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Effects of Azaspiracid-1, A Potent Cytotoxic Agent, on Primary Neuronal Cultures. A Structure−Activity Relationship Study

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Azaspiracids (AZAs) are marine phycotoxins with an unknown mechanism of action, implicated in human intoxications. We investigated the effect of azaspiracid-1 (AZA-1) on the cytosolic calcium concentration ($[Ca^{2+}]_c$), intracellular pH (pH_i), and neuron viability in neuronal cultures. AZA-1 increased $[Ca^{2+}]_c$ and decreased neuronal viability. The effects of several fragments of the AZA-1 molecule (13 different chemical structures) were examined. The *ent*-ABCD-azaspiracid-1 (**2**) showed similar potency to AZA-1 (**1**) in increasing $[Ca^{2+}]_c$ but higher cytotoxity than AZA-1. The chemical structures containing only the ABCD or the ABCDE ring domains (**3**−**8**) caused a $[Ca^{2+}]_c$ increase but did not alter cell viability. The compounds containing only the FGHI ring domain of AZA-1 (**9**−**14**) did not modify the $[Ca^{2+}]_c$ or the cell viability. Therefore, the effect of AZA-1 on $[Ca^{2+}]_c$ depends on the presence of the ABCD or the ABCDE-ring structure, but the complete chemical structure is needed to produce neurotoxic effects.

Introduction

Phycotoxins constitute a rich source of active pharmacological tools, with a wide range of mechanisms of action. One of the recently isolated marine biotoxins that has created great concern with regard to seafood poisoning and human health is azaspiracid-1 and its congeners.[1] Marine phycotoxins belonging to the azaspiracid family were first identified in The Netherlands in 1995 following cases of shellfish intoxication after consumption of mussels cultivated in Killary Harbor, Ireland.[1,2] Cases of shellfish intoxications associated with azaspiracids were recently reported in several European coastal countries, including Ireland, UK, Norway, Netherlands, France, Spain, and Italy.[1−6] The discovery of azaspiracids and its recognized health hazard led to the declaration of a new toxic syndrome, named azaspiracid poisoning (AZP). Eleven different members of the azaspiracid family of compounds have been described. AZA-1 (azaspiracid), AZA-2 (8-methylazaspiracid), and AZA-3 (22-demethylazaspiracid) are the predominant azaspiracids in nature. Other azaspiracids (i.e., AZA-4 through AZA-11) differ by the presence or lack of methyl and hydroxyl groups on the azaspiracid structure.[2,7,8]

The toxic episodes caused by AZAs show gastrointestinal illnesses. Following human consumption of AZA-contaminated shellfish, there is generally a rapid onset of symptoms very similar to those of diarrheic shellfish poisoning, including nausea, vomiting, severe diarrhea, and stomach cramps.[9] In mice and rats, intraperitoneal injections induce neurological symptoms resembling paralytic shellfish poisoning with progressive paralysis, fatigue, difficulty breathing, and subsequent death as soon as 35 min after injection.[3,10]

The AZAs are characterized by unusual structural motifs including spiro-ring assemblies, a secondary amino group, and a carboxylic acid moiety, making them unique within the nitrogen-containing marine toxins. So far, there is no information about the cellular target, the mechanism of action, or the toxicity in nervous tissues of the AZAs. Previous studies in our laboratory have focused on the effects of AZA-1 on neuroblastoma cells and human lymphocytes. This previous work suggested that the toxin reduces cellular F-actin in a nonapoptotic manner.[11] A high cytotoxicity of AZA-1 has been recently shown in several cell lines,[12] and even more recently it has been described that this toxin inhibits the electrical activity of neuronal networks;[13] however the mechanisms of action of these toxins are still unknown. Because these toxins are a serious threat to human health and could offer a new therapeutic strategy to modify the function of neuronal systems, we decided to explore the possible effects of their action on nervous tissue as well as investigate the parts of the molecule that could account for this effect. Primary cultures of cerebellar granule cells (CGC) were used as the cellular model, since these cells constitute one of the most reliable models for the study of neural function and pathology.[14,15] Cytosolic calcium concentration ($[Ca^{2+}]_c$) and intracellular pH (pH_i) are two of the primary events in the cellular response to external stimuli used to study the mechanism of action and the toxicological effects of toxins. Therefore, in this work we studied the effects of AZA-1 on the $[Ca^{2+}]_c$, intracellular pH and neuronal viability. In addition, the effects of the different domains of the AZA-1 molecule were also examined (see Figure 1) in order to elucidate the active part of the AZA-1 structure.

Results and Discussion

The AZA-1 structure (**1**), as well as the truncated fragments (**2**−**14**) of the molecule examined in this study, is shown in Figure 1. The chemical structure of the natural azaspiracid analogues is summarized in Scheme 1.

Effects of AZA-1 (1) on $[Ca^{2+}]_c$ and pH_i in Neuronal Cerebellar Granule Cells. Previous studies in our laboratory had shown that natural AZA-1 (**1**) increased the $[Ca^{2+}]_c$ without modifying pH_i levels in human lymphocytes.[11] The effects of

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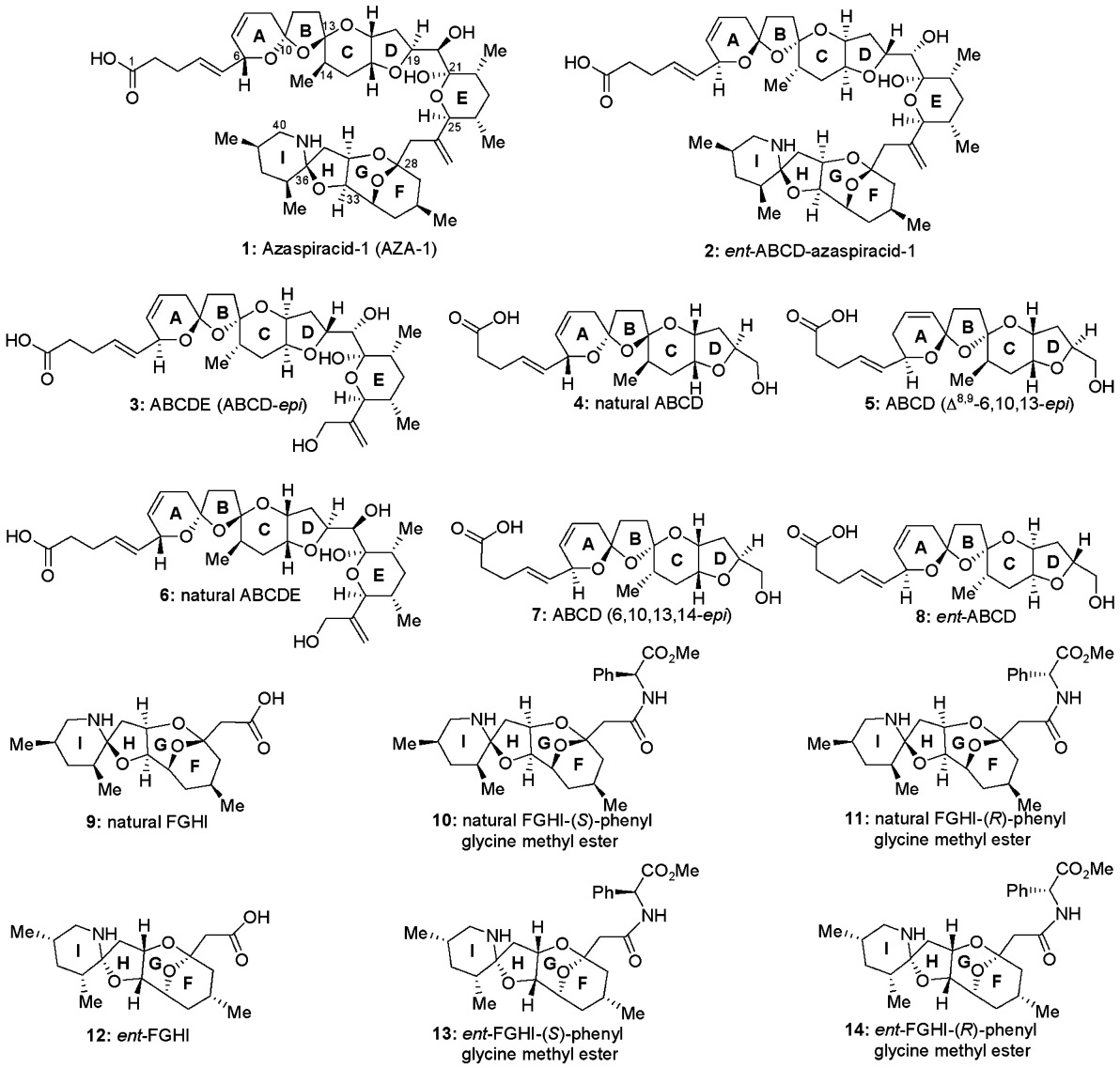
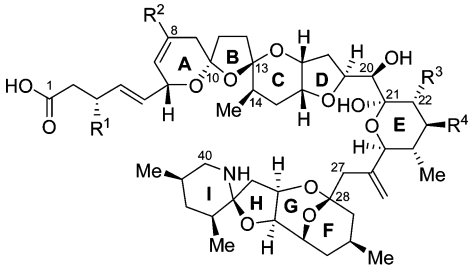


Figure 1. Chemical structure of azaspiracid-1 (AZA-1, **1**) and related structures (**2**−**14**) studied.

Scheme 1. Chemical Structures of Natural Azaspiracids 1−5



	R^1	R^2	R^3	R^4
1: azaspiracid-1	H	H	Me	H
2: azaspiracid-2	H	Me	Me	H
3: azaspiracid-3	H	H	H	H
4: azaspiracid-4	OH	H	H	H
5: azaspiracid-5	H	H	H	OH

AZA-1 (**1**) on the basal state of $[Ca^{2+}]_c$ and pH_i of primary cultures of CGC were studied. In cells incubated in a calcium-free solution and later with the calcium concentration restored, different concentrations of AZA-1 (**1**) induced a concentration-dependent rise in $[Ca^{2+}]_c$ (Figure 2A). The calcium increase caused by AZA-1 (**1**) was already significant ($p < 0.05$) at the lower concentration studied (0.25 μM). As shown in Figure 2A, AZA-1 (**1**) at concentrations from 0.25 to 3 μM did not increase $[Ca^{2+}]_c$ in a calcium-free medium, whereas it caused a modest calcium increase when calcium was restored to the bathing medium. This indicates that in cerebellar neurons AZA-1 (**1**) does not produce release of calcium from internal stores but it causes calcium influx. Under these conditions, AZA-1 (**1**) at 1 μM decreased the intracellular pH by about 0.15 units in a calcium-free medium; however, this effect was not significant. A similar tendency was observed at AZA-1 (**1**) concentrations of 3 μM, but again this effect did not reach statistical significance (Figure 2B). The intracellular acidification caused by AZA-1 (**1**) was not modified after restoring calcium to the bathing medium. No effect on pH_i was observed at AZA-1 (**1**) concentrations of 0.25 and 0.5 μM (data not shown).

For the rest of the calcium and pH_i determinations, we selected the 1 μM concentration because this concentration is enough to produce a significant rise ($p < 0.05$) in the cytosolic calcium concentration. When AZA-1 (**1**) at 1 μM is added to the cells in a calcium-containing medium (Figure 2C), a significant ($p < 0.01$) increase in $[Ca^{2+}]_c$ is observed. This effect

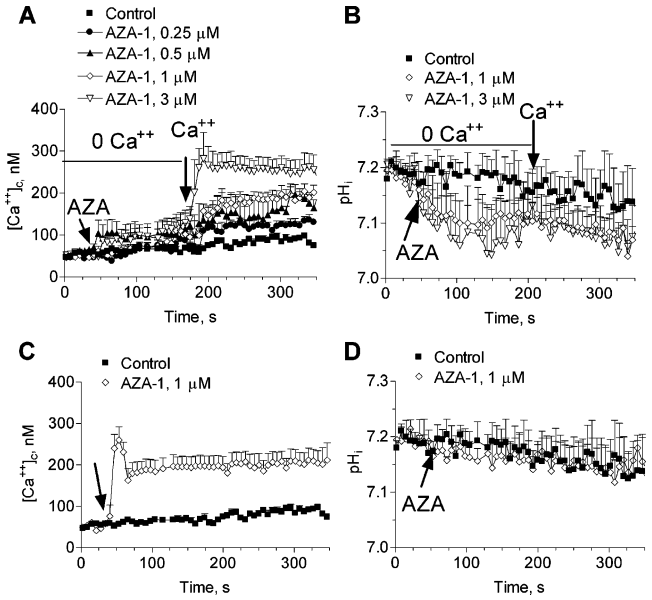


Figure 2. Effect of AZA-1 (**1**) on the cytosolic calcium concentration and intracellular pH of primary cultures of cerebellar granule cells. (A) Effect of different AZA-1 (**1**) concentrations on the cytosolic calcium concentration in a calcium-free medium and after reestablishment of calcium to the extracellular medium. (B) Effect of different AZA-1 (**1**) concentrations on intracellular pH in the absence of external calcium and after reestablishment of calcium to the bathing medium. (C) Effect of AZA-1 (**1**), at 1 μM, on the cytosolic calcium concentration in a calcium-containing medium. Addition of AZA-1 is indicated by the arrow. (D) Effect of AZA-1 (**1**), at 1 μM, on the pH_i of CGC when added in a calcium-containing medium. Values are means ± SEM of three to four independent experiments, each performed in duplicate.

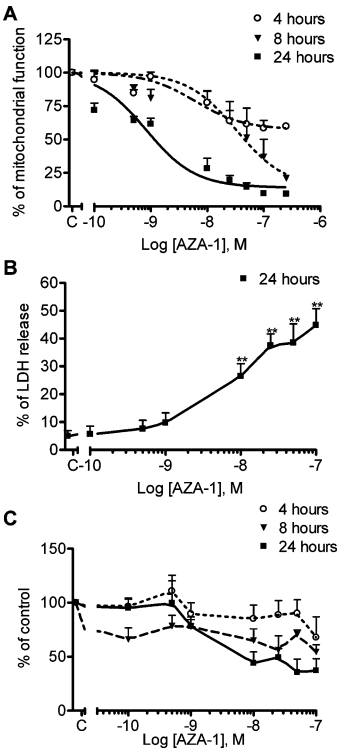


Figure 3. Effect of AZA-1 (**1**) on neuronal viability as determined with the MTT and LDH assays and effect on the F-actin cytoskeleton. (A) AZA-1 (**1**) decreased cerebellar granule cells viability in a time- and concentration-dependent manner as assessed with the MTT test. (B) Effect of different AZA-1 (**1**) concentrations on LDH leakage. (C) Effect of different AZA-1 (**1**) concentrations and exposure times on the actin cytoskeleton of CGC. Values represent means ± SEM of 3−10 experiments.

was immediate upon addition of AZA-1 (**1**) to the medium. No effect of AZA-1 (**1**) on pH_i was detected (Figure 2D).

Effects of AZA-1 (1) on Neuronal Viability and Actin Cytoskeleton. In the mouse bioassay, AZA-1 (**1**) shows clear neurotoxic symptoms that include respiratory difficulties, spasms, paralysis of the limbs, and death.[1,3,10] Although previous work in our laboratory using undifferentiated human neuroblastoma cells ruled out a possible neurotoxic effect of AZA-1 (**1**), the cytotoxic potential of this molecule in primary neuronal cultures has not yet been evaluated. The cytotoxicity of AZA-1 (**1**) was evaluated using the vital staining of the cells by MTT. This assay measures the functionality of the cell mitochondria, which correlates with cell viability. The cytotoxic effect of AZA-1 (**1**) in cerebellar granule neurons after different exposure periods to 0.5−250 nM AZA-1 (**1**) showed that AZA-1 cytotoxicity in this neuronal preparation is dependent on time as well as concentration (Figure 3A). Exposure of cerebellar neurons to AZA-1 (**1**) for 24 h in the culture medium decreased cell viability by 85% with an IC_{50} (95% confidence intervals) of 0.87 nM (0.4−1.7 nM). Treatment of cerebellar neurons with different AZA-1 (**1**) concentrations over 8 h decreased cell viability also by 90% with an IC_{50} (95% confidence intervals) of 32 nM (12−83 nM), whereas exposure of cerebellar neurons to AZA-1 (**1**) for 4 h decreased cell viability by 40% at the highest concentrations tested (100 and 250 nM). Likewise, exposure of cerebellar neurons to AZA-1 (**1**) for 24 h, resulted in an alteration of the membrane integrity as evidenced with the LDH release assay. Concentrations of AZA-1 (**1**) of 10 nM or higher caused a significant ($p < 0.01$) LDH leakage (Figure 3B) after treatment periods of 24 h.

Since previous work has indicated that the actin cytoskeleton is a possible target for the action of AZA-1 (**1**), we investigated the effect of this toxin on F-actin pools. Figure 3C shows that at 100 nM and after 4 h of exposure the toxin altered F-actin levels only at concentrations of 100 nM ($p < 0.05$). A significant effect was also observed after 8 h of exposure at AZA-1 (**1**) at concentrations of 0.5 nM ($p < 0.05$) or higher ($p < 0.01$). A similar effect of AZA-1 (**1**) on F-actin depolymerization was observed after exposure times of 24 h.

Effects of *ent*-ABCD-azaspiracid-1 (2) on $[Ca^{2+}]_c$, pH_i, Neuronal Viability and Actin Cytoskeleton. *ent*-ABCD-azaspiracid-1 (**2**), a stereoisomer of AZA-1 (**1**), increased the $[Ca^{2+}]_c$ with a potency similar to that of AZA-1 (**1**). At 1 μM, compound **2** did not modify the basal calcium level in a calcium-free medium; however, it caused a significant ($p < 0.01$) increase in the $[Ca^{2+}]_c$ of about 150 nM when calcium was restored to the bathing medium (Figure 4A). The effect of compound **2** on calcium was immediate after addition of calcium to the bathing medium and reached a plateau at about 200 nM that lasted until the end of the recording. As shown in Figure 4B, compound **2** showed a small tendency to acidify cerebellar neurons in a calcium-free medium. Although the acidification caused by compound **2** was not significant in the absence of calcium in the extracellular medium, in the presence of extracellur calcium, compound **2** produced a significant ($p < 0.01$) intracellular acidification of about 0.2 units. This observation could indicate that the stereochemical orientation of the ABCD domain could influence pH_i in neuronal cells.

Figure 5A shows that compound **2** has a higher cytotoxic potency than AZA-1 (**1**) as measured by the MTT test. Exposure of cerebellar neurons to compound **2** for 24 h in the culture medium decreased cell viability by 90% with an IC_{50} (95% confidence intervals) of 1.1 nM (0.6−2.15 nM). Treatment of cerebellar neurons with different concentrations of compound

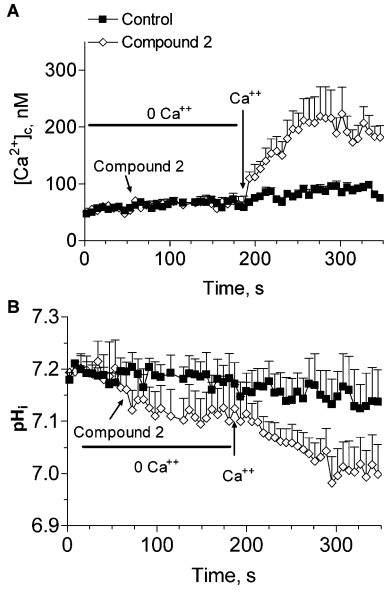


Figure 4. Effect of compound **2**, at 1 μM, on the cytosolic calcium concentration and intracellular pH of primary cultures of cerebellar granule cells. (A) Effect of compound **2** at 1 μM on the cytosolic calcium concentration in a calcium free-medium and after the addition of calcium to the bathing medium. (B) Effect on intracellular pH. Compound **2** did not modify the pH_i of CGC in the absence of calcium in the external medium; however, it caused a small acidification of CGC when calcium was added to the external medium. Values are means ± SEM of four independent experiments, each performed in duplicate.

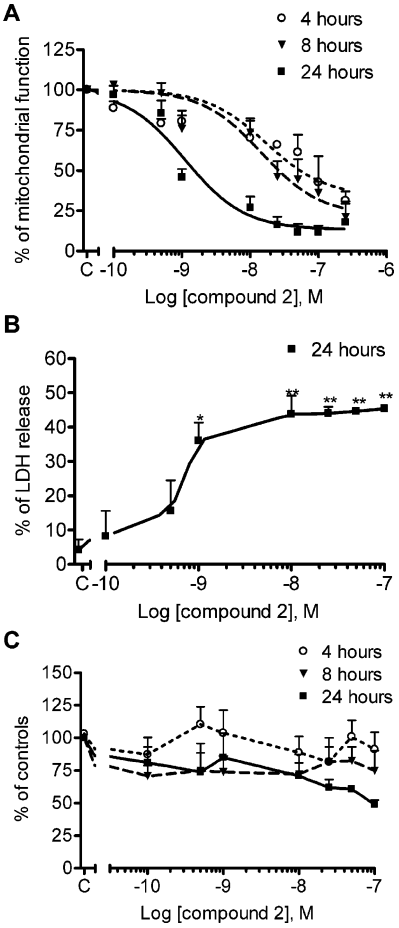


Figure 5. Effect of compound **2** on the viability of cerebellar granule cells as assessed with the MTT and LDH test and on the F-actin cytoskeleton. (A) Compound **2** decreased cerebellar granule cells viability in a time- and concentration-dependent manner as assessed with the MTT test. (B) Effect of different concentrations of compound **2** on membrane integrity as evaluated with the LDH release assay. (C) Effect of different concentrations of compound **2** on the actin cytoskeleton of CGC. Values represent means ± SEM of 4−10 determinations.

2 during 8 h decreased cell viability by about 80% with an IC_{50} of 14 nM (5.25−40.77 nM); however, treatment of neurons with compound **2** during only 4 h also decreased cell viability by 70%, with an IC_{50} (95% confidence intervals) of 17 nM (3.2−90 nM). Likewise, Figure 5B shows that exposure of neuronal cultures to compound **2** for 24 h caused concentration-dependent damage to the cell membrane, with this effect being significant at concentrations of 1 nM ($p < 0.05$) or higher ($p < 0.01$). In contrast, no significant effects of compound **2** on F-actin cytoskeleton were observed after 4 and 8 h treatment of cerebellar neurons with different concentrations of compound **2** (Figure 5C). As shown in this figure, compound **2** decreased F-actin polymerization only at concentrations of 10 nM ($p < 0.05$) and higher ($p < 0.01$) after exposure times of 24 h, conditions in which membrane integrity and neuronal viability are largely affected.

Effects of the ABCD and ABCDE Domains on the Cytosolic Calcium Concentration, Intracellular pH, and Neuronal Viability. Compound **3** [ABCDE(ABCD-*epi*)], consisting of the epimeric ABCD rings of AZA-1 (**1**) along with the correct E ring, did not influence the cytosolic calcium levels in a calcium-free medium; however, it increased ($p < 0.01$) the $[Ca^{2+}]_c$ immediately from 50 nM to about 250 nM after addition of calcium to the bathing medium (Figure 6A). However compound **3** did not modify the intracellular pH levels in the absence or in the presence of calcium in the bathing medium (data not shown).

Compound **4** (Natural ABCD), consisting of the correct ABCD domain of AZA-1, did not alter the cytosolic calcium levels in a calcium-free medium, but it increased the $[Ca^{2+}]_c$ by about 100 nM ($p < 0.05$) after reestablishment of calcium in the external medium (Figure 6B). The modest calcium increase produced by compound **4** reverted very rapidly to values similar to control values. These results suggest that small fragments of the AZA-1 structure are not sufficient for complete effects on the intracellular calcium concentration. Also, compound **4** did not modify the intracellular pH levels (data not shown).

As occurred with the other compounds tested, compound **5** [ABCD($\Delta^{8,9}$-6,10,13-*epi*)], consisting of the ABCD domain with the internal double bond at carbons 8 and 9 and epimeric at carbons 6, 10, and 13, did not modify the resting calcium levels in the absence of external calcium but increased the cytosolic calcium levels by about 130 nM in the presence of calcium in the bathing medium (Figure 6C). In the presence of calcium, compound **5** caused an immediate rise in the cytosolic calcium concentration, which reverted very rapidly to values similar to control values. However, this compound caused a small acidification of cerebellar neurons, but this effect reached statistical significance ($p < 0.05$) only in the presence of calcium in the external medium (data not shown).

As shown in Figure 6D, compound **6** (natural ABCDE), consisting of the correct ABCDE domain, did not significantly modify the basal calcium levels in a calcium-free medium. However, it significantly ($p < 0.05$) increased the $[Ca^{2+}]_c$ after addition of calcium to the external medium. The calcium increase caused by compound **6** was very rapid after addition of calcium and reached a stable plateau level of about 200 nM that lasted until the end of the recording. As in the case of its

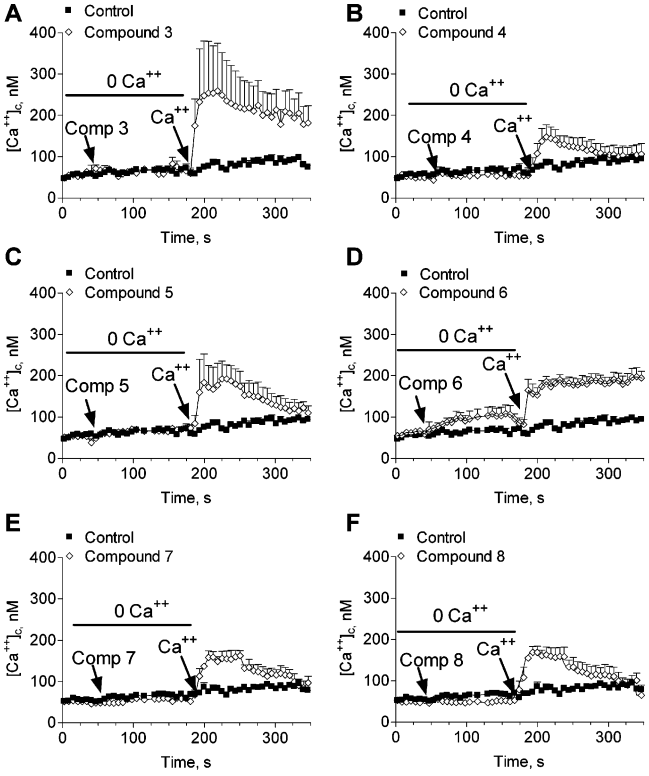


Figure 6. Effect of structures containing the ABCD or ABCDE ring domains of the AZA-1 molecule on the cytosolic calcium concentration of primary cultures of cerebellar granule cells. Effects of compounds **3**, **4**, **5**, **6**, **7**, and **8**, at 1 μM are shown in panels A, B, C, D, E, and F, respectively. Values represent means ± SEM of three or four independent determinations, each performed in duplicate.

stereoisomer, compound **3**, the intracellular pH of cerebellar granule cells was not modified in the presence of compound **6**, neither in the absence nor in the presence of calcium in the bathing medium (data not shown).

Compound **7** [ABCD(6,10,13,14-*epi*)], the epimeric ABCD domain at carbons 6, 10, 13, and 14, showed a similar behavior to its stereoisomer compound **4**. Figure 6E shows that at 1 μM, compound **7** increased the cytosolic calcium levels in cerebellar granule cells to 150 nM ($p < 0.01$). This effect was immediate after addition of calcium; however, the cytosolic calcium rise caused by compound **7** decreased very rapidly, reaching the resting calcium level of control cells before the end of the recording. This compound did not affect the intracellular pH in the absence of calcium. In the presence of external calcium, compound **7** showed a small tendency to acidify cerebellar granule cells, although this effect did not reach statistical significance (data not shown).

Compound **8** (*ent*-ABCD), the enantiomeric ABCD domain, caused a significant increase ($p < 0.01$) in the cytosolic calcium concentration in cerebellar granule cells only in the presence of calcium in the medium (Figure 6F). The effect of compound **8** was very fast after addition of calcium, reaching a peak of 150 nM calcium immediately after addition of calcium to the neurons; however, the $[Ca^{2+}]_c$ returned to basal levels before the end of the recording. Compound **8** did not modify the intracellular pH of cerebellar granule cells (data not shown).

The effects of compounds **3**−**8** on cerebellar granule cell viability, as evaluated with the MTT test, are summarized in Table 1. In spite of the modest calcium increase caused by compounds **3**−**8**, none of these compounds modified cerebellar granule cell viability, indicating that the effects of these compounds on the cytosolic calcium concentration are not related to their neurotoxicity.

Table 1. Effect of 24-h Exposure to 250 nM of Azaspiracid Compounds on Cell Viability in Cerebellar Granule Cells As Measured by the MTT Cell Viability Assay[a]

treatment	% of mitochondrial function	treatment	% of mitochondrial function
control	99.97 ± 0.397	**9**	110.15 ± 5.774
3	100.57 ± 3.629	**10**	90.49 ± 1.061
4	100.32 ± 3.232	**11**	99.25 ± 6.361
5	102.59 ± 6.784	**12**	84.95 ± 2.393
6	99.09 ± 7.952	**13**	90.02 ± 4.111
7	98.29 ± 5.186	**14**	99.40 ± 2.456
8	83.66 ± 3.939		

[a] Values represent mean ± SEM of two independent experiments, each performed in triplicate.

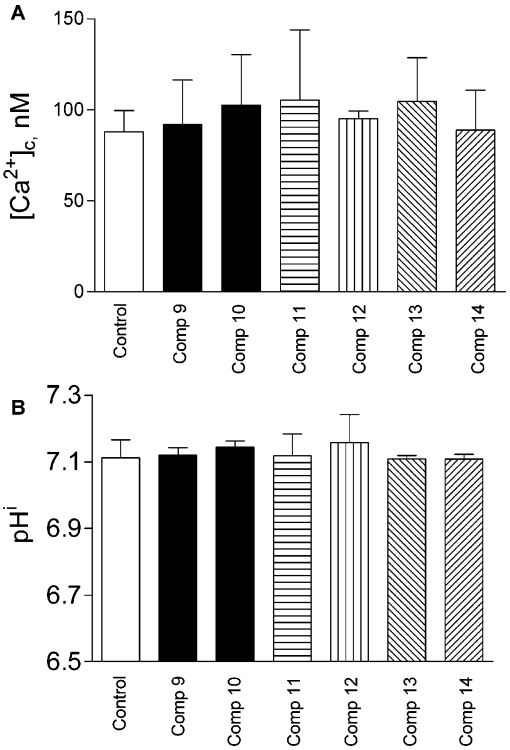


Figure 7. Effect of compounds **9**−**14** containing only the FGHI domain of azaspiracid-1, at 1 μM, on the cytosolic calcium concentration and intracellular pH of primary cultures of cerebellar granule cells. (A) Effect on the cytosolic calcium concentration. None of the compounds **9**−**14** altered the $[Ca^{2+}]_c$ in either the presence or the absence of calcium in the external medium. (B) Effect on intracellular pH. None of the compounds **9**−**14** altered the pH_i in either the presence or the absence of calcium in the external medium. Bar graphs represent the calcium and pH_i values obtained after the addition of calcium to the medium, in the time point corresponding to 200 s. Values represent means ± SEM of three or five independent experiments, each performed in duplicate.

Effects of the FGHI Domain on the Cytosolic Calcium Concentration, Intracellular pH, and Neuronal Viability. Next, we studied the effect of several partial structures of AZA-1 containing only the FGHI ring domains (see Figure 1, **9**−**14**). None of these compounds modified the basal calcium levels (Figure 7A) or the intracellular pH of cerebellar granule cells (Figure 7B) at a concentration of 1 μM. In addition, none of these structures **9**−**14** consisting of a FGHI ring modified the viability of cerebellar granule cells, after exposure of cerebellar neurons to 250 nM of each compound over 24 h, as evaluated with the MTT test (Table 1).

The main objective of this study was to evaluate the effects of AZA-1 (**1**) and several domains of the AZA-1 molecule in primary neuronal cultures. Since the cytosolic calcium concentration, intracellular pH, and neuronal viability are three of the physiological events more frequently altered during the neuronal response to external stimuli, we evaluated the effect of the different compounds in these three parameters. Primary cultures of cerebellar granule cells contain mainly glutamatergic neurons and a small proportion of GABAergic neurons[16,17] that express functional ionotropic glutamate receptors and constitute one of the most reliable neuronal models for the study of neural function and pathology.[14,15] This represents the first report on the effect of AZA-1 (**1**) and its diastereomer (**2**) as well as the different domains of the azaspiracid structure on primary neuronal cultures. AZA-1 (**1**) caused a moderate increase in the cytosolic calcium concentration in primary neuronal cultures and did not modify pH_i. Even short exposures (4 h) of cultured neurons to nanomolar concentrations of AZA-1 (**1**) significantly altered cell viability, membrane integrity, and F-actin pools. Likewise, compound **2** increased the cytosolic calcium concentration in cultured neurons with potency similar to that of AZA-1 (**1**), but it showed a higher cytotoxic effect after the shortest time of exposure examined in this work. Compound **2** only altered F-actin levels at concentrations higher than those needed to cause a cytotoxic effect and membrane damage. After evaluating the effect of different fragments of the azaspiracid molecule (compounds **3**−**14**), the data obtained so far indicates that the ABCD or the ABCDE structure (**3**−**8**) is enough to produce a significant $[Ca^{2+}]_c$ increase, whereas the fragments of the molecule containing a FGHI structure (**9**−**14**) did not modify the $[Ca^{2+}]_c$. None of the chemical structures tested showed a large effect on pH_i. Our results indicate that the complete chemical structure is necessary to alter the cell viability, since the different fragments of the molecule (**3**−**14**) did not cause cytotoxicity in this neuronal model. In addition, the data presented here constitute the first demonstration indicating a high neurotoxic effect of AZA-1 in a neuronal system, even at nanomolar concentrations and after short periods of exposure to the toxin.

AZA-1 is one of the azaspiracid analogues predominant in nature. Previous studies in our laboratory had examined the effect of AZA-1 on human lymphocytes and neuroblastoma cells. Since AZA-1 did not depolarize neuroblastoma cells, it was suggested that this compound was not neurotoxic; however, a direct evaluation of its possible cytotoxic effect was not performed.[11] It must be pointed out that AZA-1 decreased the cellular F-actin level in human neuroblastoma cells 1 or 24 h after treatment but at concentrations that were in the micromolar range,[11] that is, 1000 times higher than the IC_{50} reported in this work for the cytotoxic effects of AZ-1 after exposure times of 24 h. Supporting the results presented in this work, Twiner et al., have recently reported the time- and concentration-dependent cytotoxic effect of AZA-1 (**1**) in several types of cell lines.[12] However, the authors found that AZA-1 (**1**) needed exposure times higher than 24 h in order to produce complete cytotoxicity in most cell types, including mouse neuroblastoma cells, although its IC_{50} was in the low nanomolar range. Here, we report a complete cytotoxic effect in primary neuronal cultures after 8 h of exposure to AZA-1, with an IC_{50} in the low nanomolar range. This high neurotoxic effect of the toxin in neuronal cultures is the first report in the literature and could indicate a potential target in the central nervous system, specifically in fully differentiated neuronal cells. Supporting this idea, a recent study indicates that azaspiracid-1 at low nanomolar concentrations inhibited synaptic transmission in spinal cord neurons in a manner independent of sodium and calcium channels.[13] From the data reported here, it seems likely that neither an increase in the calcium concentration nor the F-actin cytoskeleton are the targets of AZA-1 (**1**) in neuronal tissue, since the effects on these parameters are observed only at concentrations much higher than those needed to decrease cell viability.

Only AZA-1 (**1**) and its diastereomer (**2**) were cytotoxic in primary cultures of neurons. Our data indicate that compound **2** needed shorter times of exposure and lower concentrations to cause a cytotoxic effect in cultured neurons, as assessed with the MTT and LDH assays. However, compound **2** did not modify the neuronal actin cytoskeleton. This fact could indicate that the stereochemistry of the molecule is important for the interaction with its unknown pharmacological target. Also, it could be a consequence of the intracellular acidification caused by compound **2**. However, the effect of compound **2** on neuronal pH was very weak and was observed only at concentrations 2−3 orders of magnitude higher than those needed to produce a cytotoxic effect. The data presented here show a higher cytotoxicity of AZA-1 (**1**) in primary neuronal culture than the results previously reported for undifferentiated mouse neuroblastoma cells.[12] Although this difference could be due to the source and purity of the toxin (natural versus synthetic), it is most likely due to the use of fully differentiated neuronal tissue in our experiments. The fact that compound **2** showed a higher cytotoxicity than compound **1** without alterations in the F-actin cytoskeleton further excludes the actin cytoskeleton as the target for the neurotoxic effect of the toxin.

Our results indicate that AZA-1 (**1**) and its stereoisomer (**2**) increase the cytosolic calcium concentration in cerebellar neurons. A significant calcium increase occurs at an AZA-1 (**1**) concentration of 250 nM; at this concentration, AZA-1 (**1**) and compound (**2**) decreased cell viability by 40% and 70%, respectively, after 4 h treatment. However, no effects on cell morphology where apparent during the imaging recordings with either of the two compounds. It should be highlighted here that the calcium increase produced by AZA-1 is quite small for neurons when compared with the effect of other toxins like palytoxin.[18] The increase in calcium concentration caused by AZA-1 (**1**) seems to be dependent on the presence of the ABCD or the ABCDE ring structure (**3**−**8**). However, none of the compounds containing only the ABCD or the ABCDE structure (**3**−**8**) showed a neurotoxic effect. No effect was observed from the compounds containing only the FGHI ring structure (**9**−**14**), neither in cytosolic calcium, intracellular pH, nor cell viability. Although this is the first report on the effect of the different domains of the AZA-1 (**1**) molecule on the cytosolic calcium concentration and intracellular pH, different effects of several members of the azaspiracid family in these two parameters have been previously documented. Thus, azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3) also increase the cytosolic calcium concentration even in different ways. AZA-2 induces Ca^{2+} release from intracellular pools and influx through nickel- and SKF96365-sensitive channels. AZA-3 does not empty intracellular pools but induces Ca^{2+} influx through nickel-sensitive channels. In addition, AZA-1 and AZA-2 did not modify intracellular pH in human lymphocytes, while AZA-3 increased intracellular pH.[11,19] Finally, azaspiracid-4 (AZA-4) is an inhibitor of Ca^{2+} influx through store-operated channels and AZA-5 did not modify the cytosolic calcium concentration in human lymphocytes.[20]

Conclusion

Although control of intracellular free calcium concentration is a critical component of cellular homeostasis for neurons, and an increase in the cytosolic calcium concentration can induce cell death,[21,22] this does not appear to be the case for the compounds examined in this work. The AZA-1 (**1**) molecule and compound **2** caused a modest increase in the cytosolic calcium concentration; however, this effect was found at toxin concentrations much higher than those needed to produce cytotoxic effects in neurons. In conclusion, our results indicate that AZA-1 (**1**) cytotoxicity is not dependent on the intracellular calcium increase produced by these compounds. Since no cytotoxicity was observed with the different fragments of the molecule examined (**3**−**14**), we also conclude that the whole AZA-1 (**1**) structure is needed to interact with its pharmacological target/s and to cause a cytotoxic effect. The increase in calcium caused by AZA-1 (**1**) could be a secondary physiological effect but certainly is not sufficient to reduce cell viability. This is supported by the fact that compounds **3**−**8** did not exhibited cytotoxic effect, although they all increased the cytosolic calcium concentration.

This report constitutes the first detailed study of the effects of AZA-1 (**1**) and the different domains of its molecule on primary neuronal cultures. Our data excluded several previously suggested possible targets, namely $[Ca^{2+}]_c$ increase and F-actin, as pharmacological targets for the cytotoxic effect of this toxin in neuronal cultures. However, further work is needed to elucidate the cellular pathways that could be altered after exposure of neuronal cultures to AZA-1.

Experimental Section

Chemicals and Solutions. Seven-day-old Swiss mice were obtained from the animal care facilities of the University of Santiago de Compostela. Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Fetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco's modified Eagle's medium (DMEM) was from Biochrom (Berlin, Germany). Azaspiracid fragments were synthesized by Nicolaou and co-workers.[23−25] Fura-2 acetoxymethyl ester (Fura-2-AM), BCECF-AM [2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester], and Oregon Green-514 phalloidin were from Molecular Probes (Leiden, The Netherlands). Lactate dehydrogenase (LDH) detection kit was from Roche (Barcelona, Spain). All other chemicals were reagent grade and purchased from Sigma.

Experimental solutions were based on Locke's buffer containing (in mM): 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, 3.6 $NaHCO_3$, and 10 HEPES, pH 7.4 adjusted with Tris. The pH of the buffer containing the different drugs used in this study was adjusted to 7.4 with Tris before addition to the cells.

Cell Cultures. Primary cultures of cerebellar granule cell were obtained from cerebella of 7-day-old mice as previously described.[16,26,27] In brief, cells were dissociated by mild trypsinization at 37 °C, followed by trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in DMEM containing 25 mM KCl, 31 mM glucose, and 0.2 mM glutamine supplemented with *p*-aminobenzoate, insulin, penicillin, and 10% fetal calf serum. The cell suspension was seeded in 22-mm glass coverslips precoated with poly-L-lysine and incubated in 6- or 96-multiwell plates for 8−11 days in a humidified 5% CO_2/95% air atmosphere at 37 °C. Cytosine arabinoside (20 μM) was added before 48 h in culture to prevent glial proliferation.

Cell Labeling and Determination of Intracellular pH (pH_i) and Cytosolic Calcium Concentration $[Ca^{2+}]_c$. Image Processing. Cell cultures of 8−12 days in vitro were loaded with the pH- and Ca^{2+}-sensitive fluorescent dyes BCECF-AM (0.5 μM) and Fura-2 AM (1 μM) for 10 min at 37 °C as previously described.[18,28] Briefly, after incubation the loaded cells were washed three times with cold buffer. The glass coverslips were inserted into a thermostated chamber at 37 °C (Life Science Resources), and individual cells were viewed with a Nikon Diaphot 200 microscope, equipped with epifluorescence optics (Nikon 40×-immersion UV-Fluor objective). The thermostated chamber was used in the open bath configuration, and additions were made by aspiration and addition of fresh bathing solution.

The pH_i and $[Ca^{2+}]_c$ were obtained from the images collected by quadruple excitation fluorescence with a Life Science Resources equipment. The light source was a 175 W xenon lamp, and light reached the objective with an optic fiber. The excitation wavelengths for Fura were 340 and 380 nm, with emission at 505 nm, and for BCECF excitation wavelengths were 440 and 490 nm and emission 530 nm. The calibration of the fluorescence versus intracellular calcium was made by using the method of Grynkiewicz et al.[29] The results of pH_i were expressed as a ratio of the emission fluorescence intensities 490/440; this ratio increases upon pH_i elevation. The 530-nm emission ratio resulting from 490/440 excitation (from 25−30 cells in each coverslip) was converted to a linear pH scale by means of in situ calibration between pH 6 and 9 carried out when it is necessary using the nigericin technique.[30]

Cell Viability Assays. The cytotoxic action of AZA-1 or of the several domains of the molecule was studied in cultured CGC by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test. The MTT assay was performed as described previously.[18] This test, which measures mitochondrial function, was used to assess cell viability, as it has been shown that in neuronal cells there is a good correlation between a drug-induced decrease in mitochondrial activity and its cytotoxicity.[31] Briefly, after exposure to different concentrations of AZA-1 (**1**) or its fragments added in the culture medium, cells were rinsed and incubated for 30 min with a solution of MTT (500 μg/mL) dissolved in Locke's buffer. For AZA-1 (**1**) and compound **2**, different concentrations and times of exposure were examined, whereas for the several domains of the molecule tested the cytotoxicity was evaluated after 24-h exposure to a concentration of compound of 250 nM. After washing off excess MTT, the cells were disaggregated with 5% sodium dodecyl sulfate and the colored formazan salt was measured at 590 nM in a spectrophotometer plate reader.

Lactate dehydrogenase leakage was determined following manufacturer's procedures in 50 μL of culture medium.

F-actin Assay. Cells seeded on 96-well plates were analyzed for F-actin pools using Oregon Green-514 phalloidin as a fluorescent marker for F-actin. Briefly, after treatment of the cells with different concentrations of AZA-1 (**1**) and compound **2** during 4, 8, or 24 h, cells were fixed for 20 min by the addition of 25 μL of 16% formaldehyde solution to each well (except blanks) and then washed with Locke's buffer. After permeabilization with Triton X-100, cells were washed with buffer and incubated with Oregon Green-514 phalloidin as previously described.[11]

Statistical Method. All data are expressed as means ± SEM of *n* experiments (each performed in duplicate). Statistical comparison was by two-way ANOVA followed by nonpaired Student's *t*-test. *P* values <0.05 were considered statistically significant.

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