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ORIGINAL ARTICLE

NBRI16716A, a new antitumor compound against human prostate cancer cells, produced by *Perisporiopsis melioloides* Mer-f16716

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Prostate stroma can regulate the growth and metastasis of prostate cancer through the tumor–stromal cell interactions. Thus, small molecules that modulate the tumor–stromal cell interactions will have a chance to become new antitumor drugs. In the course of our screening of the modulators, we isolated three new natural compounds, NBRI16716A (1), NBRI16716B (2) and NBRI16716C (3), from the fermentation broth of *Perisporiopsis melioloides* Mer-f16716, although compound 2 was already reported as a chemical degradation product of isotriornicin. Compounds 1 and 2 inhibited the growth of human prostate cancer DU-145 cells in the coculture with human prostate stromal cells (PrSCs) more strongly than that of DU-145 cells alone. Furthermore, both compounds showed antitumor effect against xenograft models of DU-145 cells and PrSCs *in vivo*.
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INTRODUCTION

Growing evidence supports the idea that the growth and metastasis of prostate cancer are regulated by prostate stroma.[1,2] We focused on such tumor–stromal cell interactions of prostate cancer and reported that prostate stromal cells (PrSCs) promote the growth of human prostate cancer cells through secretion of insulin-like growth factor-I.[3,4] Because small molecules that modulate the tumor–stromal cell interactions possibly show potent antitumor effect, we developed the *in vitro* coculture system of human prostate cancer cells and PrSCs, in which the growth of prostate cancer cells is increased by the coculture with PrSCs.[3,5] Using the assay method as a screening system for antitumor compounds, we have been finding several compounds that exert antitumor effects through modulation of the tumor–stromal cell interactions.[6,7] We have recently reported that leucinostatin A, a fungus natural compound, showed antitumor effect against xenograft models of human prostate cancer cells *in vivo* through the downregulation of insulin-like growth factor-I secretion from PrSCs.[8] By continuing the screening system, we have found new natural compounds from a fungal strain *Perisporiopsis melioloides* Mer-f16716. In this study we describe the isolation, structure determination and biological activity of NBRI16716A (**1**), NBRI16716B (**2**) and NBRI16716C (**3**).

RESULTS

Isolation procedure for NBRI16716A (1), NBRI16716B (2) and NBRI16716C (3)

The culture broth (12 l) was filtered and the filtrate was passed through a DIAION HP20 column (800 ml; Mitsubishi Chemical Corporation, Tokyo, Japan) pre-equilibrated with H_2O. After washing with H_2O (2 l), the active materials were eluted with 25% MeOH (2 l). The eluate was concentrated *in vacuo* to remove MeOH and diluted with H_2O up to 2 l and then extracted with BuOH. The organic layer was concentrated *in vacuo* to afford 4.1 g of dried materials. The materials were applied on a silica gel column (200 g, Wakogel C-200, 75–150 μm; Wako Pure Chemical Industries, Tokyo, Japan) prepared with $CHCl_3$, and eluted with $CHCl_3$ and $CHCl_3$-MeOH. The fractions eluted with $CHCl_3$-MeOH (2:1) were concentrated *in vacuo* to give 0.93 g of crude materials. The crude materials were purified by a reversed-phase HPLC column (Inertsil ODS-3, 20×250 mm, 6.0 ml min^{-1}; GL Sciences Inc., Tokyo, Japan) with 20% MeOH containing 0.1% TFA to afford crude 543.8 mg of **1** and crude 42.2 mg of **2**. The crude samples were applied on gel filtration chromatography of Sephadex LH-20 (50% MeOH; GE Healthcare, Tokyo, Japan) to afford pure 38.5 mg of **1**, 31.1 mg of **2** and a trace

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Table 1 Physico-chemical properties of 1–3

	1	*2*	*3*
Appearance	White powder	White powder	White powder
Molecular formula	$C_{18}H_{30}N_4O_8$	$C_{18}H_{30}N_4O_7$	$C_{18}H_{30}N_4O_7$
HR ESI-MS (m/z)			
Found	453.1937 $(M+Na)^+$	437.1990 $(M+Na)^+$	437.2031 $(M+Na)^+$
Calcd	453.1961 for $C_{18}H_{30}N_4O_8Na$	437.2012 for $C_{18}H_{30}N_4O_7Na$	437.2012 for $C_{18}H_{30}N_4O_7Na$
UVλ_{max} nm (H_2O)	End	End	End
$[\alpha]_D^{22}$ (MeOH)	-19.6°(*c* 0.3)	-21.3°(*c* 0.4)	-19.2°(*c* 0.3)

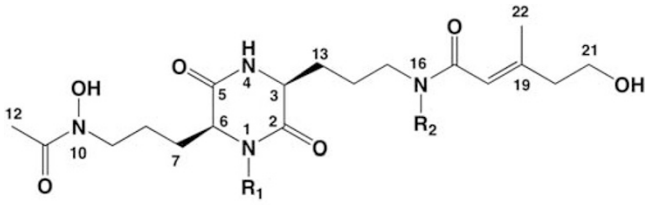

Figure 1 Structures of NBRI16716A (**1**), NBRI16716B (**2**) and NBRI16716C (**3**).

amount of **3**. To obtain more **3**, we repeated the fermentation process three times and finally obtained 1.4 mg of **3**.

Physico-chemical property

The physico-chemical properties of **1**, **2** and **3** are summarized in Table 1. Compounds **1**, **2** and **3** were obtained as white powder, which was soluble in MeOH, DMSO and water, but insoluble in $CHCl_3$ and EtOAc. The characteristic color reaction to $FeCl_3$ indicated that they were a family of siderophore.[9] The molecular formulae of **1**, **2** and **3** were determined to be $C_{18}H_{30}N_4O_8$, $C_{18}H_{30}N_4O_7$ and $C_{18}H_{30}N_4O_7$ by HR-ESI-MS, respectively. The general features of their UV and NMR spectra resembled each other, indicating structural similarities of these compounds.

Structure determination

The NMR spectra of **2** indicated that **2** was identical to the known cleavage fragment of isotriornicin isolated from *Epicoccus purpurascens*[10] as shown in Figure 1. The molecular formula of **1** was determined to be $C_{18}H_{30}N_4O_8$, which was one more oxygen atom than that of **2**. By comparing NMR data of **1** with those of **2**, it was apparent that **1** closely resembled **2** except for the ^{13}C chemical shifts of C-2, C-5, C-6 and C-7. In the ^1H NMR spectrum, compound **1** showed only one amide signal at δ_H 8.30, indicating the replacement of NH to N-OH. The connectivity between proton and carbon atoms was established by the ^{13}C-^1H HMQC spectra as shown in Table 2. The ^1H-^1H COSY and HMBC correlations of **1** are summarized in Figure 2. In the ^1H-^1H COSY spectrum, sequential proton networks were observed from NH-4 to H-15 through H-3, H-13 and H-14, and from H-6 to H-9. In the HMBC spectrum, cross peaks were observed from NH-4 to C-2 and C-6, from H-7 to C-5 and C-6, from H-13 to C-3 and C-2 and from H-3 to C-2, respectively. Thus, the planar structure of **1** was determined as shown in Figure 1.

Compound **3** was less one oxygen atom than that of **1**. The ^1H and ^{13}C NMR spectra were very similar to those of **1** except that **3** showed an additional amide signal at δ_H 7.80 in the ^1H NMR spectrum. By comparing various NMR spectra of **3** with those of **1**, the difference between **1** and **3** was ascribed to the replacement of N-OH in **1** with NH in **3** at N-16 position.

Compounds **1, 2** and **3** showed optical rotations of -19.6°, -21.3° and -19.2°, respectively. Three compounds possess asymmetric carbons at C-3 and C-6. The optical rotation of **2** is very close to that of reported value $[\alpha]_D = -22.95^\circ$ (*c* 0.78, MeOH)[10] indicating the same absolute configuration at C-3 (*S*) and C-6 (*S*). Accordingly, the absolute configuration of C-3 and C-6 in **1** and **3** was considered to be identical with **2** based on the optical rotations and biosynthetic aspect. As the ^{13}C-chemical shifts of C-22 in three compounds were almost identical to that of reported values of **2**,[10] the double bonds of C-18 in **1**, **2** and **3** were assigned to *E*-configuration.

Biological activities

The effects of **1**, **2** and **3** on coculture of human prostate cancer DU-145 cells with PrSCs were determined using rhodanile blue staining method.[5] The coculture system shows the tumor–stromal cell interactions of prostate cancer, in which the growth of DU-145 cells is increased by the coculture with PrSCs.[3,5] As shown in Figure 3, although both **1** and **2** moderately inhibited the growth of DU-145 cells cultured alone with IC_{50} values at almost 100 μg ml^{-1}, both compounds inhibited the growth of DU-145 cells in coculture with PrSCs more strongly than that of DU-145 cells alone with IC_{50} values at approximately 10 μg ml^{-1}. In contrast, compound **3** equally inhibited the growth of DU-145 cells in both culture conditions with IC_{50} values at almost 100 μg ml^{-1}. All three compounds did not show apparent cytotoxicity against PrSCs under microscopic observation (data not shown).

Acute toxicity of **1** and **2** in mice was examined using ICR female mice. When both **1** and **2** were administered intravenously, intraperitonealy or orally, no fatality was observed up to 100 mg kg^{-1}. We then examined the antitumor effects of both compounds against xenograft models. We inoculated DU-145 cells with PrSCs into nude mice subcutaneously and then injected **1** and **2** intraperitonealy. As a result, both compounds apparently inhibited the growth of tumors of DU-145 cells and PrSCs *in vivo* without any effect on body weight (Figure 4).

Compounds **1** and **2** did not show antimicrobial and antifungal activities at 100 μg ml^{-1}.

DISCUSSION

Because compound **2** was originally reported as an artificial fragment of isotriornicin cleaved by methanol-ammonia,[10] it is a very curious fact that we obtained it as a natural product. Although isotriornicin was isolated from *E. purpurascens*,[10] our compounds were isolated from the different fungus, *P. melioloides*. The biological effects of the

Table 2 The ^{13}C and ^{1}H NMR assignments of 1–3 in DMSO-d_6

	1			*2*			*3*		
Position	*^{13}C (mult.)*	*p.p.m.*	*^{1}H p.p.m. (mult., J)*	*^{13}C (mult.)*	*p.p.m.*	*^{1}H p.p.m. (mult., J)*	*^{13}C (mult.)*	*p.p.m.*	*^{1}H p.p.m. (mult., J)*
N-1						8.17 (br. s)			
C-2	162.4	(s)		167.7	(s)		162.1	(s)	
C-3	53.5	(d)	3.86 (m)	53.7	(d)	3.83 (m)	53.3	(d)	3.86 (m)
N-4			8.30 (br. s)			8.17 (br. s)			8.36 (br. s)
C-5	165.9	(s)		167.7	(s)		165.9	(s)	
C-6	62.7	(d)	4.10 (t, *J*=5.0 Hz)	53.7	(d)	3.83 (m)	62.6	(d)	4.05 (m)
C-7	27.7	(t)	1.80 (m)	30.1	(t)	1.65 (m)	27.5	(t)	1.81 (m)
C-8	21.9	(t)	1.53 (m)	21.9	(t)	1.53 (m)	21.9	(t)	1.55 (m)
C-9	46.9	(t)	3.45 (t, *J*=6.6 Hz)	46.6	(t)	3.49 (t, *J*=6.6 Hz)	46.7	(t)	3.45 (m)
N-10									
C-11	170.3	(s)		169.9	(s)		170.1	(s)	
C-12	20.4	(q)	1.93 (s)	20.2	(q)	1.96 (s)	20.2	(q)	1.97 (s)
C-13	32.1	(t)	1.65 (m)	30.1	(t)	1.65 (m)	32.0	(t)	1.65 (m)
C-14	22.4	(t)	1.53 (m)	21.9	(t)	1.55 (m)	24.9	(t)	1.45 (m)
C-15	46.9	(t)	3.45 (t, *J*=6.6 Hz)	46.6	(t)	3.49 (t, *J*=6.6 Hz)	37.7	(t)	3.05 (m)
N-16									7.80 (br. s)
C-17	166.6	(s)		165.9	(s)		165.9	(s)	
C-18	116.2	(d)	6.21 (s)	116.3	(d)	6.23 (s)	119.8	(d)	5.62 (s)
C-19	151.0	(s)		151.0	(s)		149.1	(s)	
C-20	43.9	(t)	2.22 (t, *J*=6.6 Hz)	43.7	(t)	2.22 (t, *J*=6.6 Hz)	43.5	(t)	2.17 (t, *J*=6.6 Hz)
C-21	59.2	(t)	3.52 (t, *J*=6.6 Hz)	59.1	(t)	3.53 (t, *J*=6.6 Hz)	59.0	(t)	3.53 (t, *J*=6.6 Hz)
C-22	18.3	(q)	1.98 (br. s)	18.2	(q)	2.02 (br. s)	17.7	(q)	2.07 (s)

Chemical shifts in p.p.m. from TMS as an internal standard.
The ^{13}C and ^{1}H NMR were measured at 100 and 400 MHz, respectively.

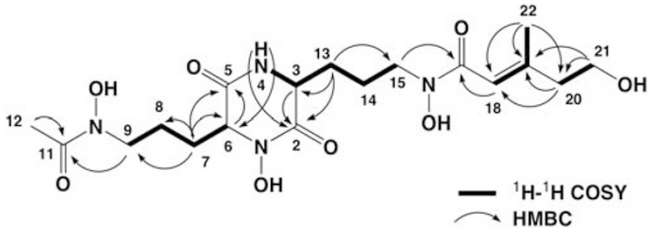

Figure 2 Structure of **1** and key ^{1}H-^{1}H COSY and HMBC correlations of **1**.

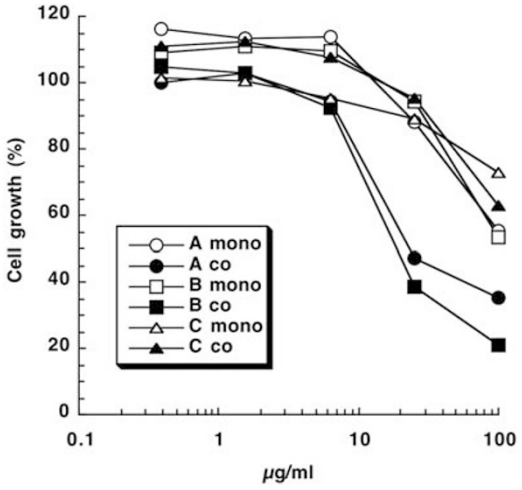

Figure 3 Effects of **1**, **2** and **3** on coculture of DU-145 cells and PrSCs. The growth of DU-145 cells cocultured with PrSCs (closed symbols) or that of DU-145 cells alone (open symbols) in the presence of the indicated concentrations of **1** (circles), **2** (squares) or **3** (triangles) was determined using rhodanile blue method. Values are means of duplicate determinations. Each s.e. is $<10\%$.

fragment and isotriornicin were not reported, but it is interesting that triornicin, an isomer of isotriornicin, has been reported to possess slight antitumor activity against Ehrlich ascites tumor *in vivo*.[9,11] It is interesting that **3** unexpectedly could not exert differential growth inhibitory activity similar to **1** and **2** (Figure 3), suggesting that N-hydroxyamide moiety at N-16 might be critical to the activity. Although the mechanisms of actions of **1** and **2** as well as that of triornicin are still unknown, they might have a similar target. We are now studying the precise mechanism of antitumor effects of **1** and **2**.

We have recently reported that leucinostatins and atpenins showed antitumor effect against tumors of DU-145 cells and PrSCs *in vivo*.[8] These compounds were also re-discovered as modulators of tumor–stromal cell interactions by our screening program.[5,6] In this paper, we have reported new natural compounds from fungal metabolite. Although the study of the precise mechanism is going on, **1** and **2** are considered to modulate the tumor–stromal cell interactions similar to other compounds reported previously.[6–8] As we obtained a new compound with potency of antitumor effect *in vivo*, we thought that our screening concept and program would be beneficial for the innovation of new antitumor drugs.

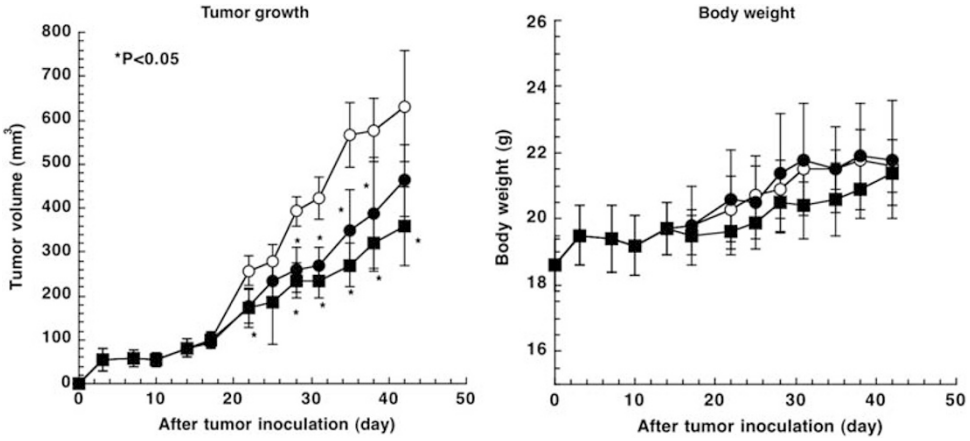

Figure 4 Effects of **1** and **2** on tumor growth of DU-145 cells *in vivo*. DU-145 cells were inoculated subcutaneously with PrSCs in female nude mice. Compound **1** (●) or **2** (■) was administered intraperitonealy at 50 mg kg^{-1} on days 17–19, 21–26, 28–33 and 35–40. The values are means ± s.d. of five mice. *$P<0.05$ versus the values with saline (○).

METHODS

Reagents

Rhodanile blue was purchased from Aldrich (Milwaukee, WI, USA). Insulin and hydrocortisone were obtained from Sigma (St Louis, MO, USA). Transferrin was obtained from Wako Pure Chemical Industries. The recombinant human basic fibroblast growth factor was purchased from Pepro Tech (London, UK).

Cells

The human prostate cancer DU-145 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), 100 units ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin at 37 °C with 5% CO_2. The human normal PrSCs were obtained from Bio Whittaker (Walkersville, MD, USA) and maintained in DMEM supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, ITH (5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin and 1.4 μM hydrocortisone) and 5 ng ml^{-1} basic fibroblast growth factor at 37 °C with 5% CO_2.

Coculture experiment

A microplate assay method for the selective measurement of epithelial tumor cells in coculture with stromal cells using rhodanile blue dye was performed as described before.[5] The PrSCs were first inoculated into 96-well plates at 5000 cells per well in 100 μl of DMEM supplemented with ITH and 0.1% fetal bovine serum in the presence of the various concentrations of test compounds. After 2 days, 10 μl of DU-145 cell suspension (5000 cells) in serum-free DMEM was inoculated onto a monolayer of PrSCs, and the cells were further cultured for 3 days. For monoculture of DU-145 cells, the assay medium alone was first incubated in the presence of test compounds for 2 days at 37 °C. Then, DU-145 cells were inoculated as described above, and cultured for further 3 days. The growth inhibitory activity was expressed as percentage of cell number compared with control cell number as 100%.

Antitumor effect *in vivo*

Female nude mice, 6 weeks old, were purchased from Charles River Breeding Laboratories (Yokohama, Japan) and maintained in a specific pathogen-free barrier facility according to our institutional guidelines. DU-145 cells (8×10^6) were trypsinized and resuspended with or without PrSCs (8×10^6) in 0.3 ml of 10% fetal bovine serum/DMEM and then combined with 0.5 ml of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA). A total of 100 μl of the cell suspension (1×10^6 cells) was injected subcutaneously in the left lateral flank of mice. Five mice were used for each experimental set. Tumor volume was estimated using the following formula: tumor volume (mm^3) = (length × width^2)/2. After the indicated times, tumors were surgically dissected.

Analytical measurement

Optical rotations were measured on a JASCO P-1030 polarimeter (JASCO, Tokyo, Japan). UV spectra were recorded on a Hitachi 228 A spectrometer (Hitachi, Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM A400 spectrometer (JEOL, Tokyo, Japan) using TMS as an internal standard. HRESI-MS spectra were measured with a JEOL JMS-T100LC spectrometer (JEOL).

Fermentation

Fungal strain, *P. melioloides* Mer-f16716, was isolated from a soil sample collected from Yakushima Island, Kagoshima prefecture, Japan. The sequence analysis of partial 28S rRNA (556 nucleotides) of this strain showed high identity with *P. melioloides* (99.3%). Thus, the strain Mer-f16716 was tentatively identified as a member of *P. melioloides*. The strain grown on an agar slant was inoculated into a 250-ml flask containing 25 ml of a seed medium consisting of 2% potato starch, 1% glucose, 2% Soypro (J-Oil Mills, Tokyo, Japan), 0.1% KH_2PO_4, 0.05% $MgSO_4$ $7H_2O$ and three glass beads, and cultured at 25 °C for 3 days on a rotary shaker at 220 r.p.m. Seed culture (1 ml) was inoculated into a 500-ml flask containing 60 ml of a culture medium consisting of 4% dextrin hydrate, 1% Bacto Peptone (BD Biosciences), 2% Pharmamedia (Traders Protein, Memphis, TN, USA), 0.5% Extract Ehlrich (Wako Pure Chemical Industries), 0.29% NaCl, 0.06% $MgSO_4$ $7H_2O$, 0.05% $MgCl_2$ $6H_2O$, 1% mineral solution (solution of 0.725% KCl, 0.097% KBr, 0.026% H_3BO_3, 0.00395% KF, 0.00008% KI, 0.001475% $CaCl_2$ $2H_2O$ and 0.0215% $SrCl_2$ $6H_2O$) (adjusted at pH 7.2 before sterilization) and cultured at 25 °C for 4 days on a rotary shaker at 220 r.p.m.

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