Toxicon 121 (2016) 61–69

Contents lists available at ScienceDirect

Toxicon

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

# Investigation of the genotoxic potential of the marine biotoxins azaspiracid 1–3

Barbara Doerr [a, b, *], John O'Halloran [b, c], Nora O'Brien [d], Frank van Pelt [a, b]

[a] Department of Pharmacology and Therapeutics, University College Cork, Cork, Ireland
[b] Environmental Research Institute, University College Cork, Cork, Ireland
[c] School of Biological, Earth and Environmental Sciences, University College Cork, Cork, Ireland
[d] School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

ARTICLE INFO

*Article history:*
Received 5 July 2016
Received in revised form
19 August 2016
Accepted 25 August 2016
Available online 27 August 2016

*Keywords:*
Azaspiracid
AZAs
AZA1
AZA2
AZA3
Genotoxicity
COMET assay
Apoptosis

ABSTRACT

Azaspiracids (AZAs) are the most recently discovered group of biotoxins and are the cause of azaspiracid shellfish poisoning (AZP) in humans. To date over thirty analogues have been identified. However, toxicological studies of AZAs are limited due to the lack of availability of toxins and toxin standards. Most data available are on acute toxicity and there are no data available on genotoxicity of AZAs. This study presents an integrated approach investigating the genotoxic potential of AZA1–3 in cell culture systems using the Comet assay combined with assays to provide information on possible apoptotic processes, cytotoxicity and changes in cell number. Results demonstrate a time and dose dependent increase in DNA fragmentation in most cell lines, indicating a genotoxic effect of AZA1–3. However, a significant reduction in cell number and a clear shift from early to late apoptosis was observed for all analogues in Jurkat T cells and HepG-2 cells; CaCo-2 cells did not show a clear apoptotic profile. Late apoptotic/necrotic cells correlate well with the percentage of tail DNA for all analogues in all three cell lines. All data taken together indicate that AZA1–3 is not genotoxic *per se* and demonstrate apoptotic/necrotic processes to be involved to some extent in AZAs toxicity. The sensitivities of cell lines and the different potencies of AZA1–3 are in agreement with the literature available. The order of sensitivity for all three AZAs tested in the present study is, in increasing order, CaCo-2 cells < HepG-2 cells < Jurkat T cells. The order of potency of AZA1–3 varies among the cell lines.



## 1. Introduction

Azaspiracids (AZAs) are the most recently discovered group of biotoxins and are the cause of azaspiracid shellfish poisoning (AZP). Since first discovered in mussels from Killary Harbour (Ireland) in 1995, AZAs have been identified in biological samples around the world, including northern Europe, Portugal, Spain, France and in the North-West of Africa (James et al., 2002; Klontz et al., 2009; Magdalena et al., 2003; Taleb et al., 2006; Torgersen et al., 2008). Symptoms of acute AZP occur within 3–18 h after consumption of contaminated shellfish and included nausea, vomiting, diarrhoea and stomach cramps. A full recovery of the clinical symptoms takes place within 2–5 days. No lethality or long term effects of AZP have been reported to date (James et al., 2010; Satake et al., 1998; Twiner et al., 2008a). AZAs are primarily produced by dinoflagellates of the species *Azadinium spinosum*, but more recently they have also been reported in the related dinoflagellate *Amphidoma languida* (Tillmann et al., 2009, 2014). To date, over thirty analogues of AZA have been identified. Most of these analogues have been either proven or suggested to be biotransformation products in shellfish (Hess et al., 2015; Jauffrais et al., 2012; McCarron et al., 2009; O'Driscoll et al., 2011) and together with the parent compounds, AZA1 and AZA2, can accumulate in shellfish. Human consumers are potentially at risk due to the increased presence of AZAs in shellfish destined for the market worldwide (Elgarch et al., 2008; James et al., 2002; Klontz et al., 2009; Magdalena et al., 2003; Torgersen et al., 2008).

Toxicological studies of AZAs are limited due to the lack of availability of toxins and toxin standards. The main target of acute AZA toxicity is the gastrointestinal tract (GI). However, AZA also targets the lymphatic system and the liver and at high

* Corresponding author. Department of Pharmacology and Therapeutics, University College Cork, Cork, Ireland.
*E-mail addresses:* barbaradoerr@hotmail.de (B. Doerr), j.ohalloran@ucc.ie (J. O'Halloran), nob@ucc.ie (N. O'Brien), f.vanpelt@ucc.ie (F. van Pelt).

http://dx.doi.org/10.1016/j.toxicon.2016.08.019
0041-0101/

concentrations can be found in the spleen, thymus and lungs (Aune et al., 2012; Ito et al., 2000, 2002). This suggests that AZAs can be absorbed by the GI system and be distributed, at least partially. Acute morphological changes observed in mice were distinctly different from other biotoxins and the onset of histopathological changes and recovery were slower (Ito et al., 2000). An acute toxicity study by Ito et al. (Ito et al., 2000) detected fluid accumulation in the small intestine, eroded villi in the *lamina propria* and epithelial cell and degenerating cells in the large intestine. Furthermore, the authors established AZA to cause steatosis (an accumulation of fat droplets) and degenerating cells in the liver, necrosis in lymphocytes and reduction in numbers of non-granulocytes in the lymphoid tissue. A recent study confirmed the findings for the GI tract, however failed to see any other changes in mice after AZA1 exposure (Aune et al., 2012). Besides diarrheic symptoms, neurological effects, spasms, respiratory difficulties, paralysis and death were observed in mice after intraperitoneal (*i.p.*) injections of mussel extract containing AZA (McMahon and Silke, 1996; 1998; Satake et al., 1998). To date, *in vitro* studies have failed to identify the cellular target of AZAs. More recent studies with neuronal networks and primary neuronal cultures showed an inhibitory effect on bioelectrical activity, a dose and time dependent cytotoxicity but only moderate effects on cytosolic calcium concentrations, F-actin and the cytoskeleton (Hjornevik et al., 2015; Kulagina et al., 2006; Vale et al., 2007; Vilarino, 2008). In general, molecular effects of AZAs in different cellular systems have been increasingly investigated over the last number of years, due to the increasing availability of standards (Hess et al., 2009). Existing data have shown AZAs to have a cytotoxic effect on various cell lines (Serandour et al., 2012; Twiner et al., 2005, 2012c; Vale et al., 2008; Vilarino et al., 2006, 2007). Cell lines, such as human lymphocytes (Jurkat T cells), epithelial colorectal adenocarcinoma cells (CaCo-2) and breast cancer cells (MCF-7), showed a clear effect on the cytoskeleton, including a rounder structure and a reduction in the amount of pseudopodia (Twiner et al., 2005; Vilarino et al., 2007; Vilarino, 2008). Further studies support those findings by showing changes in the E-cadherin pool in epithelial cells (Ronzitti et al., 2007) and reductions in the level of F-actin (Roman et al., 2002). Additionally, AZAs have been shown to increase cellular levels of cAMP (Hess et al., 2007; Roman et al., 2002, 2004), modulate intracellular pH in lymphocytes (Alfonso et al., 2006; Roman et al., 2004), modify calcium flux (Alfonso et al., 2005; Hess et al., 2007; Roman et al., 2004) and inhibit cholesterol biosynthesis (Twiner et al., 2008b). Possible implications on heart functions have been investigated recently *in vitro*, showing a blockage of hERG channels (Ferreiro et al., 2014b; Twiner et al., 2012a) and *in vivo*, demonstrating a change in heart physiology of rats leading to arrhythmias and prolonged PR intervals (Ferreiro et al., 2014a) and subsequent heart failure (Ferreiro et al., 2016).

In a later study by Ito et al. (Ito et al., 2002), some of the mice developed lung tumours after repeated treatment with AZA but data on long term effects and/or carcinogenicity of AZAs are limited. The available *in vivo* data are indicative of tumour promoter potential of AZAs but severe toxicity observed in most cases restricts the relevance of those findings (Ito et al., 2002; Twiner et al., 2008a). A study by Colman et al. (Colman et al., 2005) suggest AZA1 to be a potent teratogen to finfish, also raising concern about possible environmental effects within the marine food web and eventually long term effects for human consumers at levels around the current regulatory limits (Colman et al., 2005). To date no data on the genotoxic potential of AZAs are available in the literature. Most data available are based on acute toxicity studies, involving mainly AZA1 due to the lack of or limited availability of standards.

To address this deficit, the present study set out to investigate the possible DNA damaging effect (genotoxicity) of AZA1–3 using the COMET assay. Two additional assays were included; the Trypan Blue Exclusion assay providing information on cell viability and total cell number, and flow cytometer analysis to investigate early and late apoptosis/necrosis. Taken together, all information allowed a more precise interpretation of the observed effects. AZA1 and AZA2 are directly produced by dinoflagellates and are regularly found in shellfish samples. AZA3 occurs in lower concentrations or is often absent (Furey et al., 2003). Different potencies of AZA1–3 have been detected in *in vivo* and *in vitro* studies and results suggested AZA3 to possibly be more toxic than AZA1 and AZA2 (Cao et al., 2010; Ofuji et al., 1999; Twiner et al., 2012b). Therefore, all three analogues were included in this study. Additionally, the present study was conducted on three different cell lines, Jurkat T cells (immune system), CaCo-2 cells (intestine), HepG-2 cells (liver), representing the main target organs of AZAs toxicity.

## 2. Material and methods

### 2.1. Toxins

Certified reference standards for azaspiracid 1–3 were purchased from the National Research Council Halifax, Canada. AZAs were supplied at stock concentrations of 1.47 μM for AZA1, 1.50 μM for AZA2 and 1.25 μM for AZA3, dissolved in methanol. Stock concentrations of 1 μM were prepared and kept in a freezer at −80 °C. Prior to each experiment, working solutions were freshly prepared by serial dilution to keep the volume added to each well consistent (5.1 μL/2 mL).

### 2.2. Cell culture

Jurkat T cells (European Collection of Cell Cultures, operated by Public Health England) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 50 mg/mL gentamicin. Cells were subcultured every 2–3 days at a starting density of $1 \times 10^5$ cells/mL. CaCo-2 cells and HepG-2 cells (European Collection of Cell Cultures, operated by Public Health England) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 1% penicillin/streptomycin plus 1% non-essential amino acids for CaCo-2 cells. Cells were subcultured when reaching 80–90% confluence. All cell lines were cultured at 37 °C in a 5% $CO_2$ humidified incubator. The passage numbers used for all cell lines were between 15 and 30.

### 2.3. Cell exposure

All cell lines were exposed to a final concentration of 0.01 nM, 0.1 nM, 1 nM and 10 nM of AZA1–3. Blanks were included in each experiment, either containing the vehicle (methanol) or being vehicle free. The exposure times for all three cell lines, Jurkat T cells, CaCo-2 and HepG-2 cells, were 24 h and 48 h.

### 2.4. Trypan Blue Exclusion assay

The cell viability and total cell number were determined by the Trypan Blue Dye Exclusion assay. Equal volumes of cell suspension and 0.4% Trypan Blue reagent were mixed and applied to a haemocytometer. The viability was calculated as the percentage of viable cells (trypan blue negative) of the total cell number (trypan blue negative plus trypan blue positive) (Strober, 2001). All cell counts were performed in duplicates.