The c-Jun-N-Terminal Kinase is Involved in the Neurotoxic Effect of Azaspiracid-1

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Key Words

Azaspiracid • MAPK • JNK • Cytotoxicity • Cerebellar granule cells • MTT

Abstract

Aims: Azaspiracids (AZAs) are marine phycotoxins with an unknown mechanism of action, recently implicated in human intoxications. The predominant analog in nature, AZA-1 targets several organs *in vivo*, including the central nervous system and exhibits high neurotoxicity *in vitro*. **Methods:** We used pharmacological tools to inhibit the cytotoxic effect of the toxin in primary cultured neurons. Immunocytochemical techniques in combination with confocal microscopy were employed to examine the cellular mechanisms involved in the neurotoxic effect of AZA-1. **Results:** Several targets for azaspiracid-induced neurotoxicity, specifically the cAMP pathway, or protein kinase C and phosphatidylinositol 3-kinase activation were excluded. Interestingly, the specific c-Jun-N-terminal protein kinase (JNK) inhibitor SP 600125 protected cultured neurons against AZA-induced cytotoxicity. Immunocytochemistry experiments showed that AZA-1 increased the amount of phosphorylated JNK and caused nuclear translocation of the active protein that

was prevented by SP 600125. **Conclusion:** Our data constitute the relationship between azaspiracid-induced cytotoxicity and specific modifications in cellular transduction signals, specifically we found that JNK activation is associated with the cytotoxic effect of the toxin. These results should provide the basis to identify the mechanism of action of this group of toxins.

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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 (AZA-1) and its congeners [1]. Marine phycotoxins belonging to the azaspiracid family were first identified after an outbreak of poisoning related to the consumption of mussels (*Mytilus edulis*) in Killary Harbour, Ireland [1, 2]. Human intoxications associated with shellfish contaminated with azaspiracids have been described in several European coastal countries [1-6]. Azaspiracid poisoning in humans shares some symptoms with diarrhetic shellfish

poisoning including nausea, vomiting, severe diarrhoea and stomach cramps [7]. However, toxicity studies in mice revealed also lung tumors following chronic administration [3, 8]. Additionally, mice and rats developed neurological symptoms resembling paralytic shellfish poisoning after intraperitoneal injections with progressive paralysis, fatigue, difficulty breathing and subsequent death as soon as 35 minutes after injection [3, 8].

The total structure of AZA-1 was originally proposed by Yasumoto and co-workers¹, and was recently corrected by Nicolaou et al. [9-11]. 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, 12, 13]. The AZAs are characterized by unusual structural motifs including a trioxadispiroacetal system fused to a tetrahydrofuran ring, an azaspiro ring system, and a carboxylic acid moiety [1]. So far, there is no information about the mechanism of action of the AZAs or of their specific cellular targets. We have recently shown that AZA-1 is a potent neurotoxic agent in primary neuronal cultures showing an effect several orders of magnitude higher than the cytotoxic effects previously described in different cell lines [14, 15]. The cytotoxic action of AZA-1 in neurons was independent of an alteration on the cytosolic calcium concentration or on F-actin cytoskeleton disruption and it needed the whole molecular structure of the toxin [15]. In view of the high cytotoxic effect of AZA-1 in neuronal cultures we conducted a pharmacological and molecular approach to explore several potential pharmacological targets of azaspiracids in nervous tissue. Knowledge of the cellular mechanisms modified by these toxins is essential to discover its mechanism of action.

Materials and Methods

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). Foetal calf serum was obtained from Gibco (Glasgow, UK) and Dulbecco's Modified Eagle's medium (DMEM) was from Biochrom (Berlin, Germany). Azaspiracid-1 was synthesized by K.C. Nicolaou and co-workers [9, 11, 16, 17]. Antibodies against phospho-JNK (dually phosphorylated SAPK/JNK (Thr183/Try185)), pan-JNK, dually phosphorylated

phospho-p38 MAPK (Thr180/Try182), and phospho-ERK1/2 (Thr202/Try204) were purchased from BD Biosciences (Madrid, Spain). ERK inhibitor, SP 600125, SB 202190, and PD 98059 were from Calbiochem (Merk Biosciences Ltd. Nottingham, UK). 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was from Molecular Probes (Eugene, Oregon, USA). 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 CaCl₂, 1 MgCl₂, 5.6 glucose, 3.6 NaHCO₃ 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 [15, 18-20]. 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-amino benzoate, insulin, penicillin and 10% foetal 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% CO₂/95% air atmosphere at 37°C. Cytosine arabinoside, 20 µM was added before 48 h in culture to prevent glial proliferation.

Cell viability assays

The cytotoxic action of AZA-1 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 [15, 20, 21]. 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 [22]. Briefly, after 24 hours of exposure to different concentrations of AZA-1 alone or in combination with the different drugs tested, cells were rinsed and incubated for 30 min with a solution of MTT (500 µg/ml) dissolved in Locke's buffer. 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.

Detection of peroxides/reactive oxygen species

We used the oxidation sensitive fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) to measure the production of ROS, mainly hydrogen peroxide and hydroxyl radicals. DCFH-DA is deacetylated by esterases to dichlorofluorescein (DCFH). This nonfluorescent product is then converted by reactive species into DCF, which can easily be visualized by fluorescence at 530 nm when excited at 485 nm. Cerebellar granule cells seeded in 96-well culture plates were incubated with DCFH-DA (10 µg/ml) for 15 min in the presence of AZA-1 and fluorescence intensity was measured in a Biotek FL 600 microplate fluorescence reader (Biotek In-

Fig. 1. Effect of cAMP modulators on the AZA-1-induced cytotoxicity as determined with the MTT assay. Neurons were exposed to different AZA-1 concentrations (0.5 to 25 nM) for 24 hours in the presence of 10 μ M forskolin (A), 10 μ M SQ22,536 (B), 100 μ M dbcAMP (C) or 5 μ M H89 (D). Values are means $\pm$ SEM of three independent experiments, each performed in duplicate.

struments Inc. Winooski, VT, USA) over 3 hours in the absence or presence of AZA-1 at concentrations of 1, 10, 50, 100 and 250 nM. Average ROS production (relative to level of vehicle-treated controls) was calculated from four individual wells in at least three independent platings.

Immunocytochemistry

For immunocytochemistry, control and treated cells were fixed in 4% formaldehyde and subsequently labelled with antibodies against dually phosphorylated SAPK/JNK (p-JNK, 1:250), dually phosphorylated p38 (p-p38, 1:1000), dually phosphorylated-ERK (p-ERK 1:1000) or PKC delta (1:1000). Immunoreactivity was visualized using an Alexa 546-conjugated secondary antibody (1:1000 dilution, Molecular Probes, USA). Cells were analyzed using a laser scanning confocal microscope (Nikon, Mellville, NY), with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics KK, Japan). Immunocytochemistry controls for the specificity of the detection methods were carried out by omitting the incubation step with the primary antibodies in one of each 4 consecutive coverslips. None of the coverslips run without primary antibody showed specific labelling comparable to that obtained with the primary antibodies.

Statistical method

All data are expressed as means $\pm$ SEM of *n* experiments (each performed in duplicate). Statistical comparison was by one-way analysis of variance with the Student-Newman-Keuls test and by non-paired Student's *t*-test. *P* values < 0.05 were considered statistically significant.

Results

In this work we intended to explore the cellular pathways that could account for the azaspiracid-induced cytotoxicity in neuronal cultures. Therefore, we analysed several cellular routes frequently involved in cell death in order to elucidate their relevance for the neurotoxic effect of AZA-1. The cellular pathways analysed in this study comprised the cyclic AMP pathway, protein kinase C signalling and mitogen activated protein kinases signalling.

Role of the cAMP in the cytotoxic effect of AZA-1 in cultured neurons

It has been previously described that AZA-1 increases cAMP levels in human lymphocytes [23]. In order to investigate the role of the cAMP pathway in the cytotoxic effect of AZA-1, cerebellar neurons were exposed to different AZA-1 concentrations (0.5 to 25 nM) for 24 hours in the culture medium in the presence or absence of cAMP inhibitors and activators (Fig. 1). The effect of cAMP-active drugs was explored with the adenylate cyclase activator forskolin (10 μ M), the inhibitor SQ22,536 (10 μ M), the cAMP analog dibutyryl cAMP (dbcAMP, 100 μ M), and the protein kinase A blocker H89 (5 μ M). Figs. 1A-C show that pretreatment of cerebellar

neurons with these different drugs had no effect on the cytotoxic action of AZA-1. Only treatment of the neurons with AZA-1 in the presence of 100 μ M dibutyryl cAMP (Fig. 1D) slightly but significantly attenuated the cytotoxic effect of AZA-1 at the highest concentrations of the toxin examined (5, 10 and 25 nM, $p < 0.05$).

Effect of protein kinase C modulators on the azaspiracid-induced cytotoxicity in neurons

Several members of the protein kinase C family serve as substrates for caspases and PKC isozymes had been intimately associated with cell-death. PKC δ down-regulation was found to be associated with tumor promotion [24], suggesting that this isozyme may have a negative effect on cell survival. To examine the role of PKC we used Gö6976 and low concentrations of GF 109203X (50 nM) to inhibit calcium-dependent PKC isozymes. For calcium-independent isozymes we used GF 109203X 500 nM and 10 μ M rottlerin to specifically inhibit PKC delta. Fig 2A and 2B indicate that inhibition of the calcium-dependent PKC isozymes did not have any effect on AZA-1 induced cytotoxicity. Similarly, blockage of calcium-independent PKC isozymes with high concentrations of GF 109203X also did not alter the cytotoxic effect of AZA-1 (Fig. 2C). Inhibition of PKC delta with 10 μ M rottlerin decreased cell viability by 70%, the effect of rottlerin was not modified by azaspiracid. Similar results were found at a rottlerin concentration of 1 μ M (data not shown).

In a similar experiment we also tested the effect of inhibition of the phosphatidylinositol 3-kinase (PI₃K) with 10 nM wortmannin, this manipulation had no effect on the cytotoxic effect of AZA-1 (data not shown).

c-Jun-N-terminal kinase (JNK) is involved in AZA-1 induced cytotoxicity in primary neuronal cultures

Mitogen activated protein kinases are involved in neuronal cell death induced by several mechanisms. We evaluated the effect of several cell permeable inhibitors of MAP kinases on the cytotoxic action of AZA-1 after 24 hours of exposure of the neurons to different toxin concentrations.

Regulation of the extracellular signal-regulated kinase (ERK) cascade is distinguished by a characteristic core cascade of three kinases. The first kinase, Raf-1 and B-Raf activates the second (MEK) by serine/threonine phosphorylation. Activation of MEK leads to activation of ERK 1 and ERK 2 by phosphorylation of a threonine and a tyrosine residue [25]. Inhibition of the ERK cascade

Fig. 2. Effect of PKC modulators on the AZA-1-induced cytotoxicity as determined with the MTT assay. Neurons were exposed to different AZA-1 concentrations (0.5 to 25 nM) for 24 hours in the presence of 100 nM Gö6976 (A), 50 nM GF109203X (B), or 500 nM GF109203X (C). Values are means $\pm$ SEM of three independent experiments, each performed in duplicate.

by the MEK inhibitor (PD 98059, 20 μ M, Fig. 3A) or by the ERK inhibitor (Ste-MEK1₁₃ at 30 μ M, Fig. 3B) did not modify the cytotoxic effect of AZA-1.

To evaluate the role of the JNK/SAPK (c-Jun-N-terminal kinase/stress activated protein kinase) cascade in the cytotoxic effect of AZA-1, we used SP 600125 (Anthra[1,9-*cd*]pyrazol-6(2H)-one 1,9-pyrazoloanthrone SAPK Inhibitor II) at 5 μ M. Figure 3C shows that incubation of neurons with SP 600125 before addition of AZA-

Fig. 3. Effect of MAPKs inhibitors on the AZA-1-induced cytotoxicity as determined with the MTT assay. Neurons were exposed to different AZA-1 concentrations (0.5 to 25 nM) for 24 hours in the presence of MAPKs inhibitors. (A) Effect of the MEK inhibitor PD 98059 at 20 μ M. (B) Effect of the ERK inhibitor at 30 μ M. (C) Effect of JNK inhibition by 5 μ M SP 600125. (D) Effect of p38 inhibition by 1 μ M SB 202190. Values are means $\pm$ SEM of three independent experiments, each performed in duplicate.

1 completely prevented the cytotoxic effect of AZA-1 in neurons during the 24 hours exposure of the cells to this toxin ($p < 0.001$), thus indicating that JNK is involved in the cytotoxic effect of azaspiracid in neurons.

The p38 kinase is the most well characterized member of the MAP kinase family. It is activated in response to inflammatory cytokines, endotoxins, and osmotic stress. In this work we used SB 202190 at 1 μ M to inhibit the p38 MAPK. Fig. 3D shows that SB 202190 significantly ($p < 0.05$) modified the neurotoxic effect of AZA-1 in cultured neurons, although it only showed a partial cytoprotective effect.

Effect of AZA-1 on reactive oxygen species production

Oxidative stress or reactive oxygen species (ROS) production are capable of inducing cell death by either necrosis or apoptosis [26]. In view of the involvement of JNK in the cytotoxic effect of AZA-1, we evaluated the effect of AZA-1 on ROS production. Cultured neurons were exposed to different AZA-1 concentrations for three hours. Fig. 4 shows that at 250 nM, the highest concentration evaluated, AZA-1 did not modify the formation of peroxides detected with the fluorescent indicator CM-H₂DCFDA. Similar results were found at AZA-1 concentrations of 1, 10, 50 and 100 nM (data not shown).

Fig. 4. Treatment of the neurons with 250 nM AZA-1 did not modify the formation of peroxides detected with the fluorescent indicator CM-H₂DCFDA. Values are means $\pm$ SEM of three independent experiments, each performed in duplicate.

AZA-1 increases JNK phosphorylation and causes nuclear translocation of phospho-JNK, which is prevented by the JNK inhibitor SP 600125

It is widely documented that JNK is activated by dual phosphorylation in both tyrosine and threonine residues, after which JNK is understood to translocate to the nucleus where it activates transcription factors. In view of the protective effects of the JNK inhibitor SP

Fig. 5. Exposure of cerebellar granule cells to 50 nM AZA-1 during 4 hours alters phospho JNK staining and the cellular localization of active-JNK. (A) Control neurons show moderate p-JNK immunoreactivity localized to processes and cell bodies. (B) Treatment of the neurons with SP 600125 did not modify p-JNK immunoreactivity. (C) Cells treated with 50 nM AZA-1 showed intense p-JNK staining covering the cell soma and nuclei. (D) Treatment of the cells with SP 600125 and AZA-1 decreased the nuclear translocation of p-JNK caused by AZA-1. Scale bar is 20 μ m.

Fig. 6. Exposure of cerebellar granule cells to 10 nM AZA-1 during 24 hours alters phospho JNK expression and the cellular localization of the protein. (A) Control neurons show moderate p-JNK immunoreactivity localized to processes and cell bodies. (B) Treatment of the neurons with SP 600125 did not modify p-JNK immunoreactivity. (C) Cells treated with 10 nM AZA-1 showed intense p-JNK staining covering the cell soma and nuclei. (D) Treatment of the cells with SP 600125 and AZA-1 decreased the nuclear translocation of p-JNK caused by AZA-1. Scale bar is 20 μ m.

600125 against AZA-1 neurotoxicity, we conducted immunocytochemical experiments in order to elucidate the effect of the toxin in the subcellular localization of the protein. With this aim, cerebellar granule cells were treated either with 50 nM AZA-1 during 4 hours (conditions in which there is only partial cell death) or with 10 nM AZA-1 24 hours (to achieve complete cell death) in absence or presence of the JNK inhibitor. Fig. 5 shows p-JNK immunoreactivity in control and AZA-1 treated neurons. Control neurons showed moderate p-JNK immunoreactivity localized to processes and cell bodies and a complete absence of p-JNK staining in nuclei (Fig. 5A). The staining pattern for p-JNK was not modified by SP 600125 (Fig. 5B). However, cells treated with 50 nM AZA-1 during the same period showed intense p-JNK staining covering the cell soma and nuclei (Fig. 5C). Exposure of the cells to 50 nM AZA-1 in the presence of SP 600125 decreased the nuclear translocation of active JNK, however small punctate regions showing p-JNK immunoreactivity were more abundant in the cell processes of treated cells than in control neurons (Fig. 5D).

Treatment of cerebellar granule cells with 10 nM AZA-1 during 24 hours yielded results similar to the four hours treatment (Fig. 6A-D), thus indicating that AZA-1-induced nuclear translocation of p-JNK is associated with cell death in neurons.

The effect of AZA-1 in the total amount of JNK protein was also evaluated using a pan JNK antibody (Fig. 7). As shown in Fig. 7A cerebellar granule cells showed a high level of immunoreactivity for pan JNK in cell bodies, processes and also in nuclei. SP 600125 did not modify this pattern of expression although the staining was somehow less intense in neurites (Fig. 7B). After 4 hours of exposure to 50 nM AZA-1 pan-JNK was concentrated in the nuclei and cell soma and was almost completely absent in cellular processes (Fig. 7C). Pretreatment of the neurons with SP 600125 before addition of 50 nM AZA-1 ameliorated the effect of AZA-1 on pan-JNK immunoreactivity (Fig. 7D). In this case, although intense staining was observed in cell bodies, some staining was also observed in processes, resembling the pattern observed in control neurons.

Fig. 7. Effect of exposure of cerebellar granule cells to 50 nM AZA-1 during 4 hours on pan JNK immunoreactivity. (A) Control neurons showed intense pan JNK staining in processes, cell bodies and nuclei. (B) Treatment of the neurons with SP 600125 did not modify pan JNK immunoreactivity. (C) Treatment of the cells with 50 nM AZA-1 alters pan JNK staining. (D) Pretreatment of the cells with SP 600125 before addition of 50 nM AZA-1 ameliorated the effect of AZA-1 on pan JNK immunoreactivity. Scale bar is 20 μ m.

Fig. 8. Exposure of cerebellar granule cells to 50 nM AZA-1 during 4 hours did not modify the staining pattern of phospho p38 and phospho ERK. Phospho p38 immunoreactivity in control (A) and AZA-1 treated cells (B). Phospho ERK immunoreactivity in control (C) and AZA-1 treated cells (D). Scale bar is 20 μ m.

AZA-1 does not alter the cellular expression of phospho-p38, or phospho-ERK

Our initial results indicate a small protective effect of the p38 inhibitor SB 202190 against AZA-1 induced cytotoxicity; therefore we examined the effect of AZA-1 on p-p38 immunoreactivity in neurons. Fig. 8A shows that 4 hours exposure of cerebellar neurons to 50 nM AZA-1 did not modify the cellular expression of phospho-p38. Phospho-p38 was localized in processes and cell bodies of cerebellar granule cells with absence of staining in the nuclei, both in control and AZA-1 treated cells (Fig. 8A and 8B). Similarly, no effect on phospho-ERK immunoreactivity was observed after 4 hours exposure of the neurons to 50 nM AZA-1 (Fig. 8C and 8D). No changes in p-p38 and p-ERK staining were observed after 24 h exposure of the neurons to 10 nM AZA-1 (data not shown).

Thus, our results indicate that AZA-1 treatment specifically increases the phosphorylation state of JNK and causes nuclear accumulation of phosphorylated JNK. This effect seems to be associated with the cytotoxic effect

of AZA-1 in cultured neurons since it is blocked by the JNK inhibitor SP 600125, which also blocks AZA-1 induced cytotoxicity as assessed by the MTT test.

Discussion

The main objective of this study was to investigate the cellular pathways involved in the cytotoxic action of azaspiracid-1. We have recently excluded several possible targets of AZA-1 cytotoxicity, namely alterations in the cellular calcium homeostasis or in the F-actin cytoskeleton [15]. Taking advantage of the documented cytotoxic effect of AZA-1 in cerebellar neurons we explored the pharmacological mechanism involved in the azaspiracid-induced cytotoxicity. Primary cultures of cerebellar granule cells were used as the cellular model since they provide one of the most reliable and well characterized systems for the study of neural function and pathology [27, 28]. We found that inhibition of the mitogen activated protein kinase JNK completely prevented the cytotoxic

effect of azaspiracid-1 in neurons. Furthermore, neuronal exposure to AZA-1 caused an increase in the amount of phosphorylated-JNK and produced nuclear accumulation of the protein. This effect appeared after short times of exposure of the neurons to the toxin (4 hours) and was prevented by the specific JNK inhibitor SP 600125. Therefore, our results strongly suggest that JNK activation and nuclear translocation of active JNK is responsible for the cytotoxic effect of azaspiracid in neurons.

Despite of the different models used to study the effect of the azaspiracids, the mechanism of action of this group of toxins is still completely unknown. AZA-1 produces a complete cytotoxic effect in primary neuronal cultures after 8 hours of exposure to the toxin, with an IC₅₀ in the low nanomolar range [15]. This high neurotoxic effect could indicate a potential target in the central nervous system, specifically in fully differentiated neuronal cells. In this sense, a very recent study indicated that azaspiracid-1, at low nanomolar concentrations inhibited synaptic transmission in spinal cord neurons in a manner independent of sodium and calcium channels [29].

Human AZA poisoning produces some symptoms very similar to those of diarrhetic shellfish poisoning, including nausea, vomiting, severe diarrhoea and stomach cramps [7]. Since diarrhetic marine phycotoxins interfere with the cAMP pathway and AZA-1 increases cAMP levels in human lymphocytes [23] our initial thought was that AZA-1 could be altering the cAMP pathway. However, only the cAMP analog dcAMP produced a marginal cytoprotective effect against AZA-1 cytotoxicity. This effect is probably not due to an action of AZA-1 on the cAMP pathway since several studies have demonstrated that elevating the cAMP level attenuated cell death induced by neurotoxicity, [30]. Furthermore, the increase of intracellular levels of cAMP promotes cell survival or delays cell death induced by various conditions [31-33].

Mitogen activated protein kinases signal transduction pathways are among the most widespread mechanisms of eukaryotic cell regulation [34]. The study of the involvement of the MAPK pathway in AZA-1 cytotoxicity yielded the unexpected finding that inhibition of the JNK pathway with SP 600125 completely prevented the cytotoxic effect of AZA-1. First, we suspected that AZA-1 could bind to JNK and cause its activation. In immunocytochemistry experiments we consistently found that AZA-1 caused the accumulation of active JNK in the nucleus and that this effect was blocked in the presence of the JNK inhibitor. Although AZA-1 somehow altered the subcellular distribution of pan-JNK it did not modify

the subcellular expression of either p38 or ERK kinases. Increasing evidence implicates the JNK pathway as an important mediator of cell death in different model systems. Different intracellular and extracellular events can be responsible for JNK activation in neuronal cells [35]. One of these events could be the generation of reactive oxygen species and nitrogen species, however we demonstrated here that AZA-1 does not induce reactive oxygen species production. Several lines of evidence indicate that AZA-1 has cytotoxic effects *in vivo* as well as *in vitro* [3, 8, 14, 15], however the toxin shows a higher cytotoxicity in cultured neurons than in any other cellular system tested to date. This fact is in agreement with the observation that cerebellar granule cells in culture show JNK activity elevated considerably above that from neuron-like cell lines [36]. The enhanced JNK activity in cerebellar neurons could account for the higher cytotoxicity of azaspiracid in this system. The JNK inhibitor employed in this study, SP 600125, was developed as a competitive, specific inhibitor of JNK1, -2, and -3 [37]. Activation of JNK by AZA-1 does not result from cellular stress because the stress-activated protein kinase p38 was not activated after 4 hours of treatment with 50 nM AZA-1, however inhibition of p38 caused a partial protection against the cytotoxic effect of AZA-1 after 24 hours of exposure to the toxin. Although our immunocytochemical experiments indicated that AZA-1 does not cause an initial activation of p38, we can not completely exclude a marginal activation of this protein by longer exposures to AZA-1.

A neuroprotective effect of SP 600125 associated to decreased phosphorylation of the three JNK proteins has been previously observed [38]. In cerebellar granule cells three different pools of JNK activity have been described, among them the stress responsive translocating pool [36], which is mainly cytosolic. This pool is composed of JNK and JNK kinase (MKK4), translocates to the nucleus after activation and phosphorylates c-Jun [36]. It is interesting to note that AZA-1 induced cytotoxicity in cerebellar neurons was accompanied by neurite fragmentation, especially obvious after 24 hours of treatment. In this sense, an increased amount of phosphorylated JNK has also been described after cytoskeletal disruption. Interestingly, the tumor promoter marine phycotoxin palytoxin activates the MAPKs JNK and ERK *in vitro* [40, 41] and these effects are related with the ionic disequilibria caused by the toxin [42]. However, the cellular effects of AZA-1 on ion homeostasis have not been investigated so far.

Although this study did not provide the mechanisms

of action of the azaspiracids, we found that AZA-1 caused JNK activation in cultured neurons and this effect was observed in conditions in which AZA-1 does not produce complete cell death (4 hours of exposure to the toxin). Furthermore, inhibition of JNK prevented the cytotoxic effect of the toxin. Thus, our results identify one of the cellular mediators of the azaspiracid-induced cytotoxicity. The data presented here constitute the first work linking azaspiracids-induced cytotoxicity with specific modifications in cellular proteins. However, the complexity of the cellular routes that can ultimately produce JNK activation and the scarcity of information on the pharmacological action of these toxins prevents to suggest a potential cellular target for AZA-1. At this point, more detailed studies are required to identify the cellular routes activated by AZA-1 and responsible for the JNK activation as well as the JNK pools altered by azaspiracids and the consequences of this effect on the expression of transcription factors and genes regulated by JNK.

Abbreviations

AZA (azaspiracid); CGC (cerebellar granule cells); DMEM (Dulbecco's Modified Eagle's medium); CM-H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate);

MAPK (mitogen activated protein kinase); ERK (extracellular signal-regulated kinase); MEK (MAPK kinase); JUNK (c-Jun NH₂-terminal kinase); PKC (protein kinase C); MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); cAMP (cyclic adenosine monophosphate); dbcAMP (dibutyryl cAMP); PI3K (phosphatidylinositol 3-kinase); ROS (reactive oxygen species).

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