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ORIGINAL PAPER

Marine spongean polybrominated diphenyl ethers, selective growth inhibitors against the cancer cells adapted to glucose starvation, inhibits mitochondrial complex II

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Abstract In the course of search for selective growth inhibitors against the cancer cells adapted to nutrient starvation, two polybrominated diphenyl ethers, 3,4,5-tribromo-2-(2′,4′-dibromophenoxy)-phenol (**1**) and 3,5-dibromo-2-(2′,4′-dibromophenoxy)-phenol (**2**) were isolated from an Indonesian marine sponge of *Dysidea* sp. Compounds **1** and **2** showed the anti-proliferative activity against PANC-1 cells under glucose-starved conditions with IC_{50} values of 2.1 and 3.8 μM, respectively, whereas no growth inhibition was observed up to 30 μM in the general culture conditions. The further mechanistic analysis indicated that compound **1** might act mainly by inhibiting complex II in the mitochondrial electron transport chain.

Keywords Polybrominated diphenyl ether · Marine sponge · Cancer · Glucose starvation · Mitochondrial electron transport chain

Introduction

Tumor contains hypoxic and nutrient-starved regions due to the abnormal cell proliferation coupled with the defective structural and functional formation of vasculature [1]. In addition, the 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, 3]. Therefore, the compounds that selectively inhibit the growth of cancer cells under these conditions should have potential as anticancer drugs. Currently, some natural products have been reported to exhibit the growth inhibitory activities against the cancer cells that have adapted to the hypoxic or nutrient-starved conditions. For example, furospinosulin-1 (furanosesterterpene) and dictyoceratins-A and -C (sesquiterpene phenols) isolated from the marine sponge of *Dactylospongia elegans* were clarified to exhibit the selective growth inhibitory activity against the hypoxia-adapted human prostate cancer DU145 cells [4, 5]. Kigamicin D (polycyclic xanthone) isolated from the culture broth of an *Amycolatopsis* sp. showed the preferential cytotoxic activity against the human pancreatic carcinoma PANC-1 cells under the nutrient-starved conditions compared with those under the general culture conditions [6]. In addition, we recently isolated an 3-alkyl pyridine alkaloid named *N*-methylniphatyne A, which showed the anti-proliferative activity against the PANC-1 cells adapted to the glucose-deficient conditions, from a marine sponge of *Xestospongia* sp. [7]. In our continuous search for inhibitors against the cancer cells adapted to nutrient starvation, two polybrominated diphenyl ethers, 3,4,5-tribromo-2-(2′,4′-dibromophenoxy)-phenol (**1**) and 3,5-dibromo-2-(2′,4′-dibromophenoxy)-phenol (**2**) were isolated from an Indonesian marine sponge of *Dysidea* sp. In this paper, the

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anti-proliferative activity and mode of action of compounds **1** and **2** are presented.

Materials and methods

Materials

Dulbecco's Modified Eagle's medium (DMEM), WST-8 colorimetric reagent, and KCN were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) and Dialyzed FBS were purchased from Equitech-Bio Inc. (Kerrville, TX, USA) and Thermo Fisher Scientific Inc. (Waltham, MA, USA), respectively. Anti-Akt, Anti-phosphorylated Akt, anti-GRP78, and anti-β-tubulin antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-linked anti-rabbit IgG antibody (GE Healthcare Life Sciences, Buckinghamshire, UK) was used as secondary antibody. Mito Check Complex Activity Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) was used to evaluate the effect of compound **1** on the mitochondrial complex I–V. Rotenone, thenoyltrifluoroacetone (TTFA), antimycin A, and oligomycin mixture were obtained from Tokyo Chemical Industry Co., LTD. (Tokyo, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), LKT Laboratories, Inc. (St. Paul, MN, USA), and Cayman Chemical (Ann Arbor, MI, USA), respectively. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Kishida Chemical Co., Ltd. (Osaka, Japan).

Isolation of compounds 1 and 2

3,4,5-Tribromo-2-(2′,4′-dibromophenoxy)-phenol (**1**) and 3,5-dibromo-2-(2′,4′-dibromophenoxy)-phenol (**2**) were isolated from a marine sponge *Dysidea* sp. collected at Maumere, Indonesia (Fig. 1). Briefly, the MeOH extract [10 g, IC_{50} (Glucose Deficient Medium) = 5.7 μg/mL, IC_{50} (General Glucose Medium) = >30 μg/mL] was partitioned into a water-EtOAc mixture (1:1). The active EtOAc soluble portion [3 g, IC_{50} (Glucose Deficient Medium) = 1.6 μg/mL, IC_{50} (General Glucose Medium) = >10 μg/mL] was further fractionated by SiO_2 gel column chromatography [$CHCl_3$-MeOH-H_2O (lower phase)] to give eleven fractions (Fr. 1–Fr. 11). The active Fr. 2 [460 mg, IC_{50} (Glucose Deficient Medium) = 0.18 μg/mL, IC_{50} (General Glucose Medium) = >10 μg/mL] was then separated by HPLC [COSMOSIL $5C_{18}$-AR-II (Nacalai tesque, Kyoto, Japan); eluted with MeOH-H_2O = 8:2] to isolate compounds **1** (126 mg) and **2** (178 mg). Both compounds were identified to be 3,4,5-tribromo-2-(2′,4′-dibromophenoxy)-phenol (**1**) and 3,5-dibromo-2-(2′,4′-dibromophenoxy)-phenol (**2**) by the EI-MS and 2D-NMR analyses [8, 9].

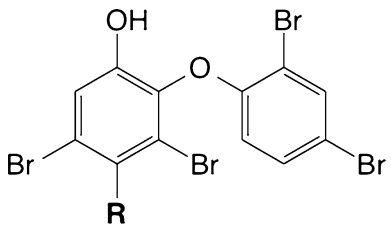

Fig. 1 Chemical structures of 3,4,5-tribromo-2-(2′,4′-dibromophenoxy)-phenol (**1**) and 3,5-dibromo-2-(2′,4′-dibromophenoxy)-phenol (**2**)

Cell culture and bioassay

Human pancreatic carcinoma PANC-1 cells were maintained in the DMEM supplemented with heat-inactivated 10 % FBS and kanamycin (50 μg/mL) in a humidified atmosphere of 5 % CO_2 at 37 °C. The nutrient-starved PANC-1 cells were cultured in the Glucose Deficient Medium [Basal Medium (25 mM HEPES buffer (pH 7.4) supplemented with 6.4 g/L NaCl, 700 mg/L $NaHCO_3$, 400 mg/L KCl, 265 mg/L $CaCl_2{\cdot}2H_2O$, 200 mg/L $MgSO_4{\cdot}7H_2O$, 125 mg/L NaH_2PO_4, 0.1 mg/L $Fe(NO_3){\cdot}9H_2O$, 15 mg/L Phenol red, 10 mL/L MEM vitamin solution (X100) (GIBCO, Carlsbad, CA), 200 mmol/L L-glutamine solution (GIBCO, Carlsbad, CA), 25 mg/L kanamycin) containing 10 % dialyzed FBS]. The General Glucose 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 the method described previously [7]. Briefly, PANC-1 cells (1×10^4 cells/100 μL in 96 well plastic plate) were pre-incubated in the DMEM supplement with 10 % FBS for 24 h. The medium was then replaced with either the General Glucose Medium or Glucose Deficient Medium to induce cells adaption to the nutrient starvation. After 12 h incubation, the serially diluted samples were added, and the cells were incubated for an additional 12 h in a humidified atmosphere of 5 % CO_2 at 37 °C. The cell proliferation was detected by using the WST-8 colorimetric reagent. The IC_{50} value was determined by linear interpolation from the growth inhibition curve. We assessed the selectivity of the anti-proliferative activity (Selective Index, SI) based on the difference in the IC_{50} values of the General Glucose and Glucose Deficient Media.

Western blotting analysis

PANC-1 cells (5×10^5 cells/2 mL in 6 well plastic plate) were pre-incubated in the DMEM supplement with 10 %

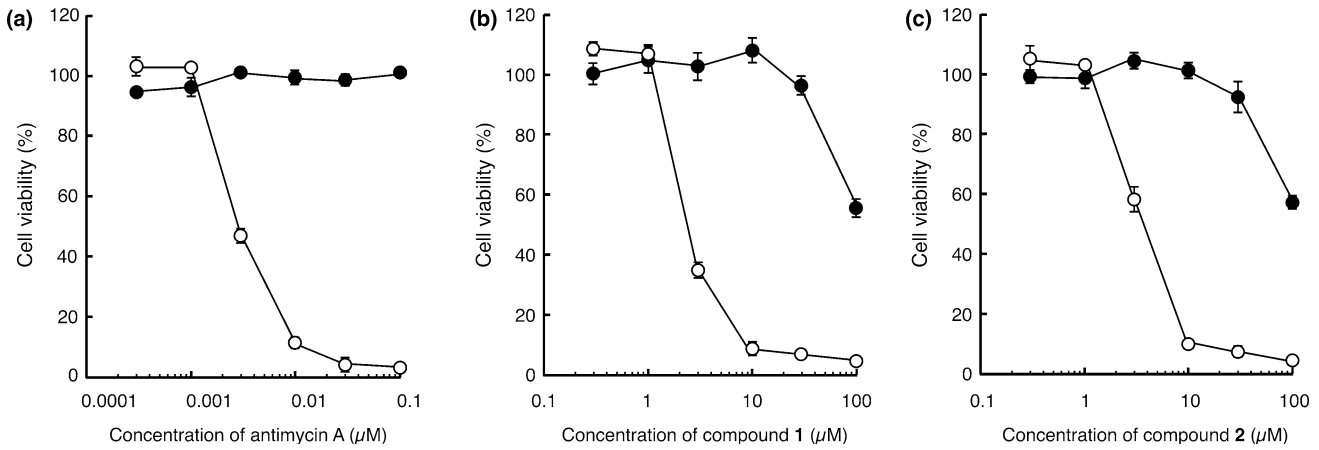

Fig. 2 Anti-proliferative activity of compounds **1** and **2** against the PANC-1 cells cultured in the Glucose Deficient and General Glucose Medium. PANC-1 cells (1×10^4 cells) were treated with indicated concentrations of **a** antimycin A (positive control), **b** compounds **1** or **c 2** in the Glucose Deficient Medium (*open circle*) or General Glucose Medium (*closed circle*) for 12 h. Cell viability was calculated by using WST-8 colorimetric reagent

FBS for 24 h. The medium was then replaced with either General Glucose or Glucose Deficient Medium. After 12 h incubation, compound **1** (10 μM) or antimycin A (3.0 nM) as a positive control was added, and the cells were incubated for an additional 8 h (for the compound **1**-treated cells) or 4 h (for the antimycin A-treated cells) in a humidified atmosphere of 5 % CO_2 at 37 °C. These experimental conditions were intended to demonstrate the 70 % viability against the PANC-1 cells cultured in the Glucose Deficient Medium. Then, the cells were rinsed with ice-cold PBS and lysed in the lysis buffer (10 mM Tris–HCl (pH 7.5) containing 135 mM NaCl, 200 mM EDTA, 1 mM NaF, 1 % Triton X-100, 1 % protease inhibitor cocktail, and 1 % phosphatase inhibitor cocktail). The cell lysate was subjected to SDS–PAGE and transferred onto PVDF membranes (GE Healthcare Life Sciences Buckinghamshire, UK). The membranes were then incubated with appropriate primary antibodies and HRP-conjugated secondary antibodies, and the immunopositive bands were visualized using an ECL kit (GE Healthcare Life Sciences). The luminescent signals were analyzed using an ImageQuant LAS4010 Scanner (GE Healthcare Life Sciences).

Result and discussion

Anti-proliferative activity of compounds 1 and 2 against the PANC-1 cells cultured under both glucose-deficient conditions and general culture conditions

The bioassay-guided separation from the active MeOH-extract of the marine sponge *Dysidea* sp. let us to isolate 3,4,5-tribromo-2-(2′,4′-dibromophenoxy)-phenol (**1**) and 3,5-dibromo-2-(2′,4′-dibromophenoxy)-phenol (**2**) (Fig. 1). We then evaluated the anti-proliferative activity of compounds **1** and **2** against the PANC-1 cells cultured under both glucose-starved and general culture conditions. We used antimycin A as a positive control, which inhibits the growth of PANC-1 cells adapted to the glucose-starved conditions through the inhibition of the mitochondrial electron transport chain [10].

In our assay system, antimycin A showed preferential anti-proliferative activity against the PANC-1 cells adapted to glucose-starved conditions with IC_{50} value of 3.0 nM. The SI value was estimated to be more than 33. Compounds **1** and **2** also showed anti-proliferative activity against the PANC-1 cells adapted to glucose starvation by cultivating in the Glucose Deficient Medium, with IC_{50} value of 2.1 and 3.8 μM, respectively, whereas both compounds did not show anti-proliferative activity at concentrations up to 30 μM in the General Glucose Medium. The SI values of compounds **1** and **2** were estimated to be more than 47 and 26, respectively (Fig. 2; Table 1). These data indicated that compounds **1** and **2** were selective growth inhibitors against the PANC-1 cells adapted to glucose starvation.

Until now, a number of polybrominated diphenyl ethers have been isolated from the marine sponges of the genus *Dysidea* [8, 11–13]. Interestingly, Unson et al. [14] reported that the polybrominated diphenyl ethers might be produced by cyanobacteria associated with marine sponge. Moreover, this class of compounds has been found to exhibit a variety of bioactivities such as anti-bacterial [13, 15, 16], anti-fungal [17], anti-viral [18, 19], as well as inhibition of microtubule assembly [20], Tie2 kinase [21], 15-lipoxygenase, inosine monophosphate dehydrogenase,

Table 1 Anti-proliferative activity of compounds **1** and **2** against PANC-1 cells under the glucose-deficient and general culture conditions

	IC_{50} (μM)		
	Glucose (−)[a]	Glucose (+)[b]	SI[c]
Compound **1**	2.1	>100	>47
Compound **2**	3.8	>100	>26
Antimycin A[d]	0.003	>0.1	>33

[a] Conditions of Glucose Deficient Medium

[b] Conditions of General Glucose Medium

[c] Selective Index

[d] Compound for positive control

and guanosine monophosphate synthetase [12]. For the first time, polybrominated diphenyl ethers were shown to exhibit the preferential anti-proliferative activity against the cancer cell adapted to glucose starvation.

Effects of compound 1 on the Akt signaling and induction of GRP78

Recent biological studies of cancer cells adapted to nutrient starvation have revealed that the activation of PI3k/Akt/mTOR signaling pathway and the unfolded protein response (UPR) such as induction of glucose-related protein 78 (GRP78) were important for the adaptation of cancer cells to nutrient starvation [22, 23]. Therefore, these processes have attracted much attention as drug targets for cancer chemotherapy. This observation prompted us to investigate the effect of compound **1** on the Akt signaling and the induction of GRP78 by utilizing a western blotting method, and we used antimycin A as a positive control for comparison (Fig. 3; Fig. S1). The PANC-1 cells cultured in the Glucose Deficient Medium were adapted to the nutrient starvation and increased the expression levels of phosphorylated Akt and GRP78 proteins compared with those cultured in the General Glucose Medium (Fig. 3a, lanes 1 and 2, b, lanes 5 and 6). The antimycin A-treated PANC-1 cells cultured in the Glucose Deficient Medium showed an inhibition of phosphorylated Akt and GRP78 proteins (Fig. 3a, lanes 1 and 3), whereas no effect was observed in the expression levels of the other investigated proteins in the PANC-1 cell cultured in the General Glucose Medium (Fig. 3a, lanes 2 and 4). Compound **1** weakly inhibited the inductions of phosphorylated Akt and GRP78 in the PANC-1 cells cultured in the Glucose Deficient Medium (Fig. 3b, lanes 5 and 7). Interestingly, compound **1** enhanced the expression levels of phosphorylated Akt accompanied by the induction of Akt expression on the PANC-1 cells cultured in the General Glucose Medium

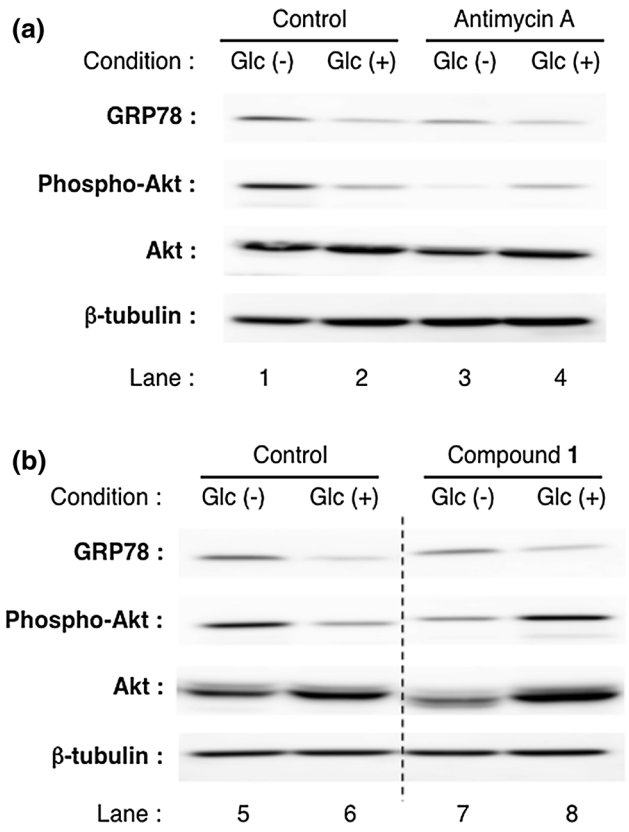

Fig. 3 Effects of compound **1** on the Akt signaling and induction of GRP78 by using western blotting analysis. Glc (−): Culture conditions of Glucose Deficient Medium. Glc (+): Culture conditions of General Glucose Medium. The PANC-1 cells cultured in the Glucose Deficient Medium were treated with **a** antimycin A or **b** compound **1** by the 70 % cell-viable conditions. Cell lysate was resolved by using SDS-PAGE and detected with antibodies against the indicated proteins

(Fig. 3b, lane 8). Although further studies on the effect of compound **1** against the expression of the Akt protein on the PANC-1 cells cultured in the General Glucose Medium would be required, the present results suggest that compound **1** showed a similar behavior with that of antimycin A against the PANC-1 cells cultured in the Glucose Deficient Medium.

Effects of compound 1 on the mitochondrial electron transport chain

Compound **1** exhibited a similar effects with antimycin A on the PANC-1 cells adapted to the glucose-deficient conditions (Fig. 3), and antimycin A is known to be an inhibitor of complex III in the mitochondrial electron transport chain. Therefore, we next examined the effect of compound **1** on the function of each mitochondrial complex (I–V) by using the Mito Check Complex Activity Assay Kit (Cayman Chemical). As shown in Table 2,

Table 2 Effect of compound **1** on the mitochondrial electron transport chain

	IC_{50} value				
	Complex I (μM)	Complex II (μM)	Complex II/III (μM)	Complex IV (μM)	Complex V
Compound **1**	3.4	0.0064	0.86	>100	10.0 μM
Positive control[a]	0.1	30	0.03	38.4	0.2 μg/mL

[a] Compounds used as a positive control are rotenone, thenoyltrifluoroacetone, antimycin A, KCN, and oligomycin for complex I, II, III, IV, and V, respectively

compound **1** strongly inhibited complexes II and III with IC_{50} values of 6.4 nM and 0.86 μM, respectively, while complexes I and V were slightly inhibited with IC_{50} values of 3.4 and 10 μM, respectively. From the reason that the assay system for complex III partly uses complex II enzyme, the major target of compound **1** was presumed to be complex II of the mitochondrial electron transport chain.

In conclusion, the selective anti-proliferative activity of the polybrominated diphenyl ethers, compounds **1** and **2**, against the cancer cells adapted to the glucose-starved conditions might be mediated by the inhibition of complex II enzyme in the mitochondria. Therefore, these compounds would be potential candidates for further development as anti-cancer drugs. Further mechanistic studies and in vivo efficacy of compound **1** are currently underway.

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