Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.8, No.3, 69-73, 2021

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Evaluation of the *in vitro* cytotoxicity of oscillatoxins E and F under nutrient-starvation culture conditions

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(Received May 19, 2021; Accepted May 31, 2021)

ABSTRACT — Oscillatoxins E (**1**) and F (**2**) are cyanotoxins isolated from cyanobacteria in the genus *Lyngbya*. We recently reported the first total synthesis of these compounds and determined their cytotoxicity in various cancer cell lines. Their anti-proliferative activities were moderate, but **2** exhibited unique cell line selectivity. In order to understand their mode of action, in this study we evaluated the cytotoxicity of **1** and **2** under nutrient-depletion culture conditions. Interestingly, **2** exhibited stronger cytotoxicity in HHUA endometrial cancer cells, especially under FBS-starvation conditions. However, its toxicity was not increased in HHUA cells precultured in FBS-depleted medium. These results suggest that **2** is not selectively toxic to nutrient-starved cells and that FBS components such as albumin more strongly neutralized the cytotoxicity of **2** relative to **1**. The protein composition of FBS varies by production lot, and the amount of FBS supplemented to culture medium is flexibly determined depending upon the cell line used and experimental objectives. Therefore, it is important to consider the detoxification activity of FBS to precisely evaluate the properties of oscillatoxins, including cytotoxic potency, cell line selectivity, and their respective structure–activity relationships.

Key words: Oscillatoxin, Cytotoxicity, Nutrient starvation, Bovine serum

INTRODUCTION

Oscillatoxins (OTXs) and aplysiatoxins (ATXs) are cyanotoxins biosynthesized by some marine cyanobacteria including *Lyngbya*, *Schizothrix*, and *Oscillatoria* spp. (Fig. 1A). This class of compounds was intensively isolated in the 1980s, and several dozen derivatives were identified. Some derivatives, such as ATX, debromo-ATX, and OTX-A, exhibit potent tumor-promoting and pro-inflammatory activities through activation of protein kinase C (PKC), a family of serine/threonine kinases that play pivotal roles in cellular signal transduction (Arcoleo and Weinstein, 1985; Fujiki and Sugimura, 1987). Some derivatives were also identified as the causative agents of skin irritation associated with contact with cyanobacteria (Mynderse *et al*., 1977; Osborne *et al*., 2001) and food poisoning caused by ingestion of red alga on which cyanobacteria grew (Nagai *et al*., 1996). In addition, because ATXs and their analogs show anti-proliferative and pro-apoptotic activity against several cancer cell lines through their activation of PKC signaling (Hanaki *et al*., 2018), they were considered potential anti-cancer candidates. Recently, OTXs and ATXs have drawn intense attention again because Han and Nagai independently isolated many new derivatives with novel carbon skeletons (Tang *et al*., 2019; Nagai *et al*., 2019a, 2019b; Zhang *et al*., 2020). However, the biological activities of these derivatives have not been fully investigated, likely due to limited availability from natural sources.

OTX-E (**1**) and OTX-F (**2**) (Fig. 1A) were isolat-

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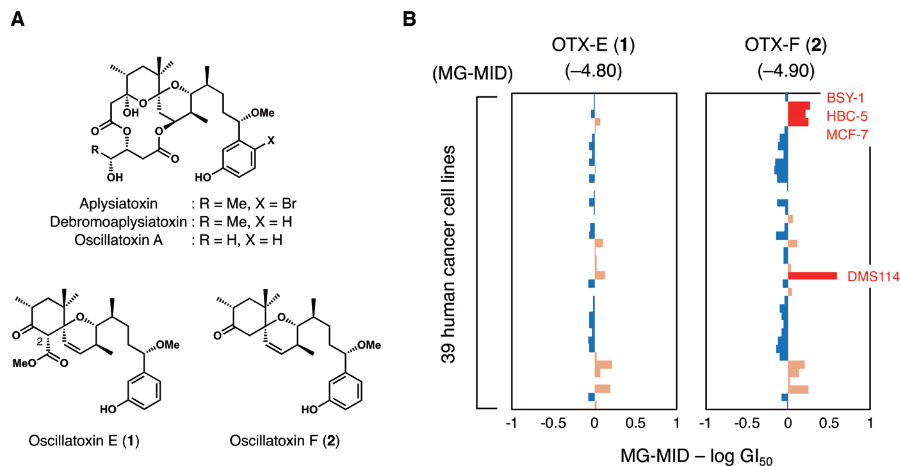

Fig. 1. **A.** Structures of aplysiatoxins and oscillatoxins. **B.** Fingerprints for 39 human cancer cell lines (Araki *et al.*, 2021). Differences between the log GI_{50} for each cell line and mean-graph midpoint (MG-MID).

ed from the cyanobacterium *Lyngbya* spp. (Tang *et al.*, 2019). Unlike ATX, they do not activate PKC, but they moderately inhibit the potassium channel Kv1.5. We recently reported the first total synthesis of these compounds and evaluated their cytotoxicity using a panel of 39 human cancer cell lines (Araki *et al.*, 2021). The growth inhibitory activity of **1** and **2** is not very strong, but interestingly, their cell line selectivity is different from each other even though their structural differences are minor; they differ only by the presence or absence of a carboxymethyl group at position 2. Compound **2** potently inhibits cell lines including DMS114, BSY-1, and MCF-7 (Fig. 1B). Because the efficacy profiles of **2** are different from those of other anti-cancer drugs or inhibitors of cellular signal transductions, we speculated that **2** might possess a novel cytotoxic mechanism.

Inspired by the unique cytotoxicity profile of **2**, we initiated a study on its mechanism of action. During the evaluation of cytotoxicity of **1** and **2** in various cell lines, including HHUA endometrial cancer cells, we noted that using different lots of fetal bovine serum (FBS) that is used to supplement culture medium slightly influences the toxic potency of **1** and **2** (data not shown). Changes in nutrient conditions affect cellular metabolism and cell cycle distribution. Hence, we hypothesized that we could identify a culture condition wherein OTXs showed stronger cytotoxicity, and these conditions might be useful for elucidating the mode of action of these compounds. Some natural products exhibit selective cytotoxicity under nutrient-starvation culture conditions (Lu *et al.*, 2004; Awale *et al.*, 2006). The interaction between serum components and OTXs is also important. Serum albumin is the main protein component of FBS, and it absorbs various toxic compounds (He and Carter, 1992). Moreover, bovine serum contains α_1-acid glycoprotein, which inhibits the activation of PKC by debromo-ATX but not that by ATX (Ueyama *et al.*, 1995). Interestingly, this activity was not observed in serum from other mammalian species. Therefore, consideration of the effects from serum is important to precisely estimate the toxicity and structure–activity relationship of novel compounds. To better understand these observations, we describe the cytotoxicity of **1** and **2** against HHUA cells in various nutrient-depleted culture medium in this letter.

MATERIALS AND METHODS

Materials

Oscillatoxin E (**1**) and oscillatoxin F (**2**) were synthesized as reported previously (Araki *et al.*, 2021). Their CLog P values were calculated by ChemDraw® Professional version 19.0 (PerkinElmer, Waltham, MA, USA). Ham's F-12 culture medium with or without L-glutamine (N6658, N4888) was obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM culture medium without D-glucose (042-32255), a penicillin–streptomycin solution, a trypsin-EDTA solution, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Fetal bovine serum (lot 27419001) was obtained from Corning (Corning, NY, USA).

Cell culture

The human endometrial cancer cell line, HHUA (RCB0658), was provided by RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED (Ibaraki, Japan). The cells were cultured in Ham's F-12 medium with 10% heat-inactivated FBS, 1 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 humidified incubator maintained at 37°C.

Cytotoxicity assay under nutrient-starvation culture conditions

HHUA cells were seeded in 96-well plates at a density of 5,000 cells/well in 200 μL of medium and were allowed to adhere overnight. After the medium was replaced with Ham's F-12 medium without FBS or L-glutamine or DMEM medium without D-glucose, a test compound in 0.5 μL of DMSO was added to each well, and the cells were incubated for 24 hr. A solution of MTT (100 μg) in PBS buffer (20 μL) was then added to each well, and the plate was incubated for an additional 4 hr. The medium was removed by aspiration, the generated formazan was dissolved in 150 μL of DMSO, and the absorbance was measured at 570 nm using a multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The cell viability (%) in the presence of each concentration of test compound was plotted as a percentage relative to the vehicle group, and IC_{50} was determined by processing these values.

Cytotoxicity assay with pre-starved cells

HHUA cells were seeded in 96-well plates at a density of 5,000 cells/well in 200 μL of medium, and cells were allowed to adhere overnight. After the medium was replaced with Ham's F-12 medium without FBS, cells were incubated for an additional 24 hr. After the FBS-depletion medium was replaced with standard medium, the cytotoxicity of **1** and **2** was evaluated using a MTT assay as described above.

RESULTS

Glucose, glutamine, and FBS are essential for *in vitro* cell culture, and depletion of these nutrients alters cellular metabolic conditions. Therefore, we first determined the toxicity of **1** and **2** in HHUA cells in glucose-, glutamine-, and FBS-depleted medium. The viable cell number was estimated using a MTT assay, and the results were plotted as a percentage of the untreated control (Fig. 2). The IC_{50} value in each culture condition is listed in Table 1. In standard medium, **1** and **2** exhibited similar growth inhibitory activities (Fig. 2A). Similar dose–response curves were observed in glucose- and glutamine-depleted medium (Fig. 2B, C). However, based on IC_{50} values, **2** showed 4.5 times stronger cytotoxicity in FBS-depleted medium than in standard medium (Fig. 2D and Table 1).

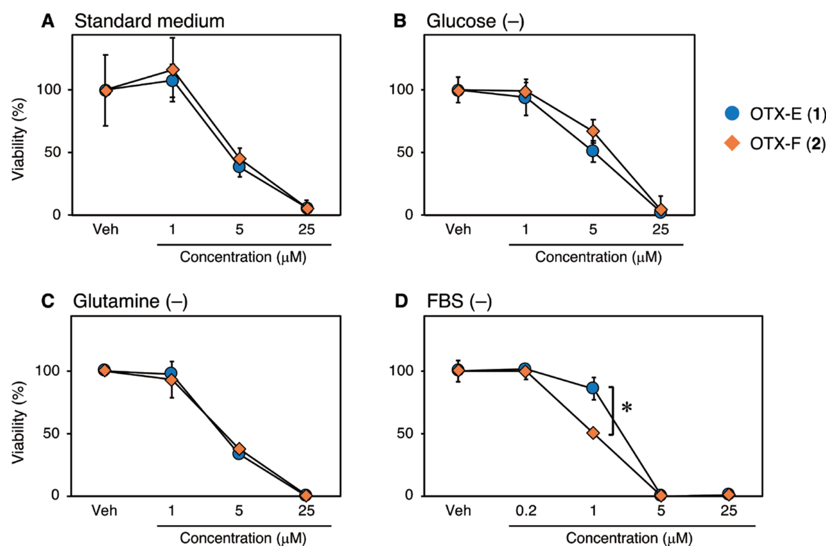

Fig. 2. Cytotoxicity of **1** and **2** in HHUA cells under various culture conditions. HHUA cells were treated with the indicated concentrations of **1** or **2** for 24 hr in various culture mediums. Thereafter, cell number was determined using a MTT assay. Cell viability is plotted as a percentage relative to the vehicle (Veh) group. The average and standard deviation are presented (n = 3 or 4). *P < 0.01 (Student's t test).

Table 1. IC_{50} values for **1** and **2** in HHUA cells in various culture mediums.

		Standard medium	Glucose (–)	Glutamine (–)	FBS (–)
IC_{50} (μM)	OTX-E (**1**)	3.8	5.2	3.3	1.9
	OTX-F (**2**)	4.5	7.8	3.5	1.0

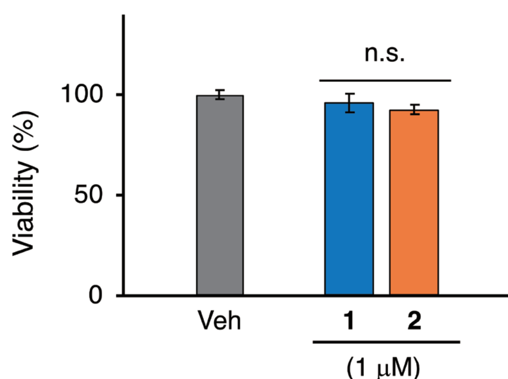

Fig. 3. Cytotoxicity of **1** and **2** in HHUA cells precultured in FBS-depleted medium. HHUA cells were cultured in FBS-depleted medium for 24 hr, and the culture medium was then replaced with standard medium containing 10% FBS. The precultured cells were immediately treated with 1 μM **1** or **2** for 24 hr. Cell number was determined using a MTT assay, and cell viability is plotted as a percentage relative to the vehicle (Veh) group. The average and standard deviation are presented ($n = 4$). n.s., not significant.

Treatment with 1 μM of **2** inhibited cell viability by 50%. The cytotoxicity of **1** also increased in FBS-depletion conditions, but the increase was more modest than what was observed for **2**. Cell viability was still more than 80% in the presence of 1 μM of **1**.

Because stimulation by serum starvation is generally used to synchronize the cell cycle at the G_0/G_1 phase (Davis *et al*., 2001), we predicted that **2** would exhibit selective toxicity against serum-starved cells in this cell cycle phase. Therefore, we precultured HHUA cells in FBS-depleted medium for 24 hr and evaluated the effect of treatment with 1 μM of **1** and **2** in standard medium containing 10% FBS (Fig. 3). If **2** exhibited cell cycle–selective toxicity, **2** would potently decrease the viability of the precultured cells. However, cell viability was still more than 90%, and no significant difference in cytotoxicity between **1** and **2** was observed. Hence, we conclude that **2** does not have selective toxicity against serum-starved cells.

DISCUSSION

In this study, we demonstrate that **2** exhibits potent cytotoxicity in FBS-depleted medium, but it does not have specific toxicity against cells precultured in serum-depleted medium. These results suggest that serum components absorb OTXs and inhibit their access to the cell surface. Interestingly, FBS more strongly neutralized the toxicity of **2** relative to **1** in spite of their structural similarity. Because serum albumin absorbs toxic molecules on its hydrophobic motif, the hydrophobicity of **1** and **2** might be a critical factor. Although the predicted LogP values of these compounds are almost the same (CLogP: **1**, 5.13; and **2**, 5.12), their experimentally determined HPLC retention times indicate that **2** is slightly more hydrophobic than **1** (Tang *et al*., 2019). Kamachi *et al*. (2013) reported that the growth inhibitory activity of ATX analogs was dependent upon their molecular hydrophobicity. They noted that analogs with LogP values outside the range from 4.0 to 4.5 exhibited weaker anti-proliferative activity because a highly hydrophobic analog was trapped by the cellular membrane, but hydrophilic analogs could not penetrate the membrane. Our study suggests that interaction with serum albumin is a considerable factor that influences the biological activity of hydrophobic derivatives of ATXs and OTXs. Moreover, Ueyama *et al*. (1995) reported that α_1-acid glycoprotein in bovine serum specifically interacted with debromo-ATX. This protein could also bind to **2** because debromo-ATX and **2** share a common structural motif. In future studies we will more precisely investigate the structural factors of OTXs that contribute to trapping by serum component proteins.

Although the detoxification function of serum albumin is well-known, this effect is usually disregarded in studies investigating the cytotoxicity of natural products. The type and concentration of serum supplemented to culture medium are individually optimized for each cell line. Moreover, the components of serum differ among production lots. This inconstancy in serum-supplemented conditions is a pitfall in determining the properties of novel compounds, including cell line selectivity, structure–activity relationships, and data reproducibility. We previously evaluated the cytotoxicity of OTXs in the

L1210 leukemia cell line in medium with horse serum and in other cancer cell lines in medium with FBS (Araki *et al*., 2021). Hence, we recently began to reevaluate the biological activity of OTXs with careful consideration of the effects from serum-specific detoxification.

ACKNOWLEDGMENTS

The authors thank Prof. Masashi Sato at Kagawa University for generous support in the laboratory. The authors are grateful to Prof. Noriyuki Sueyoshi at Kagawa University for valuable comments on the cytotoxicity assay under serum-depleted conditions. This work was supported by JSPS KAKENHI (grant number 21K14793 to Y.H., 19H02896 to T.N., and 18H04400 to T.N.).

Conflict of interest---- The authors declare that there is no conflict of interest.

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