

Comparative Effects of the Marine Algal Toxins Azaspiracid-1, -2, and -3 on Jurkat T Lymphocyte Cells

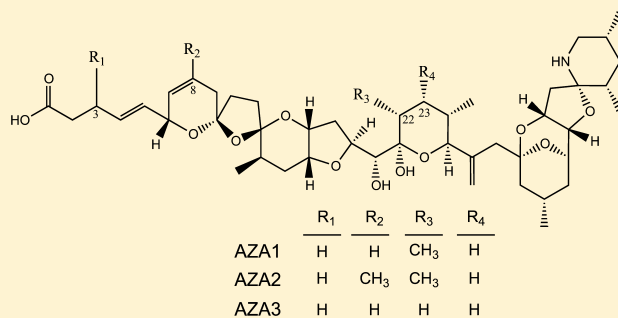
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ABSTRACT: Azaspiracids (AZA) are polyether marine toxins of dinoflagellate origin that accumulate in shellfish and represent an emerging human health risk. Although monitored and regulated in many European and Asian countries, there are no monitoring programs or regulatory requirements in the United States for this toxin group. This did not prove to be a problem until June 2009 when AZAs were identified in US seafood for the first time resulting in human intoxications and further expanding their global distribution. Efforts are now underway in several laboratories to better define the effects and mechanism(s) of action for the AZAs. Our investigations have employed Jurkat T lymphocyte cells as an *in vitro* model to characterize the toxicological effects of AZA1, AZA2, and AZA3. Cytotoxicity experiments employing a metabolically based dye (i.e., MTS) indicated that AZA1, AZA2, and AZA3 each elicited a lethal response that was both concentration- and time-dependent, with EC₅₀ values in the sub- to low nanomolar range. On the basis of EC₅₀ comparisons, the order of potency was as follows: AZA2 > AZA3 > AZA1, with toxic equivalence factors (TEFs) relative to AZA1 of 8.3-fold and 4.5-fold greater for AZA2 and AZA3, respectively. Image analysis of exposed cells using Nomarski differential interference contrast (DIC) imaging and fluorescent imaging of cellular actin indicated that the morphological effects of AZA1 on this cell type are unique relative to the effects of AZA2 and AZA3. Collectively, our data support the growing body of evidence suggesting that natural analogues of AZA are highly potent and that they may have multiple molecular targets.



INTRODUCTION

Azaspiracids (AZAs) are a class of polyether marine toxins produced by the marine dinoflagellate *Azadinium spinosum*.¹ AZA toxins were originally found in shellfish off the coast of Ireland and have caused multiple human intoxication events throughout Europe (reviewed by Furey et al.²) and most recently in the United States.³ Reports of *Azadinium* species and/or AZAs have now been documented for many other parts of the world, including Morocco,⁴ Chile,⁵ and Canada (M. Quilliam, Pers. comm). AZAs were also recently detected in marine sponges.⁶ Human symptoms following intoxication include nausea, vomiting, and stomach cramps,⁷ but thus far, no deaths have been attributed to AZAs.

The AZA group now includes more than 20 analogues that are produced either by phytoplankton, through biotransformation in shellfish, or as a byproduct formed during processing and storage of toxin-containing tissues.^{7–9} Although there is geographical variation, plankton samples are dominated by AZA1 and AZA2 (Figure 1), with the former often constituting over 80% of the toxin profile.^{1,10} The recent isolation and culturing of *A. spinosum* has further confirmed these findings,^{1,11} but interestingly, other nontoxicogenic *Azadinium* species have now been described.¹² Naturally contaminated shellfish often remain above the European regulatory action

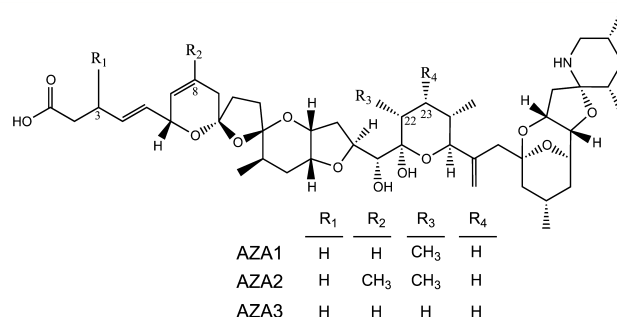


Figure 1. Chemical structures of AZA1, AZA2, and AZA3.

level for many months following exposure to AZAs.^{13,14} Toxin binding proteins may be responsible, in part, for this slow rate of depuration in certain shellfish.^{15,16}

Studies in mice exposed orally to AZA1 revealed severe gastrointestinal tract deterioration and adverse histopathologies of the liver, spleen, and thymus, as well as a small incidence of tumor formation.^{17,18} The minimal lethal doses for AZA1 are ca. 500 μg/kg (oral)¹⁹ and ca. 150 μg/kg (i.p.).²⁰ Relative

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Table 1. Cytotoxicity EC₅₀ Values (nM) and 95% Confidence Intervals for AZA1, AZA2, and AZA3

AZA analogue	24 h		48 h		72 h		mean EC ₅₀	rel. pot.
	EC ₅₀	95% CI	EC ₅₀	95% CI	EC ₅₀	95% CI		
AZA-1	2.1	0.56–8.3	2.7	1.2–6.2	2.6	1.4–4.9	2.5	1.0
AZA-2	0.38	0.10–1.4	0.28	0.15–0.52	0.25	0.14–0.43	0.30	8.3
AZA-3	0.44	0.15–1.3	0.58	0.29–1.2	0.63	0.32–1.2	0.55	4.5

analogue potencies (via i.p.) are as follows: AZA2 > AZA3 > AZA1 > AZA4 > AZA5.^{7,21,22} Note that AZA4 and AZA5 are the 3- and 23-hydroxyl analogues, respectively, of AZA3. Although these experimental data are extremely valuable, their clear interpretation has been hindered due to the use of material of unknown purity. Nonetheless, this information has provided the guidance needed to propose toxic equivalence factors (TEFs) (1.0, 1.8, and 1.4 for AZA1, AZA2, and AZA3, respectively) that are used for determining the total AZA equivalent concentrations in European shellfish.²³ The EU regulatory limit is 0.16 µg/g “total” AZAs in shellfish as detected by LC-MS/MS. Only AZA1–3 are currently regulated.

Multiple studies have demonstrated the *in vitro* cytotoxic potential of AZA1 wherein every cell type tested to date appears to be susceptible (see Twiner et al.⁷ for a review). AZAs are very potent cytotoxic compounds,^{6,24,25} induce irreversible cytoskeletal rearrangements in mammalian cells,^{24,26,27} stimulate secondary messenger molecules,^{28,29} deplete cellular ATP,³⁰ inhibit neuronal ion flux and bioelectrical activity,^{31,32} induce cell volume reductions followed by c-Jun N-terminal kinase (JNK) activation,^{33,34} inhibit membrane protein endocytosis,³⁵ and up-regulate low density lipoprotein receptor as well as stimulating cholesterol biosynthesis.³⁶ AZA1 has also been shown to be a potent teratogen in developing fish embryos.³⁷ In many cases, the toxin concentrations necessary to observe these various effects are in the low nanomolar range,^{7,31,34,35} but for some end points, they approach or exceed micromolar concentrations.^{31,38} Despite the efforts of many investigators, the mechanism(s) of action of AZAs has not yet been determined.

The possible involvement of an apoptotic pathway in AZA1 cytotoxicity is complicated and remains unresolved. Results of an *in vitro* study assessing mitochondrial membrane potential in neuroblastoma cells³⁸ are suggestive of a nonapoptotic pathway; however, up-regulation of caspase activity in neuroblastoma cells³⁹ and neocortical neurons³¹ is supportive of apoptosis activation. Furthermore, apoptotic lymphocyte cells have been observed in the spleens of mice exposed to AZA1.⁴⁰ Nonetheless, such discrepancies may be explained, in part, by AZA1 causing simultaneous induction of both necrotic and apoptotic mechanisms.³¹

Because of difficulties in obtaining sufficient amounts of purified material, much less information is available regarding the toxicological properties of the other natural AZA analogues. At relatively high concentrations (i.e., 50–1000 nM), AZA2, AZA3, AZA4, and AZA5 have been shown to have various effects on intracellular pH, cytosolic calcium, and cAMP in lymphocytes.^{29,41,42} In neocortical neurons, AZA1, AZA2, and AZA3 inhibited spontaneous Ca²⁺ oscillations at moderate concentrations (EC₅₀ values ranged from 138 to 445 nM), with 48 h cytotoxicity EC₅₀ values of 43, 48, and 10 nM, respectively.³¹ This study provided the first side-by-side evidence for potency differences among these regulated toxins.

Clearly, structure–activity relationship (SAR) studies are very important from a regulatory perspective, but to date, have been hindered by the lack of pure material. As such, many of the previous SAR studies have been confined to the study of AZA1 and its synthetic fragments;^{43,44} however, purified AZA2 and AZA3 have recently become available commercially.⁴⁵ This supply of new material enabled the present comparative study in which we characterized the toxicological effects of the currently regulated AZAs (i.e., AZA1, AZA2, and AZA3) in an *in vitro* model system based on the Jurkat T lymphocyte cell line.

MATERIALS AND METHODS

Toxin Isolation. AZA1, AZA2, and AZA3 were isolated from whole cooked mussel tissue (*M. edulis*) collected in 2005 from Bruckless, Donegal, Ireland. The toxins were purified using a 7 step isolation procedure, the details of which have recently been reported.⁴⁶ Toxin purity (>95%) was confirmed by LC-MS/MS, and NMR spectroscopy and this material was used to produce commercially available standards now distributed by the Certified Reference Materials Program at the National Research Council of Canada (Institute for Marine Biosciences, Halifax, NS, Canada). Toxin dilutions prior to cell exposure were performed in phosphate-buffered saline (PBS; pH 7.4)/10% methanol. Toxin/methanol additions never exceeded 1% vol/vol.

Cell Culturing. Human Jurkat E6-1 T lymphocyte cells (American Type Culture Collection TIB-152; Manassas, VA, USA) were grown as described in Twiner et al.²⁴ Briefly, cells were grown in RPMI medium (cat. #11875-093, Invitrogen, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; cat. #26140, Invitrogen, CA, USA) and maintained in a humidified incubator (Sanyo 18AIC-UV) with 5%:95% CO₂/air at 37 °C. Cells were subcultured with fresh medium at an inoculum ratio of 1:4 every 3 to 4 days by transferring 2.5 mL of cells to 7.5 mL of fresh supplemented medium in 75 cm² screw cap culture flasks.

Cytotoxicity Assay. T lymphocytes were diluted in fresh medium to (3–4) × 10⁵ cells/mL and seeded in 96-well plates containing 100 µL per well ((3–4) × 10⁴ cells/well). Cells were allowed to settle for 18–24 h prior to AZA additions to each set of three replicate wells for 24, 48, or 72 h of continuous exposure. The final concentration of AZA1 ranged from 9.48 × 10^{−8} to 1 × 10^{−12} M, and the final concentrations of AZA2 and AZA3 ranged from 1.5 × 10^{−8} to 5 × 10^{−12} M. Vehicle control wells with an equivalent amount of 10% methanol in PBS were used to normalize the viability data. Cellular viability/cytotoxicity was assessed using the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; cat. no. G358A, Promega Biosciences, CA, USA). Absorbance of each well was measured at 485 nm using a Fluostar microplate reader (BMG Technologies, NC, USA). All data (mean ± SE) from 3 or more separate experiments were normalized to a percentage relative to vehicle controls.

Morphological Characteristics. T lymphocytes were diluted in fresh medium to (3–4) × 10⁵ cells/mL and seeded in 24-well plates containing 500 µL per well (1.5–2) × 10⁵ cells/well. Cells were exposed to AZA concentrations approximately twice the EC₅₀ concentration (Table 1 and Figure 2). Final concentrations were 5 × 10^{−9} M AZA1, 5 × 10^{−10} M AZA2, and 1 × 10^{−9} M AZA3. Vehicle control wells contained an equivalent amount of 10% methanol in PBS. Cells were continuously exposed for 24, 48, or 72 h prior to

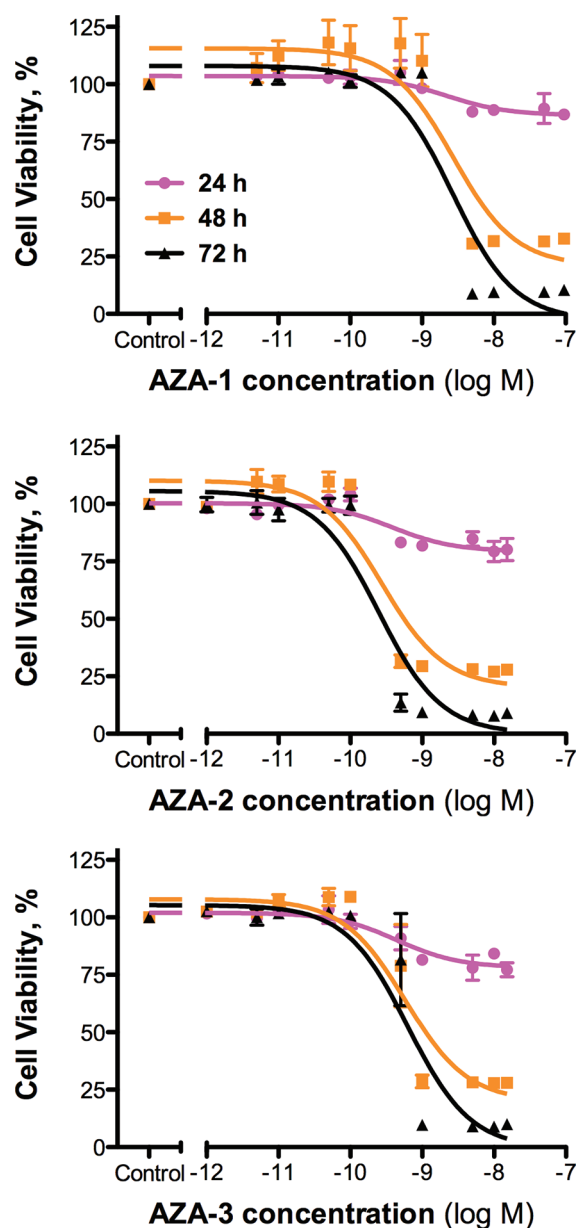


Figure 2. Effect of various AZA analogues on cell viability. Lymphocyte T cells were exposed to AZA1, AZA2, or AZA3 for 24, 48, or 72 h, and viability was assessed using the MTS assay. All data (mean \pm SE; $n \geq 3$) were normalized to the control (methanol vehicle) and analyzed using nonlinear sigmoidal dose–response (variable slope). Calculated EC_{50} values are shown in Table 1.

microscopic examination and imaging. Differential interference contrast (DIC) and epi-fluorescence photomicrographs were taken using an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY, USA). DIC images were taken directly using aliquots of suspended cells. Epi-fluorescence images of intracellular actin were obtained by preparing the cells as described in Twiner et al.²⁴ and staining with Alexa Fluor Phalloidin (cat. # 12379, Invitrogen, CA, USA). Images were captured using a Canon EOS 5D (EF 24–105 L IS USM) camera.

Statistical Analysis. Data are presented as the means \pm SE of at least three separate experiments. In addition, each cytotoxicity experiment was performed using triplicate wells. Cytotoxicity data were blank corrected and normalized to the vehicle control (% viability). EC_{50} and 95% confidence interval determinations were calculated using three parameter, variable slope, nonlinear regression analysis (GraphPad Prism, version 5.0c, San Diego, USA).

RESULTS

Effect of AZA Analogues on Cell Viability. Jurkat T lymphocyte cells were exposed to natural analogues of AZA (i.e., AZA1, AZA2, and AZA3) to assess cellular viability and determine relative potencies. AZAs were tested across a range of concentrations and time points (24, 48, and 72 h). Each AZA analogue induced time- and concentration-dependent cytotoxicity to the lymphocyte cells (Figure 2) but differed in their relative potencies. Cytotoxicity was minimal at 24 h with cell viability ranging from 78%–86% for the three AZA analogues. Longer exposure times of 48 and 72 h for each AZA analogue resulted in a stepwise reduction of viability causing more complete cytotoxicity (20–21% viability at 48 h and 7–12% viability at 72 h) at AZA1 concentrations >1 nM, AZA2 concentrations >0.1 nM, and AZA3 concentrations >0.5 nM. EC_{50} values (including 95% confidence intervals) are presented for each AZA analogue at each time point in Table 1. The mean EC_{50} value for each AZA analogue across all three time points was 2.5 nM for AZA1, 0.30 nM for AZA2, and 0.55 nM for AZA3. As such, AZA2 and AZA3 were 8.3-fold and 4.5-fold more potent than AZA1, respectively (Table 1).

Morphological Changes of Lymphocyte Cells Following Exposure to AZA Analogues. Photomicrographs of control T lymphocyte cells illustrated that they were clearly intact with no signs of lysis and exhibited cytosolic extensions that have been identified as pseudopodia or false feet (Figure 3A–C; see arrows). However, lymphocyte cells exposed to the AZA analogues suggest a variety of cytolytic effects. Cells exposed to 5 nM AZA1 displayed a lack of cellular integrity (particularly at 48 and 72 h) as revealed by lysed cells and debris, organelle protrusion with concurrent flattening of cells, and a distinct lack of pseudopodia (Figure 3D–F). Although cell diameter was significantly ($p < 0.05$) increased at 72 h, this appears to be an artifact related to the flattening of the cells during cytolysis and not due to cellular swelling or blebbing.

Cells treated with AZA2 and AZA3 also showed time-dependent cytolysis with some features (i.e., lack of cellular integrity, organelle protrusion, flattening of cells) in common with AZA1-treated cells (Figure 3G–I and J–L, respectively). However, despite clear cytolysis, pseudopodia were still present in each of these treatment regimes (as indicated by arrows). Apparent increases in cell diameter were also statistically significant ($p < 0.05$) for AZA2 at 72 h and AZA3 at 48 and 72 h (data not shown), but similar to AZA1, these changes are attributed to cell flattening and not actual swelling.

Effects of AZA Analogues on Arrangement of F-Actin. Photomicrographs of fluorescently stained control cells illustrate their spheroid-like shape with irregular, F-actin filled pseudopodial extensions (Figure 4A–C). Upon exposure to AZA1 for 24, 48, or 72 h, cells became more rounded with a loss of pseudopodial extensions (Figure 4D, E, and F, respectively). Lysed or lysing cells were not apparent, likely due to the loss of exuded material or debris during the staining procedure. Cells exposed to AZA2 and AZA3 for 24, 48, or 72 h looked remarkably similar to control cells with respect to the continued presence of pseudopodia (Figure 4G, H, I, and J, K, L, respectively). Similarly, the presence of cells undergoing lysis was not expected due to the staining procedure.

Quantitative Effects of AZA Analogues on Pseudopodia. Jurkat T lymphocyte cells were exposed to AZA1 across a range of concentrations for 48 h prior to enumeration of pseudopodial extensions. AZA1 caused a concentration-

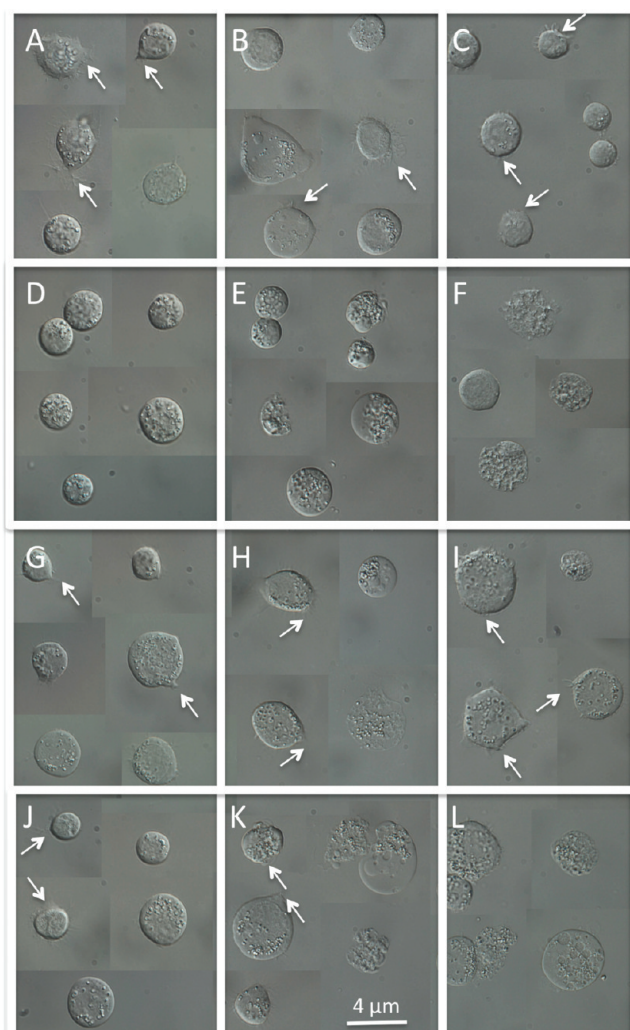


Figure 3. Effect of various AZA analogues on cell morphology. Jurkat T lymphocyte cells were exposed to AZA analogues at ca. twice the EC_{50} concentrations for 24, 48, and 72 h (from left to right) prior to photographs being taken. Panels A–C illustrate control cells exposed to equivalent volumes of methanol, panels D–F illustrate cells exposed to 5 nM AZA1, panels G–I illustrate cells exposed to 0.5 nM AZA2, and panels J–L illustrate cells exposed to 1 nM AZA3. Arrows indicate the location of pseudopodia. Note: Each panel is a composite of various individual DIC images/cells and not a single field of view, thus illustrating a representative range of cell morphologies.

dependent reduction in visible pseudopodia per cell with an IC_{50} value of 0.68 nM (0.54–0.84 nM 95% confidence intervals) (Figure 5). Control cells consistently had visible pseudopodia (mean = 2.1 ± 0.18 ; $n = 50$ cells), whereas cells treated with ≥ 10 nM AZA1 had no discernible pseudopodia. When cells were treated for 48 h with AZA2 or AZA3 under the same conditions outlined in Figures 3 and 4 (0.5 nM AZA2 and 1 nM AZA3), there was an average of 2.2 ± 0.19 and 2.1 ± 0.17 ($n = 50$) visible pseudopodia per cell, respectively (Figure 6).

DISCUSSION

Human AZA shellfish poisonings have now been reported in multiple European countries and in the United States, caused by the import of contaminated Irish shellfish.^{2,3} Although more than 20 AZA analogues have been identified, only three (AZA1, AZA2, and AZA3) are currently thought to be of toxicological concern and as such are regulated in European seafood

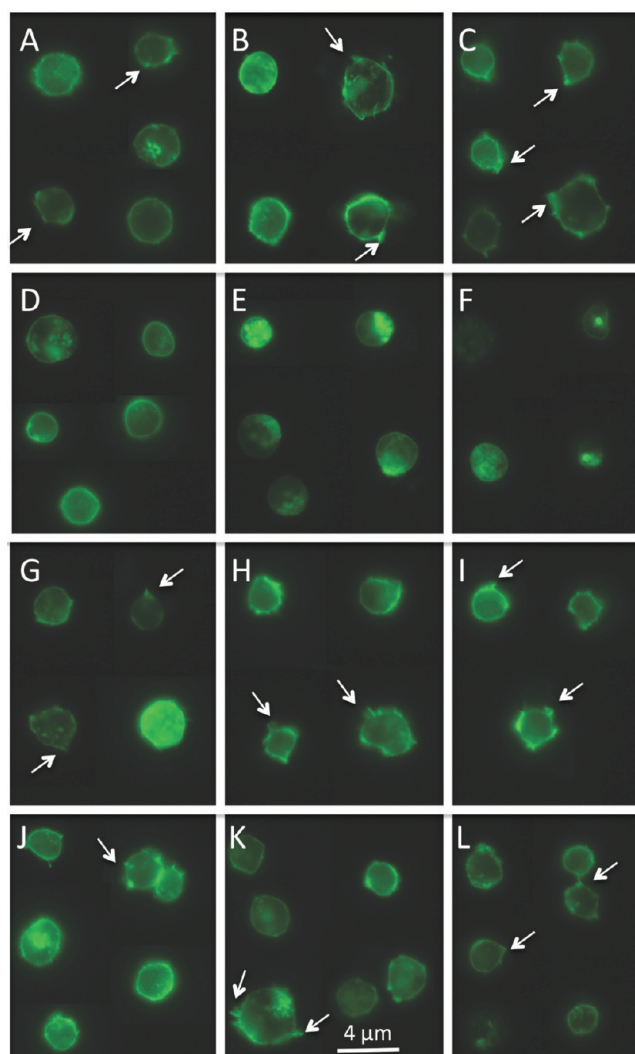


Figure 4. Fluorescence staining and visualization of F-actin in Jurkat T lymphocyte cells following exposure to various AZA analogues. Jurkat T lymphocyte cells were exposed to AZA analogues at ca. twice the EC_{50} concentrations for 24, 48, and 72 h (from left to right) prior to fluorescent staining and visualizing cellular F-actin using Alexa Fluor 488 phalloidin. Panels A–C illustrate control cells exposed to equivalent volumes of methanol, panels D–F illustrate cells exposed to 5 nM AZA1, panels G–I illustrate cells exposed to 0.5 nM AZA2, and panels J–L illustrate cells exposed to 1 nM AZA3. Arrows indicate the location of pseudopodia. Note: Each panel is a composite of various individual epi-fluorescence images/cells and not a single field of view, thus illustrating a representative range of cell morphologies.

products at a maximal level of $0.16 \mu\text{g AZA}_{\text{total}}/\text{g}$ shellfish meat.²³ The determination of $\text{AZA}_{\text{total}}$ (i.e., AZA1, AZA2, and AZA3) is primarily performed via LC-MS/MS quantification and the application of toxic equivalence factors (TEFs) to account for potency differences between the AZA analogues. On the basis of mouse intraperitoneal injections, TEFs of 1.0, 1.8, and 1.4 are currently employed for AZA1, AZA2, and AZA3, respectively,^{21,47} thereby implying that the *in vivo* order of potency is $\text{AZA2} > \text{AZA3} > \text{AZA1}$. Our focus herein was to compare these *in vivo* findings with *in vitro* cytotoxicity-based data, as well as assess the morphological effects of AZAs in a T lymphocyte model cell line as a means to better understand their potential toxic effects.

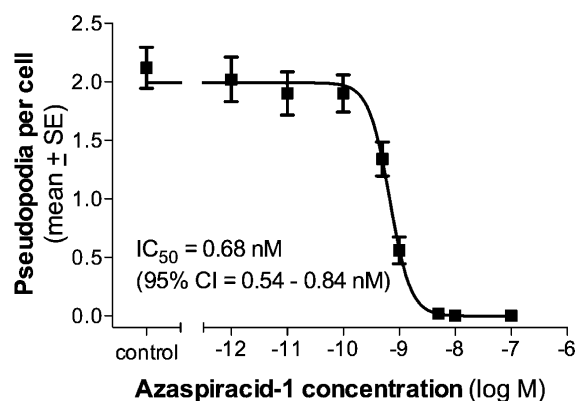


Figure 5. Effect of AZA1 on the abundance of pseudopodia. Lymphocyte T cells were exposed to various concentrations of AZA1 for 48 h, and the number of pseudopodia per cell were counted using DIC microscopy. All data (mean \pm SE; n = 50 individual cells) were analyzed using nonlinear sigmoidal dose–response (variable slope).

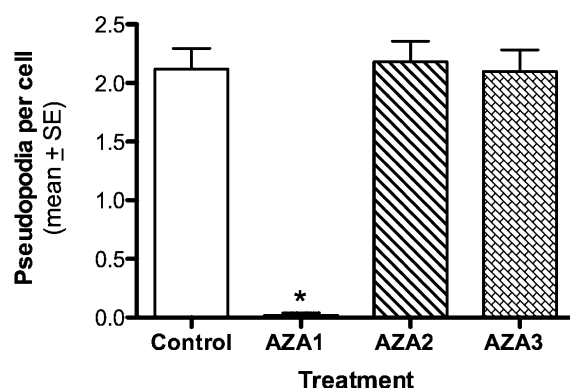


Figure 6. Effects of various AZA analogues on the abundance of pseudopodia. Lymphocyte T cells were exposed to AZA1 (5 nM), AZA2 (0.5 nM), or AZA3 (1 nM) for 48 h, and the number of pseudopodia per cell were counted using DIC microscopy. Data are illustrated as the mean \pm SE (n = 50 individual cells), and an asterisk (*) indicates a significant difference from the vehicle control (p < 0.05).

The cytotoxic potential of AZA1 toward various cell types is well established and has been unambiguously demonstrated using microscopy techniques,^{24,27,34,39} cellular protein content,²⁷ DNA content and synthesis,^{27,48} release of cytosolic enzymes,^{31,36} and mitochondrial activity.^{24,31} Within the current study, the cytotoxic potential of AZA1, as well as AZA2 and AZA3, was confirmed using human Jurkat T lymphocytes as the model cell line, selected based on previous work demonstrating its high level of sensitivity to AZA1.²⁸ Each analogue induced cytotoxicity in a time- and concentration-dependent manner, exhibiting EC_{50} concentrations in the sub-to low-nanomolar (0.25–2.7 nM) range, which are remarkably lower (i.e., more potent) than the EC_{50} values for these same AZA analogues tested against murine neocortical neurons (42.7, 48.0, and 9.88 nM for AZA1, AZA2, and AZA3, respectively)³¹ but very similar to AZA2 studies using murine lymphoblast cells (EC_{50} = 0.84 nM).⁶

Consistent with previous AZA1 studies, increased exposure time to the AZA analogues did affect cell viability, but it did not change the calculated EC_{50} values for time points up to 72 h.²⁴ On the basis of EC_{50} values for each analogue, AZA2 and AZA3

are 8.3-fold and 4.5-fold more potent than AZA1, suggesting the order of *in vitro* potency as AZA2 > AZA3 > AZA1. This order is consistent with the corresponding *in vivo* results reported by Satake et al.⁴⁷ and Ofuji et al.²¹ (based on minimal lethal concentrations) that have been implemented by the EU for regulatory purposes.²³ However, the TEFs estimated based on these *in vivo* studies²³ suggest less drastic potency differences between these three AZA analogues as compared to the *in vitro* data. In contrast, the relative cytotoxic potencies of these same AZA analogues differ when assessed in neocortical neurons (i.e., AZA3 > AZA2 \sim AZA1) by cytotoxicity (via LDH release) and calcium ion oscillation suppression³¹ (i.e., AZA3 > AZA2 > AZA1), further supporting cell type differences in sensitivity. As such, methylation at C8 (AZA2) confers the highest degree of cytotoxic potency in T lymphocytes, whereas demethylation at C22 (AZA3) confers the highest degree of potency in neocortical neurons, suggesting that structural changes in analogues yield variable toxicities in different cell lines.

An interesting and rather unusual observation compared to other phycotoxin classes is that the various AZA analogues showing structural differences at the C3, C8, C22, and/or the C23 positions elicit different cellular effects and in turn may be affecting more than one molecular target. This was suspected early on by the Botana group when studying secondary messenger systems^{28,29,38,41} and also reported more recently by the Murray group when they observed a discrepancy between the effects of AZA1 and fragments of AZA1 on cytotoxicity and spontaneous calcium ion oscillations.³¹ We have documented previously the total retraction of pseudopodia in T lymphocyte cells caused by AZA1 in a time-dependent manner that was specific to AZA1 but not elicited by a wide variety of other marine phycotoxins.²⁴ In the present study, AZA1 caused a similar retraction of pseudopodia in a concentration-dependent manner with no observed effect on ruffle formation. On the contrary, exposure of T lymphocyte cells to AZA2 and AZA3 had no effect on morphological characteristics or number of pseudopodia. The mechanisms involved in pseudopodia assembly are not completely understood, but they are known to involve nucleation of actin filaments by nucleation factors such as formin proteins.⁴⁹ Despite evidence that AZA1 does not directly alter actin polymerization and/or depolymerization,⁷ actin nucleation factors such as formin FRL2 have been shown to be directly involved in the formation and length of pseudopodia in Jurkat T lymphocyte cells⁵⁰ and may be affected by AZA1. The functional relevance of pseudopodia inhibition by AZA1 has not yet been examined but may compromise immune function in organisms exposed to AZAs.

With the possibility of multiple targets, discerning the structure–activity relationships (SAR) of AZAs becomes very complex. Although varying in toxic potency, AZA1, AZA2, AZA3, AZA4, and AZA5 are lethal to mice following i.p. injection,^{21,22,47} and AZA1, AZA2, and AZA3 have been shown to be highly cytotoxic *in vitro*.^{6,24,25,31} Prior to the recent availability of other natural AZA analogues, SAR studies have benefited tremendously from the *in silico* organic synthesis of AZA1⁴⁴ and the use of various fragments produced during this process. AZA1 fragments containing the ABCD/ABCDE rings yielded increased cytosolic calcium ion concentrations without affecting viability in murine cerebellar granule cells (CGCs).²⁵ These fragments also did not affect the growth of a lung carcinoma cell line, nor the morphology and cytoskeletal features of a neuroblastoma cell line.²⁷ The same ABCD/

ABCDE fragments tested against neocortical neurons did not elicit any effects on calcium ion oscillations or cellular viability.³¹ SAR studies using the FGHI rings revealed no effects on cytosolic calcium ion concentrations or viability in CGCs,²⁵ yet inhibited calcium ion oscillations and reduced cellular viability in neocortical neurons.³¹ The relative potencies were particularly high for the FGHI rings when attached to a phenyl glycine methyl ester moiety, a functional component of the natural AZA1, AZA2, and AZA3 analogues. On the contrary, the same set of fragments provided by Nicolaou et al. and applied *in vivo* suggested that the full AZA structure, with its correct stereochemistry, is necessary to induce lethality at levels similar to the natural analogue.⁴⁰

Many of these differences in SAR observations may be due to the respective model cell lines/organisms employed, coupled with the possibility of multiple molecular targets among the AZA toxin class. The existence of distinct molecular targets may also help explain the simultaneous appearance of cellular markers for both apoptosis and necrosis across a variety of cell lines^{7,31,38,39} and following *in vivo* exposure.^{17,18}

CONCLUSIONS

Our data provide evidence that AZA1, AZA2, and AZA3 are highly cytotoxic to T lymphocytes and that there are significant differences in their relative potencies that correlate well with *in vivo* studies. Furthermore, specific cellular effects such as pseudopodial retraction are not conserved across all analogues within this toxin class but thus far are only observed for AZA1. Collectively, our data support the growing body of evidence suggesting that there may be several molecular targets for the AZAs, and it is likely that these are differentially affected by the various AZA analogues.

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Notes

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ABBREVIATIONS

AZA, azaspiracid; CGC, cerebellar granule cells; DIC, differential interference contrast; EC₅₀, 50% maximal effective concentration; i.p., intraperitoneal; LDH, lactate dehydrogenase; SAR, structure–activity relationship; TEF, toxic equivalence factor

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