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Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines

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Abstract

Azaspiracid-1 (AZA-1) is a newly identified phycotoxin reported to accumulate in molluscs from several northern European countries and documented to have caused severe human intoxications. The mechanism of action of AZA-1 is unknown. Our initial investigations have shown that AZA-1 is cytotoxic to a range of cell types. Cytotoxicity was evident in all seven cell types tested, suggesting a broad-spectrum mode of action, and was both time- and concentration-dependent. However, AZA-1 took an unusually long time (>24 h) to cause complete cytotoxicity in most cell types, with the exception of the rat pituitary GH_4C_1. Extended exposure times did not always lower the EC_{50} value for a given cell line, but always resulted in more complete cytotoxicity over a very narrow concentration range. The Jurkat cell line (human lymphocyte T) appeared to be very sensitive to AZA-1, although the EC_{50} values (24–72 h) for all the cell types were in the low nanomolar range (0.9–16.8 nM). The effect of AZA-1 on membrane integrity was tested on Jurkat cells and these data confirm our visual observations of cytotoxicity and necrotic cell lysis following exposure of Jurkat cells to AZA-1 and suggest that AZA-1 has some properties unique among marine algal toxins. Additionally, there were dramatic effects of AZA-1 on the arrangement of F-actin with the concurrent loss of pseudopodia, cytoplasmic extensions that function in mobility and chemotaxis. Although these phycotoxin-specific effects of AZA-1 suggest a possible site of action, further work using cell-based approaches is needed to determine the precise mode of action of AZA-1.

Keywords: Azaspiracids; Lymphocyte; Shellfish; Phycotoxins; Cytotoxicity

1. Introduction

Azaspiracids (AZA) are nitrogen-containing polyether toxins with a unique spiral ring assembly, a cyclic amine, and a carboxylic acid, and were first detected in mussels (*Mytilus edulis*) in Ireland in 1995. This toxin has

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subsequently been detected in other bivalve species, including oysters (*Crassostrea gigas*, *Ostrea edulis*), scallops (*Pecten maximus*), clams (*Tapes phillipinarium*), cockles (*Cardium edule*), and razor fish (*Ensis siliqua*) (Hess et al., 2001; Furey et al., 2003; Hess et al., 2003). Cases of AZA intoxications and/or contaminated shellfish in several countries, including Ireland, UK, Norway, Netherlands, France, Spain, and Italy have been documented (Satake et al., 1998a; Ito et al., 2002; James et al., 2002a; Magdalena et al., 2003a). Although the human symptoms resembled those of diarrhetic shellfish poisoning (DSP), the illness was subsequently named azaspiracid poisoning (AZP) once azaspiracid was identified as a novel toxin (Ito et al., 1998; Ofuji et al., 1999). The regulatory limit for AZA in shellfish in the European Union has been set at 160 μg azaspiracid equivalents per kg (European Commission, 2002).

To date, 11 different congeners have been identified and named AZA-1 through AZA-11 (Ito et al., 1998; Satake et al., 1998b; James et al., 2003b). Consistent with their highly oxygenated polyether structure and the seasonal occurrence of shellfish toxicity, AZAs are thought to originate from a dinoflagellate source (Ofuji et al., 1999). Initially, AZA was identified in association with a heterophic dinoflagellate, *Protoperidinium* sp (Yasumoto, 2001) later reported as *P. Crassipes* (James et al., 2003a); however, definitive confirmation that this *Protoperidinium* species produces AZAs de novo will require demonstration of AZA synthesis in laboratory cultures grown on defined prey species.

Current studies are aimed at assessing the distribution and long-term dynamics of AZA accumulation in shellfish. There is some evidence that AZAs, unlike the DSP toxins, may accumulate not only in the hepatopancreas (digestive glands), but may also occur in the gonads (roe) (Magdalena et al., 2003b) and the adductor muscle (meat) (James et al., 2000; 2002b). Yet, given the extensive variation between individual animals and among shellfish species, no general trend has been established. The fact that AZAs can persist in *M. edulis* for up to 8 months following the initial exposure, possibly reflecting tissue migration and a slow depuration rate of the toxin (James et al., 2000), does indicate that protection of shellfish consumers will require vigilant monitoring.

Following human consumption of AZA-contaminated shellfish, there is generally a rapid onset of symptoms very similar to those of DSP, including nausea, vomiting, severe diarrhea, and stomach cramps (McMahon and Silke, 1996; 1998). In mice and rats, intraperitoneal (i.p.) injections induce neurological, 'PSP-like' symptoms with progressive paralysis, fatigue, difficulty breathing, and subsequent death as soon as 35 min following injection (Ito et al., 1998; McMahon and Silke, 1998; Satake et al., 1998a). Pathological effects include histological alterations in the liver, pancreas, spleen, and necrotic lymphocytes in the thymus. More detailed in vivo studies with mice have since demonstrated that oral AZA-1 exposure induces a variety of changes in the intestinal tract, including accumulation of fluid, necrosis and edema in the lamina propria of the mid-intestinal tract, and fused, shortened villi. Necrosis of T and B lymphocytes were also documented in the spleen and thymus, as well as fatty changes in the liver, hyperplasia of the epithelial lining in the stomach, and tumors in the lungs (Ito et al., 1998; 2000; 2002).

Recent efforts to determine the mode of action of AZA have been conducted by several investigators using in vitro techniques. Due to the similar nature of the symptoms of AZP with DSP, a mode of action similar to the DSP toxins on protein phosphatase (PP) activity was proposed by Flanagan et al. (2001). However, unlike DSP toxins, bioactive extracts containing AZAs did not have any inhibitory effect on PP1 activity, yet were clearly cytotoxic to both human hepatoblastoma and bladder carcinoma cell lines (Flanagan et al., 2001). This suggests that the AZA-1 mode of action differs from that of the DSP toxins, although human symptomatology of these two syndromes is similar. Using a combination of neuroblastoma cells and human lymphocytes, it was shown that AZA-1 reduces cellular F-actin content in a non-apoptotic manner following the elevation of cytosolic calcium and cAMP levels (Roman et al., 2002). The authors suggested that AZA-1 might be targeting the cytoskeleton. In contrast to AZA-1, the congeners AZA-2 and AZA-3 have different effects on the levels of cytosolic calcium, possibly suggesting a unique mode of action for each toxin analogue (Botana, 2002).

Herein we report on our investigation of the AZA-1 mode of action using cytotoxicity assays and morphological analyses. Following initial experiments examining the effects of AZA-1 on protein phosphatase 2A, we used a combination of in vitro cell-based approaches to screen a panel of seven mammalian cell lines for cytotoxicity to assess cell-specific sensitivities to AZA-1. Detailed experiments were then performed on an AZA-1-sensitive, Jurkat lymphocyte T cell line in order to characterize AZA-induced effects on cellular morphology and cytoskeletal arrangements. We anticipate that our findings will lead to the development of new methods for AZA detection and aid in defining the AZA-1 mode of action, which will, in turn, facilitate monitoring of the ecological and human health effects associated with the AZAs. Ultimately, this information may also be of use in the development of treatments for humans exposed to AZAs.

2. Materials and methods

2.1. Toxins and chemicals

Azaspiracid (AZA-1) was extracted from 2 kg of mussels (*M. edulis*) that were collected in 1996 from Killary Harbour, on the west coast of Ireland and in 1999 from Bantry Bay, on the southwest coast of Ireland.

Toxins were extracted in 2001, as described by Satake et al. (1998b) and Ofuji et al. (1999) with slight modifications. Stock AZA-1 (2.4 mg) was determined to be >93% pure by NMR and showed <1% impurity of other AZA subtypes/congeners by liquid chromatography–mass spectrometry (LC–MS). Subsequent LC–MS analysis comparing this lot of AZA-1 to a previously prepared lot found no statistically significant differences.

Okadaic acid, saxitoxin, and domoic acid were purchased from IMB/NRC, Halifax, NS Canada and known to be free of impurities. Maitotoxin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and brevetoxin-3 from Calbiochem (San Diego, CA, USA). Dinophysistoxin-1 was purified in the laboratories of the Marine Biotoxins Program (Charleston, SC, USA) and ciguatoxin was a gift from Dr Robert Dickey (US Food and Drug Administration, AL, USA).

2.2. Cell lines

The non-adherent or suspension human cell lines Jurkat, Raji, and THP-1 were each grown in RPMI medium supplemented with 10% (v/v) fetal bovine serum (FBS). The adherent HEK-293 and A549 cells were grown in DMEM medium supplemented with 10% (v/v) FBS, while GH_4C_1 and Neuro-2A cells were grown in MEM medium supplemented with 10% (v/v) FBS. See Table 1 for details of the cell lines. All cells were maintained in humidified 5%:95% CO_2:air at 37 °C. Non-adherent cell lines were subcultured with fresh medium at an inoculum ratio of 1:10 every 5 to 7 days by transferring 1 ml of cells to 9 ml of fresh supplemented medium in 75 cm^2 screw cap culture flasks. The adherent cell lines (HEK-293, A549, Neuro-2A) were subcultured by first washing with phosphate-buffered saline (PBS) followed by release with a trypsin/EDTA solution (Life Technologies, Gaithersburg, MD, USA). GH_4C_1 cells were released and used as an inoculum by gentle agitation following a wash with PBS solution (no trypsin/EDTA).

2.3. Protein phosphatase 2A inhibition assay

To determine the effect of AZA-1 on protein phosphatase 2A (PP2A) activity, we employed a fluorescent 96-well assay (Vieytes et al., 1997). Briefly, the assay tests the ability of okadaic acid (OA) standards and unknown samples to inhibit activity of purified PP2A against the fluorimetric substrate, 6,8-difluro-4-methyl umbelliferyl phosphate (DiFMUP). All AZA-1 samples were diluted 20-fold in 10% methanol to reduce the interference of methanol on PP2A activity. For the assay, 5 μl $NiCl_2$ (40 mM), 5 μl bovine serum albumin (BSA) (1 mg ml^{-1}), 17 μl reaction buffer (50 mM Tris–HCl pH 7.0, 0.1 mM $CaCl_2$), 33 μl of diluted AZA-1 samples (1.7 pM to 83 nM final) or OA certified reference standards (10 pM to 10 nM final) and 20 μl purified PP2A enzyme (0.005 Units $well^{-1}$ final) (Upstate Biotechnology, Lake Placid, NY) were added to triplicate wells of a black 96-well plate (Costar, Corning, NY). To start the reaction, 120 μl DiFMUP (50 μM final) (Molecular Probes, Eugene, OR, USA; cat. no. D-6567) was added to each well and the reaction was allowed to proceed for 30 min at 25 °C with constant shaking (600 rpm). PP2A activity was determined by measuring the fluorescence (excitation 360 nm, emission 460 nm) of each well using a fluorometric plate reader (Fluostar, BMG Lab Technologies, Durham, NC, USA). The detection limit of the fluorimetric phosphatase inhibition assay was approximately 0.1 nM OA-equivalents.

2.4. Cytotoxicity assay

Cells were seeded in a volume of 100 μl of the appropriate supplemented medium at a density of 30,000 cells per well (unless noted otherwise) in white, sterile, 96-well culture plates for 24 h to allow for attachment or settling. A range of final AZA-1 concentrations were then added for 24, 48, or 72 h of continuous exposure prior to assessment of cytotoxicity. Parallel controls of equivalent amounts of methanol were used to normalize the viability data for each treatment. Cellular viability/cytotoxicity was assessed using the MTS assay (Promega Biosciences, San Luis Obispo, CA, USA; cat. no. G5421). Like other tetrazolium-based assays, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) in the presence of an electron coupling reagent (phenazine methosulfate; PMS) measures cellular viability

Table 1
Details of the mammalian cell lines used in the current study

Tissue type	Cell line	ATCC #	Origin	Adherent or suspension
Monocyte	THP-1	TIB-202	Human	S
B Lymphocyte	Raji	CCL-86	Human	S
T Lymphocyte	Jurkat E6-1	TIB-152	Human	S
Human embryonic kidney	HEK-293	CRL-1573	Human	A
Lung epithelial	A549	CCL-185	Human	A
Pituitary epithelial	GH_4C_1	CCL-82.2	Rat	A
Neuroblast	Neuro-2A	CCL-131	Mouse	A

Included are tissue types from which the cell lines were originally derived, American Type Culture Collection (ATCC) number, organism of origin, and growth properties (adherent = A, suspension = S).

by determining the activity of mitochondrial dehydrogenases (Mosmann, 1983). As a substrate for dehydrogenases, MTS becomes reduced into a soluble, purple dye that can be quantified colorimetrically to determine the relative level of cellular viability/cytotoxicity per well. Following exposure of the cells to AZA-1 for a specified period of time, each well received 10 µl of a PMS/MTS (1:20) solution. Cells were incubated for 1 to 3 h, depending on cell type, after which absorbance readings at 485 nm were obtained using a FluoStar microplate reader (BMG Lab Technologies). Data were corrected for the blanks, normalized to the control, and expressed as a percentage.

2.5. F-actin assay

Jurkat cells were plated in 48-well tissue culture plates and exposed to 10 nM AZA-1 for 2, 4, 8, 12, 18, and 24 h. At the appropriate time, cells were transferred to 1.5 ml Eppendorf tubes and centrifuged at 2000×*g* for 3 min. The supernatant was then removed and the cells resuspended in 400 µl of 3.7% formaldehyde. After 10 min of incubation, cells were centrifuged (2000×*g*, 3 min) and the supernatant removed. The cells were then resuspended in 400 µl of Triton X-100 (0.1% final) for 4 min prior to being centrifuged (2000×*g*, 3 min) and resuspended in 400 µl of phosphate buffered saline (PBS) with BSA (1% w/v final) for 20 min. Oregon Green 488 phalloidin (1% w/v final; ~66 nM) was then added to each sample, followed by incubation in the dark for an additional 20 min. The cells were centrifuged, resuspended in 100 µl of PBS, and cellular F-actin was visualized using an, Axiovert S100 epifluorescence microscope (excitation 480 nm, emission 535 nm; Carl Zeiss, Inc., Thornwood, NY, USA).

2.6. Statistics

Data are presented as means±SE of at least three separate experiments; each experiment being done in triplicate wells. Significant differences between means were assessed by an ANOVA followed by a Tukey multiple *t*-test and EC_{50} values were obtained following variable-slope sigmoidal concentration-response analysis as performed by GraphPad Prism™ software (v. 4.01; GraphPad Software, Inc., San Diego, CA, USA). Pseudopodial counts were obtained by counting 50 selected cells using Nomarski differential interference contrast imaging techniques at different focal planes.

3. Results

3.1. Effect of AZA-1 on protein phosphatase 2A activity

OA inhibited PP2A activity in a concentration-dependent manner (EC_{50} = 1.5 nM), whereas it is clear that AZA-1 did not have any effect on PP2A activity at all concentrations tested (Fig. 1).

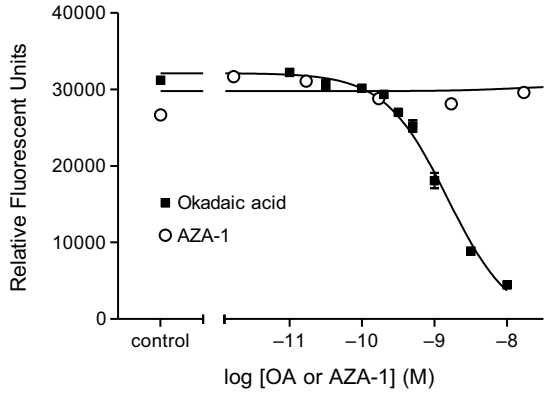

Fig. 1. Effect of AZA-1 and okadaic acid on protein phosphatase 2A activity. AZA-1 and okadaic acid (OA) were incubated in an in vitro microplate fluorescence assay at various concentrations (1.7 pM to 83 nM AZA-1 or 10 pM to 10 nM OA). Protein phosphatase 2A activity was measured as a function of dephosphorylation of the DiFMUP substrate into a fluorescent molecule following 30 min of incubation (Vieytes et al., 1997). Data are expressed as relative fluorescent unit means±SEM of triplicate incubations. Note, in some cases the symbol is larger than the error bars.

3.2. Effect of AZA-1 on cell line viability

Screening experiments were conducted on seven mammalian cell lines (Table 1) to determine the cell type-specificity and sensitivity of AZA-1-induced cytotoxicity. After 24 h of exposure, AZA-1 concentrations of ≤1 nM did not affect viability for any of the seven mammalian cell lines tested. However, all cell types, with the exception of the lung epithelial A549 cells, reduced in viability as AZA-1 concentrations approached 100 nM (data not shown). The Jurkat (Fig. 2, Table 2) and GH_4C_1 (Table 2) cells were particularly sensitive at 24 h such that viability was reduced to 43 and 6% of the control cells, respectively, at the highest AZA-1 concentrations. EC_{50} values of 3.5±1.2 and 16.8±6.5 nM were calculated for the Jurkat and GH_4C_1 cell lines, respectively (Table 2). Longer exposure times of 48 and 72 h decreased viability for all cell lines (data not shown). This step-wise reduction in viability for each cell line was time-dependent and appeared to cause more complete cytotoxicity at AZA-1 concentrations >1 nM with increased exposure time. For the seven cell lines over the three exposure periods, there was a relatively narrow range in cytotoxic EC_{50} values from 0.9 to 16.8 nM AZA-1 (Table 2) with no difference among individual cell line EC_{50} values. The Jurkat cell line was selected for use in all subsequent experiments based on its sensitive, cytotoxic response to AZA-1 exposure as well previous reports that AZA-1 is toxic to T lymphocytes (Ito et al., 2000, 2002).

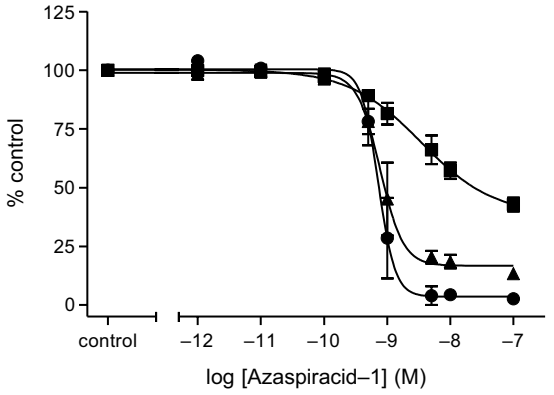

Fig. 2. Effect of AZA-1 on cytotoxicity of Jurkat lymphocyte T cells as determined by the mitochondrial MTS protocol. A range of concentrations of AZA-1 (1 pM to 100 nM) were continuously applied for 24 h (■), 48 h (▲), or 72 h (●) prior to cytotoxicity testing. Data (means ± SEM; triplicate wells for three independent experiments) are expressed as a percentage of control, where equivalent amounts of methanol were used as the vehicle. See Table 2 for EC_{50} values.

3.3. Morphological changes of Jurkat cells following exposure to AZA-1

Photomicrographs of control Jurkat cells illustrated that they are clearly intact without exhibiting any signs of lysis and exhibited cytosolic extensions that have been identified as pseudopodia or lamellipodia (Fig. 3A–C). Jurkat cells exposed to 10 nM AZA-1 for 24, 48, and 72 h displayed a lack of cellular integrity as revealed by lysed cells and debris, organelle protrusion with concurrent flattening of cells, and a distinct lack of pseudopodia (Fig. 3D–F). Cytolysis of Jurkat cells by AZA-1 was time-dependent.

Jurkat cells were exposed to a variety of other phycotoxins for 24 h, followed by a morphological assessment (Fig. 4). Cells were exposed to phycotoxin concentrations similar to their corresponding reported K_d (the concentration of radiolabeled toxin that produces 50% of the maximum receptor occupancy following equilibrium) or EC_{50} (half maximal effective concentration) value. The effects of AZA-1 (EC_{50} = 3 nM), domoic acid (K_d = 5 nM) (Hampson and Manalo, 1998), dinophysistoxin-1 (EC_{50} = 2 nM) (Sheppeck et al., 1997), maitotoxin (EC_{50} = 0.7 nM; M. Twiner, unpublished data), okadaic acid (EC_{50} = 2 nM; this study) (Sheppeck et al., 1997), brevetoxin-3 (K_d = 1 nM) (Bottein Dechraoui and Ramsdell, 2003), and saxitoxin (K_d = 2.3 nM) (Bottein Dechraoui and Ramsdell, 2003) on Jurkat cells are shown in Fig. 4B–H, respectively. Methanol (1% final) was used as a control (Fig. 4A). While some of these toxins had obvious cytotoxic effects on the Jurkat cells (e.g. cellular lysis caused by maitotoxin), the absence of pseudopodia was only observed in cells exposed to AZA-1.

Table 2
Effective concentrations of AZA-1 that elicit half-maximal viability (EC_{50}) for each cell line following continuous exposure for 24, 48, or 72 h

Cell line	AZA-1 Cytotoxicity EC_{50} (nM) (mean ± SE)		
	24 h	48 h	72 h
Jurkat	3.5 ± 1.2	1.1 ± 0.5	0.9 ± 0.6
Raji	n/c	1.6 ± 0.8	1.1 ± 0.5
A549	n/c	1.5 ± 0.4	2.0 ± 0.7
Neuro-2A	n/c	2.3 ± 0.1	2.3 ± 0.2
THP-1	n/c	2.4 ± 0.4	2.5 ± 0.3
HEK-293	n/c	4.6 ± 2.5	2.8 ± 1.5
GH_4C_1	16.8 ± 6.5	7.9 ± 2.7	5.5 ± 2.1

Mean EC_{50} values ± SE (nM) of at least 3 independent experiments in triplicate wells were determined using the MTS viability assay. For some 24 h time treatments, the cytotoxic response was insufficient to determine an EC_{50} value and was not calculated (n/c).

3.4. Effects of AZA-1 on arrangement of F-actin

Fig. 5A–F illustrate a time course of Jurkat cells following exposure to methanol vehicle (1% v/v final) for 2, 4, 8, 12, 18, and 24 h. Cells in each of the control treatments displayed extended pseudopodia at all time points. In cells exposed to 10 nM AZA-1 for the same time periods (Fig. 5G–L), pseudopodia were observed at the 2, 4, and 8 h time points, but following 12 h of exposure, there appeared to be a retraction or shortening of each pseudopodium. No pseudopodia were present in the Jurkat cells exposed to AZA-1 for either 18 or 24 h (Fig. 5K and L).

Quantification of pseudopodia following 2, 4, 8, 12, 18, and 24 h of exposure to AZA-1 (10 nM) determined that the average number of pseudopodia per cell declined from ~1.5 for the 2, 4, and 8 h exposures to ~1.0 pseudopodium per cell for 12 h of exposure, to 0.7 for 18 h of exposure, and then to 0.2 after the 24 h exposure (Fig. 6). These data are expressed as percentage of control. The untreated cells consistently maintained an average of 1.5 to 1.9 pseudopodia per cell for all time treatments. The difference between treatment and untreated cells following a 24 h exposure represented ~9-fold reduction in pseudopodia per cell.

4. Discussion

Since AZA was first detected during a 1995 outbreak in Ireland, a considerable amount of work has been carried out on the structural characterization of AZA-1 and its analogs, the development of LC–MS based analytical methods for AZA detection and quantification, as well as ecological studies related to its spatial and temporal distribution. However, no studies have investigated the toxicological effects of AZA on aquatic organisms, no AZA specific bioassays have been developed, and only a few in vivo and in vitro studies have attempted to address the mode(s) of action for AZA. Although previous work provides some

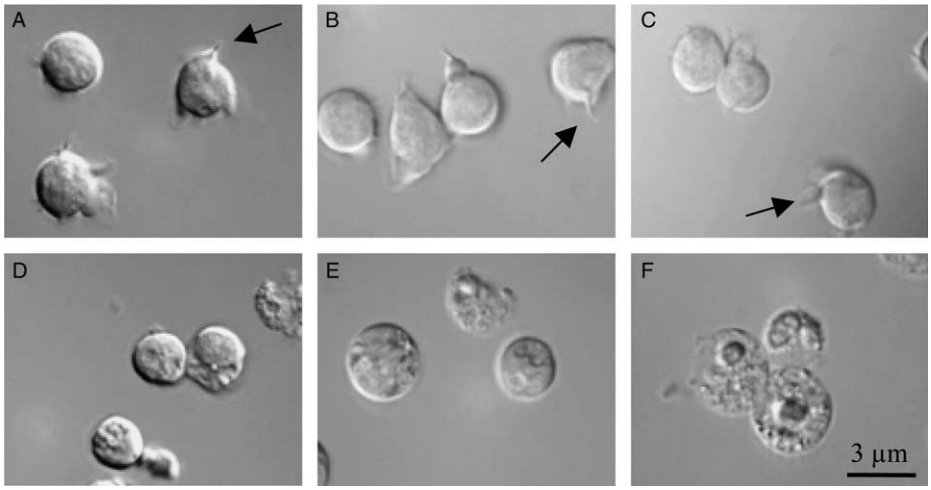

Fig. 3. Photomicrographs of Jurkat T lymphocyte cells following exposure to AZA-1. Jurkat cells were exposed to AZA-1 for 24, 48, and 72 h prior to photographs being taken. Panels A–C illustrate control cells 24, 48, and 72 h after incubation with equivalent volumes of methanol vehicle, respectively. Panels D–F illustrate cells exposed to 10 nM AZA-1 for 24, 48, and 72 h; respectively. Arrows indicate location of pseudopodia.

insight into the toxicity of AZAs, the specific mode of action for AZA-1 remains unknown. Herein, we have examined several potential modes of action for AZA-1, including protein phosphatase 2A inhibition, including cytotoxic effects a panel of mammalian cell lines, and modifications to the morphology and cytoskeleton of a human T lymphocyte cell line.

In humans and laboratory animals, AZA intoxication is characterized primarily by gastrointestinal symptoms very similar to those reported for okadaic acid and other DSP toxins (McMahon and Silke, 1996, 1998). Nausea, vomiting, severe diarrhoea, and stomach cramps were common in the initial AZP outbreak following consumption of contaminated shellfish. However, DSP toxin- and phytoplankton-analysis revealed only trace levels of okadaic acid and dinophysistoxin-2 (DTX-2) and no known toxic phytoplankton species were detected in the water column (McMahon and Silke, 1998). Additionally, pathophysiological data from orally exposed mice have also suggested gastrointestinal alterations also characteristic of DSP (Ito et al., 2000). Although Flanagan et al. (2001) reported that toxic shellfish extracts containing AZAs did not inhibit serine/threonine protein phosphatase type 1 (PP1), activity against protein phosphatase type 2A (PP2A) could not be ruled out, given its ca. 100-fold greater sensitivity to DSP toxins and the fact that AZA levels were not quantified by

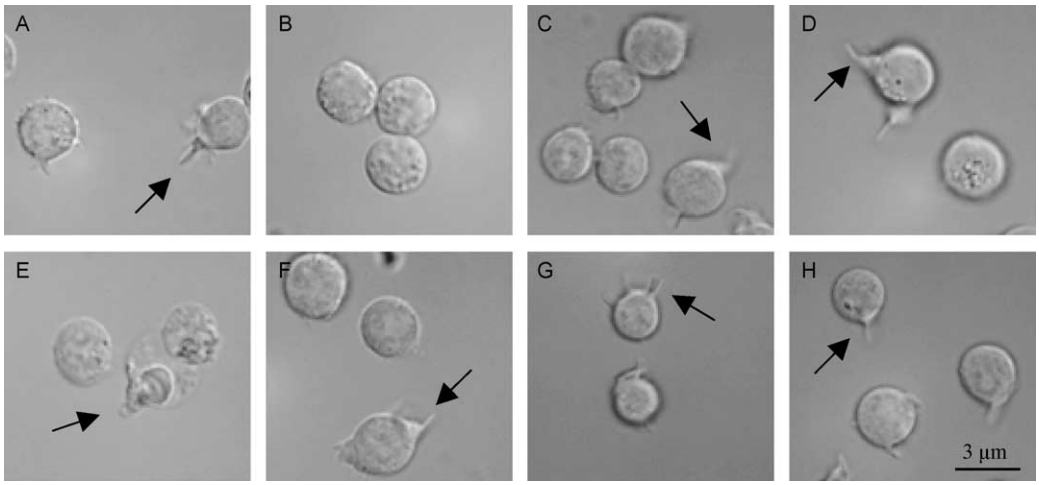

Fig. 4. Photomicrographs of Jurkat T lymphocyte cells following exposure to various phycotoxins. Jurkat cells were exposed to (A) methanol vehicle (1% v/v final), (B) 3 nM AZA-1, (C) 5 nM domoic acid, (D) 2 nM dinophysistoxin-1, (E) 0.7 nM maitotoxin, (F) 2 nM okadaic acid, (G) 1 nM brevetoxin-3, and (H) 2.3 nM saxitoxin for 24 h prior to photographs being taken. Arrows indicate location of pseudopodia.

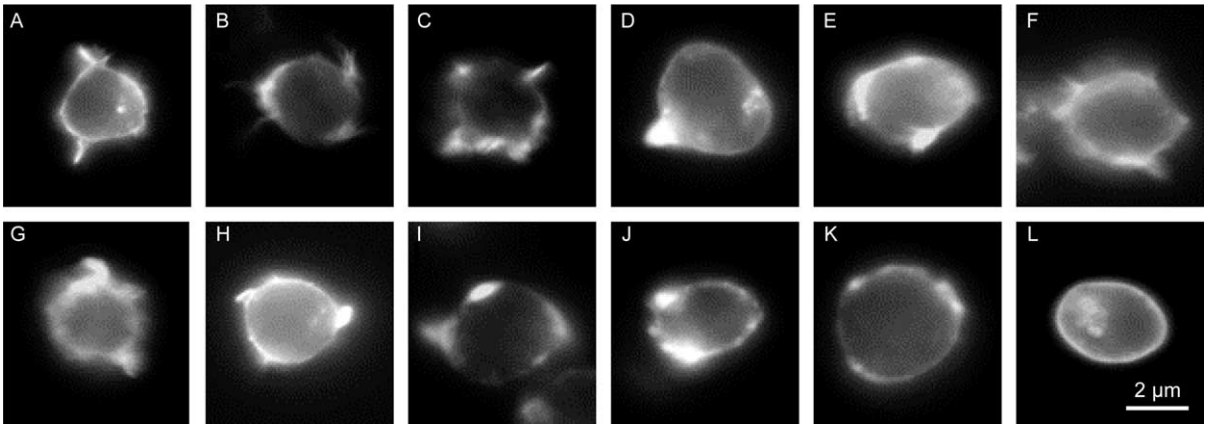

Fig. 5. Fluorescence staining and visualization of F-actin in Jurkat T lymphocyte cells following exposure to AZA-1. Jurkat cells were exposed to methanol vehicle (1% v/v final) for (A) 2 h, (B) 4 h, (C) 8 h, (D) 12 h, (E) 18 h, and (F) 24 h prior to fluorometrically staining visualizing for F-actin using Oregon Green 488 phalloidin. Similarly, cells were exposed to 10 nM AZA-1 for (G) 2 h, (H) 4 h, (I) 8 h, (J) 12 h, (K) 18 h, and (L) 24 h.

Flanagan and co-workers. We have demonstrated that AZA-1 concentrations up to 83 nM, almost two orders of magnitude greater than the cytotoxicity EC_{50} for all cell types tested in this study, have no effect on PP2A activity. Collectively, these results confirm that the AZA-1 mode of action is different from that of the DSP toxins with respect to protein phosphatase inhibition (PP1 and PP2A).

Exposure of mice to AZA can induce a number of effects on a range of tissue types (Ito et al., 2000, 2002). Although the most dramatically affected tissues following oral exposure appear to be those directly exposed to AZA (e.g. the gastrointestinal system), putative distribution of toxin throughout the animal appears to cause effects on other tissues. As an initial approach to evaluating tissue-type AZA-1 sensitivity, seven mammalian cell lines were screened for cytotoxicity. Data from a series of exposures clearly illustrated that AZA-1 is cytotoxic to all cell types tested in a concentration- and time-dependent manner. Although a 24 h exposure of AZA-1 caused significant cytotoxicity in all cell lines except the A549 lung epithelial cells, only the Jurkat T lymphocyte and the GH_4C_1 pituitary cells were sufficiently affected to yield a sigmoidal dose–response curve with a calculated EC_{50}. However, after 48 and 72 h of exposure to AZA-1 levels ≥ 1 nM, all cell types showed markedly reduced viability with concurrent EC_{50} values in the low nanomolar range. Of particular note was the observation that cytotoxicity occurred over a very narrow concentration range (i.e. 48 h Hill slopes ranging from -1.1 to -5.8) such that cytotoxicity became more complete with longer exposure time. EC_{50} values for each of the cells lines did not differ ($p > 0.05$); however, values decreased slightly with longer exposure time. This response is distinctly different from OA, for which EC_{50} values change with increased exposure time (Tubaro et al., 1996).

While the most sensitive cell lines were the Jurkat T lymphocytes and the GH_4C_1 rat pituitary cells, AZA-1 cytotoxicity appeared to be broad-spectrum in action. Interestingly, these data correlate well with in vivo observations, where immune cell types (e.g. T and B lymphocytes) undergo necrosis in tissues such as the spleen (Ito et al., 2000). Moreover, T lymphocyte cells were also used in the in vitro study of Roman et al. (2002) in which it was reported that AZA-1 had marked effects on cytosolic Ca^{2+}, cAMP and F-actin, leading the authors to suggest a non-apoptotic, multiple site mechanism of action. It has been shown that AZA-1 cytotoxicity extends beyond mammalian cell types, as similar concentration- and time-dependent effects have been observed on Sf9 insect cells (M. Twiner, unpublished data).

AZA-1 caused physiological alterations in all cell types tested, but the Jurkat cells were the only cells with overt morphological alterations. A significant number of Jurkat cells showed morphological effects over the 24, 48, and 72 h

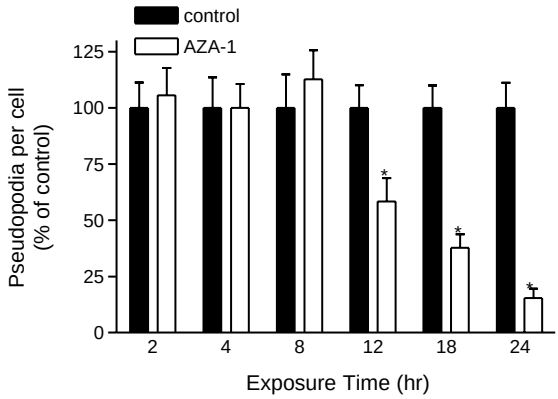

Fig. 6. Effect of AZA-1 on pseudopodia number of Jurkat T lymphocyte cells. Jurkat cells were exposed to methanol vehicle (1% v/v final) or 10 nM AZA-1 for 2, 4, 8, 12, 18, and 24 h and pseudopodia per cell were enumerated for 50 arbitrarily chosen cells. An asterisk (*) denotes a significant difference ($p < 0.05$) between the control and the AZA-1 treatment for a given time point according to Tukey *t*-test.

time courses. Cells typically became rounder and flatter with accentuated internal organelles (i.e. nucleus), displayed changes in membrane permeability as confirmed by increased concentrations of extracellular cytosolic glucose-6-phosphate dehydrogenase (data not shown), and most interestingly, lacked the cytosolic-extending pseudopodia. Pseudopodia are actin-containing appendages that aid in mobility, chemotaxis, and cell-to-cell signaling (Wehrle-Haller and Imhof, 2003). These morphological alterations were time-dependent and were not found to occur in response to several other phycotoxins. While phycotoxins such as maitotoxin induced cell lysis, pseudopodia were still observed on the remaining non-lysed cells and even on some of the remnants of the lysed cells. Neither domoic acid, dinophysistoxin-1, okadaic acid, brevetoxin-3, nor saxitoxin had any effect on the presence or number of pseudopodia in Jurkat cells. Quantitative observations indicated that Jurkat cells have an average of two visible pseudopodia per cell. However, AZA-1 exposures resulted in a time-dependent reduction in cellular pseudopodia whereby after 24 h, these appendages were virtually undetectable. This is a particularly important result in the context of the development of an AZA-specific bioassay. Shellfish samples that are contaminated with AZAs also may contain significant amounts of okadaic acid and/or dinophysistoxin (Hess et al., 2003) and traditional animal assays are non-specific and unable to distinguish between types of toxins present. An assay based on the reduction of cellular pseudopodia could provide an alternative biological method to screen for AZA.

F-actin is a semi-flexible polymer protein that plays a role in all processes that are essential to the function of an activated immune cell, including cell movement, cell shape, cell division, and T cell activation (Samstag et al., 2003). Roman et al. (2002) suggested that F-actin may be the cellular target of AZA-1; however, the toxin concentrations leading to changes in F-actin levels were $\sim$5 μM. Nonetheless, we were able to induce changes over time in F-actin organization within Jurkat cells upon exposure to 10 nM AZA-1. Visualization of this cytoskeletal component in Jurkat cells by epifluorescence microscopy revealed that F-actin is concentrated primarily around the membrane periphery, with particularly high densities in the extended pseudopodia of untreated cells. However, prolonged exposure ($\geq$12 h) to AZA-1 induced contraction of the pseudopodial extensions, concurrent with an apparent spatial reorganization of F-actin within the cells. Such in vivo cytoskeletal changes in lymphocytes may affect cellular functions that are particularly important during an immune response. Alternatively, the reorganization of F-actin may be downstream of the toxic effects of AZA-1. However, if AZA-1 does target ubiquitous cytoskeletal components such as F-actin, in vivo toxicity will not be confined only to lymphocytes but may affect all exposed cell and tissue types following ingestion of the toxin. Our work using a range of mammalian cell lines demonstrates the broad spectrum toxicity of AZA-1, which may explain in part the nearly ubiquitous pathophysiological effects observed by Ito et al. (2000, 2002). However, a direct effect of AZA-1 on cytoskeletal components such as actin, microtubules, or intermediate filaments, and thus the specific mode of action for this toxin, remain to be elucidated.

The broad spectrum of AZA-1 toxicity may have ecological consequences to other aquatic life (e.g. zooplankton, fish, marine mammals, etc.). Although there are no reports of AZA accumulation in non-bivalve marine organisms, exposure of certain species to these toxins may reveal carcinogenic and/or teratogenic potential. A recent study by our group has shown the potential teratogenic effects of AZA-1 on microinjected embryos of Japanese medaka (*Oryzias latipes*) (Colman et al., in press). Doses of $>$40 pg (48 fmol), the equivalent amount of toxin contained in $\sim$26 *Protoperidinium crassipes* cells (James et al., 2003a), caused significant depressions in heart rate, development, hatching success, and survival. Thus, in addition to the well-established public health effects of AZA, trophic transfer of this toxin through aquatic food webs could adversely impact various components of ecosystem structure and function. Our results suggest a possible site of action for a wide variety of mammalian cell types, although further work using cell-based approaches is needed to determine the precise mode of action of AZA-1.

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