Harmful Algae 32 (2014) 64–72

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

Effect of 5-fluoro-2′-deoxyuridine on toxin production and cell cycle regulation in marine dinoflagellate, *Alexandrium tamarense*

Yuko Cho [a,*], Motoo Ogawa [b], Mari Yotsu-Yamashita [a], Yasukatsu Oshima [b]

[a] Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan
[b] Graduate School of Life Sciences, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan

ARTICLE INFO

Article history:
Received 14 August 2013
Received in revised form 18 December 2013
Accepted 18 December 2013

Keywords:
Alexandrium tamarense
5-Fluoro-2′-deoxyuridine (FUdR)
Paralytic shellfish toxins

ABSTRACT

The effect of metabolic inhibitor, 5-fluoro-2′-deoxyuridine (FUdR) on toxin production and the cell cycle of marine dinoflagellate, *Alexandrium tamarense*, was investigated. Compared to untreated cells, FUdR at 3 μM ($p < 0.05$) to 300 μM ($p < 0.01$) inhibited the cell proliferation and toxin production in a dose-dependent manner for *A. tamarense* cultured in modified T_1 medium. FUdR at 203 μM resulted in cell cycle arrest at the S phase at day 4 and toxigenesis was inhibited after day 2. The toxin profiles of the FUdR-treated cultures were similar to those of the control culture. These results suggest that FUdR inhibits saxitoxin (STX) biosynthesis in the early stage of the pathway. This report is the first to demonstrate the inhibition of toxin production in *A. tamarense* by a nucleoside analog.

1. Introduction

Saxitoxin (STX), which is one of the most common paralytic shellfish toxins (PSTs), acts as a voltage-gated sodium channel blocker (Kao, 1966; Bordner et al., 1975; Schantz et al., 1975; Bricelj and Cembella, 1995). STX and its analogs are of interest from the perspective of a public health issue, and understanding the biochemical mechanisms that control the production of STX and its analogs is being urgently pursued. PSTs (including STX) are produced primarily by marine algae and are associated with harmful algal blooms (HABs) reported in many areas of the world, with the main causative organisms being marine dinoflagellates in the genera *Alexandrium, Gymnodinium* and *Pyrodinium* (Smayda, 2007; Hallegraeff, 2010; Trainer et al., 2010; Anderson et al., 2012). Of these, many species in genus *Alexandrium* have been found to produce PSTs: *Alexandrium acatenella* (Cembella et al., 1987), *Alexandrium andersoni* (Ciminiello et al., 2000), *Alexandrium catenella* (Schantz et al., 1966; Hallegraeff et al., 1988), *Alexandrium fundyense* (Anderson et al., 1990), *Alexandrium lusitanicum* (Mascarenhas et al., 1995), *Alexandrium minutum* (Chang et al., 1997), *Alexandrium ostenfeldii* (MacKenzie et al., 2004), *Alexandrium tamarense* (Buckley et al., 1976; Oshima and Yasumoto, 1979), and *Alexandrium tamiyavanichii* (Hashimoto et al., 2002). Interestingly, some freshwater cyanobacteria have been reported to produce PSTs (Llewellyn, 2006).

The unique characteristics of the dinoflagellate genome had made the isolation and determination of the genes encoding the proteins involved in the PSTs synthesis difficult (Plumley, 1997). Recently, the complete sequence of the putative *sxt* gene cluster (>35 kb, genes designated *sxtA–sxtZ*) encoding 26 proteins in the toxin-producing cyanobacterial strain, *Cylindrospermopsis raciborskii* T3, was determined (Kellmann et al., 2008). Subsequently, the STX biosynthesis pathway in cyanobacteria was elucidated through the determination of the molecular and bioinformatic characteristics of the STX gene clusters in the genomes of several other cyanobacteria (Mihali et al., 2009; Moustafa et al., 2009; Stüken and Jakobsen, 2010; Soto-Liebe et al., 2010). Since only 14 genes (termed the "core genes") are found in common in all cyanobacterial STX gene clusters, it seems likely that all 26 genes in the *C. raciborskii* STX cluster are not necessary for STX production (Murray et al., 2011). In dinoflagellates, almost all of the core genes for STX production have been identified, the structures of these genes have been partially determined, and limited information about the likely regulation of STX production in the dinoflagellate genome has been elucidated (Stüken et al., 2011; Orr et al., 2013a,b). Nine cyanobacterial *sxt* genes (*sxtA*, *sxtB*, *sxtF/M*, *sxtG*, *sxtH/T*, *sxtI*, *sxtR*, *sxtS*, *sxtU*) were found in the transcripts of *Alexandrium minutum*. Eight of these genes were also found in *Alexandrium fundyense* (Stüken et al., 2011). Thirteen cyanobacterial *sxt* genes (*sxtA*, *sxt*B, *sxt*D, *sxtF/M*, *sxtG*, *sxtH/T*, *sxtI*, *sxtL*, *sxtN*, *sxtP*, *sxtS*, *sxtU*, and *sxtX*) were identified among the *Alexandrium*

Abbreviations: dc, decarbamoyl; FUdR, 5-fluoro-2′-deoxyuridine; GTX, gonyautoxin; PI, propidium iodide; PST, paralytic shellfish toxin; STX, saxitoxin; TS, thymidylate synthetase.

* Corresponding author. Tel.: +81 22 717 8973; fax: +81 22 717 8922.
E-mail address: choyuko@m.tohoku.ac.jp (Y. Cho).

1568-9883/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.hal.2013.12.007

tamarense transcripts (Hackett et al., 2013). These genes represent almost all of the core complements found in cyanobacteria, except for three homologs, which may have been replaced by genes with similar function in dinoflagellates. Previous work based on the differential expression of these genes during toxin-producing and non-producing periods in *A. fundyense* identified three genes encoding S-adenosylhomocysteine hydrolase (SAHH), methionine aminopeptidase (MAP), and a histone-like protein (HLP), one of which, SAHH was proposed to be involved in toxin biosynthesis (Taroncher-Oldenburg and Anderson, 2000). Another study on gene expression and toxin production in dinoflagellates has identified S-adenosyl methionine synthetase, MAP and SAHH as being differentially expressed genes. Based on the reactions catalyzed by these enzymes, the authors speculate that they might be involved in the synthesis of STX in *Alexandrium catenella* (Harlow et al., 2007). Subtractive hybridization of cDNA between toxin-producing and non-producing subclones from the same parental toxin-producing strains of *A. tamarense* was performed to identify genetic differences associated with the production of toxins. The differentially expressed gene fragments, however, did not exhibit a direct relationship with toxin production, as for other subclones (Cho et al., 2008). A microarray-based comparison of toxin-producing and non-producing strains of *A. minutum* detected several unique genes in the toxin-producing *A. minutum* strains (Yang et al., 2010, 2011). However, the relationship of these genes to toxin biosynthesis is unclear. Searching for *sxt* genes in dinoflagellates is difficult due to the large genome size (LaJeunesse et al., 2005), the presence of multiple copies of genes (Li et al., 1997; Stüken et al., 2011), dispersion of functionally related genes (Monroe and Van Dolah, 2008), and significant genetic variation in clonal cultures (Cho et al., 2008). Proteomics studies have identified nine proteins with known functions that may be involved in toxin biosynthesis and that vary significantly at different toxin biosynthesis stages in *A. catenella* (Wang et al., 2013): methionine S-adenosyltransferase (MAT), chloroplast ferredoxin-NADP reductase (FNR, encoded by *sxtW* in cyanobacteria), S-adenosylhomocysteinase (SAH), adenosylhomocysteinase (AdoHcy), ornithine carbamoyltransferase (OTC, encoded by *sxtI* in cyanobacteria), inorganic pyrophosphatase (PPi), sulfotransferase (SULT, encoded by *sxtN* in cyanobacteria), alcohol dehydrogenase (ADH, encoded by *sxtU* in cyanobacteria), and arginine deiminase (ADI). The identification of these genes and proteins is valuable for the understanding of the biosynthetic pathway of STX in dinoflagellates; however, the relationship of these genes and proteins in saxitoxin biosynthesis has not yet been fully confirmed.

Previously, we tested metabolite inhibitors as tools for regulating cell proliferation in *Alexandrium tamarense* (Cho et al., 2011). Results of our screening revealed that not all inhibitors can be used for the study of the toxin biosynthetic pathway in *A. tamarense* because some inhibitors are lethal, show no effect, or form white precipitates when mixed with T_1 modified medium. Of the seven nucleic acid synthesis inhibitors, six compounds (mitomycin C, bleomycin hydrochloride, hydroxyurea, aphidicolin, phenethyl alcohol and ethidium bromide) showed lethal effects at high concentrations. Among six cell division inhibitors (isopropyl N-(3-chlorophenyl)-carbamate (CIPC), demecolcin, 2-(4-thiazolyl)-1H-benzimidazol, nocodazole, vinblastine, and colchicine), five showed lethal effects, but CIPC had no effect on the growth of *A. tamarense*. The four tested nucleic acid base analogs (2-thiouracil, 8-azaguanin, 6-azauracil, 6-methylpurine) did not affect growth; in contrast, the nucleoside analog, 5-fluoro-2′-deoxyuridine (FUdR), did inhibit growth. Of the 18 metabolite inhibitors tested, four compounds (mytomycin C, colchicine, bleomycin hydrochloride and FUdR) were found to be applicable for the study of *A. tamarense*. The concentration at which cultures did not increase in cell number but cells remained alive and motile (IC_{100}) was determined to be at the following concentrations: mitomycin C, 2 μM; bleomycin hydrochloride, 15 μM; colchicine, 2 mM; and FUdR, 30 μM. Moreover, the effect of mytomycin C and colchicine on toxin production and cell cycle progression was investigated. Mytomycin C arrested cells in the S phase, but toxin production continued. High concentrations of colchicine arrested cells in G_1 phase and inhibited toxin production in a reversible manner. The mechanisms of the effect of these compounds on toxin production remain to be elucidated.

FUdR, which is a metabolite of 5-fluoro-uracil (5-FU), an anti-cancer agent, of which the main action was reported to be the DNA synthesis inhibition (Valeriote and Santelli, 1984; Parker and Cheng, 1990; Elstein et al., 1997) is examined here. We compared the effect of FUdR with that of mitomycin C, which is also known to be a DNA synthesis inhibitor by a different mechanism. Based on these findings, we propose a putative mechanism for the influence of FUdR on STX biosynthesis.

2. Materials and methods

2.1. Materials

The marine dinoflagellate, *A. tamarense* Balech strain Axat-2, was used in this study. Strain Axat-2 was re-isolated by Dr. Omura at Tokyo University of Marine Science and Technology in 1996 from strain OF935-AT6, which was isolated by Dr. Ogata of Kitasato University from sea water collected at Ofunato, Japan in 1993 (Omura et al., 2003; Cho et al., 2006). Based on 28S rDNA sequence analysis, this strain belongs to *A. tamarense* species complex Group I.

2.2. Culture conditions

Axat-2 was maintained and grown in modified T_1 medium prepared in artificial sea water in 250-ml tissue culture flasks under the following culture conditions (Cho et al., 2006): 12 h light/12 h dark photocycle with light provided by cool white bulbs (100–150 μmol photons m^{-2} s^{-1}) at 15 °C. These culture conditions were also used for the experiments performed in this study.

2.3. Analytical methods

2.3.1. Cell count

Cell number was counted in diluted subsamples (100 μl, sampled in triplicate or more for each flask or well) under a light microscope using a counting plate with 1 mm rules (RIGO Co., Ltd., Tokyo, Japan). The average number of cells per milliliter was calculated for three culture flasks or assay wells.

2.3.2. Cell volume

Cell volume was determined using 10 μl of culture sample concentrated about 10-fold and placed in a counting chamber (Improved Neubauer, 0.1 mm deep, Erma, Tokyo, Japan). Photographs of 100 cells or more from each sample were taken under an inverted microscope equipped with a digital camera and analyzed using image analysis software (NIH image). Under the assumption that *A. tamarense* cell is spherical, cell radius was calculated using calibration curve prepared by areas of circles drawn using the picture of stage micrometer.

2.3.3. Flow cytometric analysis

Cells were stained for flow cytometry using the method described previously (Cho et al., 2011). Briefly, cells were fixed with 500 μl of 5% (vol/vol) formaldehyde-filtered sea water. After removing pigments by extraction with 1 ml of ice cold MeOH, cells were stored at −30 °C until analysis. After washing twice with 1×