

2005 Progress report on ASTOX – research on Azaspiracid standards and toxicology

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Introduction

The ASTOX project is a 3-year NDP-funded project and began in 2003, due to finish in mid 2006. Irish partners are the Marine Institute and University College Dublin, outside partners (not Irish funded) are official control laboratories for marine biotoxins and university collaborators in Japan as well as the Center for Coastal Environmental Health & Biomolecular Research (NOAA/NOS/NCCOS), US. Additional collaborations were also sought during the project and the outcome of those is also described in this manuscript.

The structure of this outline follows the project modules, which were designed to fulfil the major objectives:

- Obtain pure standards of AZAs + DTX-2 for instrument calibration and method validation
- Obtain pure standards of AZAs for toxicological studies confirming & extending previous work
- Conducting studies into the functional toxicity of azaspiracids at the cellular level
- Conducting studies into the effects of azaspiracids on gene expression of cellular models

The first 2 objectives are supported by WP1 (retrieval of contaminated shellfish) to obtain material for purification and preparation of reference materials. The objectives 3 and 4 will eventually be used in a WP6 on the establishment of safe levels of azaspiracids in shellfish for human consumption.

WP 1: Retrieval of contaminated shellfish materials

Several bulk shellfish tissue materials were obtained during the course of the ASTOX project. These materials served both for the isolation and purification of the toxins from shellfish and for the preparation of shellfish tissue reference materials that can be used in method development and validation.

Shellfish initially harvested in 2001 have been obtained, stored and used by the project for a number of studies until 2005. Also, three additional bulk lots of shellfish have been made available to this project, originating from 1999 and 2000. The year 2005 has been particularly toxic and 3 further bulk materials have been obtained, processed and are available now to the project.

WP 2: Preparation of stable and homogenous reference materials for AZA- and DTX-contaminated shellfish

Previous reports in this forum have primarily focussed on the study of parameters affecting homogeneity of shellfish tissue reference materials. Here, we report on studies of the factors influencing stability of shellfish tissue reference materials and how these can be optimised. Apart from slow oxygenation of the analyte in the matrix over a long time, the degradation of shellfish tissues can be accelerated by microbiological processes, in particular at temperatures above 0 °C. We have studied two separate processes that potentially are capable of preventing this microbial degradation, through suppression of microbial activity:

- a) Heat treatments
- b) Gamma-irradiation

While heat treatments had been previously studied at conditions below 100 °C (Hess et al., 2005), where no degradations were observed, the total removal of microbial activity typically requires autoclaving of tissues at ca. 120 °C. Therefore, we tested this condition for shellfish tissues contaminated with AZAs (Fig 1.). This harsher treatment at 120 °C leads to destruction of more than 50 % of the total AZA-content in the shellfish tissue. Therefore, heat treatment was not considered a practicable approach for the stabilisation of shellfish tissue reference materials for AZAs.

Subsequently, we tested gamma-irradiation as a technique and several doses from 5 to 20 kGy did not show any significant effect on the concentration of AZAs in shellfish tissues (Fig. 2). Also, gamma-irradiation has a less detrimental effect on the other, major constituents of the matrix and therefore, we recommend to further investigate the use of irradiation as a stabilisation technique of shellfish tissue materials as reference materials for AZAs.

WP 3: Isolation of Azaspiracids and Dinophysistoxin-2

In the first part of the study we implemented the procedure for isolation of AZAs as developed initially. Subsequently, we clarified and optimised this procedure to obtain the best possible procedure adapted to our laboratory environment. Over the past year, isolation of approximately 1 mg of AZA-1 was achieved. A spectrum acquired using nuclear magnetic resonance was used to determine the purity (Figure 3), and purity was confirmed to be in excess of 97 %. This purity is sufficient to use the compound either as calibrant or in toxicology studies. Current studies make use of the higher contaminated shellfish obtained in 2005 and are investigating the efficacy of the procedure by comparison with a procedure developed at the National Research Council, Canada.

Also, we have isolated in this project approximately 1 mg of DTX-2, which was characterised for its purity by NMR and found to be of sufficient purity for toxicology studies.

Both AZAs and DTX-2 have been made available to the European Community and National Reference Laboratories and to other research projects funded by the EU (see BIOTOX).

WP 4: Establishment of a functional in vitro assay for AZA-1

The aims of this study were to examine the effect that AZA-1 had on gastrointestinal cells and to determine potential mechanisms of action with a view to establishing an *in vitro* functional assay for AZA-1 detection. The human colon cell line - Caco-2 cells - were selected for these studies due to their ability to form tight junctions and allow for measurements of transepithelial electrical resistance (TEER). TEER was measured using an electrical resistance measurement device across a monolayer of Caco-2 cells adhered to a semi-permeable membrane. When confluent monolayers of Caco-2 cells were exposed to increasing concentrations of AZA-1 there was a dose- and time-dependent decrease in TEER. The robustness of our model was tested using AZA-1 of lower purity. No alteration in sensitivity was observed compared to AZA-1 of higher purity.

In order to address the potential problems of matrix interference in our model a range of experiments examining the effect of uncooked mussel extract (UME) on Caco-2 cells were carried out. No effect on TEER was observed with a 10% concentration of UME or lower. The effect of UME in combination with a range of toxins was also examined. UME did not appear to have any significant effect on the toxicity of AZA-1, OA or PTX-2. The effect of UME on DTX-2 needs to be clarified. The data to date suggests that UME will not interfere with the sensitivity of our model.

Additional marine biotoxins were examined in our model; okadaic acid (OA), DTX-2 and PTX-2. OA (100 nM) and DTX-2 (100 nM) both reduced TEER significantly at 24 h, with OA (500 nM) reducing TEER to basal levels. DTX-2 (500 nM) did not reduce TER to basal levels until 48 h, this was significantly less than the equivalent concentration of OA ($p \leq 0.05$). PTX-2 (100 nM) reduced TEER to basal levels at 24 h, while a significant decrease was observed as early as 4 h.

WP 5: Analysis of AZA-1 induced gene alterations by genechip microarray

Gene microarray experiments using the Affymetrix human genome array U133A 2.0 have been carried out in Dublin and bioinformatic analysis of gene expression alteration by AZA-1 is under way (Fig. 4). This data is enabling us to examine changes in gene expression after exposure to AZA-1. A concentration of 10 nM AZA-1 was selected with 24 and 48 h timepoints. Our current analysis has identified 132 genes significantly upregulated and 15 downregulated at 24 h and 209 genes upregulated and 18 downregulated at 48 h. Gene microarray experiments with the Jurkat - lymphocyte T cell line have also been carried out. We are exchanging data in an attempt to identify common mechanisms of action of AZA-1 in our different models.

Bioinformatic analysis has highlighted many biological pathways from our gene microarray data. Some of those identified are reported to be involved in response to stress, growth, differentiation, metabolism and structural integrity. Further analysis of these genes in combination with existing literature is necessary to attribute specific mechanisms to AZA-1.

By highlighting the modes of action of AZA-1 these studies will aid in the replacement of the *in vivo* mouse bioassay with the development of alternative *in vitro* test systems.

WP 6: Establishment of a NOAEL

This work-package is the last in the ASTOX project, to be completed at UCD. Results of this work-package will be reported in the final report.

Additional collaborations

An additional collaboration was possible during 2005 with a group in Norway. The toxicity of DTX-2 had previously been questioned, since mouse bioassays in Ireland and Norway were negative even though doses of DTX-2 were comparable to the lethal dose of okadaic acid. The toxicity of DTX-2 was investigated using intraperitoneal injection into mice and a relative toxicity factor of ca. 0.6 compared to OA was established.

Conclusions

A number of bulk samples of toxic shellfish have been gathered, processed and stored by the ASTOX project. These raw materials have been used successfully in the isolation of both AZA-1 and DTX-2, and have allowed us to study the parameters affecting the preparation of stable shellfish tissue reference materials. The isolated toxins and homogenous and stable reference materials are essential requisites in the development and validation of non-animal test methods for lipophilic shellfish toxins, and these materials will be used in further projects. The isolated DTX-2 was used in the study of its intraperitoneal toxicity in mice and a relative toxicity factor of DTX-2 versus OA was established (0.6). *In-vitro* toxicology studies using transepithelial electrical resistance suggest that a functional assay may be developed for azaspiracids using this technique. Such a functional assay may be helpful in replacing the current mouse bioassay, either directly, or by cross-validation of other more rapid and cost-effective techniques. Bioinformatic analysis of changes in gene expression induced by AZA-1 using micro-array gene-chips have aided in determining possible mechanisms of action. In the future, this may allow an evaluation of some of the earlier indications of the carcinogenicity of azaspiracids. Work package 6 will focus on establishing a safe level of azaspiracids in shellfish destined for human consumption.

Figure 1. Effect of heat treatment (120 °C) of tissue on AZA-concentration

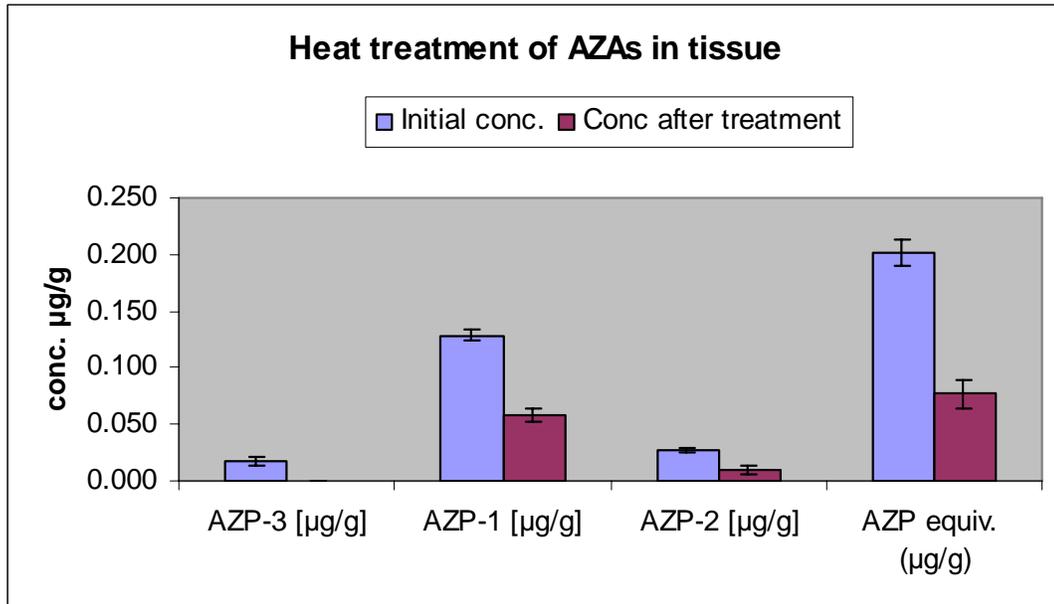


Figure 2. Effect of gamma-irradiation of tissue on AZA-concentration

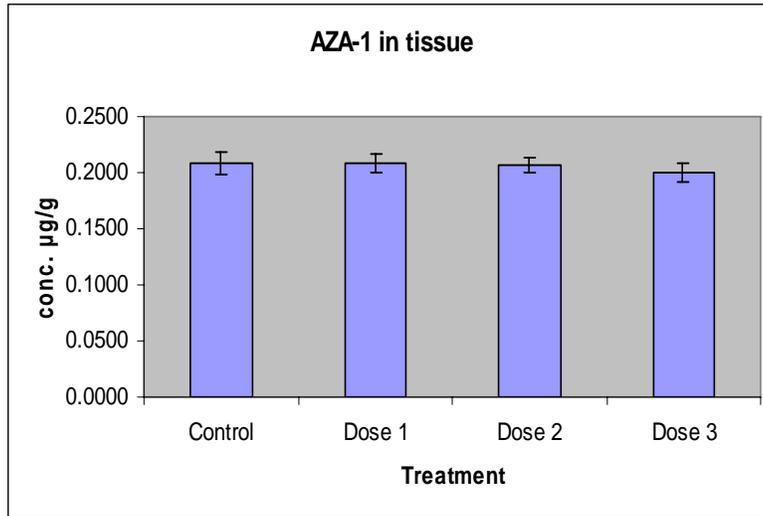


Figure 3. Purity of AZA-1 isolated at MI as characterised by NMR spectrum (Yasumoto and Naoki, 2005).

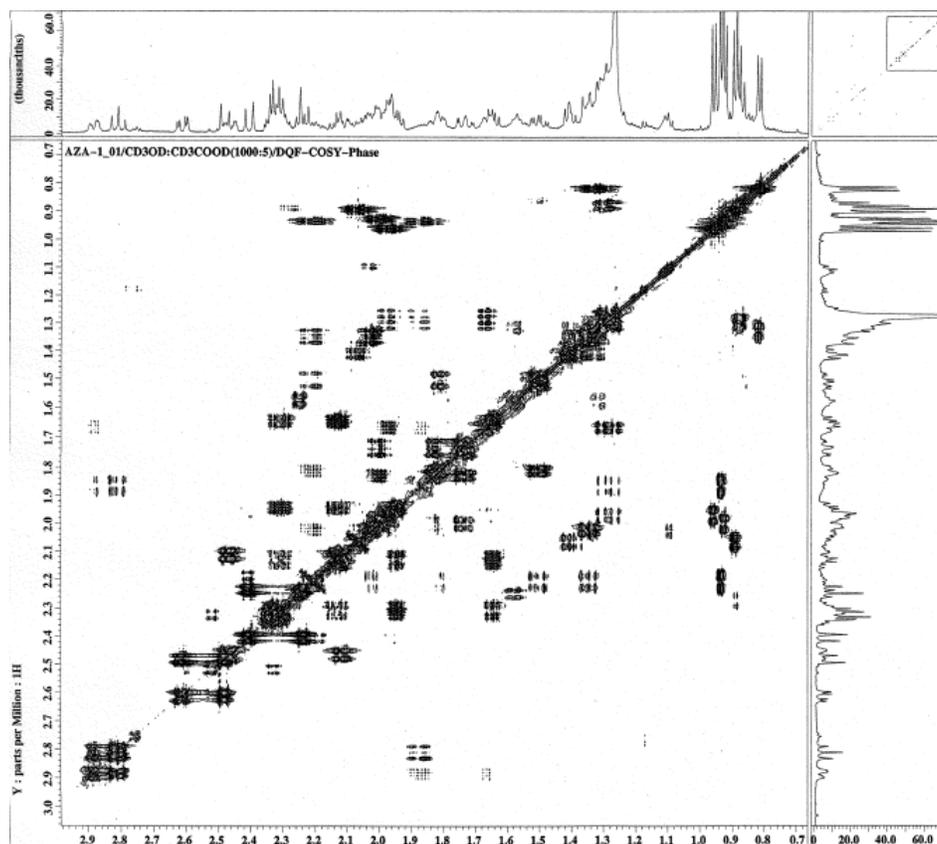


Figure 4. Representative display of gene changes using Affymetrix gene microarrays at 24 and 48 hours after exposure to 10 nM AZA-1.

