was purified by preparative HPLC (COSMOSIL 5C18-AR-II, 250 × 20 mm i.d. 50% CH_3CN in distilled water), and the collected fraction was concentrated to give **4** (2.3 mg, 23% yield): IR (neat) ν_{max} 3265, 1605 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 0.81 and 0.82 (total 3H, each d, J = 7.0 Hz), 0.85 (3H, d, J = 7.0 Hz), 0.86 (6H, d, J = 7.0 Hz), 0.99–1.40 (13H, m), 1.46–1.65 (4H, m), 3.96 (1H, m), 4.13 (1H, m), 6.21 (1H, d, J = 2.0 Hz), 6.23 (1H, d, J = 2.0 Hz), 6.73 (1H, t, J = 8.0 Hz), 6.81 (1H, dd, J = 8.0, 1.0 Hz), 7.12 (1H, dd, J = 8.0, 1.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 19.2, 19.3, 22.1, 22.2, 24.4, 25.0, 28.3, 29.9, 30.06, 30.14, 33.0, 34.9, 35.0, 37.3, 37.4, 37.5, 37.6, 39.7, 47.7, 100.2, 101.4, 116.8, 121.1, 122.5, 125.6, 127.8, 134.6, 142.5, 146.3, 148.8, 153.7, 170.6; HR-ESITOFMS m/z $[M+Na]^+$ 491.2880 (calcd for $C_{28}H_{40}N_2O_4Na$, 491.2880).
13. In the same manner as described for **4**, 5.6 mg of **5** was obtained from **3** (10 mg, 0.020 mmol) in 55% yield: IR (neat) ν_{max} 1617 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.79 and 0.80 (total 3H, each d, J = 8.5 Hz), 0.86 (6H, d, J = 8.5 Hz), 0.87 (3H, d, J = 8.5 Hz), 0.97–1.32 (13H, m), 1.43–1.70 (4H, m), 3.75 (3H, s), 3.86 (3H, s), 3.89 (3H, s), 4.02 (1H, m), 4.10 (1H, m), 6.30 (1H, d, J = 3.0 Hz), 6.36 (1H, d, J = 3.0 Hz), 6.85 (1H, dd, J = 10, 3.0 Hz), 6.87 (1H, t, J = 10 Hz), 7.00 (1H, br s), 7.42 (1H, dd, J = 9.0, 3.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.66, 19.70, 22.6, 22.7, 24.3, 24.8, 28.0, 30.46, 30.50, 32.8, 35.0, 35.1, 37.2, 37.3, 37.4, 39.4, 47.68, 47.72, 55.7, 56.3, 56.5, 96.1, 100.2, 112.9, 120.9, 124.1, 125.6, 128.8, 134.7, 142.2, 148.1, 150.7, 155.9, 168.5; HR-ESITOFMS m/z $[M+Na]^+$ 533.3352 (calcd for $C_{31}H_{46}N_2O_4Na$, 533.3350).
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