Harmful Algae 10 (2011) 235–244

Contents lists available at ScienceDirect

Harmful Algae

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

Effects of mitomycin C and colchicine on toxin production and cell cycle regulation in the dinoflagellate *Alexandrium tamarense*

Yuko Cho [a,*], Motoo Ogawa [b], Mayumi Hirota [c], Yasukatsu Oshima [b,1]

[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
[c] Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan

ARTICLE INFO

Article history:
Received 31 March 2010
Received in revised form 9 October 2010
Accepted 9 October 2010
Available online 15 October 2010

Keywords:
Mitomycin C
Colchicine
Alexandrium tamarense
Paralytic shellfish toxins

ABSTRACT

Paralytic shellfish toxin (PST) production and cell proliferation in the dinoflagellate *Alexandrium tamarense* were investigated under the influence of two metabolic inhibitors (mitomycin C and colchicine) that have different mechanisms of action. Intracellular PST levels in cells treated with 2 μM mitomycin C increased gradually, reaching a maximum of 176 fmol/cell (a 6-fold increase). High concentrations of colchicine prolonged G_1 phase in *A. tamarense* cells, even though colchicine arrests several other eukaryotic cell types in M phase. The cells in prolonged G_1 phase under the influence of colchicine were apparently unable to produce PST. Cell proliferation and toxin production recovered after removal of colchicine; in contrast, the effect of mitomycin C was irreversible. While the timing of toxin production within the cell cycle was not conclusively determined, *A. tamarense* cells in S phase were able to produce PST.

1. Introduction

Paralytic shellfish toxins (PSTs), represented by saxitoxin (STX), are potent neurotoxins that possess a saxitoxin skeleton (a trialkyl tetrahydropurine) modified with different functional groups. Ingestion of PST-contaminated shellfish causes paralytic symptoms (paralytic shellfish poisoning). Dinoflagellates that produce PSTs contaminate shellfish and cause paralytic shellfish poisoning. Most toxic dinoflagellate species are members of the genus *Alexandrium*, including *A. andersoni* (Ciminiello et al., 2000), *A. catenella* (Hallegraeff et al., 1988), *A. fundyense* (Anderson et al., 1990), *A. lusitanicum* (Mascarenhas et al., 1995), *A. minutum* (Chang et al., 1997), *A. ostenfeldii* (MacKenzie et al., 2004), *A. tamarense* (Buckley et al., 1976; Oshima and Yasumoto, 1979), and *A. tamiyavanichii* (Hashimoto et al., 2002). Other dinoflagellate species, including *Gymnodinium catenatum* (Oshima et al., 1987) and *Pyrodinium bahamense* var. *compressum* (Harada et al., 1982), also produce PSTs.

Feeding experiments revealed that the trialkyl tetrahydropurine skeleton is not produced by the usual purine metabolic pathway, but rather that arginine and acetate are incorporated into a STX skeleton and the carbon side chain is derived from the methyl group of S-adenosyl methionine (Shimizu, 1993). Many factors, including nutrients (nitrogen, phosphate, amino acids) and environmental conditions (salinity, light, and temperature), are known to affect the growth rate and toxin production in dinoflagellates (Ogata et al., 1987; Anderson et al., 1990; Siu et al., 1997; Taroncher-Oldenburg et al., 1999; Lim and Ogata, 2005). In recent years, the understanding of STX biosynthesis has been enhanced by the molecular and bioinformatic characterization of STX gene clusters in genomes of cyanobacteria, another group of organisms with species known to produce PSTs (Kellmann et al., 2008). Genetic studies have also enhanced our understanding of the PST biosynthetic pathways in cyanobacteria (Mihali et al., 2009). In contrast, the genes involved in the biosynthesis of PSTs in dinoflagellates are less well characterized, in spite of the fact that dinoflagellates are the most common causative agents of paralytic shellfish poisoning cause by marine shellfish and have been studied by many researchers for more than three decades. Some attempts have been made to identify genes involved in toxin production in dinoflagellates using molecular biological methods. By differential display of genes expressed during the period of toxin production and genes expressed during the period with no toxin production in synchronized *A. fundyense* cultures, three genes (S-adenosylmethionine synthetase, methionine aminopeptidase, and S-adenosylhomocysteine hydrolase) were identified as candidate STX biosynthesis genes (Taroncher-Oldenburg et al., 1997). However, the role of these genes in toxin synthesis in

Abbreviations: PSTs, paralytic shellfish toxins; GTX, gonyautoxin; STX, saxitoxin; dc, decarbamoyl; CIPC, isopropyl N-(3-chlorophenyl)-carbamate; FudR, 5-fluoro-2′-deoxyuridine; PI, propidium iodide.

* Corresponding author. Tel.: +81 22 717 8973; fax: +81 22 717 8922.
E-mail address: choyuko@biochem.tohoku.ac.jp (Y. Cho).
[1] Present address: School of Marine Biosciences, Kitasato University, Sanriku-cho, Ofunato-shi, Iwate 022-0101, Japan.

1568-9883/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2010.10.003

dinoflagellates has not been established. Molecular and genetic analysis of dinoflagellates is unusually challenging because of their large genome size (LaJeunesse et al., 2005), the existence of multiple copies of genes (Li et al., 1997), dispersed functionally related genes (Monroe and Van Dolah, 2008), and significant genetic variation in clonal cultures (Cho et al., 2008). Moreover, results on the timing of toxin production within the cell cycle are not consistent; for example, toxin is produced during S phase in *A. catenella* (Siu et al., 1997), in early G_1 phase in *A. fundyense* (Taroncher-Oldenburg and Anderson, 2000), and during $G_2 + M$ phase in *A. catenella* (Harlow et al., 2007).

We have taken a different approach to studying the relationship between the cell cycle and toxin production; specifically, we have used metabolic inhibitors to induce synchronous cell cycle arrest. Several metabolic inhibitors with known mechanisms of action result in synchronous cultures that arrest at specific points in the cell cycle of eukaryotic cells. However, little was known about the use of such metabolic inhibitors in dinoflagellates. Therefore, we selected metabolic inhibitors in different categories to find the compounds applicable to *A. tamarense*. Here we present the results of our screens for growth inhibitors that cause synchronous cell cycle arrest in *A. tamarense* and our investigations on the effects of two such inhibitors, mitomycin C and colchicine, on cell proliferation, cell cycle progression, and toxin production.

2. Materials and methods

2.1. Materials

The dinoflagellate *A. tamarense* Balech, strains T-1 and Axat-2, were used in this study. The strain T-1 was isolated at Funka bay, Japan in 1978 by Dr. Oshima. The strain Axat-2 was generated by Dr. Omura at Tokyo University of Marine Science and Technology in 1996 from the strain OF935-AT6, which was isolated by Dr. Ogata of Kitasato University from natural sea water from Ofunato, Japan in 1993 (Omura et al., 2003).

2.2. Culture conditions

Screening for metabolic inhibitors of *A. tamarense* was performed using the strains T-1 and Axat-2. The strain T-1 was cultured in Guillard F medium, and the strain Axat-2 was cultured in modified T_1 medium (Cho et al., 2006). Cultures were maintained and grown under a 12-h-light/12-h-dark photo cycle with light provided by cool white bulbs (100–150 μmol photons m^{-2} s^{-1}) at 15 °C in 250-ml tissue culture flasks. Bacterial contamination of the axenic strain was monitored by incubation of the culture media with Marine Broth 2216 or epifluorescent microscopic observation of DAPI (4′-6-diamidino-2-phenylindole)-stained culture media.

2.3. Screening of metabolic inhibitors

Reagents were purchased from Sigma–Aldrich Japan (Tokyo, Japan) unless otherwise stated. Eighteen substances were tested. Seven nucleic acid synthesis inhibitors (mitomycin C, bleomycin hydrochloride, hydroxyurea, nalidixic acid (Wako, Osaka, Japan), ethidium bromide, aphidicolin, and phenethyl alcohol), six cell division inhibitors (isopropyl N-(3-chlorophenyl)-carbamate (CIPC), demecolcine, 2-(4-thiazolyl)-1H-benzimidazol (Wako, Osaka, Japan), nocodazole, vinblastine, and colchicine), four nucleic acid base analogues (2-thiouracil (Wako, Osaka, Japan), 8-azaguanin, 6-azauracil, 6-methylpurine), and one nucleoside analogue (5-fluoro-2′-deoxyuridine (FUdR)) were screened. The stock solutions were prepared by dissolving each substance with culture medium, except for demecolcine (1 M HCl) and nocodazole (DMSO). The concentrations used for screening were as follows. Mitomycin C: first screening at 1, 10, 50, 500, and 5000 μM; second screening at 0.05, 0.125, 0.25, 0.5, 1, and 2 μM; bleomycin hydrochloride at 15 and 75 μM; hydroxyurea at 75, 750, 7500, and 75,000 μM: nalidixic acid at 20, 200, and 2000 μM; aphidicolin at 0.5, 5, and 50 μM; phenethyl alcohol at 200, 2000, and 20,000 μM; ethidium bromide at 0.5, 5, and 50 μM; CIPC at 20, 200, and 2000 μM; demecolcine at 0.5, 5, 50, 500, 100, and 1000 μM; 2-(4-thiazolyl)-1H-benzimidazol at 0.025, 0.05, 0.5, 5, 50, 500, and 5000 μM; nocodazole at 1, 50, and 100 μM; vinblastine at 5.5, 55, and 550 μM; first colchicine screening at 1, 5, 10, 50, 100, 500, and 1000 μM; second colchicine screening at 1, 2, 4, 6, 8, and 10 mM; 2-thiouracil, 8-azaguanin, 6-azauracil, and 6-methylpurine at 10, 100, and 1000 μM; and first FUdR screening at 100, 200, 400, 600, 800, and 1000 μM, with a second FudR screening at 0.3, 3, 30, and 300 μM. The experiments were performed in parallel with control cultures that contained no metabolic inhibitor, only vehicle. In the demecolcine screens, acid was used as a vehicle, and the pH was adjusted with 1 M sodium hydroxide.

Cells were cultured in 2 ml of culture media containing a single metabolic inhibitor or control medium for 7 days in test tubes for the strain T-1 or 10 days in 12-well culture plates for the strain Axat-2. Cell proliferation was observed under the microscope every other day. When the cell number in inhibitor-treated medium was indistinguishable from that in control medium, the inhibitor at the given concentration was designated as having no effect. The concentration at which all cells were dead at the end of observation period was designated as LD_{100} (lethal concentration). The concentration at which there was no increase in cell number but cells were alive and motile was designated as IC_{100} (inhibition concentration).

2.4. Metabolic inhibitor treatment

Axat-2 cell suspensions (210 ml each) were mixed with 420 ml of modified T_1 (control) media or 420 ml modified T_1 medium containing 3 μM mitomycin C (inhibited). Cells were inoculated at an initial cell density of 2×10^3 cells ml^{-1} with a final mitomycin C concentration of 2 μM. Each cell suspension was divided into three 210-ml cultures in T-75 flasks and cultured for 21 days. Aliquots (8 ml for toxin analysis and 2 ml for cell counts) were collected from each flask at the end of a dark period every day until the 7th day and every other day from day 7 to day 21 to assess toxin production and cell density. After appropriate dilution (4–10 fold) of harvested sample, cell numbers of diluted subsamples (100 μl each, more than three subsamples per flask) were counted under light microscopy. The average number of cells per ml was calculated for three flasks. In another series of experiments, initial cell density was adjusted to approximately 6×10^3 cells ml^{-1}, and the final concentrations of mitomycin C and colchicine were 2 μM and 2 mM, respectively. Aliquots (8 ml for toxin analysis and 2 ml for cell count) were collected from each flask at the end of a dark period every day until the 7th day. A cell cycle study was also conducted over the course of one 7-day incubation. In this study, seven culture flasks for each inhibitor and eight control flasks (including a day-0 control) were prepared for harvest of 200-ml samples for flow-cytometric analysis, harvest of 8-ml samples for toxin analysis, and harvest of 2-ml samples for cell counts every day.

2.5. Block and release study

2.5.1. Mitomycin C

A 30-ml sample of cell suspension treated with 1 μM of mitomycin C for 6 days was centrifuged, and approximately 29 ml of supernatant was discarded. The residual 1 ml was re-suspended with 29 ml of fresh modified T_1 medium to make the concentration