Cytotoxic Activity of Abietane-Type Diterpenes Isolated From *Taxodium distichum* Against Cancer Cells Adapted to Nutrient-Starved Conditions

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Abstract

The mechanisms of cancer cell adaptation to tumor microenvironmental conditions, such as hypoxia and nutrient starvation, are currently receiving much attention as possible therapeutic targets. In an attempt to identify selectively cytotoxic substances against cancer cells adapted to nutrient starvation, 4 abietane-type diterpenes, sugiol (1), 6-α-hydroxysugiol (2), cryptojaponol (3), and 6-hydroxy-5,6-dehydrosugiol (4), were isolated from the bark of *Taxodium distichum* L. Rich var. *distichum* (bald cypress). Compounds 1, 2, and 4 showed potent cytotoxic activity against PANC-1 cells adapted to nutrient-starved conditions with half-maximal effective concentration (EC₅₀) values of 6.4-9.2 µM, whereas the EC₅₀ values of these compounds against PANC-1 cells under general culture conditions were more than 100 µM. Alternatively, compound 3, which we report for the first time in the genus *Taxodium*, showed moderate cytotoxicity against PANC-1 cells under nutrient-starved conditions with an EC₅₀ of 37.9 µM. The selective index (S.I.), which compared the activity under nutrient-starved conditions with that under general culture conditions, was low (7.9). Further investigation revealed that the selective cytotoxic activity of compound 2 might be affecting the mitochondria.

Keywords

terpenoids, bioactivity, *Taxodium distichum*, cancer, microenvironment, nutrient starvation

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Tumors contain hypoxic and nutrient-starved regions due to abnormal cell proliferation coupled with defective structural and functional vascularization.¹ Cancer cells that have adapted to this tumor environment are assumed to stimulate the pathological progression of cancer by promoting tumor growth, angiogenesis, metastasis, and drug resistance.² In addition, the hypoxic and nutrient-starved environment in a tumor is unlike that found in normal tissues. Therefore, compounds that selectively inhibit the growth of cancer cells under these conditions have potential as cancer cell-selective drugs.

Currently, some natural products have been reported to exert selective inhibition against cancer cells that have adapted to hypoxic or nutrient-starved conditions; for example, furospinosulin-1 (furanseseterterpenes) and dictyoceratin A and C (sesquiterpene phenols) isolated from the marine sponge, *Dactylospongia elegans*, selectively inhibit the growth of hypoxia-adapted human prostate cancer DU145 cells.³,⁴ Kigamicin D (polycyclic xanthone) and ancistrolikokine E3 (naphthylisoquinoline alkaloid) isolated from the culture extract of *Amycolatopsis* sp. and the plant *Ancistrocladus likoko*, respectively, showed cytotoxic activity against the nutrient-starved PANC-1 cells.⁵,⁶ Arctigenin (lignin) originally isolated from plant *Arctium lappa*, has been proven to have nutrient starvation selective cytotoxicity against cancer cells and has entered the clinical trial.⁷ In addition, we recently demonstrated that DC1149B (epidithiodiketopiperazine), N-methylniphatin A (novel 3-alkyl pyridine alkaloid), and biakamides (unique novel polyketide) from the marine fungus, *Trichoderma lixi*, and the marine sponges, *Xestospongia* sp. and *Petrosaspongia* sp., respectively, display cytotoxic activity against human pancreatic carcinoma PANC-1 cells adapted to glucose deficiency.⁸,⁹,¹¹

*Taxodium distichum* L. Rich var. *distichum* (bald cypress) of the family Cupressaceae is native to the eastern United States as well as South America, Europe, and East Asia.¹²-¹⁴ The trees'
wood is of high quality and has been used traditionally in the manufacture of vats, boats, boundaries, flooring, and houses. In Egypt, T. distichum trees are cultivated for the same economic purposes and the production of volatile oil from their fruits. Taxodium leaves and cones, which are mainly rich in essential oils, are used for the treatment of skin, gastrointestinal and respiratory diseases, inflammation, and infections. Burnt bark pieces are applied onto sores, burns, and ulcerations of the skin. In this study, we rediscovered 4 abietane-type diterpenes, sugiol (1), 6-α-hydroxysugiol (2), cryptojaponol (3), and 6-hydroxy-5,6-dehydrosugiol (4), from the bark of T. distichum L. Rich var. distichum (Bald cypress) as selective cytotoxic compounds against cancer cells adapted to glucose-starved conditions. Moreover, their mode of action was proposed.

The 10% EtOAc in n-hexane extract prepared from the bark of T. distichum showed selective cytotoxicity against human pancreatic carcinoma PANC-1 cells adapted to glucose starvation. Bioassay-guided separation of the 10% EtOAc in n-hexane extract led to the isolation of sugiol (1), 6-α-hydroxysugiol (2), cryptojaponol (3), and 6-hydroxy-5,6-dehydrosugiol (4), from the bark of T. distichum L. Rich var. distichum (Bald cypress) as selective cytotoxic compounds against cancer cells adapted to glucose-starved conditions. Moreover, their mode of action was proposed.

Cytotoxicity assays using isolated compounds 1-4 were then performed on PANC-1 cells in both glucose-starved conditions and general culture conditions using antimycin A as a reference compound. As shown in Table 1, sugiol (1), 6-α-hydroxysugiol (2), and 6-hydroxy-5,6-dehydrosugiol (4) showed potent cytotoxic activity against PANC-1 cells adapted to glucose-starved conditions with half-maximal effective concentration (EC_{50}) values in the range of 6.4-9.2 µM, whereas the EC_{50} values of these compounds under general conditions were more than 100 µM. The selective index (SI), which compared the activity under glucose-starved conditions with that under general culture conditions, was determined to be 16.9-27. Compound 1 showed the most potent activity and selectivity; in contrast, cryptojaponol (3) showed moderate cytotoxic activity against the PANC-1 cells under glucose-starved conditions (EC_{50} = 37.9 µM) and exhibited lower selectivity (SI = 7.9) than compounds 1, 2, and 4. These results indicated that the hydroxyl moiety at R^1 increased the cytotoxicity against PANC-1 cells under general culture conditions and decreased the SI. The olefin at the C-5 position increased the cytotoxicity under both glucose-starved and general culture conditions and resulted in decreased SI. In addition, it was suggested that the presence of a substituent on R^3 reduced activity and selectivity; moreover, it may also confer the selective cytotoxicity that R^3 has with a hydroxyl group. We further evaluated the cytotoxic activity of compounds 1-4 against normal human dermal fibroblast (NHDF) cells. As a result, the cytotoxicity of all compounds against the NHDF cells was more than 1.5 times lower than that against PANC-1 cells cultured under glucose-starved conditions. In particular, compound 1 showed 7.6-fold higher cancer cell selectivity than the other compounds.

It has been reported that the inhibitors of the mitochondrial electron transport chain, such as antimycin A, show selective cytotoxicity against PANC-1 cells adapted to glucose-starved conditions. Therefore, we investigated the effect of 6-α-hydroxysugiol (2) on the mitochondria. As shown in Figure 2, antimycin A decreased the adenosine triphosphate (ATP) levels in the PANC-1 cells cultured in media containing glucose and

**Figure 1.** Chemical structures of compounds 1-4.

**Table 1.** Cytotoxic Activity of Compounds 1-4 on the PANC-1 and NHDF Cells.

| Compounds | PANC-1 | NHDF |
|-----------|--------|------|
|           | EC_{50} (µM) | SI^{a} | EC_{50} (µM) | SI^{b} |
| 1         | 9.0 | 243 | 27.0 | 68.0 | 7.6 |
| 2         | 9.2 | 155 | 16.9 | 18.4 | 2.0 |
| 3         | 37.9 | 303 | 7.9 | 64.4 | 1.7 |
| 4         | 6.4 | 114 | 18.3 | 9.8 | 1.5 |

Abbreviations: SI, selective index; NHDF, normal human dermal fibroblast; EC_{50}, half-maximal effective concentration.

^{a}SI = IC_{50} (Glc+)/IC_{50} (Glc−).

^{b}SI = IC_{50} (NHDF)/IC_{50} (PANC-1, Glc−).

^{c}Glucose-starved conditions

^{d}General culture conditions
galactose as the carbon source. Similarly, compound 2 reduced the intracellular ATP levels in a dose-dependent manner without cytotoxicity. This suggested that the selective cytotoxic effect of compound 2 against PANC-1 cells adapted to glucose-starved conditions might be due to its effect on the mitochondria.

To date, it has been reported that some abietane-type diterpenes, 6-α-hydroxysugiol (2), taxodione, ferruginol, and 14-deoxcoleon U, showed cytotoxic activity against human colon cancer cell HT-29. Additionally, sugiol (1) and cryptoponol (3) exhibited cytotoxic activity against human pancreatic cancer cells (MIA Paca-2) in general culture conditions. Furthermore, it has been reported that compound 1 inhibited topoisomerase I as a mode of action. Alternatively, compounds 1-4 have lower SI (7.9-28) than antimycin A (SI = 960 000), which is an inhibitor of the mitochondrial electron transport chain III (complex III), as shown in Table 1. Based on these observations, the isolated compounds showed selective cytotoxicity against PANC-1 cells cultured under glucose-starved conditions. Furthermore, these compounds inhibited mitochondrial function and showed cytotoxicity against PANC-1 cells cultured under general culture conditions by targeting other molecules, such as topoisomerase I.

To our knowledge, this is the first study to report isolated abietane-type diterpenes, particularly compound 1, possessing selective cytotoxic activity against PANC-1 cells adapted to glucose starvation, which simulates a typical condition of the tumor microenvironment. Although a detailed study of the structure–activity relationships using synthetic analogs is necessary to improve the selectivity, our findings suggest that these compounds isolated from T. distichum have potential as active ingredients in anticancer drugs.

**Experimental**

**General**

NMR spectra, referenced to tetramethylsilane or residual solvent peaks, were measured on a Varian Inova 600 NMR system (6H: 600 MHz, 13C: 150 MHz). ESI-TOF-MS was recorded on
a Q-Tof Ultima API mass spectrometer (Waters Co., MA, USA). Infrared (IR) spectra and specific rotations were obtained on a JASCO FT/IR-5300 (potassium bromide pellets). Ultraviolet (UV) spectra were obtained on a UV-2450 spectrophotometer (SHIMADZU, Kyoto, Japan). Column chromatography was performed on Silica gel BW-200 (Fuji Silysia Aichi, Japan) and COSMOSIL 5C18-MS-II (Nacalai tesque, Kyoto, Japan). High-performance liquid chromatography (HPLC) was performed using a Hitachi L-6000 pump equipped with a Hitachi L-4000H UV detector (Hitachi High-Tech Science Corporation, Tokyo, Japan). Thin-layer chromatography (TLC) analysis was carried out using precoated TLC plates (Merck, 60 F254). Spots on the TLC plates were detected by spraying acidic p-anisaldehyde solution (p-anisaldehyde: 25 mL, c-H2SO4: 25 mL, AcOH: 5 mL, and EtOH: 425 mL) or phosphomolybdic acid solution (phosphomolydbic acid: 25 g and EtOH: 500 mL) with subsequent heating. The following reagents and materials were used for cell culture and bioassays: Dulbecco’s Modified Eagle’s medium (DMEM) and WST-8 colorimetric reagent were purchased from Nacalai Tesque, Inc. (Kyoto, Japan); fetal bovine serum (FBS) and dialyzed FBS colorimetric reagent were purchased from Nacalai Tesque, Inc. (Kyoto, Japan); fetal bovine serum (FBS) and dialyzed FBS (7 mg), (1.9 mg), and (15 mg), and 4 (22 mg). Compounds 1-4 were identified by ESI-TOF-MS and NMR analyses as well as comparison with authentic spectral data.20,21

**Cytotoxicity of the Compounds on the PANC-1 Cells**

Human pancreatic carcinoma PANC-1 cells were maintained in DMEM supplemented with heat-inactivated 10% FBS and kanamycin (50 µg/mL) in a humidified atmosphere of 5% CO2 at 37°C. Nutrient-starved PANC-1 cells were cultured in glucose-deficient medium (basal medium [25 mM HEPES buffer, pH 7.4, supplemented with 6.4 g/L NaCl, 700 mg/L NaHCO3, 400 mg/L KCl, 265 mg/L CaCl2·2H2O, 200 mg/L MgSO4·7H2O, 125 mg/L NaH2PO4·12H2O, 0.1 mg/L Fe(NO3)3·9H2O, 15 mg/L phenol red, 10 mL/L MEM vitamin solution [×100] [GIBCO, Carlsbad, CA, USA], 200 mM/L L-glutamine solution [GIBCO, Carlsbad, CA, USA], and 25 mg/L kanamycin] containing 10% dialyzed FBS). General culture medium (basal medium supplemented with 10% FBS and 2.0 g/L glucose [final 25 mM]) was also used in the bioassay for comparison. The bioassay was carried out according to a previously described method.8 Briefly, PANC-1 cells (1 × 104 cells/100 µL in 96-well culture plates) were preincubated with DMEM supplemented with 10% FBS for 24 hours. The medium was then replaced with either normal glucose-containing medium or glucose-deficient medium to induce cell adaptation to nutrient starvation. After 12 hours of incubation, serially diluted samples of compounds 1-4 were added, and the cells were incubated for an additional 12 hours in a humidified atmosphere of 5% CO2 at 37°C. Cell proliferation was evaluated using the WST-8 colorimetric reagent. The EC50 value was determined by linear interpolation from the generated growth inhibition curve. We assessed the selectivity of antiproliferative activity (SI) based on the difference in the EC50 values obtained in the presence and absence of glucose.

**Cytotoxicity of the Compounds on the NHDF Cells**

NHDF cells were purchased from Kurabo Ind. Ltd. (Tokyo, Japan) and grown in fibroblast growth media with growth supplements (Kurabo Ind. Ltd., Tokyo, Japan). A suspension of NHDF cells in the culture medium was plated into each well of 96-well plates (5 × 104 cells/well/100 µL) and incubated for 24 hours in a humidified atmosphere of 5% CO2 at 37°C. After 24 hours, serially diluted samples of compounds 1-4 were added; then, the plates were incubated for an additional 48 hours in a humidified atmosphere of 5% CO2 at 37°C. The cytotoxicity was detected by WST-8 colorimetric reagent. The EC50 value was determined by linear interpolation from the growth inhibition curve. We assessed the selectivity of cytotoxic activity from the differences of EC50 values against the NHDF and PANC-1 cells cultured under glucose-starved conditions.
Effect of Compound 2 on the Mitochondrial Function

The toxicity of 6-z-hydroxysugiol (2) on the mitochondria was assessed using the Mitochondrial ToxGlo Assay (Promega, Madison, WI, USA) as per the manufacturer’s instructions. Briefly, PANC-1 cells (1 × 10⁴ cells/100 µL in 96-well culture plate) cultured in the general culture medium or the glucose-deficient medium supplemented with 10 mM galactose were treated with the indicated concentrations of compound 2 for 90 minutes. Thereafter, bis-AAF-R110 substrate, which is used to detect cell membrane damage, was added to the plate, which was incubated for 30 minutes in a humidified atmosphere of 5% CO₂ at 37°C. After 30 minutes, the cytotoxicity based on was incubated for 30 minutes in a humidified atmosphere of cell membrane damage was measured by intracellular fluorescence intensity at Ex 485 nm and Em 530 nm. Subsequently, ATP Detection Reagent was added following cell lysis, and luminescence intensity was measured using an Infinite M1000 microplate reader (Tecan Group Ltd., Mannendorf, Switzerland) to evaluate intracellular ATP levels.

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Declaration of Conflicting Interests

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