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ORIGINAL ARTICLE

New atpenins, NBRI23477 A and B, inhibit the growth of human prostate cancer cells

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The growth and metastasis of prostate cancer are regulated by prostate stroma through the tumor–stromal cell interactions. Small molecules that modulate the tumor–stromal cell interactions will be new anticancer drugs. In the course of our screening of the modulators, we isolated two new atpenins, NBRI23477 A (4) and B (5), from the fermentation broth of *Penicillium atramentosum* PF1420. Compounds 4 and 5 as well as atpenin A4 (1), A5 (2) and B (3) inhibited the growth of human prostate cancer DU-145 cells in the coculture with human prostate stromal cells more strongly than that of DU-145 cells alone.
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INTRODUCTION

The growth and metastasis of prostate cancer are regulated by prostate stroma.[1,2] We have reported earlier that prostate stromal cell (PrSC) promotes the growth of human prostate cancer cells through the secretion of insulin-like growth factor-I.[3,4] There is a possibility that small molecules could inhibit cancer cell growth by modulating tumor–stromal cell interactions. We developed the *in vitro* coculture system of human prostate cancer cells and PrSC, in which the growth of prostate cancer cell is increased by the coculture with PrSC.[3,5] Using the assay method, we have been searching for the modulators of the tumor–stromal cell interactions. In the course of our screening of the modulators, we isolated new atpenins, NBRI23477 A (4) and B (5), along with the known compounds, atpenin A4 (1), A5 (2) and B (3).[6,7] Here we describe the isolation, structure determination and biological activity of 4 and 5. We also report the activity of 1, 2 and 3 on our assay.

MATERIALS AND 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 (Tokyo, Japan). 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 American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), $100 \, U \, ml^{-1}$ penicillin G and $100 \, \mu g \, ml^{-1}$ streptomycin at 37 °C with 5% CO_2. The human normal PrSCs were obtained

from Bio Whittaker (Walkersville, MD, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, $100 \, U \, ml^{-1}$ penicillin G, $100 \, \mu g \, ml^{-1}$ streptomycin, ITH ($5 \, \mu g \, ml^{-1}$ insulin, $5 \, \mu g \, ml^{-1}$ transferrin and 1.4 μM hydrocortisone) and $5 \, ng \, ml^{-1}$ human 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] PrSCs were first inoculated into 96-well plates at 5000 cells per well in 100 μl of Dulbecco's modified Eagle's medium supplemented with ITH and 0.1% fetal bovine serum in the presence of various concentrations of the test compounds. After 2 days, 10 μl of DU-145 cell suspension (5000 cells) in serum-free Dulbecco's modified Eagle's medium was inoculated onto a monolayer of PrSC, 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.

Analytical measurement

Melting points were obtained on a Yanagimoto micro melting point apparatus (Yanagimoto, Kyoto, Japan). 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). ^1H- and ^{13}C-NMR spectra were measured on a JEOL JNM A400 spectrometer (JEOL, Tokyo, Japan) using TMS as an internal standard. High resolution electrospray ionization mass spectrometry (HR-ESI-MS) spectra were measured with a JEOL JMS-T100LC spectrometer (JEOL).

Fermentation of fungal strain PF1420

Penicillium atramentosum PF1420 was isolated from a soil sample collected in Iwamizawa, Hokkaido, Japan. A slant culture of *P. atramentosum* PF1420 was used to inoculate 100-ml Erlenmeyer flasks. Each contained 20 ml of a seed

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medium consisting of 2.0% soluble starch, 1.0% glucose, 0.2% soybean meal, 0.6% wheat germ, 0.5% polypeptone, 0.3% yeast extract and 0.2% $CaCO_3$ in deionized water adjusted to pH 7.2 with NaOH solution before sterilization. The flasks were incubated at 25 °C for 72 h on a rotary shaker at 220 r.p.m. Portions of 1.0 ml of this seed culture were transferred into six 500-ml Erlenmeyer flasks, each of which contained 100 ml of a seed medium. The flasks were incubated at 25 °C for 48 h on a rotary shaker at 220 r.p.m. Portions of 150 ml of this seed culture were transferred into four stainless vats, each of which contained 2.5% soybean meal and water-absorbed rice (4 kg) as solid production medium. The stainless vats were thoroughly stirred and then statically cultured at 25 °C for 14 days. After incubation, 16-kg portion of the obtained culture was extracted with 32 l of 67% aqueous acetone.

RESULTS

Isolation procedure for atpenins

The 16-kg culture broth of *P. atramentosum* PF1420 was extracted with 32 l of 67% aqueous acetone. The filtrate of the extracts was concentrated *in vacuo* to remove acetone. The aqueous solution (5 l, pH 7) was applied on an HP-20 column. After washing the column with H_2O and 50% MeOH, active ingredients were eluted with 100% MeOH. The eluate was concentrated *in vacuo,* dissolved in 600 ml H_2O and then extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to afford 9.92 g of dried material. The materials were applied on a silica gel column (450 g, Wakogel C-200, 75–150 μm; Wako, Osaka, Japan) prepared with $CHCl_3$, and eluted with $CHCl_3$ and $CHCl_3$-MeOH. The fractions eluted with $CHCl_3$-MeOH (25:1) were concentrated *in vacuo* to give 4.13 g of viscous material. The viscous material was applied on gel filtration chromatography of Sephadex LH-20 (MeOH). The fractions containing atpenins were concentrated *in vacuo* to give 2.76 g of crude material. The crude material was purified by a reversed-phase HPLC column (Inertsil ODS-3, 20×250 mm, 6.0 ml min^{-1}) with 50% CH$_3$CN to afford crude atpenins, 46.4 mg of **1**, 119.8 mg of **2**,

111.1 mg of **3** and **4**, and 14.5 mg of **5**. The crude sample of **5** was applied on a Sephadex LH-20 column (MeOH) to afford pure 8.0 mg of **5**. The crude mixture of **3** and **4** was further purified by a reversed-phase HPLC column (Inertsil ODS-3, 10×250 mm, 3.0 ml min^{-1}) with 75% CH$_3$CN and 0.1% TFA, and then the fractions containing

Table 2 The ^{13}C- and ^{1}H-NMR assignments of 4 and 5 in pyridine-d_5

	4		5	
Position	^{13}C p.p.m. (mult.)	^{1}H p.p.m. (mult., J(Hz))	^{13}C p.p.m. (mult.)	^{1}H p.p.m. (mult., J(Hz))
2[a]	162.7 (s)		162.8 (s)	
3	100.6 (s)		101.0 (s)	
4[a]	165.9 (s)		165.8 (s)	
5	125.0 (s)		124.9 (s)	
5-OCH$_3$	60.7 (q)	3.84 (s)	60.7 (q)	3.83 (s)
6	160.2 (s)		160.1 (s)	
6-OCH$_3$	54.4 (q)	3.90 (s)	54.4 (q)	3.89 (s)
1'	210.6 (s)		211.3 (s)	
2'	42.2 (d)	4.36 (m)	42.2 (d)	4.42 (m)
2'-CH$_3$	16.4 (q)	1.35 (d, 6.4)	18.1 (q)	1.33 (d, 6.8)
3'	36.8 (t)	2.06–2.13 (m)	41.0 (t)	1.42 (m)
				2.10 (m)
4'	48.0 (d)	2.40 (m)	36.6 (d)	2.33 (m)
4'-CH$_3$	15.8 (q)	1.34 (d, 6.4)	20.5 (q)	1.07 (d, 6.8)
5'	97.9 (s)		145.2 (d)	5.79 (ddd, 7.6, 10.4, 17.2)
6'	35.7 (q)	2.14 (s)	114.4 (t)	4.95 (dd, 1.2, 10.4)
				4.99 (dd, 1.2, 17.2)

[a]Assignments may be interchanged.
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.

Table 1 Physicochemical properties of 4 and 5

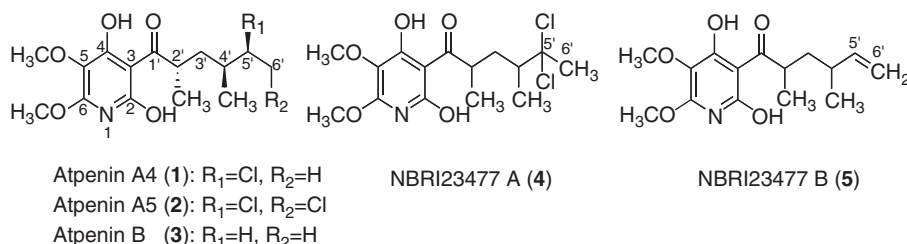|	4	5
Appearance	White powder	Hygroscopic solid
Melting point	130–132 °C	—
Molecular formula	C$_{15}$H$_{21}$NO$_5$Cl$_2$	C$_{15}$H$_{21}$NO$_5$
HR-ESI-MS (m/z)		
Found	364.0713 (M-H)$^-$	294.1361 (M-H)$^-$
Calcd.	364.0719 for C$_{15}$H$_{20}$NO$_5$Cl$_2$	294.1342 for C$_{15}$H$_{20}$NO$_5$
UVλ_{max} (nm) (MeOH)	236, 273, 329	235, 271, 331
$[\alpha]_D^{20}$ (c 0.2, EtOH)	−37°	−39°
IRυ_{max} (KBr) (cm^{-1})	2935, 1651, 1597, 1448, 1325, 1198, 1163, 995	2931, 1651, 1597, 1452, 1325, 1196, 1163, 995
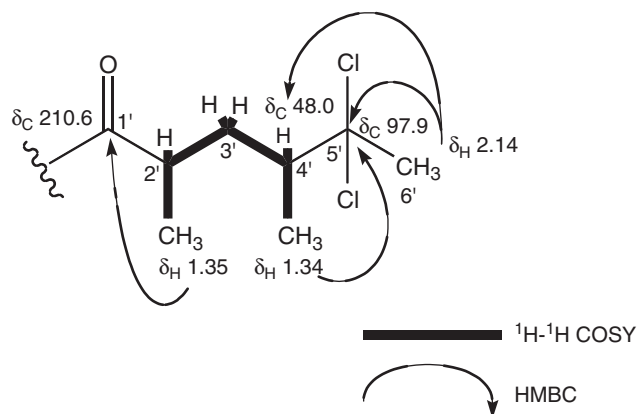
Figure 2 Partial structure of **4**.

Figure 1 Structures of **1**–**5**.

Atpenin A4 (**1**): R$_1$=Cl, R$_2$=H
Atpenin A5 (**2**): R$_1$=Cl, R$_2$=Cl
Atpenin B   (**3**): R$_1$=H, R$_2$=H

NBRI23477 A (**4**)   NBRI23477 B (**5**)

3 and 4 were applied on a Sephadex LH-20 column (MeOH) to afford pure 72.4 mg of 3 and 4.5 mg of 4, respectively. The crude mixture of 1 and 2 was further purified by a reversed-phase HPLC column (Inertsil ODS-3, 10×250 mm, 3.0 ml min^{-1}) with 60% CH$_3$CN and 0.1% TFA, and then the fractions containing 1 and 2 were applied on a Sephadex LH-20 column (MeOH) to afford pure 15.5 mg of 1 and 26.1 mg of 2, respectively. Separation by analytical HPLC (Inertsil ODS-3, 3 μm, 4.6×150 mm, 1.0 ml min^{-1}) with 75% CH$_3$CN and 0.1% TFA gave the following retention times (in minutes): 4.38 (1), 4.40 (2), 5.29 (3), 4.95 (4) and 4.28 (5).

Physicochemical properties

The physicochemical properties of 4 and 5 are summarized in Table 1. They are soluble in MeOH and DMSO. The molecular formulae of 4 and 5 were determined to be C$_{15}$H$_{21}$NO$_5$Cl$_2$ and C$_{15}$H$_{21}$NO$_5$, respectively, by HR-ESI-MS. The general features of their UV and NMR spectra resembled each other, indicating structural similarities of these compounds. Compounds 1–3 were identified by NMR spectra as atpenin A4 (1), A5 (2) and B (3), respectively (Figure 1).[6,7]

Structure determination of NBRI23477 A (4)

The ^{1}H- and ^{13}C-NMR data of 4 (Table 2) were similar to those of 2.[8] However, the signals of 5′-methine and 6′-methylene of 2 were not observed in the ^{13}C-NMR spectrum of 4, but a quaternary carbon (δ 97.9) and a methyl carbon (δ 35.7) appeared in 4. The partial structure of 4 was established by analyses of ^{1}H-^{1}H correlation spectroscopy (COSY) and heteronuclear multiple bond connectivity (HMBC) spectra (Figure 2). ^{1}H-^{1}H COSY spectrum revealed the following fragment: -CH(CH$_3$)-CH$_2$-CH(CH$_3$)-. In the HMBC spectrum, singlet methyl protons (6′-H, δ 2.14) correlated to a

quaternary carbon (C-5′, δ 97.9) and a methine carbon (C-4′, δ 48.0). Methyl protons (δ 1.35) connecting to C-2′ methine correlated to a carbonyl carbon (C-1′, δ 210.6). Therefore, the total structure of 4 was found to be a new family of atpenin (Figure 1).

Structure determination of NBRI23477 B (5)

The ^{13}C- and ^{1}H-NMR data of 5 (Table 2) were similar to those of 3.[6,7] However, the signals of 5′-methylene and 6′-methyl of 3 were not observed in the ^{13}C-NMR spectrum of 5. On the other hand, an olefine carbon (δ 145.2) and a terminal olefine carbon (δ 114.4) were observed in 5. The partial structure of 5 was established by analyses of the ^{1}H-^{1}H COSY and HMBC spectra (Figure 3). ^{1}H-^{1}H COSY
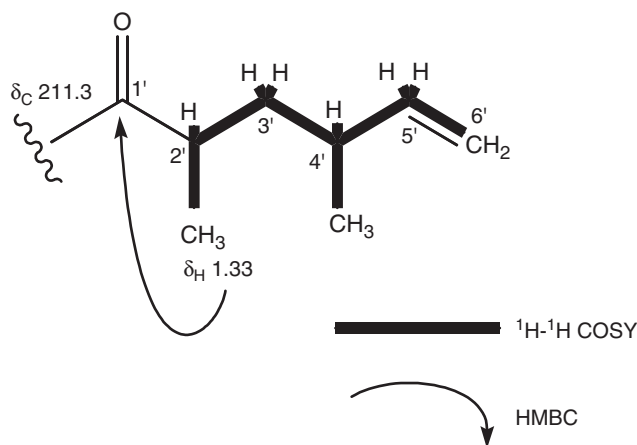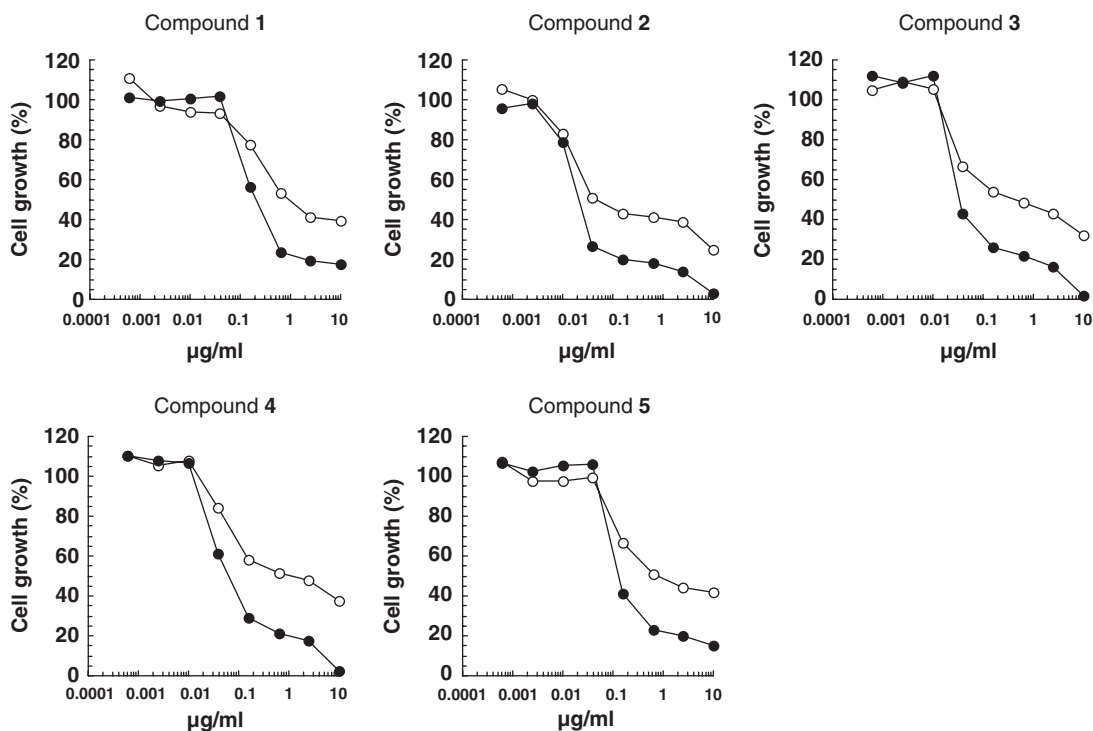
Figure 3 Partial structure of 5.

Figure 4 Effects of 1–5 on coculture of DU-145 cells and PrSC. The growth of DU-145 cells cocultured with PrSC (●) or that of DU-145 cells alone (○) in the presence of the indicated concentrations of 1–5 was determined using rhodanile blue method. Values are means of duplicate determinations. Each s.e. is less than 10%.

spectrum revealed the following fragment: -CH(CH$_3$)-CH$_2$-CH(CH$_3$)-CH=CH$_2$. In the HMBC spectrum, a methyl proton (δ 1.33) connecting to C-2′ methine correlated to a carbonyl carbon (C-1′, δ 211.3). Therefore, the total structure of **5** was found to be a new family of atpenin (Figure 1).

Biological activities

The effects of **1–5** on coculture of human prostate cancer DU-145 cells with PrSC were determined using rhodanile blue staining method.[5] In the coculture, the growth of DU-145 cells is increased by PrSC.[3,5] As shown in Figure 4, all compounds showed selective growth inhibitory activities and inhibited the growth of DU-145 cells in coculture with PrSC more strongly than that of DU-145 cells alone. The IC$_{50}$ values of **1–5** against the growth of DU-145 cells in coculture were 0.21, 0.021, 0.034, 0.064 and 0.13 μg ml^{-1}, respectively, whereas those of **1–5** against the growth of DU-145 cells alone were 0.85, 0.048, 0.54, 0.95 and 0.71 μg ml^{-1}, respectively. All compounds did not show apparent cytotoxicity against stromal cells under microscopic observation (data not shown).

DISCUSSION

In this study, we have also obtained three structurally related compounds in addition to **1–5**. The HR-ESI-MS spectra revealed that the molecular formulae of these compounds were C$_{15}$H$_{22}$NO$_6$Cl, C$_{15}$H$_{23}$NO$_6$ and C$_{15}$H$_{20}$NO$_5$Cl$_3$, respectively. Among them, a compound having the molecular formula of C$_{15}$H$_{20}$NO$_5$Cl$_3$ may be identical to reported WF-16775 A2,[8] but we could not elucidate the structures of additional three compounds further due to their trace amounts. Ōmura *et al.*[6] reported that there were atpenins A1, A2 and A3 along with A4, A5 and B, but they did not obtain A1, A2 and A3 in pure form and did not show any structural information. We cannot exclude the possibility that **4** and **5** would be identical to one of them. However, we have actually presented here two new structures of atpenins.

Atpenins A4, A5 and B were originally isolated as antifungal antibiotics.[6,7] Thereafter, atpenin B was found to decrease the cellular adenosine 5′-triphosphate.[9] Furthermore, it is reported that atpenins specifically inhibit mitochondrial complex II (succinate–ubiquinone oxidoreductase).[10] Mitochondria is now considered as a rational target for cancer therapy.[11] Although there is a possibility that atpenins modulate tumor–stromal cell interactions by inhibiting mitochondrial functions, the elucidation of the precise mechanism of action needs to be studied further. We are now studying the effects of atpenins on tumor growth *in vivo* using mouse xenograft models.

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