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# **Investigation of the genotoxic potential of the marine biotoxins okadaic acid and azaspiracids.**

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**A thesis submitted in fulfilment of the requirements for the degree of**

**Doctor of Philosophy**

**National University of Ireland, Cork**

**Department of Pharmacology and Therapeutics**

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## **Declaration**

I hereby certify that this material, which I submit for assessment on the programme of study leading to the award of PhD are the result of my work. The material has not been submitted for any other degree or qualification at University College Cork or elsewhere.

Signed: \_\_\_\_\_

Date: \_\_\_\_\_

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## Abstract

The present study investigated the genotoxic potential of the marine biotoxins okadaic acid (OA) and azaspiracids (AZAs). Harmful algae blooms (HABs) are an increasing global problem with implications for the ecosystem, economy and human health. Most data available on human intoxication are based on acute toxicity. To date, limited data has been published on possible long term effects, carcinogenicity and genotoxicity. To investigate genotoxicity in the present study, DNA fragmentation was detected using the COMET assay. In contrast to most other available studies, two further endpoints were included. The Trypan Blue Exclusion assay was used to provide information on possible cytotoxicity and assess the right concentration range. Flow cytometer analysis was included to detect the possible involvement of apoptotic processes. In house background data for all endpoints were established using positive controls. Three different cell lines, Jurkat T cells, CaCo-2 cells and HepG-2 cells, representing the main target organs, were exposed to OA and AZA1-3 at different concentrations and exposure times. Data obtained from the COMET assay showed an increase in DNA fragmentation for all phycotoxins, indicating a modest genotoxic effect. However, the data obtained from the Trypan Blue Exclusion assay showed a clear reduction in cell viability and cell number, indicating the involvement of cytotoxic and/or apoptotic processes. This is supported by data obtained by flow cytometer analysis. All phycotoxins investigated showed signs of early/late apoptosis. Therefore, the combined observations made in the present study indicate that OA and AZA1-3 are not genotoxic *per se*. Apoptotic processes appear to make a major contribution to the observed DNA fragmentation. The information obtained in this study stresses the importance of inclusion of additional endpoints and appropriate positive controls in genotoxicity studies. Furthermore, these data can assist in future considerations on risk assessment, especially regarding repeated exposure and exposure at sub-clinical doses.

## **Chapter 1: General Introduction**

### ***Biotoxins***

#### **Background**

Approximately 4000 phytoplankton species have been identified to date and about 300 of them can occur in high enough numbers to form so called harmful algae blooms (HABs). HABs is a broad term and includes visible (surface) blooms so called red tides and non-visible blooms with too small a population to discolour the water or which occur in deeper water levels [1]. Over the last few decades the frequency and intensity of HABs has increased as well as the geographical regions in which they have been reported [1-4]. The exact reasons for HABs remain unknown but suggestions have been made towards both natural mechanisms and human influence. Natural changes in the environment, for example increased temperature, light penetration and nutrient availability have been proposed as possible contributing factors for rapid population growths. Climate change, eutrophication, commercial shipping and the increased usage of coastal waters for aquaculture could also be held account for it, as well as a general increase in awareness and monitoring programs [5-11]. Of the species involved in HABs, approximately 60-80 are potential toxin producers [1, 12]. Some produce toxins at population densities as low as 100 cells/l, others at densities at  $1 \times 10^6$  cells/l or higher. Toxin production has been suggested as a mechanism to improve the ability of species to compete for space, avoid predation and overgrowth; however the exact reasons remain unclear [8]. Both toxic and non-toxic blooms can have negative impacts on the environment. The increase in biomass can lead to oxygen depletion, reduced light penetration and disruption of food web dynamics [6, 13]. Phycotoxins can have a direct impact on the marine fauna causing mortalities in fish, birds and marine mammals [6, 14, 15]. Plankton species release phycotoxins into the water but also serve as a food source for filter feeding shellfish and the larvae of some crustaceans and finfish allowing accumulation throughout the food web. Mussels (Mytilidae), oysters (Ostreidae), clams (Veneridae)

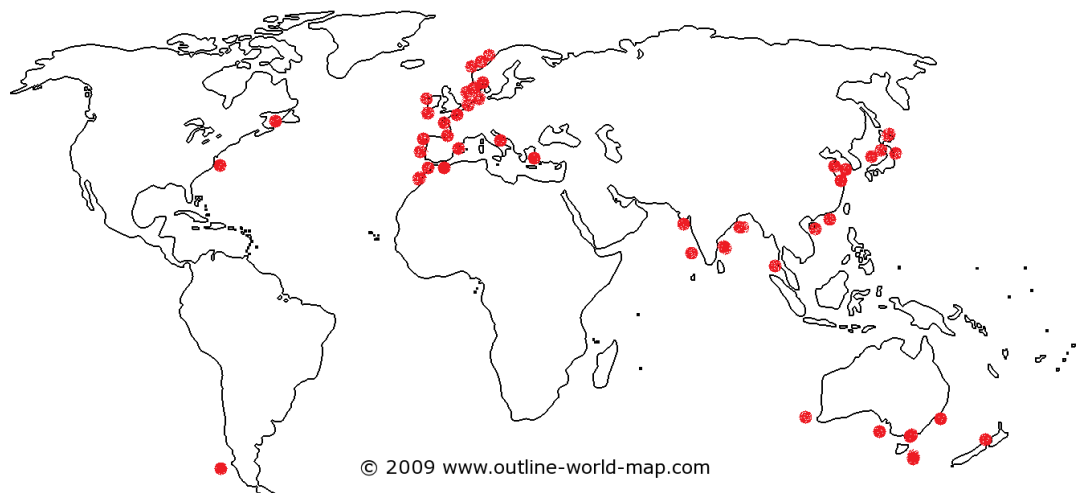
and scallops (Pechinidae) are the main bivalve species affected and are able to accumulate phycotoxins in their digestive glands up to levels that pose health implications to human consumers [1, 2, 5]. Acute intoxication has been reported with a variety of gastrointestinal and neurological symptoms but little is known about the impact of chronic exposure on humans [8]. Phycotoxins are a diverse group of chemicals with different structures, physical properties, mechanisms of action, potencies and toxic effects [16]. Historically they were organised in groups due to their symptoms caused in humans, however recently they have been re-grouped based on their chemical structure (**Table 1.1.**) [8, 9, 17].

**Table 1.1.** Overview of the biotoxin groups organised historically by their toxic syndrome/clinical symptoms and by their chemical structure. Acute symptoms in humans and cellular targets are listed, as far as they have been reported in the literature [9, 16, 18, 19].

<b>Toxin group</b> <b>Chemical structure</b>	<b>Historical classification</b>	<b>Reference compound/ analogues</b>	<b>Acute symptoms in humans</b>	<b>Cellular target</b>
Azaspiracid	Azaspiracid Shellfish Poisoning (AZP)	AZA1 ≥ 20 analogues	Gastrointestinal (Neurological)	Unknown
Brevetoxin	Neurotoxic Shellfish Poisoning (NSP)		Gastrointestinal Neurological	α-subunit of voltage sensitive Na-channels
Cyclic imines		Gymnodimine, Spirolide Pinnatoxins Prorocentrolide Spirocentrimine	None reported	Muscle/neuronal types of nicotine acetyl- choline receptors
Domoic acid	Amnesic Shellfish Poisoning	Domoic acid	Gastrointestinal Neurological	Kainate receptors
Okadaic acid	Diarrhetic Shellfish Poisoning (DSP)	Okadaic acid Dynophysistoxins ≤ 10 analogues	Gastrointestinal	Proteinphosphatase 1 and 2A
Pectenotoxin	Diarrhetic Shellfish Poisoning (DSP)	Pectenotoxin-2 ≥ 13 analogues	None reported	Actin
Saxitoxin	Paralytic Shellfish Poisoning (PSP)	Saxitoxin ≥ 30 analogues	Respiratory paralysis Death	Block voltage-gated Na-channels (site 1)
Yessotoxin	Diarrhetic Shellfish Poisoning (DSP)	Yessotoxin ≥ 36 analogues	None reported	Phosphodiesterase isoenzymes

## Okadaic acid

Okadaic acid (OA) and its analogues dynophysistoxins (DTX) are the most common phycotoxins involved in human intoxication and are the cause of Diarrhoeic Shellfish Poisoning (DSP) [20]. The earliest reports on DSP date back to 1961 in the Netherlands. The first confirmed incident of DSP however was in Japan in the late 70s [19]. Since then, thousands of cases of human poisoning have been reported worldwide, including Asia, Canada, United States, New Zealand and Europe (**Figure 1.1.**) [1, 22]. The areas most affected by OA seem to be Europe and Japan [23].

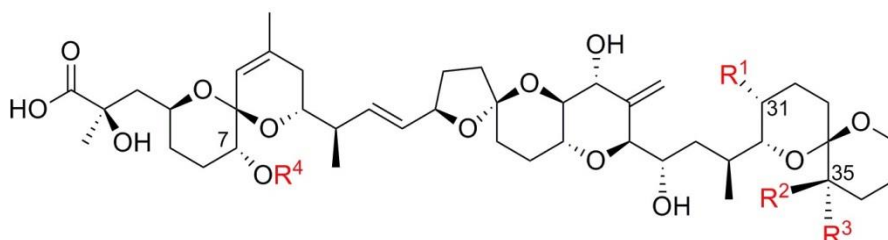


**Figure 1.1.** Worldwide occurrences of Diarrhoeic Shellfish Poisoning (DSP) are marked in red [6].

OA is a heat stable polyether fatty acid and is produced by dinoflagellates of the genus *Dynophysis* sp. and *Prorocentrum* sp.. Together with its analogues, DTX1-3 it forms the OA-toxin group. They differ in the position and number of methyl groups (**Figure 1.2.**), thereby DTX3 is a collective of the acylated forms of OA, DTX1 and DTX2 [24]. The acylated forms are quite unstable and have been suggested to be metabolic products as they have only been detected in shellfish and not in the toxin producing dinoflagellates



[25, 26]. The toxic equivalent factor<sup>1</sup> (TEFs) for OA and DTX1 is 1, for DTX2 0.6. The values for DTX3 are based on its unesterified equivalents [21].



	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>
<b>OA</b>	CH <sub>3</sub>	H	H	H
<b>DTX1</b>	CH <sub>3</sub>	CH <sub>3</sub>	H	H
<b>DTX2</b>	H	H	CH <sub>3</sub>	H
<b>DTX3</b>	CH <sub>3</sub> /H	CH <sub>3</sub> /H	CH <sub>3</sub> /H	Fatty acid

**Figure 1.2.** Chemical structure of Okadaic acid and its analogues.

Due to their lipophilic properties OA-toxins are able to accumulate in the hepatopancreas (digestive gland) of various species of filter-feeding shellfish [28]. The most common species are bivalve molluscs, consumption of these posing a risk to human consumers. Acute symptoms of DSP include diarrhoea, nausea, vomiting and abdominal pain. Symptoms occur within a few minutes to hours after consumption and a full recovery of the clinical symptoms normally occurs within a few days [17, 23, 29]. No lethality has been reported with the severity of the effects depending on the amount of toxin ingested [8, 23]. The main acute effects of OA in mice and rats, under laboratory conditions, are intestinal injury and lethality, oral administration

<sup>1</sup> The TEF is defined by the relative toxicity of an individual congener to either the most studied congener of the group, or if sufficient data are available, the most toxic compound. The latter is thereby assigned a value of 1 [27].

being 2-10 times less toxic than intraperitoneal (*i.p.*) administration [21, 30]. Administration by gavage<sup>2</sup> showed OA to be well absorbed from the gastrointestinal (GI) tract and a distribution among all internal organs within a very short time period. Intestinal content, urine, intestinal tissue, lung, liver, stomach, kidney and blood all contained OA, in descending order. 24 hours after administration the tissue and contents of the GI tract still showed high amounts of OA, indicating slow elimination. OA was further present in the liver and bile which together with the wide distribution throughout all organs indicates enterohepatic circulation to have taken place [23, 32]. A more recent study by Ito et al. [30] confirmed the distribution pattern, finding OA in lung, liver, small and large intestine, heart and kidney after oral administration. Additionally, the authors detected lung injuries and oedema in and erosion of intestinal villi as well as hypersecretion after single dosing of up to 250 µg OA per kg body weight. In contrast to rats receiving OA intragastrically and human cases, no diarrhoea could be seen in mice as fluids and eroded tissues were re-absorbed efficiently [33]. After administration, OA could be detected for another two weeks in liver and blood and for another four weeks in excretions from the intestine. A study by Tripuraneni et al. [35] failed to show OA as secretagogues yet reduction in resistance across cell monolayers could be detected. The authors concluded OA to disrupt the barrier function and increase paracellular permeability rather than directly stimulate secretion. *In vitro* studies have identified OA to be a potent inhibitor of serine/threonine phosphatase PP1 and PP2A in mammalian cells [35, 36]. The resulting hyperphosphorylation of proteins leads to a change in many cellular processes, including proliferation, differentiation and apoptosis [37-40]. Morphological and cytoskeletal changes have frequently been reported, including cell-cell and cell-surface detachment, cell rounding and effects on F-actin organisation and cytokeratin network [41-45]. A variety of studies have looked into the genotoxic potential

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<sup>2</sup> Gavage is a method by which a nutritional substance is directly supplied into the stomach of an animal by using a small plastic tube [31].

of OA. No mutagenic effect in the Ames test was detected, with or without metabolic activation but experiments with Chinese hamster lung (CHL) cells showed OA to induce a strong genotoxic effect without metabolic activation [46]. A significant increase in sister-chromatid exchange (SCEs) and mitotic cells, characterized by chromosome condensation could be identified in human lymphoblastoid cells and Chinese hamster ovary (CHO) cells. OA furthermore induced chromosome fragmentation in human lymphoblastoid cells and fragmented nuclei in CHO cells [47]. Using a  $^{32}\text{P}$ -postlabelling method OA was found to induce a dose-dependent DNA adduct formation in BHK21 C13 fibroblasts and HESV keratinocytes at a non-cytotoxic concentration range. Both cell lines showed the highest effect in the middle range of the concentrations used, HESV cells being overall more sensitive to OA. The authors suggested differences in the cell cycle, the accessibility of OA to the cell lines and possible biotransformation potential to be responsible for the earlier DNA adduct formation in HESV cells. Based on the DNA adduct formation in both cell lines the authors concluded OA to have a direct effect on the DNA [48]. In contrast, no direct effect on the DNA could be identified in other studies using CHO-K1 cells and CaCo-2 cells [49, 50]. The micronucleus (MN) assay in combination with fluorescence *in situ* hybridisation (FISH) showed OA to significantly induce MN at non-cytotoxic concentrations in CHO-K1 cells. The detected MN were centromere-positive, hence the authors suggested OA to be aneugenic rather than directly genotoxic [49]. OA also induced mononucleated and/or binucleated CaCo-2 cells with centromere-positive MN, in the absence of cytotoxicity. Again, the loss of whole chromosomes suggests an aneugenic potential of OA [38, 50]. Further studies using the mammalian cell forward mutation test and *in vitro* unscheduled DNA synthesis (UDS) in rat hepatocytes and the MN assay in human lymphocytes confirmed the lack of primary/direct DNA damage. The authors detected a change in chromosome number (aneuploidy) which they suggested contributed to the carcinogenic effect of OA [38, 51]. A study on colon epithelial cells of mice *in vivo* was inconclusive whether or not OA has a direct genotoxic or an aneugenic potential [38]. Other studies have linked apoptotic/necrotic processes [43, 52-54], oxidative damage and the

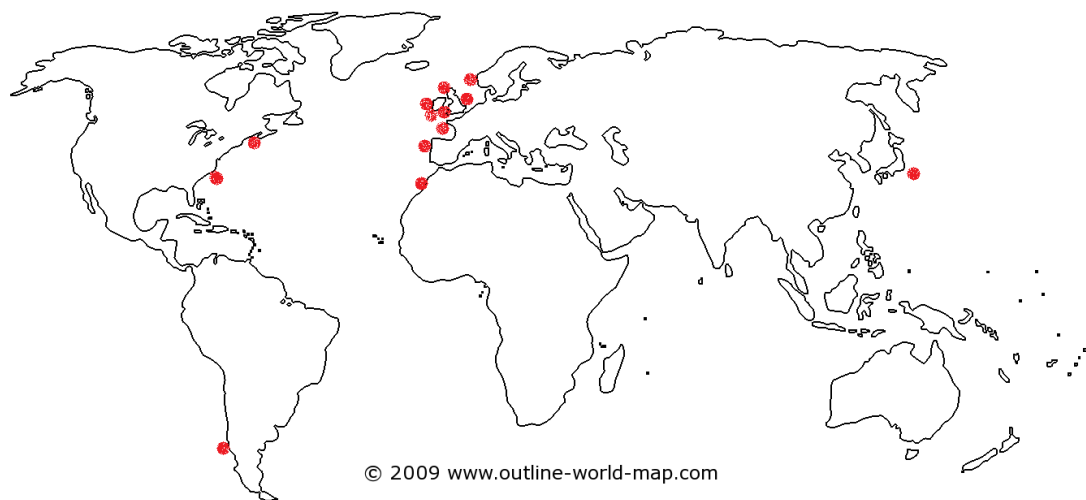
possibility of metabolic activity [37, 55, 56] to OA toxicity. Souid-Mensi [28] proposed that the effect of OA might be cell line dependent. A 2-stage carcinogenesis experiment with a single application of 7,12-dimethylbenz[*a*]anthracene (DMBA) followed by repeated application (twice a week) of OA to mouse skin prompted tumour development in 93% of the animals after 16 weeks. After 30 weeks an average of 2.6 tumours per mouse were detectable, hence the authors suggested OA to be a potent tumour promoter [57]. Further studies identified OA to also induce tumour promotion in rat glandular stomach after initiation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and to prompt tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) gene expression in mouse skin [58, 59]. No additive or synergistic effect could be detected after simultaneous application of OA and teleocidin, a 12-*O*-tetradecanoylphorbol-13 acetate (TPA) type tumor promoter [59, 60]. Together with the understanding that the inhibition of PP1 and PP2A alters gene expression, data suggests that OA has the potential to act as a non-TPA-type tumour promoter [59, 61, 62]. However, data on long term effects are limited. Most information is based on acute toxicity and therefore no tolerable daily intake (TDI) can be established. For this reason the European Food Safety Authority (EFSA) panel on Contaminants in the Food chain decided on an acute reference dose<sup>3</sup> (ARfD) of 0.3  $\mu\text{g}$  OA equivalents per kg body weight [23]. Shellfish meant for human consumption is controlled by the Regulation (EC) No 853/2004 and the maximum amount of OA equivalents allowed in shellfish meat has been limited to 160  $\mu\text{g}$  per kg. Due to the lack of long term data, concern has been expressed recently about potential effects of OA below the current regulation limit [37, 64].

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<sup>3</sup> ARfD is an estimate of a substance in food or drinking water that can be ingested over a short time period, such as one meal or over one day, without an appreciable health risk to the consumer. The ARfD is thereby expressed on a body weight base [63].

## Azaspiracid

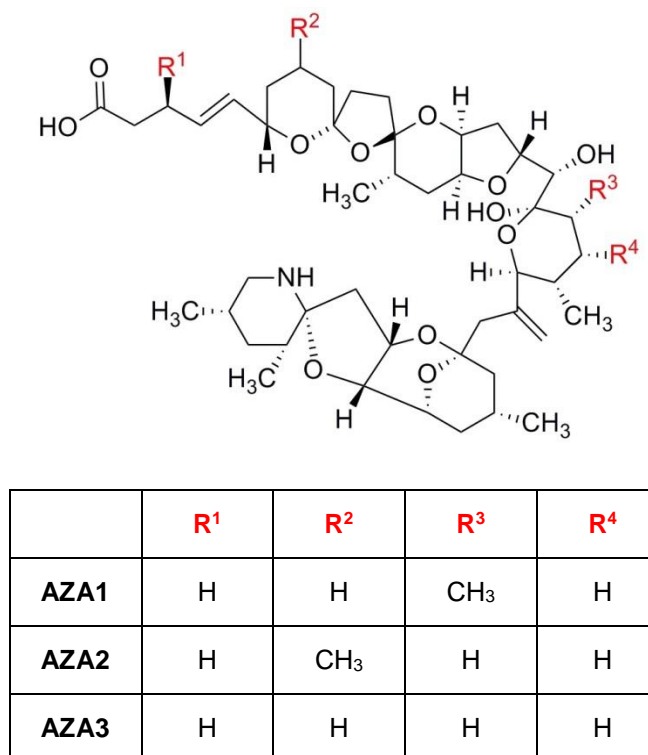
The azaspiracid group (AZA) is the most recently discovered group of biotoxins and is the cause of azaspiracid shellfish poisoning (AZP). It was first detected in 1995 by an outbreak of human illness in the Netherlands after consumption of mussels from Killary Harbour, Ireland. The symptoms associated with the outbreak were similar to DSP; however levels of DSP toxins were below the regulatory limit [65, 66]. The toxin was later identified as a novel marine toxin and named azaspiracid. Since its first discovery, AZAs have been identified in numerous outbreaks around the world, including northern Europe, Spain, France and recently Japan, Morocco, South America, eastern Canada and the United States (**Figure 1.3.**) [67-71]. In contrast to other biotoxins, blooms have also been detected during the winter months [66, 72].



**Figure 1.3.** Worldwide occurrences of Azaspiracid Shellfish Poisoning (AZP) are marked in red [67-71, 73].

AZAs are primarily produced by dinoflagellates of the genus *Azadinium spinosum* [74]. *Azadinium* comprises of six species, three of which have demonstrated toxin production to date. Recently, AZAs production has also been reported in the related dinoflagellate *Amphidoma languida* [73]. AZAs

are nitrogen-containing polyether toxins, their heterocyclic amine or aza group, unique tri-spiro-ring assembly and aliphatic carboxylic acid group are name giving. AZA1 was the first one to be identified and since then more than twenty further analogues have been discovered. AZA1-3 only differ in the number of methyl groups (**Figure 1.4.**). Most of these analogues are believed to be biotransformation products in shellfish and only AZA1 and AZA2 are said to be directly produced in *Azadinium spinosum* [73, 75-78]. AZA1 is heat stable (up to 100°C), colourless and at physiological pH electrically neutral, but contains both a negative and positive charge (zwitterion), AZA3 appears to be the most easily acid degradable of the analogues [76, 79]. Based on the limited toxicity data available TEFs have been established relative to AZA1; TEFs are AZA1 = 1, AZA2 = 1.8 and AZA3 = 1.4. AZA4 and AZA5, hydroxyl analogues of AZA3 are less toxic with TEFs of 0.4 (AZA4) and 0.2 (AZA5) [79].



**Figure 1.4.** Chemical structure of AZA1-3 [79].

AZAs are able to accumulate in filter-feeding bivalve molluscs, such as mussels, oysters, clams and scallops [75]. Recently AZAs have also been discovered in crustaceans from Scandinavia [69]. Based on the occurrence and TEFs AZA1-3 have the highest biological relevance. The majority of AZAs in shellfish samples detected to date are AZA1 or AZA2. AZA3 is generally present at lower concentrations or absent. AZAs accumulate in the digestive gland of shellfish and from there can migrate to other parts of the shellfish tissue [72]. Ingestion of contaminated shellfish can lead to AZP in humans. Acute symptoms are similar to DSP and include vomiting, nausea, diarrhoea and stomach cramps. Symptoms occur within a few hours after consumption and last for 2-3 days before a full recovery of the clinical symptoms is seen. No lethality or long term effects have been reported to date [80]. In contrast to DSP, *in vivo* studies in mice also showed neurotoxin-like symptoms, including respiratory difficulties, spasms, paralysis and death after *i.p.* injection with mussel extract [65, 66, 81]. The main target of AZA toxicity is the gastrointestinal tract. However, AZA1 administration to mice via gastric intubation also recognised the lymphatic system and the liver as target organs. At high concentrations AZA1 can also be found in other organs, including spleen, kidneys and lungs [82, 83]. This suggests that AZAs can be absorbed by the GI system and be distributed at least partially. Acute morphological changes in mice are distinctly different from other biotoxins. A study by Ito et al. [82] detected fluid accumulation in the small intestine, eroded villi in the lamina propria and epithelial cell and degenerating cells in the large intestine. Induction of histopathological changes and recovery were slower than in other biotoxins. Furthermore, the authors established AZA to cause fatty changes 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 [83]. To date, *in vitro* studies have failed to identify the cellular target of AZAs. A variety of morphological changes in cell lines have been reported, such as loss of cell membrane integrity, flattening of cells and reduction of pseudopodia [84]. Alterations of the cytoskeleton, accompanied

with changes in cell shape and loss of cell-cell / cell-surface interactions are suggested to be the result of changes in the E-cadherin pool and F-actin levels [85-87]. Furthermore, AZAs have proven to act on the activity of neurons [88], decrease viability in a variety of cell lines [88-91], inhibit cholesterol biosynthesis [92] and change cellular cAMP levels [93-95], intracellular pH [94, 96] and calcium flux [94-96]. Possible implications on heart functions have been investigated recently *in vitro*, showing a blockage of hERG channels [97] and *in vivo*, demonstrating a change in heart physiology of rats [98]. Exposure in the latter study occurred via single intravenous injection at concentrations of 11 µg and 55 µg per kg body weight. Limited data are available on long-term toxicity and/or carcinogenicity of AZAs. The above mentioned study by Ito et al. [82] also investigated the long term effects of repeated exposure to AZA1 by oral gavage in mice. AZA1 was administered at concentrations ranging from 1 µg to 50 µg per kg body weight, twice a week, up to 40 times within 145 days. Animals in the higher dose groups that died or had to be sacrificed during the treatment showed a loss in body weight, accumulation of gas in the gastrointestinal organs and a range of pathological changes. The latter included inflammation of liver and lung, erosion in the stomach and shortened villi in the small intestine. A few lung tumours were observed but not further considered due to the high toxic effects. No illness, weakness or lung tumours were detectable in the animals of the lower dose groups, neither at the end of treatment nor after an additional three months at the end of the treatment. No tumours were observed after eight months of treatment in a follow-up study by the same authors [99] but lymphatic nodules in the lung of about 1/3 of the animals were detected. One quarter of the animals that were kept on up to a year developed malignant lymphomas or lung tumours within that time frame, in the control group one out of fifty-two animals. The limited *in vivo* data available are indicative of tumour promoter potential of AZAs but severe toxicity observed in most cases restricts the relevance of those findings [79, 80]. A study in Japanese medaka (*Coryzias latipes*) mimicking maternal-egg transfer investigated the teratogenic potential of AZA1. Results showed dose-dependent effects on heart and developmental rate, hatching



success and the overall survival of the embryo. Further features included a reduced somatic growth and yolk absorption and a delayed onset of blood circulation and pigmentation. Hence the authors 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 below the regulatory limit [100]. To date no data on the genotoxic potential of AZAs are available in the literature [79]. Most data available are based on acute toxicity studies, involving mainly AZA1 due to the lack of or limited availability of standards. For this reason the European Food Safety Authority (EFSA) decided on an acute reference dose (ARfD) of 0.2 µg AZA1 equivalent per kg body weight. Shellfish meat for human consumption is regulated by the Regulation (EC) No 853/2004 and states 160 µg AZA1 equivalent per kg shellfish meat as the maximum amount permitted [79, 95].

### **Detection methods**

To protect the consumer from possible effects of phycotoxins, monitoring programs have been established in many European countries. These monitoring programs normally cover a wide range of toxins as contamination in shellfish is generally not restricted to one phycotoxin [8]. Both the rat bioassay and the mouse bioassay were regulated and standardized as the two main mammalian bioassays in the EU Commission Regulation (EC) No 2074/2005 [23, 101]. The rat bioassay (RBA) does not require the extraction of phycotoxins as shellfish hepatopancreas or meat is mixed with regular rat food or directly fed to pre-starved female rats. The consistency of faeces and amount of food eaten is observed and marked as -, +/-, +, ++ and +++. Responses in rats rated as + or ++ are considered equivalent to severe complaints in humans involving diarrhoea and nausea. In contrast, the mouse bioassay (MBA) includes the extraction of phycotoxins from shellfish hepatopancreas or whole flesh with solvents. Mice are exposed to the extract via *i.p.* injection and the survival is monitored over time giving a simple positive or negative response [23, 102]. The MBA is costly, non-specific,

solvent dependent, lacks sensitivity and is prone to inaccuracies in detection and procedural variations [5]. Biological functional assays, immunological assays and chemical analytical assays rely on structural and chemical properties as opposed to toxicity and therefore the overall toxicity has to be calculated with the help of toxic equivalent factors (TEFs). Biological functional assays are based on receptors or cells and use the mechanism of action to quantify toxicity [5, 101]. As receptors are not necessarily specific for one toxin group, results can only indicate toxin activity and not unambiguously identify the toxin. Immunological assays rely on specific antibodies. Based on the structure of the antibody either a specific toxin can be identified or all members of a toxin family. Hence cross-reactions can be beneficial or a limitation, depending on the test reason. Both methods are rapid, simple and easy to use. Chemical analytical methods include liquid chromatography (LC) with fluorescence (FL), ultra violet (UV) or mass spectrometer (MS) detection. Although they require trained personnel, toxic standards which can be limited in availability and are relatively expensive, these chemical analytical methods, especially LC-MS are effective methods for the detection and quantification of phycotoxins. For this reason LC-MS has been adopted in 2011 by the European Commission Regulation as a replacement for the MBA for the monitoring of the four major phycotoxin families, OA, PXT, YXT and AZAs [5, 101].

### **Implications and assessment**

Phycotoxins do not only display an environmental and public health problem but also pose an economic problem [1]. Aquacultures and harvesting sites can be closed for a prolonged time due to the occurrence of HABs. Mortalities of wild or farmed fish and shellfish and implications on tourism have been reported. The economic impact has been estimated to be millions of dollars around the world [6, 9, 13, 15, 103]. Maximum levels of toxins permitted in shellfish are regulated in many countries and monitoring programs have been set in place. Recent reports on acute intoxication are few or non-existent [80]. However, these regulation limits and ARfDs are

often based on very few studies and acute toxicity data only. In 2009 the EFSA panel on Contaminants in the Food chain concluded, on request from the European Commission (EC), that the current regulation limits in the European Union for OA, AZAs, STX and DA are not sufficient to protect human consumers [61]. This conclusion was based on the comparison of the current EU limits for shellfish meant for the market and the acute reference doses (ARfDs) as recommended by the EFSA panel. Establishing 400 g of shellfish meat as a realistic estimate of a large portion, exposure to OA and the AZA-group would exceed the recommended ARfDs 3- and 5-fold, respectively. For STX and DA the exposure would be 10- and 4-fold, respectively above the recommended ARfDs. No long term reference values could be established due to the lack of long-term toxicity data. The panel proposed in its concluding remarks that the reporting system for human illnesses should be improved. For some toxin groups, additional information such as mechanism of toxicity and genotoxicity is required to fully assess potential risks to human consumers [23, 61, 79, 104].

### ***Genotoxicity***

#### **Background**

Testing for genotoxicity is an essential part of hazard identification and is defined as the process in which the structure and/or information of the DNA gets altered. Such alterations to the genome can be spontaneous or through exposure to genotoxic agents. Genotoxicity can lead to permanent changes in the amount/structure of the genetic material but this is not an inevitable consequence [105, 106]. However, changes in the genetic material can trigger cell death, disturb cell homeostasis, alter cell regulation and has been linked to a variety of genetic diseases [31, 107]. The accumulation of DNA damage in cells has been proposed to play a role in degenerative conditions, such as immune dysfunctions and cardiovascular and neurodegenerative diseases. Mutations may cause cancer if DNA damage/changes occur in tumor suppressor cells and/or DNA response genes. Genetic alteration in

germ cells can result in infertility or inheritable damage which could have consequences for subsequent generations [31]. Carcinogenicity studies are relatively expensive and time consuming. Therefore genotoxic studies are often used as part of safety assessments to provide information on the potential damage to genetic material [31]. A range of *in vitro* and *in vivo* assays have been developed to identify substances which could trigger genotoxicity, inheritable damage or are able to identify the mechanism of action of such compounds. No assay *per se* is able to provide all the required information but can under- or overestimate the effect. This can be resolved using a multiple test system or so called test battery. Such test batteries include a variety of assays; the exact composition is dependent on the type of study and regulatory protocol involved. Yet all individual assays included complement each other, allowing for a better understanding of findings and more accurate recommendations concerning hazard identification [31, 108, 109]. The sensitivity, the chance of correctly identifying a genotoxic compound, increases with increasing numbers of tests. Conversely, the specificity decreases. The higher the number of different assays performed the greater the likelihood of false positive results [31, 108, 110]. *In vitro* assays have a higher sensitivity than *in vivo* assays and the exposure of the target cells is guaranteed, also they do not have an ethical component. They are designed to detect either micro-lesions (for example point-mutations) or macro-lesions (clastogenic effects). Micro-lesions can be detected by assays such as the bacterial reverse mutation test in *Salmonella typhimurium* and *Escherichia coli* (Ames test, OECD guideline 471) and the *in vitro* mammalian cell gene mutation test [31, 111]. The main principle of the Ames test is the reversion of originally present mutations in the bacterial strains and their re-found ability to synthesize an essential amino acid. While parent strains need amino acid supplementation, if gene mutation has occurred, the daughter generation is able to grow without. It is a quick, easy and widely used method. However, it uses prokaryotic cells which are different to mammalian cells in a variety of factors such as their chromosome structure, DNA repair processes and metabolism. The *in vitro* mammalian cell gene mutation test (OECD guideline 476) on the other hand uses a variety of

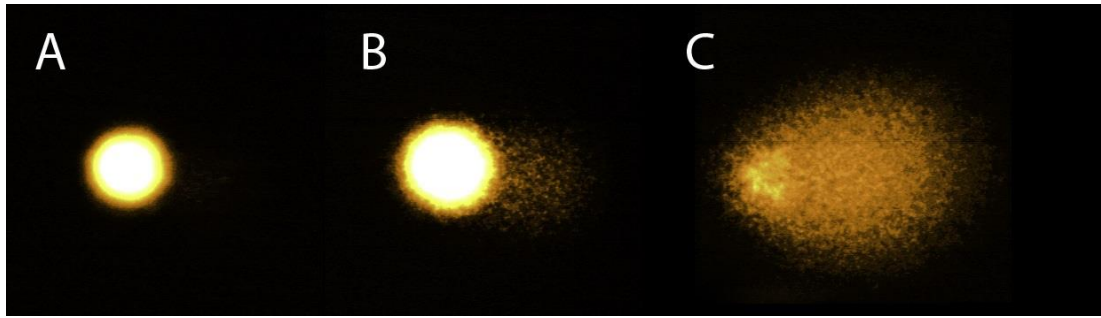
mammalian cell lines to detect gene mutations, such as base-pair substitutions or frame shifts. Preference is often given to the L5178Y mouse lymphoma cell line assay, which additionally can detect other genetic events such as large deletions or mitotic recombination [31]. Macro-lesions can be detected by assays such as the *in vitro* mammalian chromosomal aberration (CA) test, the sister chromatid exchange (SCE) assay, the *in vitro* mammalian cell micronucleus (MN) test and the COMET assay [31]. The CA test detects structural aberrations, also numerical changes (polyploidy) while the SCE test detects, as the name states, the exchange of genetic material between sister chromatids, visualized through staining techniques. If an exchange has occurred, chromosomes have stained and non-stained areas and are therefore called “harlequin chromosomes”. Both assays are time consuming and require training [31, 112]. The MN assay is a method to detect clastogens and aneugens alike. Isolated or broken chromosomes form micronuclei if they are not excluded during cell division and can be made visible through DNA staining. Additional to the standard protocol (OECD guideline 474), kinetochore staining and fluorescent *in situ* hybridisation (FISH) can give extra mechanistic information, for example about non-disjunction. Cytochalasin B (cytoB) addition allows assessment of cell proliferation. The COMET assay (described in detail below) detects overall DNA damage and as the MN assay, is quick and easy to perform [31, 105]. All *in vitro* tests are designed to detect one or more of the main genotoxic endpoints a) gene mutation b) alterations in chromosome structure (clastogenicity) and c) alterations in chromosome number (aneuploidy) [31, 113]. A possible drawback with *in vitro* systems is the general lack of metabolism. No cultured cell line is able to reproduce the full biotransformation capacity of tissues used in *in vivo* tests or the whole animal [109]. To overcome this potential challenge, metabolic activation systems are often included. The most frequently used system is a cofactor-supplemented post-mitochondrial liver fraction (S9) of animals treated with cytochrome P450 enzyme inducing agents, most commonly from rats [31, 105, 109, 114]. Literature suggests that a metabolic activation system is not necessarily required for all phycotoxins [46, 51, 55]. For example, OA showed a

genotoxic effect in Chinese hamster lung cells without metabolic activation [46]. A variety of assays has been established to detect genotoxicity *in vivo*. The transgenic rodent somatic and germ cell gene mutation assay (OECD guideline 488) has been established for the detection of gene mutations. The assay uses transgenic rats and mice to measure point mutations, insertions and small deletions in genetically neutral marker genes, genes of no immediate consequence to the animal. Both the mammalian erythrocyte micronucleus test (OECD guideline 474) and bone marrow chromosome aberration test (OECD guideline 475) have been established to detect chromosome damage. The latter detects only structural aberrations in bone marrow, while the mammalian erythrocyte micronucleus test detects structural and numerical chromosome damage in somatic cells. The COMET assay and unscheduled DNA synthesis (UDS) test with mammalian liver cells (OECD guideline 486) have been established for the detection of primary DNA damage. The endpoint of the UDS test is measured by the uptake of labelled nucleosides and indicative of DNA adduct removal by repair mechanisms [31, 105].

Recommendations have been made by several agencies such as the UK Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) [105], the U.S. Food and Drug Administration (FDA) [108] and EFSA [31] to use a set number of genotoxic tests with different endpoints, two *in vitro* assays and if necessary a third *in vivo* assay. The U.S. Environmental Protection Agency (EPA) has such a test battery in place, comprising of a) the Ames test, b) the *in vitro* mammalian cell gene mutation assay and c) either the *in vivo* bone marrow mammalian chromosome aberration test or the *in vivo* erythrocytes micronucleus assay [31, 105, 108]. Internationally recognized protocols for both, *in vitro* and *in vivo* tests are available through the Organisation for Economic Co-operation and Development (OECD), the International Workshops on Genotoxicity Testing (IWGT), the U.S. Environmental Protection Agency and the EU test methods regulation (EC 440/2008) [31, 105].

### **The COMET assay**

The COMET assay or single-cell gel electrophoresis (SCGE) is an established method for the detection of DNA damage and has been extensively used in various (research) areas, including biomonitoring, ecotoxicology, fundamental DNA damage and repair research and genotoxicity testing [115, 116]. The assay was first developed by Ostling and Johanson [117] in 1984 and later modified by Singh et al. [118] in 1988. The general principle behind the assay is that negatively charged DNA fragments will migrate towards the anode if an electrical field is applied [119]. In short, exposed cells are embedded in agarose on a microscope slide, lysed, the DNA uncoiled and placed in an electrical field for a short time frame. The DNA is afterwards stained with a fluorescent dye, most commonly Ethidium bromide (EtBr) and analysed under the microscope [116]. The assay got its name from the appearance of the DNA of a single cell. The undamaged high molecular weight DNA forms the comet head, the migrated DNA fragments form the comet tail (**Figure 1.5.**). Analysis can be done visually or with the help of software packages, which identify fluorescent parameters of manually selected comets. The parameters used most commonly are tail length, percentage tail DNA and tail moment. The tail length increases when the COMET tail is first being established at relatively low damage. However, with increasing damage the tail intensity increases but not the tail length. Tail moment is the sum of the tail length and the tail intensity. Both, the tail moment and the tail length do not show a linear dose-response and are more likely to be effected by thresholds and background settings. Percentage tail DNA is considered the most reliable of the three parameters, as it has a linear relationship to strand break frequency and allows discrimination over the widest range [116, 120].



**Figure 1.5.** Imaging of cells after performance of the COMET assay a) a cell without DNA fragmentation, b) a cell with minor DNA fragmentation and c) a cell with major DNA fragmentation. Cells are stained with Ethidium bromide and images were taken under a fluorescence microscope (Nikon EFD-3, magnification: 40x).

Two different variations of the COMET assay are in use, one in neutral conditions and one in alkaline conditions. The neutral assay can detect only single-strand breaks and double-strand breaks, while the alkaline version is able to detect single- and double-strand breaks as well as incomplete repair sites, alkali labile sites and with further modifications DNA-protein and DNA-DNA cross links [116, 119, 121]. The COMET assay has many advantages compared to other tests. It is a simple, easy to use, cost effective quantitative and qualitative assay. It requires minimum amounts of test sample [122], which is especially important in relation to biotoxins. Often only small amounts of toxin sample isolated from shellfish extract are available and standards can be expensive. Detection is at a single cell level and it can be applied to any eukaryotic or prokaryotic cells or tissues given that a single cell suspension is possible. It is non-invasive when used *in vivo*, and shows a higher flexibility compared to other assays as it can be applied to proliferating cells as well as non-proliferating cells [115, 120, 122, 123]. The COMET assay (*in vitro* and *in vivo*) has shown some variability within and between experiments. Automated scoring systems have minimized the interpretation error but selection of comets is still done manually [115, 120, 122]. Results given by the COMET assay reflect the overall damage in the cellular DNA based on strand breaks, independent of the mode of action. To



assess whether the damage visualized is based on direct genotoxicity or other factors, for example cytotoxicity, apoptosis or necrosis, additional assays should be included in the study design. This allows for the appropriate interpretation of the DNA fragmentation detected and its biological relevance [116, 119, 124].

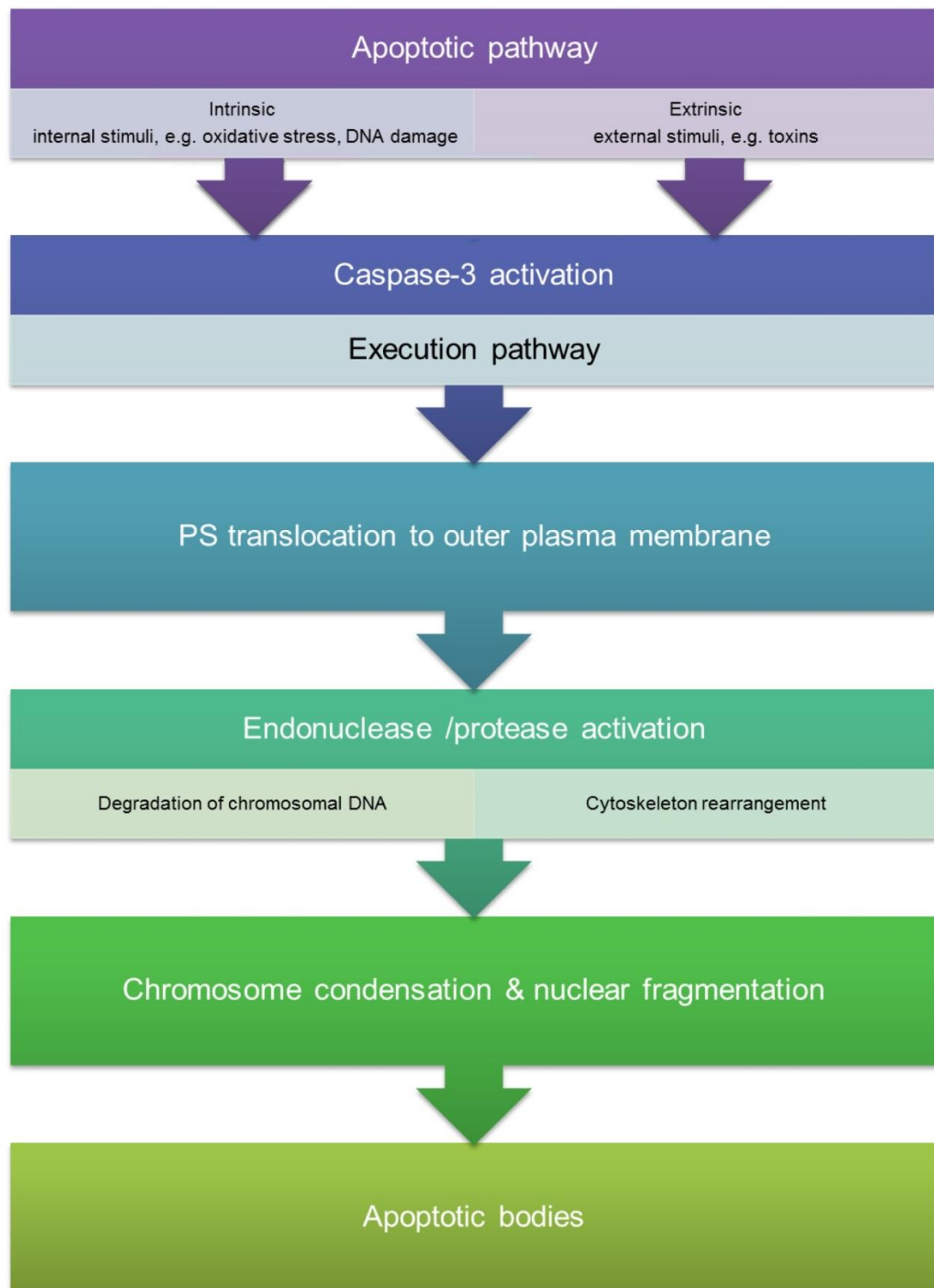
### **Cell death**

An integral part of *in vitro* assays are cell viability tests. They are essential to interpret data from other endpoints correctly, such as genotoxicity. Cell death can be a result of natural events or external factors. One of the main questions surrounding cell death is, “when is a cell dead?” The Nomenclature Committee on Cell Death (NCCD) has recommended that cells should be considered dead when 1) the cell membrane integrity is lost 2) the cell and nucleus are fully disintegrated and/or 3) the cell has been engulfed by a neighbouring cell [125]. The loss of cell membrane integrity can be assessed *in vitro* by the exclusion of certain dyes, for example propidium iodide (PI) or trypan blue. Viable cells are impermeable to trypan blue due to their intact cell membrane, whereas dead cells have a deficient cell membrane and are permeable to trypan blue. Excessive cytotoxicity has been shown to give a number of false positive results in a variety of assays, including the MN assay, CA test and COMET assay [109, 121, 126, 127]. Ideally a wide concentration range should be included, a highest concentration with a clear cytotoxic effect as well as a lower concentration which does not cause cytotoxicity (viabilities between 90% - 100%). If these requirements are met, one can be confident that observed positive results, in the absence of overt cytotoxicity, represent a genotoxic effect caused by the test compound and equally that negative results are due to lack of genotoxicity of the test compound and not due to limitation of the concentration range investigated [109, 115, 121]. An additional indication for cell death is the cell number. While the cell number is unchanged in case of genotoxic damage, the cell number is reduced during cell death based on the full disintegration of cells (125, 128).

Part of the process of cell death is the activation of biochemical cascades that result in a variety of morphological changes, for instance the display of apoptotic or necrotic features [125]. Apoptosis or “programmed cell death” occurs under normal circumstances as part of a balanced process to maintain cell populations and tissues during development and aging. However, it can also be triggered during immune responses or when cells are damaged due to diseases or external stimuli, such as toxic substances [129, 130]. To date, two main apoptotic pathways are distinguished 1) the extrinsic pathway or death receptor pathway a result of external stimuli, and 2) the intrinsic pathway or mitochondrial pathway, a result of internal stimuli, for example oxidative stress and DNA damage. While there are significant differences between those two pathways, some features are common (**Figure 1.6.**). Both share a range of morphological and molecular changes that are reversible until the so called “point of no return” [125, 129, 131, 132]. Furthermore, both pathways lead on to the same execution pathway, triggered by the activation of caspase-3, a member of the cysteine-dependent aspartate-specific protease family. Caspase-3 plays a major role in the cleavage of a range of cellular proteins [133]. An early marker after the onset of the execution pathway is the exposure of phosphatidylserine (PS). PS is a phospholipid component normally located in the inner leaflet of the plasma membrane. Due to the plasma membrane changing its structure in the process of apoptosis PS becomes exposed on the outer leaflet of the membrane. There it functions as a specific marker for macrophages and phagocytes [134]. This is generally followed by protease activation and endonuclease activity which lead to the degradation of chromosomal DNA and structural changes in the cytoskeleton. Chromatin condensation and nuclear fragmentation are later steps in the apoptotic process and finally lead to the formation of apoptotic bodies [132, 134].

In contrast to apoptosis, necrosis is characterized by an increase in cell volume and an early loss in cell membrane integrity. Until recently necrosis has been considered only an accidental and uncontrolled event but evidence is growing that it frequently is a well regulated process. Under special

conditions, ligation of death receptors, excitotoxins or alkylating DNA damage can trigger regulated necrosis [131, 132].



**Figure 1.6.** Overview of apoptotic events, external or internal stimuli trigger a cascade of changes. Both pathways share the activation of an execution pathway and the translocation of phosphatidylserine (PS) to the outer membrane, an early marker of apoptosis and DNA fragmentation, a late event.

A vast variety of methods are available to detect parameters associated with cell death *in vitro* or *in vivo*. Based on the aim of the study it could be feasible to apply a combination of complementary tests. A single test might not precisely demonstrate the aspect of cell death which is of interest [125]. The methods vary in their specificity, sensitivity, detection range, cell stage, death parameter and throughput. For example, light microscopy is a quick and easy method but lacks specificity. One of the more convenient methods is cytofluorometry. Different protocols have been developed using a variety of dyes for different endpoints. Annexin V, a phospholipid binding protein is not able to penetrate the plasma membrane but it has a high affinity for PS. On translocation of PS from the inner to the outer leaflet of the plasma membrane in the apoptotic process, Annexin V can bind and if conjugated with fluorescein isothiocyanate (FITC), a fluorescence dye, can be made visible in a flow cytometer. As PS translocation is considered an early process in the apoptotic event, Annexin V binding / FITC positive staining is used as an early marker of apoptosis. To discriminate apoptotic processes from necrotic processes membrane impermeable DNA stains such as PI are used in combination with Annexin-FITC. PI is unable to penetrate intact cell membranes and therefore cells that stain FITC positive but PI negative can be considered (early) apoptotic. Cells which stain both FITC positive and PI positive have lost their membrane stability and are therefore either necrotic or late apoptotic [125, 129, 134].

### **Objectives**

Most information on the toxicity of phycotoxins is based on acute toxicity. Data on genotoxicity and low level exposure including long term effects are limited. OA has shown some genotoxic potential, however data are often contradictory and the involvement of cytotoxicity in the detected effects cannot be eliminated. As a result the data available are difficult to interpret (23). No long term toxicity/carcinogenicity studies have been reported but OA

is identified as a tumor promoter in rodents [57-60]. No genotoxicity data for AZAs has been reported to date [79]. Repeated toxin administration over a longer duration in rodents identified occasional lung tumours. These findings coincided with doses causing severe toxicity and therefore may be of limited relevance [79, 80, 82]. To fully assess the potential risk of phycotoxins on the environment and human consumers, information on those aspects are important factors.

The aims of the present study are to investigate the genotoxic effects of OA and the AZA group using the COMET assay in cell lines representing the main target organs of these biotoxins. The COMET assay is a direct method that requires only a short time frame to complete, depending on the sample size. It can be adopted for small amounts of test substances and for a variety of cell lines making it ideal for biotoxin studies. The cell lines selected in this study were 1) Jurkat T cells (human T cell lymphoblasts), 2) CaCo-2 cells (human epithelial colorectal adenocarcinoma cells) and 3) HepG-2 cells (human hepatocellular cells). Besides representing the main target organs of OA and AZAs, published data indicates cell line specificity of biotoxins. The COMET assay analysis was complemented with cytotoxicity and apoptosis analysis. These assays provide information on whether the biotoxin-induced DNA fragmentation in the COMET assay coincides with an increase in cytotoxicity and/or apoptosis. Taken together, all information will allow a more precise interpretation of the observed effects.

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## Chapter 2: Positive controls

### ***Introduction***

The main goal of hazard identification is the realistic assessment of potential risks caused by drugs and chemicals [1]. One essential part of these safety assessments are tests for genotoxicity. A variety of *in vitro* and *in vivo* assays have been developed which are mostly easy to perform, relatively inexpensive, faster than carcinogenicity studies and detect a variety of genotoxic effects using a range of endpoints. Genotoxic compounds might not test positive in all assays; however there is a good overall correlation between the different assays [2]. In order to appropriately interpret the findings of any of these tests some requirements have to be met. A suitable concentration range has to be established, with a clear and definite positive and negative effect. A dose-response relationship should be detectable and the results should be reproducible [3]. Furthermore, negative controls and appropriate positive controls should be included. The crucial functions of negative and positive control data are the in-house establishment of the test system and the development of in-house background data. If provided for each test system, the data supports the verification of new studies or compounds tested [3, 4]. Usually the solvent of the test substance is used as the negative control. Results found in the test system are compared to the data found with the negative control study. Differences identified can be regarded as the test compound effect. Untreated samples are also often included to rule out any effect of the solvent on the test system [4-6]. Positive controls assess if the test system used is capable of detecting a known genotoxic agent under the current conditions. The concentration of the compound should give a clear positive result. However, it should not be associated with excessive cytotoxicity. The exact compound used, thereby depends on the experimental aim and the test assay. Some examples of positive controls used in the absence of metabolic activation (S-9 mix) are 4-nitroquinoline-N-oxide, ethylnitrosourea (ENU), methylmethanesulfonate (MMS) and ethylmethanesulfonate (EMS) [3, 5, 6]. EMS has frequently been

used in DNA repair studies and is widely used as a positive control for genotoxicity testing *in vitro* and *in vivo*. It is a direct acting genotoxic agent which causes carcinogenic effects in mammals and mutagenic effects in animals and plants [3, 7, 8]. Cotelle et al. [9] found EMS to significantly increase DNA migration in plant cells. Other studies have found EMS to significantly increase the number of mutations in the HCO/HPRT assay [10], increase the frequency of micronuclei (MN) in flow cytometer analysis and DNA damage in the COMET assay [11]. EMS acts through the addition of a methyl group to DNA nucleotides and has been stated to cause DNA adduct formation, resulting in single DNA strand breaks [11-13]. Cadmium chloride ( $\text{CdCl}_2$ ) is another compound that has been reported as a good positive control for genotoxic testing [14-17]. It is a highly toxic metal compound [18, 19] but has also been described in the literature as mutagenic [18], genotoxic and carcinogenic in human and animal cell lines [14, 19-21]. However, the DNA damaging effects identified in various studies are often accompanied by either excessive cytotoxicity [15] or reactive oxygen species (ROS) formation. This has led various authors to suggest that the DNA damage detected might be caused by indirect interactions [18, 20, 22-24]. It has also been proposed that  $\text{CdCl}_2$  may induce necrosis [15] or apoptosis in various cell lines [18, 21, 25]. Staurosporine, an alkaloid isolated from *Streptomyces* sp., is a potent inhibitor of phospholipid/calcium dependent protein kinase. It has the ability to rapidly induce apoptosis, via mitochondrial caspase activation in mammalian cells, in the absence of genotoxicity. Staurosporine is therefore frequently used as an apoptosis inducing agent [26, 27].

The aim of the present study was the establishment of in house reference data for the assays used in subsequent studies on marine biotoxins. The positive controls chosen are characterized above. The assays and the decision as to which cell lines to use have been described in greater detail in Chapter 1. In brief, the COMET assay was selected as the detection method for genotoxicity. It has many advantages to other assays, such as being quick, easy to use and requiring only little test compound. It can furthermore be performed on various cell types if a single cell suspension is possible.

This is of importance when investigating differences in tissue responses to compounds. The additional analysis of cytotoxicity assisted in assigning the correct concentration range as well as with the interpretation of data. It allowed determining whether DNA damage detected was a positive genotoxic result or the result of overt cytotoxicity. The use of flow cytometer analysis was included to investigate early and late apoptosis as an alternative explanation for the observed DNA damage.

### ***Materials & Methods***

#### **Chemicals & Reagents**

All chemicals were purchased from Sigma-Aldrich, Ireland unless otherwise indicated. Annexin V and Annexin V detection kit, flow tubes and flow cytometer fluids were obtained from BD Bioscience, UK. Microscope slides and cover slips were purchased from Fisher Scientific, Ireland. All plastic ware was acquired from Sarstedt, Ireland.

#### **Cell culture**

Jurkat T cells (human T cell lymphoblasts), CaCo-2 cells (human epithelial colorectal adenocarcinoma cells) and HepG-2 cells (human hepatocellular cells) were obtained from the European Collection of Cell Cultures (ECACC, operated by Public Health England). Jurkat T cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 50 mg/mL gentamicin. CaCo-2 cells and HepG-2 cells were maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. Additionally, 1% non-essential amino acids (NEAA) were added for CaCo-2 cells. All cell lines were cultured at 37°C in a 5% CO<sub>2</sub> humidified incubator (Forma Scientific Infrared CO<sub>2</sub> incubator, Biosciences, Ireland). Non-adherent cells were kept in upright standing 25 cm<sup>2</sup> polystyrene tissue culture flasks; adherent cell lines were kept in 75 cm<sup>2</sup>

polystyrene tissue culture flasks. The passage numbers used for all cell lines were between 15 and 30.

Adherent cells were passaged when reaching 80-90% confluence. The medium was aspirated and the cells were washed with phosphate buffered saline (PBS). PBS was aspirated and cells were incubated for 5 minutes with trypsin-EDTA (0.25%) to allow cell detachment. Trypsin was neutralised by addition of complete medium and the cell suspension was then centrifuged at 400 g for 5 minutes. The cell pellet was re-suspended in complete medium and 10% of the cells were then re-seeded in a new flask containing complete medium. Non-adherent cells were passaged every 2-3 days at a starting density of  $1 \times 10^5$  cells/mL.

### **Cell exposure**

The stock solutions of 1 mM and 5 mM cadmium chloride ( $\text{CdCl}_2$ ) were made up in double distilled water and 50 mM and 250 mM of ethylmethanesulfonate (EMS) were prepared in serum free medium. Both stocks were kept at 4° C until use. Stock solutions of EMS were prepared fresh after a month due to the half-life given by the product information sheet provided by Sigma-Aldrich, Ireland. Staurosporine was purchased as 1 mM solution dissolved in DMSO. The chemicals were added at 2% v/v of the total volume in the well; serial dilutions were performed where needed to keep the added volume consistent.

For experiments, cells were seeded in 6 well plates at a density of  $2 \times 10^5$  cells/mL, with a total of 2 mL per well. Adherent cells were seeded the night before (to allow re-attachment) while non-adherent cells were seeded 4 hours prior to exposure. The final concentrations initially used for  $\text{CdCl}_2$  were 20  $\mu\text{M}$  and 100  $\mu\text{M}$ . Lower concentrations were included for CaCo-2 (5  $\mu\text{M}$ ) and HepG-2 cells (1  $\mu\text{M}$  and 5  $\mu\text{M}$ ) due to low viabilities/reduction in cell number observed at 20  $\mu\text{M}$  and 100  $\mu\text{M}$  in initial experiments. All three cell lines were exposed to 1 mM and 5 mM EMS. Based on concentrations established in the literature [27, 28, 29] Jurkat T cells were furthermore

exposed to Staurosporine at a final concentration of 2.5  $\mu\text{M}$  (0.25% v/v) and 5  $\mu\text{M}$  (0.5% v/v). Blanks were included in each experiment, either containing the corresponding vehicle or being vehicle free. The exposure time for Staurosporine was 2 hours. The initial exposure time for all other experiments was 24 hours. Based on initial results, exposure times of 48 hours for Jurkat T cells and CaCo-2 cells and 12 hours for HepG-2 cells were included in this study.

### **Trypan Blue Dye Exclusion assay**

The cell viability was determined by the Trypan Blue Dye Exclusion assay following the protocol by Strober [30] with slight modifications. Aliquots (100  $\mu\text{L}$ ) of the cell suspension were transferred to Eppendorf cups, mixed with a 0.4% trypan blue solution (1:1) and applied to a haemocytometer. The viability was calculated<sup>4</sup> as the percentage of viable cells (trypan blue negative) of the total cell number (trypan blue negative plus trypan blue positive). All cell counts were performed in duplicates.

### **COMET assay**

Alkaline single cell gel electrophoresis was performed following the protocol by Woods et al. [31]. In brief, the exposure medium (2mL) was transferred to Eppendorf tubes and centrifuged, the adherent cells were detached as described before. Cell pellets from the exposure medium, if existent, and detached cells were re-suspended together in 1 mL of complete medium. Non-adherent cell samples were taken directly out of the wells. In the case of low cell numbers the suspension was centrifuged (400 g, 5 minutes) and re-suspended in 100  $\mu\text{L}$  medium. Microscope slides were pre-coated with 30  $\mu\text{L}$  1% normal-melting agarose (NMA) in PBS and allowed to dry. Onto those

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<sup>4</sup> Calculation of total cell number:

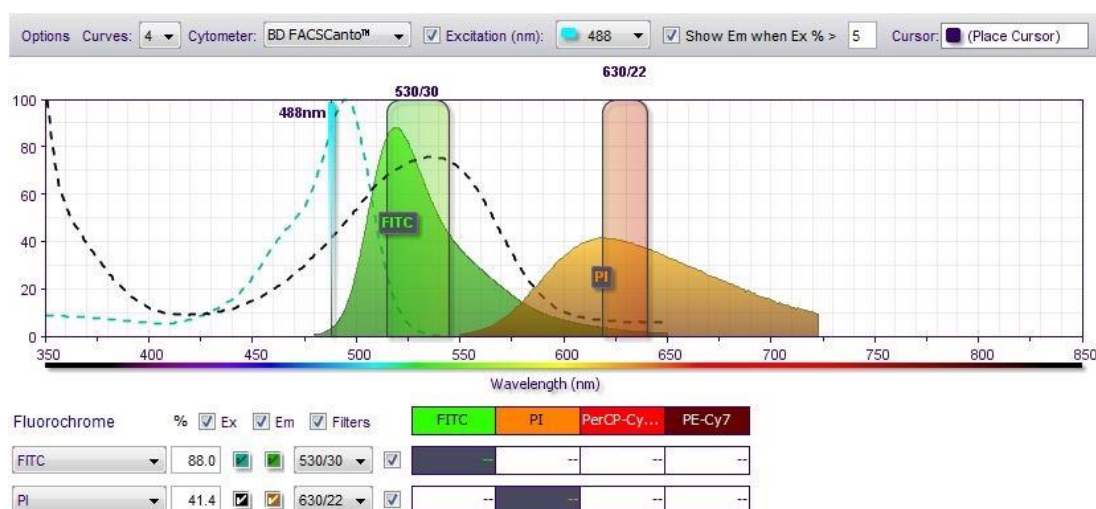
$\text{Cells/mL} = (\text{average count/square}) \times 2 (\text{dilution factor}) \times 10\,000 (\text{chamber conversion factor})$

slides 100  $\mu\text{L}$  of NMA was added, a cover slip applied (22 x 22 mm) and allowed to solidify on ice. The cover slip was removed and an aliquot of the cell suspension (30  $\mu\text{L}$ ) was mixed with 1% low-melting point agarose (LMA, 70  $\mu\text{L}$ ) in PBS and transferred to the prepared microscope slide. The slides were allowed to solidify on ice before being immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 90 mM sodium sarcosinate, containing 10 % (v/v) DMSO and 1 % (v/v) Triton X-100) for a minimum of 1 hour at 2-8°C in the dark. The lysis solution was stored at 4°C for at least 1 hour prior to use. Slides were then transferred to a horizontal electrophoresis tank and immersed in an alkaline solution (200 mM EDTA (pH 10), 10 N NaOH ) for 30 minutes. Electrophoresis was carried out for exactly 25 minutes at 22 V and 300 mA. Slides were then neutralised three times for 5 minutes with 0.4 M Tris (pH 7.5) and stained with Ethidium bromide (EtBr, 20  $\mu\text{g}/\text{mL}$ ) for 5 minutes. Slides were washed with double distilled water (ddH<sub>2</sub>O) for 5 minutes before being stored in a fridge until analysis. Storage under damp conditions gave reliable data up to 4 days. In total 50 cells per slide were scored and the percentage of tail DNA was used to determine the degree of DNA fragmentation. All samples were analysed in duplicate and a total of four independent experiments were performed for each cell line, chemical and exposure time. The analysis was performed using imaging analysis software package Komet 4.0 (Kinetic Imaging Ltd).

### **Flow cytometer analysis**

Samples for flow cytometer analysis were prepared according to the instruction on the technical data sheet provided by BD Pharming, with slight modifications [32]. Non-adherent and adherent cells were transferred to 15 mL tubes and washed twice with cold PBS. The cells were re-suspended in 400  $\mu\text{L}$  binding buffer, 200  $\mu\text{L}$  if the cell pellet was small. An aliquot of 100  $\mu\text{L}$  was then transferred to a flow tube. Five  $\mu\text{L}$  of FITC Annexin V and 5  $\mu\text{L}$  of PI staining solution were added to each sample and stored at room temperature, in the dark for 15 minutes. After staining 400  $\mu\text{L}$  of binding buffer was added and samples were analysed on a flow cytometer within 1

hour. Analysis was carried out on a FACSCanto (Becton Dickinson, UK), a two-laser (Octagon, 488 nm blue laser; Trigon, 633 nm red laser), six-colour instrument. The excitation wavelength used for both dyes was 488 nm. The emission for FITC was detected in the FL1 channel (Filter 530/30), for PI in the FL2 channel (Filter 630/22) (**Figure 2.1.**) [33]. All samples were analysed in duplicates and a total of four independent experiments were performed for all cell lines, chemicals and exposure times.

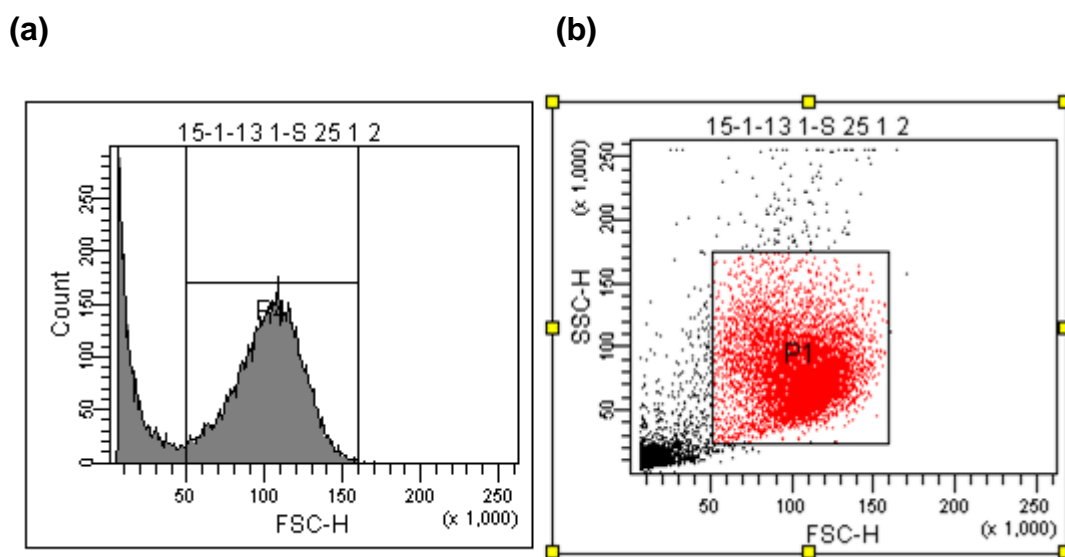


**Figure 2.1.** Overview of the fluorescence spectra of fluorescein isothiocyanate (FITC) and propidium iodide (PI). The dotted curves represent the excitation spectra of FITC and PI and the solid colour curves the emission spectra. The excitation wavelength for both dyes on a FACSCanto is 488 nm; the emission for FITC is detected in the FL1 channel (530/30) and for PI in the FL2 channel (630/22) [33].

The FACS Diva program allows the display of results either as pulse area, width or height. The pulse area is the total signal given by the particle, while the pulse height is the maximum signal intensity. The pulse width gives the transit time and therefore the size or aggregation of cells. The pulse area for two cells stuck together however is double the pulse area for a single cell, yet the pulse height is essentially the same [34]. In this study pulse height

was chosen as display method to exclude any possible interference of cell aggregation.

Gating was used as a method to provide differentiation between the actual cell population and cell debris. To define the borders of the cell population more precisely, results were displayed in histograms (forward scatter (FSC) vs. cell count); the gained information was then applied to gate off the main cell population. The gate for the main cell population was first defined on the two blanks. Taking the information of the histograms into account, all other samples were then double checked to see if their main cell population would fall within the gate. Once this was established the gate for the main cell population was kept constant. Defining the borders of the cell population had to be repeated for each independent experiment (**Figure 2.2.**).

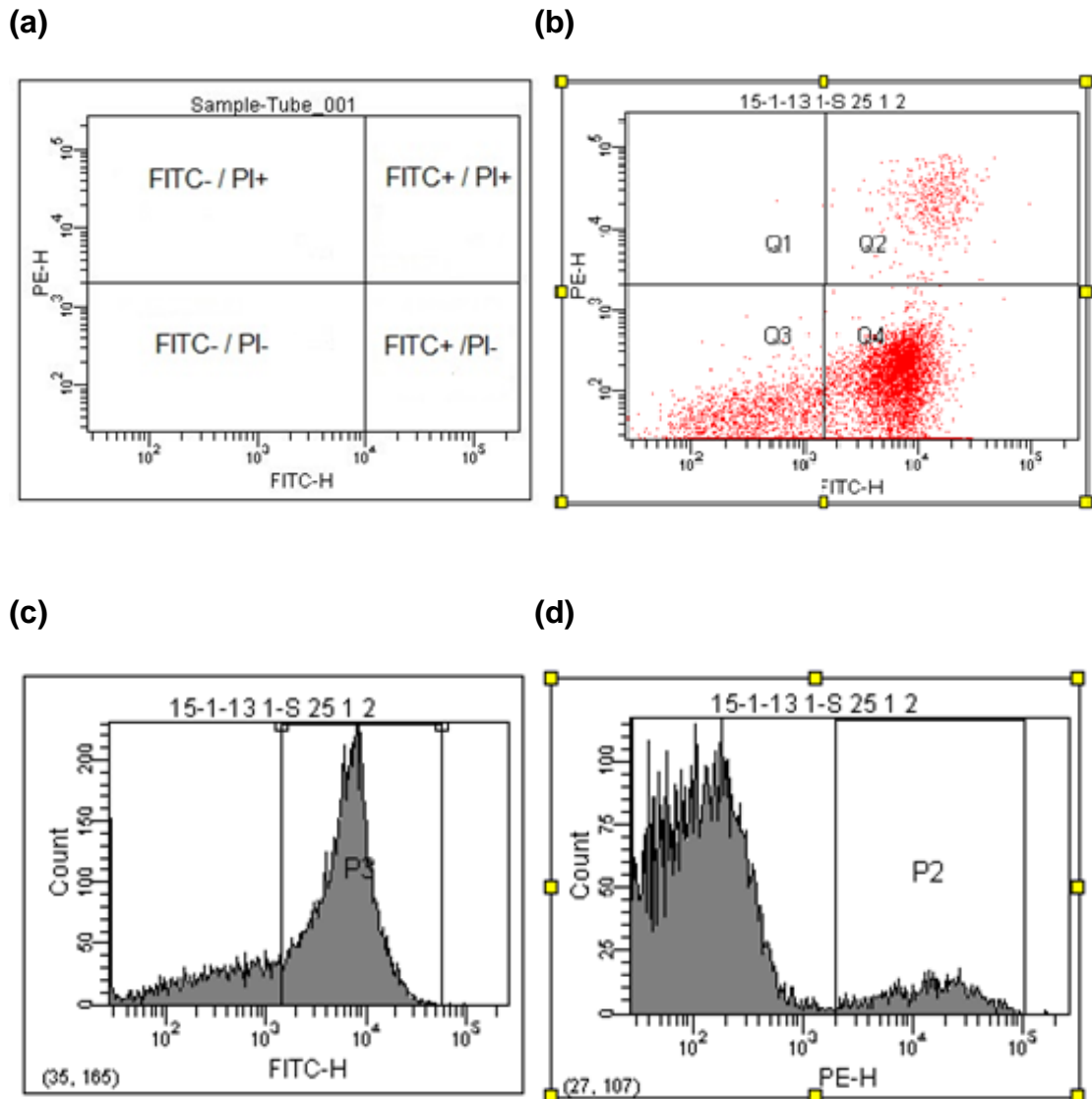


**Figure 2.2.** Example of flow cytometer data obtained in this study. Data is displayed a) as histogram (FSC vs. cell count) and b) as cluster. The cluster display includes the gate for the main cell population which is based on the borders indicated in the histogram.

For both stains the borders for classification of positive and negative cells in the main population, were established using histograms of stains vs. cell



count. Using the histograms as guideline allowed a more precise and consistent definition of the quadrant borders within one experiment. The quadrant borders had to be defined for each independent experiment. Once applied, the percentage of cells in each quadrant could be calculated and displayed (**Figure 2.3.**).



**Figure 2.3.** Example of flow cytometer data obtained in this study. Data is displayed a) schematic, b) as cluster, c) as histogram for FITC (vs. cell count) and d) as histogram for PI (vs. cell count). The cluster (b) shows the actual quadrants which are based on the borders given in the two histograms.

Viable cells would stain both FITC and PI negative, early apoptotic cells would stain FITC positive only, late apoptotic/necrotic cells would stain both FITC and PI positive. Technically no cells should stain PI positive only. However, in some cases a small amount of cells could be detected in the upper left quadrant. This is most likely due to physical damage caused by the treatment prior to analysis [35].

A total of 10,000 cells were counted per sample. All samples were performed in duplicate and a total of four experiments were performed for each cell line, chemical and exposure time.

### **Statistical analysis**

Statistical analysis was carried out using IBM SPSS Statistics 20. Data were expressed as mean  $\pm$  standard deviation (SD) of four independent experiments. Outliers were identified by box plots and where clearly related to an experimental error, removed. Differences between means were established using the non-parametric Kruskal-Wallis-Test with Mann-Whitney U test for pairwise comparison. Results were considered significantly different with a p-value  $\leq 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*). Correlation between cell viability based on Trypan Blue Exclusion assay data and on flow cytometer data (FITC- / PI- plus FITC+ / PI-) was analysed using the Spearman's rank correlation in Graph Pad Prism 5.

### **Results**

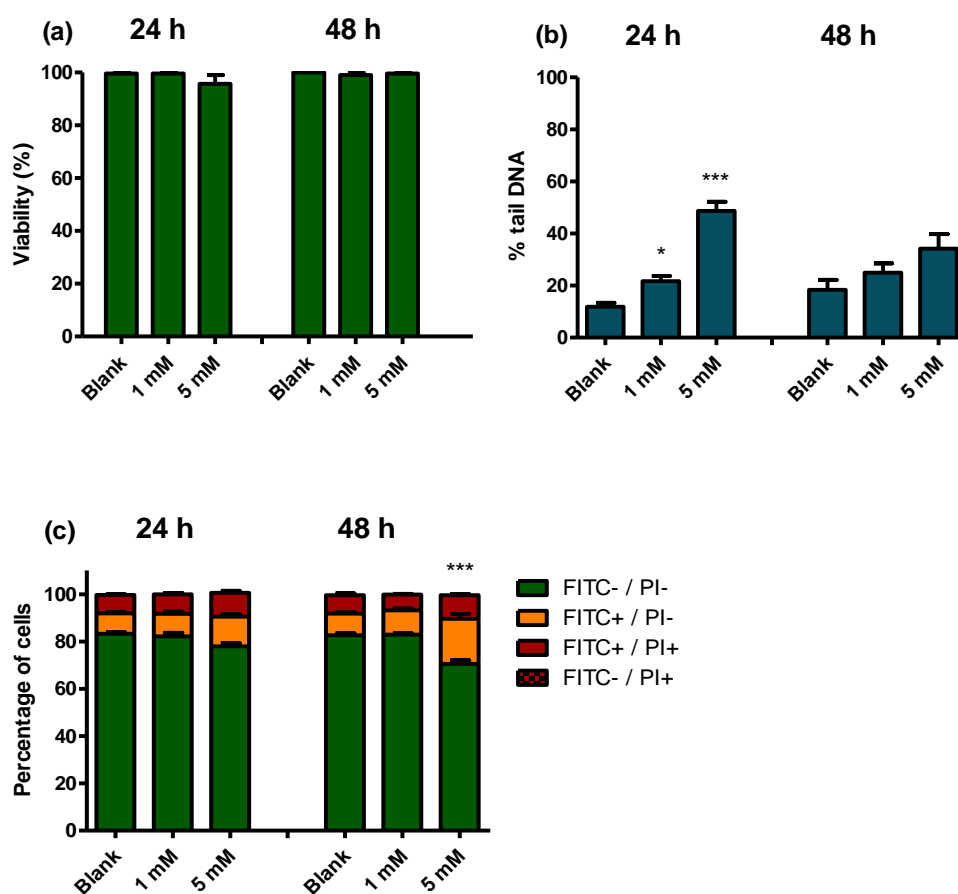
#### **The effect of positive controls on Jurkat T cells.**

Jurkat T cells were exposed to different concentrations of EMS for 24 and 48 hours. No statistically significant ( $p \geq 0.05$ ) effect on the cell number could be seen after 24 hours of exposure. The cell number at 5 mM however was reduced to 79%. A significant reduction ( $p \leq 0.01$ ) could be shown for both concentrations at 48 hours (**Table 2.1.**)

**Table 2.1.** Cell number of Jurkat T cells, expressed as % of the blank, after exposure to EMS at 2 different concentrations for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*).

Assay	Exposure Time	Blank	EMS 1 mM	EMS 5 mM
<i>Trypan Blue Exclusion assay</i>	24 hours	100	87 $\pm$ 25	79 $\pm$ 10
	48 hours	100	82 $\pm$ 11 (**)	71 $\pm$ 17 (**)

No reduction in cell viability ( $p \geq 0.05$ ) could be detected at either time point (**Figure 2.4.a**). Comparing viability data from the Trypan Blue Exclusion assay and flow cytometer analysis (sum of FITC- / PI- and FITC+ / PI- stained cells) gave correlation coefficients ( $r$ ) of 1 (ns) for 24 hours and 0.8 (ns) for 48 hours. A significant increase ( $p \leq 0.001$ ) in FITC+ / PI- stained cells (early apoptosis) could only be noticed after 48 hours for EMS at a concentration of 5 mM (**Figure 2.4.c**). A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be detected at the highest concentration at both time points (24 hours  $p \leq 0.01$ , 48 hours  $p \leq 0.01$ ). A significant increase in DNA fragmentation could be seen for both concentrations (1 mM  $p \leq 0.05$ , 5 mM  $p \leq 0.001$ ) of EMS after 24 hours, whereas no significant difference ( $p \geq 0.05$ ) could be identified after 48 hours exposure (**Figure 2.4.b**).



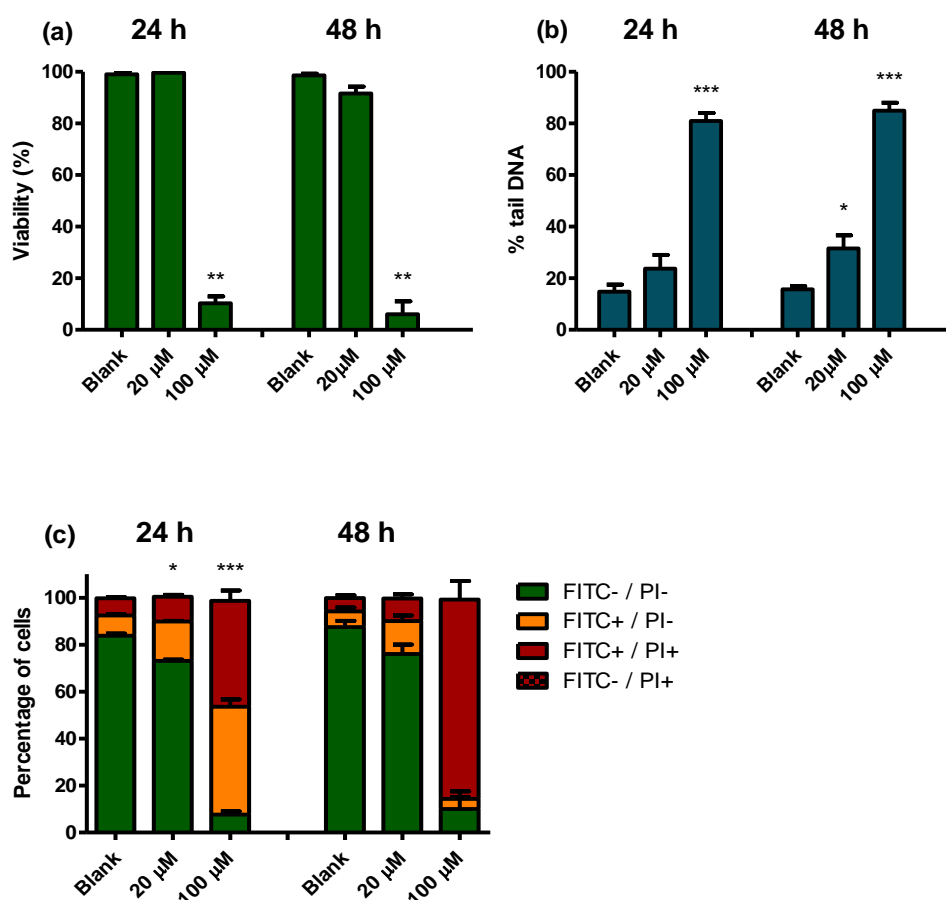
**Figure 2.4.** The effect of EMS on Jurkat T cells after 24 and 48 hour exposure a) on cell viability b) on DNA fragmentation and c) on apoptosis/necrosis. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for FITC+ / PI-stained cells (early apoptosis). Results presented are the mean  $\pm$  SD of 4 independent experiments.

Exposure to different concentrations of  $\text{CdCl}_2$  showed a significant reduction ( $p \leq 0.05$ ) in cell number at the highest concentration ( $p \leq 0.05$ ) after 24 hours and at both concentrations after 48 hours (20  $\mu\text{M}$   $p \leq 0.05$  and 100  $\mu\text{M}$   $p \leq 0.01$ ) (**Table 2.2.**).

**Table 2.2.** Cell number of Jurkat T cells, expressed as % of the blank, after exposure to CdCl<sub>2</sub> at 2 different concentrations for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value < 0.05 (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Assay	Exposure Time	Blank	CdCl <sub>2</sub> 20 $\mu$ M	CdCl <sub>2</sub> 100 $\mu$ M
<i>Trypan Blue Exclusion assay</i>	24 hours	100	95 $\pm$ 17	48 $\pm$ 18 (*)
	48 hours	100	69 $\pm$ 20 (*)	20 $\pm$ 4 (**)

A strong significant decrease ( $p \leq 0.01$ ) in cell viability could be detected for 100  $\mu$ M at both time points (**Figure 2.5.a**). Comparing viability data given by flow cytometer analysis (FITC- / PI- plus FITC+ / PI-) and the Trypan Blue assay showed a correlation coefficient of  $r = 0.5$  (ns) for 24 hours and  $r = 1$  (ns) for 48 hours. A significant increase in FITC+ / PI- stained cells (early apoptosis) could be detected for 20  $\mu$ M ( $p < 0.05$ ) and 100  $\mu$ M ( $p < 0.01$ ) after 24 hour exposure. However, no significant increase ( $p \geq 0.05$ ) in FITC+ / PI- stained cells (early apoptosis) could be seen after 48 hours. This lack of FITC+/PI- stained cells (early apoptosis) coincides with a significant increase ( $p < 0.001$ ) in FITC+ / PI+ stained cells (late apoptosis/necrosis) (**Figure 2.5.c**). Cells displayed a significant increase in DNA fragmentation for 100  $\mu$ M ( $p \leq 0.001$ ) at 24 hours and for 20  $\mu$ M ( $p \leq 0.05$ ) and 100  $\mu$ M ( $p \leq 0.001$ ) at 48 hours (**Figure 2.5.b**). The increase in tail DNA in the COMET assay coincides with the above mentioned decrease in viability as well as the increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) detected by flow cytometer analysis.



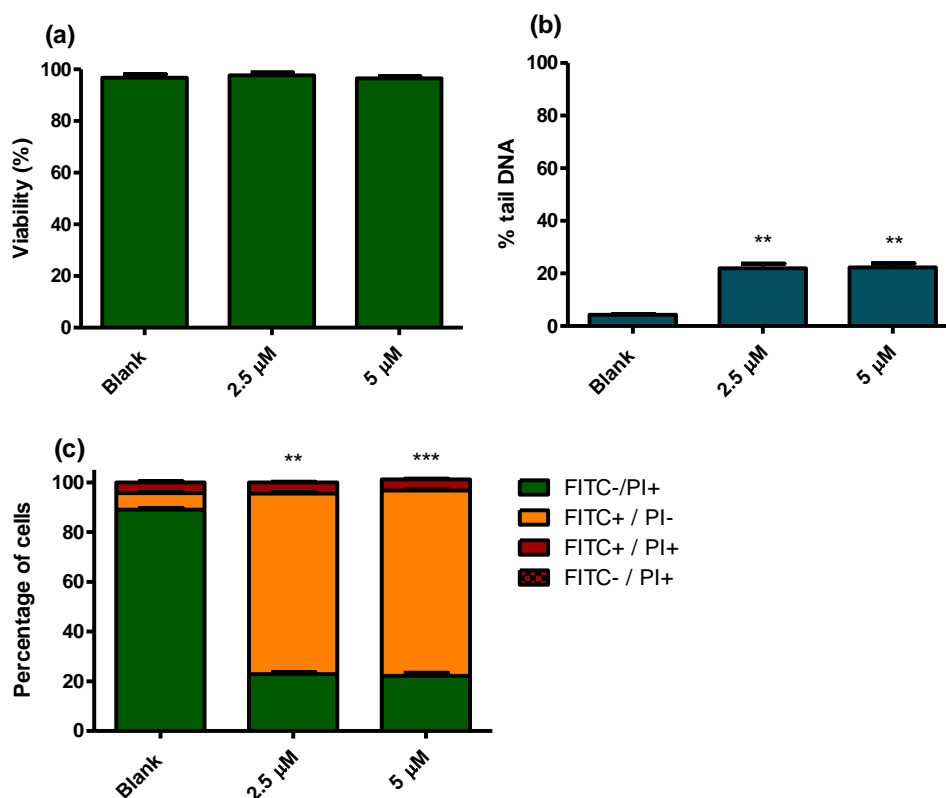
**Figure 2.5.** The effect of  $\text{CdCl}_2$  on Jurkat T cells after 24 and 48 hour exposure a) on cell viability b) on DNA fragmentation and c) on apoptosis/necrosis. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for FITC+ / PI-stained cells (early apoptosis). Results presented are the mean  $\pm$  SD of 4 independent experiments.

Exposure to different concentrations of Staurosporine for 2 hours showed no significant ( $p \geq 0.05$ ) effect on cell number (**Table 2.3.**) but a reduction to approximately 80% compared to the blank could be identified.

**Table 2.3.** Cell number of Jurkat T cells, expressed as % of the blank, after exposure to Staurosporine at 2 different concentrations for 2 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*\*).

Assay	Exposure Time	Blank	Staurosporine 2.5 $\mu$ M	Staurosporine 5 $\mu$ M
<i>Trypan Blue Exclusion assay</i>	<i>2 hours</i>	100	81 $\pm$ 16	82 $\pm$ 24

No effect of Staurosporine could be seen on cell viability (**Figure 2.6.a**). Correlation analysis between viability data from flow cytometer analysis (FITC- / PI- plus FITC+ / PI-) and the Trypan Blue Exclusion assay gave a coefficient of  $r = 0.9$  (ns). A significant increase in FITC+ / PI- stained cells (early apoptosis) could be detected for both concentrations (2.5  $\mu$ M  $p \leq 0.01$  and 5  $\mu$ M  $p \leq 0.001$ ) by flow cytometer analysis (**Figure 2.6.c**). No change in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be detected. A significant increase ( $p \leq 0.01$ ) in DNA fragmentation could be seen for both concentrations (**Figure 2.6.b**).



**Figure 2.6.** The effect of Staurosporine on Jurkat T cells after 2 hour exposure a) on cell viability b) on DNA fragmentation and c) on apoptosis/necrosis. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for FITC+ /PI-stained cells (early apoptosis). Results presented are the mean  $\pm$  SD of 4 independent experiments.

### The effect of positive controls on CaCo-2 cells.

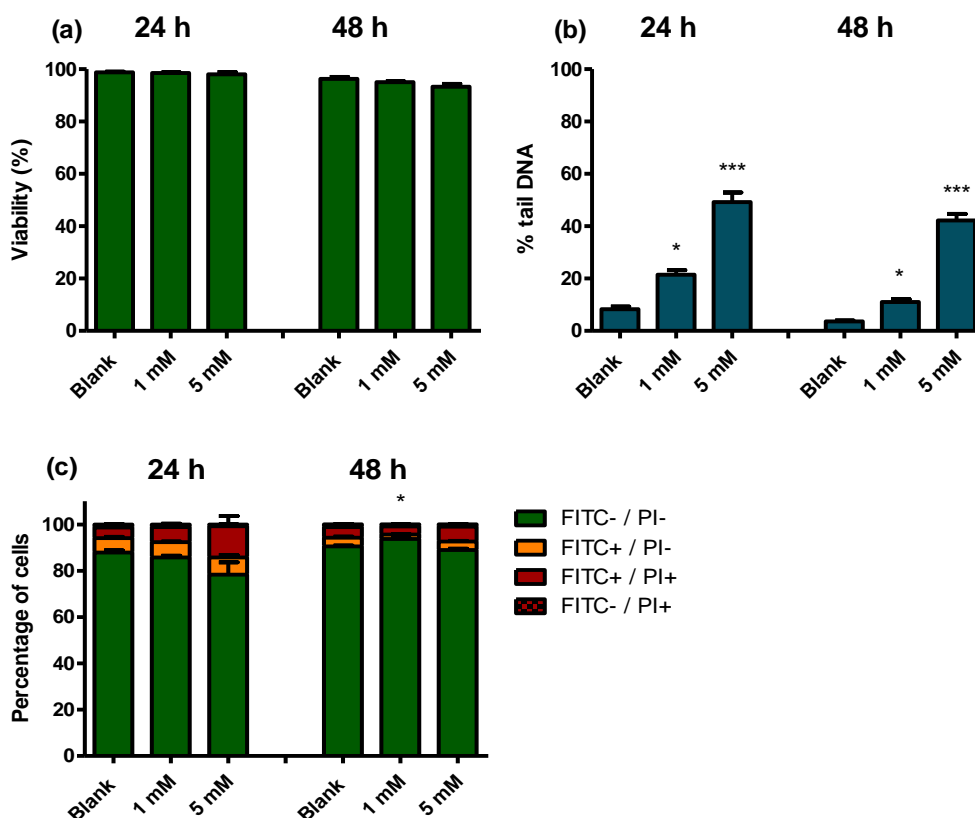
CaCo-2 cells were exposed to different concentrations of EMS for 24 and 48 hours. No significant decrease ( $p \geq 0.05$ ) in cell number could be detected at 24 hours; however the cell number at a concentration of 5 mM was 71%. A significant reduction ( $p \leq 0.01$ ) in cell number could be seen at 5 mM after 48 hours of exposure (**Table 2.4.**).



**Table 2.4.** Cell number of CaCo-2 cells, expressed as % of the blank, after exposure to EMS at 2 different concentrations for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*).

Assay	Exposure Time	Blank	EMS 1 mM	EMS 5 mM
<i>Trypan Blue Exclusion assay</i>	24 hours	100	98 $\pm$ 20	71 $\pm$ 14
	48 hours	100	98 $\pm$ 23	67 $\pm$ 15 (**)

No significant reduction ( $p \geq 0.05$ ) in cell viability could be detected at any time point (**Figure 2.7.a**). Comparing the viability data from flow cytometer analysis (FITC- / PI- plus FITC+ / PI- data) and Trypan Blue Exclusion assay gave a correlation coefficient of  $r = 1$  (ns) for 24 hours and  $r = 0.5$  (ns) for 48 hours exposure. A significant change ( $p \leq 0.05$ ) in FITC+ / PI- stained cells (early apoptosis) could be detected after 48 hours for EMS at a concentration of 1 mM (**Figure 2.7.c**). No significant changes could be detected for FITC+ / PI+ stained cells (late apoptosis/necrosis). After 24 and 48 hours a significant increase in DNA fragmentation could be identified for both concentrations (1 mM  $p \leq 0.05$  and 5 mM  $p \leq 0.001$ ) (**Figure 2.7.b**).



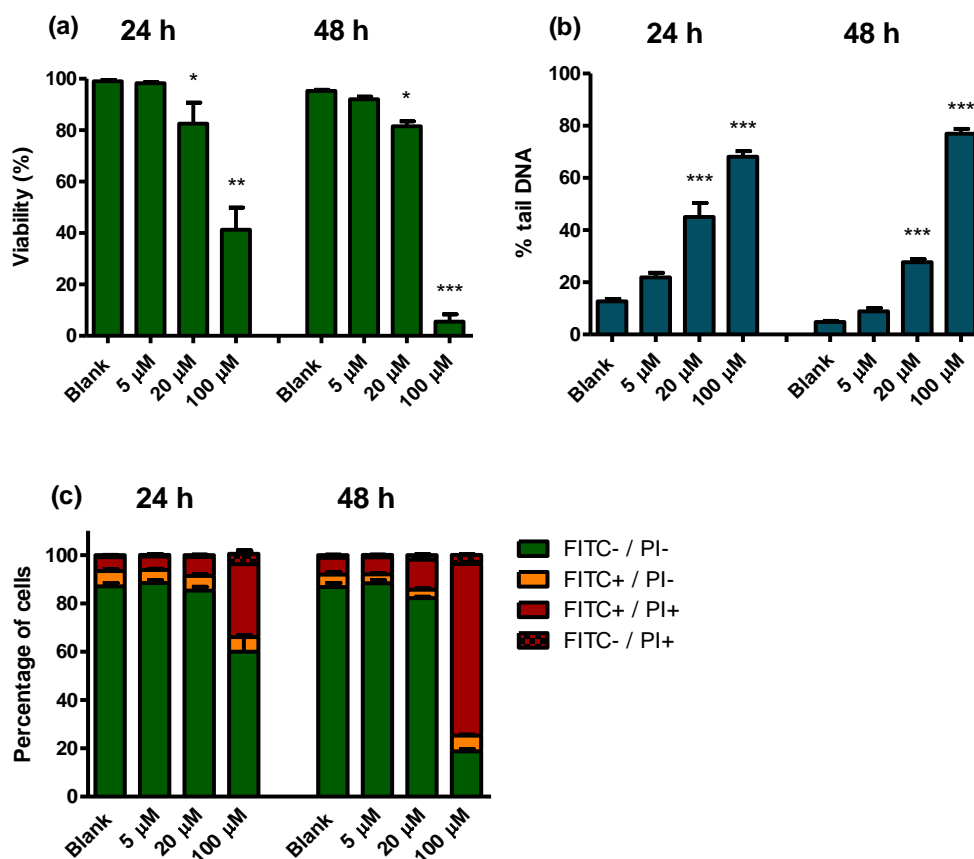
**Figure 2.7.** The effect of EMS on CaCo-2 cells after 24 and 48 hour exposure a) on cell viability b) on DNA fragmentation and c) on apoptosis/necrosis. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for FITC+ / PI-stained cells (early apoptosis). Results presented are the mean  $\pm$  SD of 4 independent experiments.

Exposure to different concentrations of  $\text{CdCl}_2$  for 24 and 48 hours showed a significant reduction ( $p \leq 0.01$ ) in cell number for the two highest concentrations at 24 hours and for 100  $\mu\text{M}$  after 48 hours ( $p \leq 0.01$ ). Although not statistically significant ( $p \geq 0.05$ ), a reduction in cell number could also be seen at 20  $\mu\text{M}$  at 48 hours (**Table 2.5.**).

**Table 2.5.** Cell number of CaCo-2 cells, expressed as % of the blank, after exposure to CdCl<sub>2</sub> at 3 different concentrations for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value < 0.05 (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Assay	Exposure Time	Blank	CdCl <sub>2</sub> 5 $\mu$ M	CdCl <sub>2</sub> 20 $\mu$ M	CdCl <sub>2</sub> 100 $\mu$ M
<i>Trypan Blue Exclusion assay</i>	24 hours	100	93 $\pm$ 9	70 $\pm$ 10 (**)	24 $\pm$ 11 (**)
	48 hours	100	106 $\pm$ 6	68 $\pm$ 12	38 $\pm$ 5 (**)

A significant decrease in cell viability could be detected at both time points for 20  $\mu$ M ( $p \leq 0.05$ ) and 100  $\mu$ M (24h  $p \leq 0.01$ , 48h  $p \leq 0.001$ ) (**Figure 2.8.a**). Correlation analysis between the Trypan Blue Exclusion assay and the viability data given by flow cytometer analysis (FITC- / PI- plus FITC+ / PI-) gave coefficients of  $r = 0.8$  (ns) for 24 hours and  $r = 1$  (ns) for 48 hours exposure. No significant increase ( $p \geq 0.05$ ) in FITC+ / PI- stained cells (early apoptosis) could be identified (**Figure 2.8.c**). A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be shown for 100  $\mu$ M CdCl<sub>2</sub> ( $p \leq 0.01$ ) at 24 hours and 20  $\mu$ M ( $p \leq 0.01$ ) and 100  $\mu$ M ( $p \leq 0.001$ ) at 48 hours (**Figure 2.8.c**). A minor percentage of FITC- / PI+ stained cells could be identified for 100  $\mu$ M at 24 hours and 20  $\mu$ M and 100  $\mu$ M at 48 hours of exposure. A significant increase ( $p \leq 0.001$ ) in DNA fragmentation could be detected at 24 hours and 48 hours at the two highest concentrations (**Figure 2.8.b**) This increase coincides with the above mentioned decrease in cell viability and increase in FITC+ / PI+ stained cells (late apoptosis/necrosis).



**Figure 2.8.** The effect of  $\text{CdCl}_2$  on CaCo-2 cells after 24 and 48 hour exposure a) on cell viability b) on DNA fragmentation and c) on apoptosis/necrosis. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for FITC+ / PI-stained cells (early apoptosis). Results presented are the mean  $\pm$  SD of 4 independent experiments.

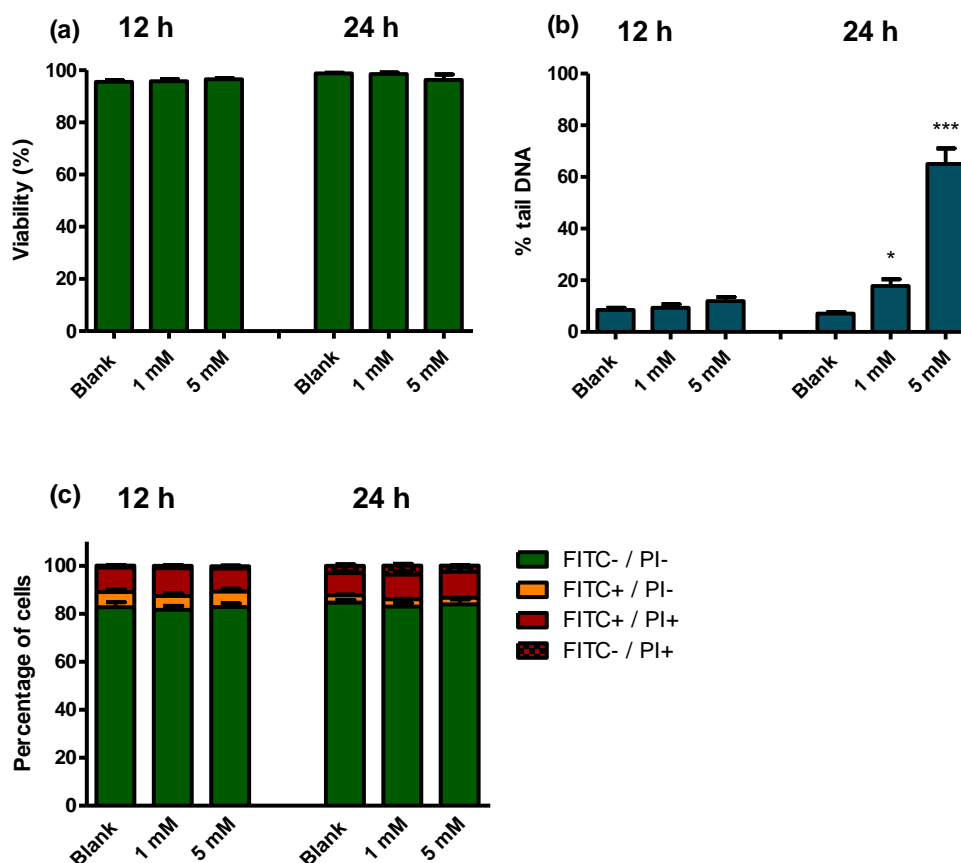
### The effect of different positive controls on HepG-2 cells.

HepG-2 cells were exposed to different concentrations of EMS for 12 and 24 hours. No significant decrease ( $p \geq 0.05$ ) in cell number could be seen at any concentration or time point. However, the cell number at 5 mM after 48 hours is 62% (**Table 2.6.**).

**Table 2.6.** Cell number of HepG-2 cells, expressed as % of the blank, after exposure to EMS at 2 different concentrations for 12 hours and 24 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*\*).

Assay	Exposure Time	Blank	EMS 1 mM	EMS 5 mM
<i>Trypan Blue Exclusion assay</i>	24 hours	100 $\pm$ 0	106 $\pm$ 16	97 $\pm$ 30
	48 hours	100 $\pm$ 0	92 $\pm$ 29	62 $\pm$ 26

No reduction in cell viability could be detected (**Figure 2.9.a**). Comparing the viability given by flow cytometer analysis (sum of FITC- / PI- and FITC+ / PI- stained cells) and the Trypan Blue Exclusion assay showed coefficients of  $r = 0.8$  (ns) for 12 hours and  $r = 0.5$  (ns) for 24 hours of exposure. No significant increase ( $p \geq 0.05$ ) in either, FITC+ / PI- (early apoptosis) or FITC+ / PI+ stained cells (late apoptosis/necrosis) could be seen (**Figure 2.9.c**). At 24 hours of exposure all concentrations, including the blank, show a minor percentage of FITC- / PI+ stained cells. A significant increase in DNA fragmentation could be identified for both concentrations (1 mM  $p \leq 0.05$ , 5 mM  $p \leq 0.001$ ) of EMS after 24 hours (**Figure 2.9.b**).



**Figure 2.9.** The effect of EMS on HepG-2 cells after 12 and 24 hour exposure; a) on cell viability b) on DNA fragmentation and c) on apoptosis/necrosis. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for FITC+ / PI- (early apoptotic) cells. Results presented are the mean  $\pm$  SD of 4 independent experiments.

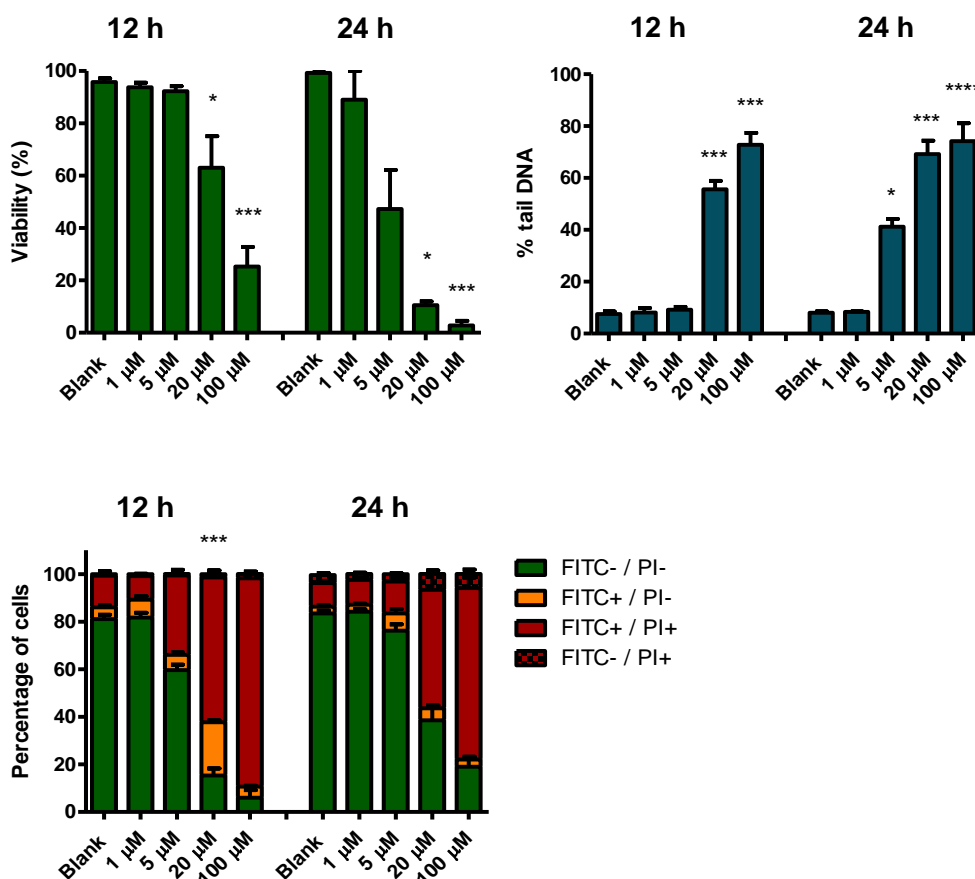
Exposure to different concentrations of  $\text{CdCl}_2$  for 12 and 24 hours showed a significant reduction in cell number at the two highest concentrations ( $20 \mu\text{M}$   $p \leq 0.05$  and  $100 \mu\text{M}$   $p \leq 0.01$ ) after 12 hours and at  $5 \mu\text{M}$  and  $100 \mu\text{M}$  ( $p \leq 0.05$ ) after 24 hours. Although not significant ( $p \geq 0.05$ ), the cell number at 48 hours for a concentration of  $20 \mu\text{M}$  is 49% (**Table 2.7.**).

**Table 2.7.** Cell number of HepG-2 cells, expressed as % of the blank, after exposure to CdCl<sub>2</sub> at 4 different concentrations for 12 hours and 24 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value < 0.05 (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Assay	Exposure Time	Blank	CdCl <sub>2</sub> 1 $\mu$ M	CdCl <sub>2</sub> 5 $\mu$ M	CdCl <sub>2</sub> 20 $\mu$ M	CdCl <sub>2</sub> 100 $\mu$ M
Trypan Blue Exclusion assay	12 hours	100 $\pm$ 0	99 $\pm$ 13	70 $\pm$ 3	34 $\pm$ 7 (*)	31 $\pm$ 16 (**)
	24 hours	100 $\pm$ 0	122 $\pm$ 20	34 $\pm$ 011 (*)	49 $\pm$ 18	41 $\pm$ 11 (*)

A significant decrease in cell viability could be detected at both time points for 20  $\mu$ M ( $p \leq 0.05$ ) and 100  $\mu$ M ( $p \leq 0.001$ ) (**Figure 2.10.a**). Although not statistically significant ( $p \geq 0.05$ ), a clear decrease can also be seen for 5  $\mu$ M at 24 hours. Comparing the viability data obtained by Trypan Blue Exclusion Assay and flow cytometer analysis (FITC- / PI- plus FITC+ / PI- data) gave correlation coefficients of  $r = 0.9$  (ns) at both time points. A significant increase in FITC+ / PI- stained cells (early apoptosis) could only be identified for 20  $\mu$ M ( $p \leq 0.001$ ) after 12 hours (**Figure 2.10.c**). A minor percentage of FITC- / PI+ stained cells could be identified for all concentrations, including the blank at 24 hours of exposure. A significant increase in DNA fragmentation could be detected for 20  $\mu$ M and 100  $\mu$ M at 12 hours ( $p \leq 0.001$ ) and 5  $\mu$ M ( $p \leq 0.05$ ), 20  $\mu$ M ( $p \leq 0.001$ ) and 100  $\mu$ M ( $p \leq 0.001$ ) at 24 hours (**Figure 2.10.b**). The increase in tail DNA coincides strongly with the above mentioned decrease in cell viability and with a significant increase in FITC+ / PI+ stained cells (late apoptosis/ necrosis) as measured by flow cytometer analysis. The only exception is CdCl<sub>2</sub> at a concentration of 5  $\mu$ M. The viabilities given by the Trypan Blue Exclusion assay and the tail DNA given by the COMET assay coincide. However, the percentage of FITC+ / PI+ stained cells (late apoptosis/necrosis) at 12 hours is higher than the

viabilities would indicate and at 24 hours lower than the viabilities would indicate.



**Figure 2.10.** The effect of  $\text{CdCl}_2$  on HepG-2 cells after 12 and 24 hour exposure; a) on cell viability b) on DNA fragmentation and c) on apoptosis. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for FITC+ / PI- stained cells (early apoptosis). Results presented are the mean  $\pm$  SD of 4 independent experiments.



## ***Discussion***

The principle of the COMET assay is the visualization of DNA damage. On application of an electrical field, low weight DNA strands will migrate away from the nucleus and form the comet tail while the undamaged DNA does not migrate and forms the comet head. This migration can then be visualized with fluorescent dyes and informs about DNA damage caused by the test compound(s). The main purpose of the present study was to establish and validate a) the methodologies used and b) to generate in house reference data for the interpretation of future genotoxic studies. Three cell lines, Jurkat T, CaCo-2 and HepG-2 were exposed to EMS, a direct genotoxic agent [7], CdCl<sub>2</sub> a compound widely used as positive control in genotoxic testing [14-17] and Staurosporine, a non-genotoxic apoptosis inducer [34]. The COMET assay, Trypan Blue Exclusion assay and flow cytometer analysis were performed on all cell lines and compounds.

Attempts were made to ensure that the mixing of cells and the sampling of aliquots for the Trypan Blue Exclusion assay were as constant as possible. Slight variations are likely. While this would not affect the cell viability *per se*, it might well account for higher standard deviations (SD) in cell numbers.

EMS caused no significant decrease in viability in all three cell lines. The percentage of viable cells determined by flow cytometer analysis was lower than by the Trypan Blue Exclusion assay. The reason for this discrepancy is most likely due to the preparation of samples for flow cytometer analysis. This involves several centrifugation and pipetting steps which might introduce some physical damage to the cell membrane [35]. No cytotoxic effect could be identified at the concentration range tested (1 mM and 5 mM). Normally in toxicity testing a concentration range up to cytotoxicity is applied. Concentrations for EMS are well established in the literature and followed in this study. Furthermore, no additional concentrations were necessary based on the detection of DNA damage in the COMET assay at both concentrations, in the absence of cytotoxicity. In case of HepG-2 cells, no DNA fragmentation could be detected at 12 hours of exposure to EMS. A significant dose dependent increase in DNA fragmentation of 20% for 1 mM

EMS and 50-65% for 5 mM EMS after 24 hours was detected in all three cell lines (**Figure 2.4.b, 2.7.b, 2.9.b**). The percentage in tail DNA after 48 hours of exposure for Jurkat T cells and CaCo-2 cells is lower than after 24 hours. Repair mechanisms following EMS exposure have been reported in the literature *in vivo* and *in vitro* [11, 37-39] and could account for the observations made in the present study. One criterion for genotoxic damage and possible repair mechanisms is an unchanged cell number [40]. In the current study, the cell numbers for 1 mM, especially at 24 hours but also in most cases for 48 hours (**Table 2.1., 2.4., 2.6.**) are above 80% (compared to the blank) and therefore support the suggested DNA repair. For 5 mM, especially for HepG-2 cells the cell number is between 60% and 80% compared to the blank. Cell loss could be a possible explanation. Floating cells in the exposure media could have been missed before active detachment. In the present study, flow cytometer analysis showed no significant increase in early apoptosis (FITC+ / PI-) in all three cell lines, at both concentrations and all time points. EMS has been widely used as a positive control for genotoxicity testing *in vitro* and *in vivo* in various organisms, including human cell lines. It is a direct acting, DNA damaging agent which causes a concentration dependent increase in DNA damage [7, 12, 41, 42]. The clear positive genotoxic response achieved with EMS in the present study is in agreement with the above literature and established the COMET assay as an in-house method to detect DNA damage in subsequent biotoxin studies.

All three cell lines were exposed to a concentration range of CdCl<sub>2</sub> that has been reported widely in the literature and demonstrated a significant dose and time dependent decrease in cell viability [22, 24, 43]. These findings could be confirmed. CaCo-2 cells and HepG-2 cells seemed to be the most affected in the present study. The exposure concentration had to be lowered to 5 µM and 1 µM to allow for a broader concentration range, showing cytotoxic and non-cytotoxic effects (**Figures 2.5.a, 2.8.a, 2.10.a**). Comparison between viability data obtained by the Trypan Blue Exclusion assay and flow cytometer analysis showed good correlation with coefficients

close to 1. Although not statistically significant, this is most likely of biological relevance. Within the concentration range (1  $\mu\text{M}$  – 100  $\mu\text{M}$ ), the COMET assay showed a significant increase in DNA fragmentation at both time points for all three cell lines (**Figures 2.5.b, 2.8.b, 2.10.b**). However, HepG-2 cells seemed to be the most sensitive cell line showing a statistically significant increase in DNA fragmentation at concentrations of 5  $\mu\text{M}$  after 24 hours. This is in agreement with other published findings [14, 18, 19, 44]. No data on cytotoxicity or cell number were reported in these publications. In the current study, the cell number for all three cell lines decreased substantially in a concentration and time dependent matter (**Tables 2.2, 2.5, 2.7**). In case of direct genotoxic damage the cell number should stay consistent [40]. Therefore, the cell loss described here indicates rather apoptotic mechanisms to have taken place. This is supported by flow cytometer analysis undertaken in the present study. Results showed a significant increase in early apoptosis (FITC+ / PI-) in Jurkat T cells after 24 hour exposure at both concentrations (20  $\mu\text{M}$  and 100  $\mu\text{M}$ , **Figure 2.5.c**). HepG-2 cells showed a significant increase in early apoptosis after 12 hours at a concentration of 20  $\mu\text{M}$  (**Figure 2.10.c**). The lack of a significant percentage of early apoptotic cells at 48 hours for Jurkat T cells and 24 hours for HepG-2 cells indicated a time dependent shift from early apoptosis to late apoptosis. CaCo-2 cells showed no indication of early apoptosis, but a significant increase in late apoptosis/necrosis (**Figure 2.8.c**). Recent papers have demonstrated that Cd/CdCl<sub>2</sub> can not only induce DNA strand breaks but also induce apoptosis in various cell lines [22, 25, 43, 44]. DNA fragmentation is also a biochemical marker of apoptosis [47-49] and can therefore be detected in the COMET assay. The percentage of tail DNA detected in the present study correlates well with the percentage of late apoptotic cells (FITC+ / PI+). Bacso and Eliason [50] found that FITC+ / PI+ stained Jurkat cells (late apoptosis/necrosis) had measurable comets in the COMET assay while only few comets could be detected for FITC+ / PI- stained cells (early apoptosis). This is supported by Elmore et al. [47] and other publications on apoptosis [51-53], which state DNA fragmentation as a late event in apoptosis. The data obtained for CdCl<sub>2</sub> in the present study seems to

indicate a cytotoxic as well as apoptotic effect in at least two of the three cell lines. This effect is most likely responsible for the DNA damage detected in the COMET data. To verify the possible connection between apoptosis and visible DNA fragmentation in the COMET assay Jurkat T cells were exposed to Staurosporine, a known apoptosis inducer which does not have a direct effect on the genome [36]. Flow cytometer analysis showed a significant increase in early apoptosis for both concentrations of Staurosporine (**Figure 2.6.c**) verifying it as an apoptosis inducer. No significant reduction in cell viability could be shown for any of the concentrations (**Figure 2.6.a**). Also, no significant reduction in cell number could be detected after 2 hours at either concentration (**Table 2.3.**). The decrease in cell number to about 80% at 2.5 and 5  $\mu$ M is more likely a result of the handling procedure than of great biological relevance. However, the COMET assay showed a statistically significant yet modest increase in DNA fragmentation for both concentrations (**Figure 2.5.b**). DNA fragmentation is a late apoptotic event; however no significant amount of late apoptotic cells (FITC+ / PI+) could be detected in this study. The concentrations and exposure durations are in agreement with the literature and so are the results detected in the present study. DNA fragmentation as a result of Staurosporine has been demonstrated by Bertrand et al. [26] and Belmokhtar et al. [29], the latter who concluded that Staurosporine induces cell death via a caspase dependent pathway in Jurkat T cells and DNA fragmentation being a biochemical marker of apoptosis. The percentage of tail DNA detected in the current study is about 20%, while the amount of early apoptotic cells (FITC- / PI+) is approximately 80%. CdCl<sub>2</sub> also shows an increase in early apoptotic cells (FITC+ / PI-) in the absence of cytotoxicity and only minimal amounts (~ 10%) of late apoptotic cells (FITC+ / PI+). One possible explanation could be that these cells have undergone the complete apoptotic process and are almost completely fragmented and do not consist of a cell membrane anymore. Therefore, they would not have stained FITC+ / PI+ in the present study and would not have been recognised. The findings in the present study are supported by Roser et al. [54]. The authors found the relative numbers of apoptotic cells to be three fold higher than the DNA fragmentation detected in HT-29 colon

adenocarcinoma cells. They concluded that apoptosis does not necessarily coincide with DNA damage shown by the COMET assay. However, the ratio may differ depending on cell line and concentration.

Comparing the different cell lines with each other, EMS gave similar time and response profiles in all three cell lines for all endpoints. Lung, kidney and liver are reported in the literature as main target organs for Cd toxicity [24, 43, 55]. Skipper et al. [56] showed a 24h-LD<sub>50</sub> of 3.6 µg/mL for CdCl<sub>2</sub> and a significant increase in DNA fragmentation, concluding CdCl<sub>2</sub> to be highly cytotoxic to HepG-2 cells. This is in agreement with results found in the current study. HepG-2 cells showed the earliest and strongest response of all three cell lines used. A significant decrease in cell viability and a significant increase in DNA fragmentation and early apoptosis could be detected after 12 hours of exposure. Jurkat T cells and CaCo-2 cells showed DNA fragmentation of similar percentages after 24 hours, Jurkat T cells also showed a significant increase in early apoptosis. No increase in early apoptosis could be detected for CaCo-2 cells even after 48 hours; however, an earlier significant decrease in cell viability could be seen. This is in agreement with findings by Boveri et al. [15]. In their study CaCo-2 cells showed necrotic cell death after 24 hours at a concentration of 50 µM, while no variation in apoptosis could be detected. Apoptosis induction is often linked to oxidative stress [24, 19]; however, Boveri et al. [15] and Noda et al. [56] were unable to link CdCl<sub>2</sub> exposure to oxidative stress in CaCo-2 cells. Another possible explanation for the different responses detected in this study could be variable amounts of endonucleases, which in turn lead to different levels of apoptosis induction [43].

Technically no cells should stain FITC- / PI+. However, a small percentage could be detected for CaCo-2 cells at the highest concentration of CdCl<sub>2</sub> at both time points and HepG-2 cells with all chemical and concentrations at 24 hours, including the blank. This might be due to handling and preparation of cells (centrifugation, re-suspension) prior to flow cytometer analysis [33]. Again there are differences between the cell lines, no FITC- / PI+ stained

cells could be detected for Jurkat T cells. This might indicate possible damage due to cell detachment.

### **Conclusion**

The COMET assay is a reliable and rapid method for the detection of genotoxic effects. It has been generally assumed that the DNA fragmentation seen in the COMET assay coincides with the damage done to the genome [58]. Recent studies, including this one have shown that DNA fragmentation which occurs during apoptosis/necrosis can be detected in the COMET assay [29, 58-60]. Therefore, the results presented here indicate the importance of the right positive control. In this study the COMET assay could be verified as a method for DNA damage using EMS, a direct DNA damaging compound. However,  $\text{CdCl}_2$  which is also widely used as a positive control may be inducing DNA fragmentation indirectly through apoptotic processes and could therefore lead to false positive results in the COMET assay. Results of this study stress the importance of including cytotoxicity and apoptosis studies in genotoxicity testing to avoid false positive results due to other factors than direct DNA strand breaks.

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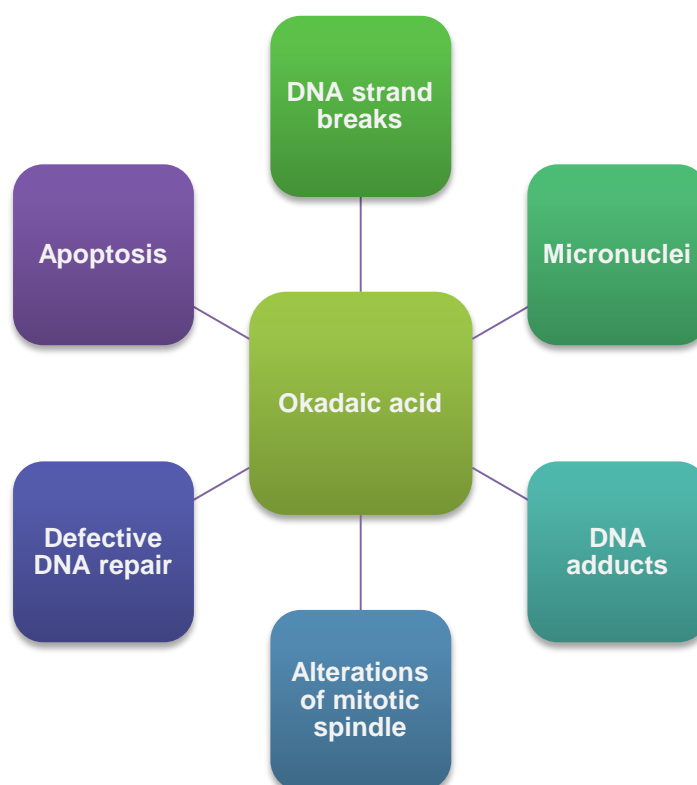
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## Chapter 3: Okadaic acid

### ***Introduction***

Okadaic acid (OA) is a marine biotoxin produced by the dinoflagellates of the genus *Dynophysis sp.* and *Prorocentrum sp.*. It has been reported globally but the main areas where it seems to occur are Europe and Japan [1, 2]. OA is a polyether fatty acid, heat stable and due to its lipophilic nature able to accumulate in shellfish, mainly in filter feeding molluscs. It is the main cause for Diarrhoeic Shellfish Poisoning (DSP) in humans causing acute symptoms including diarrhoea, vomiting, stomach cramps, nausea and abdominal pains. Symptoms occur rapidly within minutes to hours and last up to a few days [3-5]. Human intoxication with OA is an increasing global problem and occurs mainly through the consumption of fishery produce. Not all cases are severe and therefore reported, leading researchers to believe that the amount of affected individuals is higher than recorded [6]. Information on acute toxicity is available for humans and European regulations have been set in place, focusing on the gastrointestinal symptoms. *In vivo* studies have shown OA to be widely distributed in mice after oral administration, the gastrointestinal tract being the main target [7-9]. It has also been detected in the liver as soon as 5 minutes after oral or *i.p.* administration [5] and enterohepatic circulation has been suggested to have taken place [10]. However, liver damage in general has not been reported [5, 11]. Data on the genotoxic potential *in vitro* are often contradictory and no data on chronic or subchronic effects in humans have been reported [6]. *In vitro* studies have identified OA to be a potent inhibitor of serine/threonine protein phosphatases (PP1 and PP2A) in mammalian cells. The resulting hyperphosphorylation of proteins leads to a disruption in many cellular processes and can result in a total collapse of regulatory processes [4, 6, 9, 12]. Morphological changes have frequently been reported as a consequence of OA toxicity, including cell rounding, cell-cell and cell-surface detachment and disruption of the cytoskeleton [2, 12-14]. Various studies have examined the genotoxic potential of OA (**Figure 3.1.**), which have been

described in greater detail in Chapter 1. In brief, the Ames test proved negative while the Chinese hamster lung (CHL) cells, Chinese hamster ovary cells and human lymphoblastoid cells showed direct genotoxic effects. These toxic effects have been identified through sister-chromatid exchange and chromosome condensation [15, 16]. Data by Fessard et al. [17] support those findings. The authors detected DNA adduct formation at non-cytotoxic levels. Other studies have come to different conclusions. Le Hegerat et al. showed OA to disturb the mitotic spindle and induce premature sister chromatid separation. The authors suggested an aneugenic potential rather than a direct mutagenic potential of OA [9, 18]. This was supported by data from other studies which showed the loss of whole chromosomes, centromere-positive micronuclei and confirmed the lack of primary DNA damage [8, 9, 19].



**Figure 3.1.** Schematic overview of the genotoxic effects of okadaic acid (OA) [20].

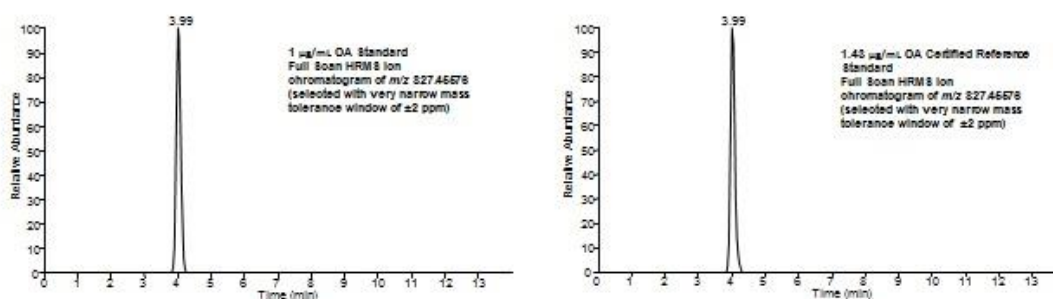
Romero et al. [20] and Valdiglesias et al. [6] reported OA to interfere with DNA repair mechanisms. Various publications have linked both oxidative damage and effects on metabolic/anabolic pathways to OA toxicity [4, 21-23]. Decrease in membrane potential and the activation of caspase-3 have been associated with OA, leading authors to conclude that OA might induce apoptosis/necrosis [13, 24-26]. Some data indicates the need for metabolic activation for OA to exert its mutagenic effect [27] while others showed OA to act directly [28]. A study by Soud-Mensi et al. [29] along with other reports have proposed that the effect of OA is cell line dependent [22, 29-31]. It has been suggested that the contradicting information on genotoxicity of OA might reflect the complex mechanisms involved [6]. OA has been identified as a tumour promoter in rodents [3, 32, 33]. A 2-stage carcinogenesis study by Suganuma et al. [32] found OA to induce tumours on the skin of mice and in the stomach of rats. Furthermore OA was found to prompt tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ). The tumour promotion effect of OA raises concern about the effects for human shellfish consumers, including chronic exposure and exposure to concentrations below the current regulation limit [4, 34].

The purpose of the present study was to investigate the possible genotoxic effects of OA using the COMET assay. To explain possible DNA damage detected correctly, additional assays have been included (see previous chapters). Based on reports suggesting that the effect of OA is cell line dependent, work was conducted on three different cell lines, Jurkat T cells (immune system), CaCo-2 cells (intestine), HepG-2 cells (liver). They were chosen because they represent the main target organs of OA toxicity.

## Materials & Methods

### Chemicals & Reagents

Okadaic acid (OA) was purchased from LC Laboratories, USA and verified using a certified standard solution of OA (14.3 µg/mL) obtained from the National Research Council Halifax, Canada (**Figure 3.2.**). The verification was performed by the Mass Spectrometry Research Centre for Proteomics and Biotoxins (PROTEOBIO), Cork Institute of Technology. All chemicals were obtained from Sigma-Aldrich, Ireland except otherwise indicated. Annexin V and Annexin V detection kit, flow tubes and flow cytometer fluids were purchased from BD Bioscience, UK. Microscope slides and cover slips were acquired from Fisher Scientific, Ireland. All plastic ware was purchased from Sarstedt, Ireland.



**Figure 3.2.** Standardization of OA via Liquid Chromatography-Mass Spectroscopy (Thermo Scientific Quantum Discovery Max triple quadrupole mass spectrometer, heated electrospray ionization source, hyphenated to a Thermo Scientific Accela LC system). The analysis was conducted by the Mass Spectrometry Research Centre for Proteomics and Biotoxins (PROTEOBIO), Cork Institute of Technology [35].

### Cell culture

Jurkat T cells (human T cell lymphoblasts), CaCo-2 cells (human epithelial colorectal adenocarcinoma cells) and HepG-2 cells (human hepatocellular carcinoma cells) were provided by the European Collection of Cell Cultures

(ECACC, operated by Public Health England) and cultured as described in Chapter 2.

### **Cell exposure**

OA was dissolved in methanol at a stock concentration of 70 µg/mL. Prior to each experiment, working solutions were freshly prepared by serial dilution to keep the volume added to each well consistent.

For experiments, cells were seeded in 6 well plates at a density of  $2 \times 10^5$  cells/mL. Adherent cells were seeded the night before (to allow re-attachment) while non-adherent cells were seeded 4 hours prior to exposure. All cell lines were exposed to a final concentration of 3 ng/mL, 10 ng/mL, 33 ng/mL and 100 ng/mL OA. Blanks were included in each experiment, either containing the vehicle (methanol) or being vehicle free. The exposure times for Jurkat T cells and CaCo-2 cells were 24 hours and 48 hours, the exposure times for HepG-2 cells were 12 hours and 24 hours. The reduced exposure times for HepG-2 cells was based on a substantial reduction in cell number at 24 hours in initial experiments.

### **Assay analysis**

The Trypan Blue Exclusion assay, COMET assay and flow cytometer analysis were performed as described in Chapter 2. All cell counts and sample analysis were performed in duplicate and a total of four independent experiments were performed for each cell line and exposure time.

### **Statistical Analysis**

Statistical analysis was carried out using IBM SPSS Statistics 20. Data were expressed as mean  $\pm$  standard deviation (SD) of four independent experiments. Outliers were identified by box plots. If the outlier could clearly be identified as the result of experimental error, it was removed. Differences



between means were established using the non-parametric Kruskal-Wallis-Test with Mann-Whitney U test for pairwise comparison. Results were considered significantly different with a p-value < 0.05 (\*), 0.01 (\*\*) and 0.001 (\*\*\*). Correlation between Trypan Blue Exclusion assay data and flow cytometer data (FITC- / PI- plus FITC+ / PI-) was analysed using the Spearman's rank correlation in Graph Pad Prism 5.

## Results

### The effect of okadaic acid on Jurkat T cells.

Jurkat T cells were exposed to four different concentrations of OA for 24 hours and 48 hours. No significant differences ( $p \geq 0.05$ ) between the blank and vehicle blank could be detected at any time point for all endpoints. Therefore the vehicle blank is used as reference blank when presenting the findings of this study. A significant reduction ( $p \leq 0.01$ ) in cell number could be seen for 33 and 100 ng/mL of OA at both time points. Although not statistically significant ( $p \geq 0.05$ ) a substantial reduction in cell number could also be seen for 10 ng/mL of OA at both time points (**Table 3.1.**).

**Table 3.1.** Cell number of Jurkat T cells, expressed as % of the blank, after exposure to OA at four different concentrations for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value < 0.05 (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Assay	Exposure Time	Blank	OA 3 ng/mL	OA 10 ng/mL	OA 33 ng/mL	OA 100ng/mL
<i>Trypan Blue Exclusion assay</i>	24 hours	100	102 $\pm$ 30	69 $\pm$ 30	53 $\pm$ 6 (**)	50 $\pm$ 17 (**)
	48 hours	100	108 $\pm$ 10	34 $\pm$ 14	15 $\pm$ 5 (**)	16 $\pm$ 4 (**)

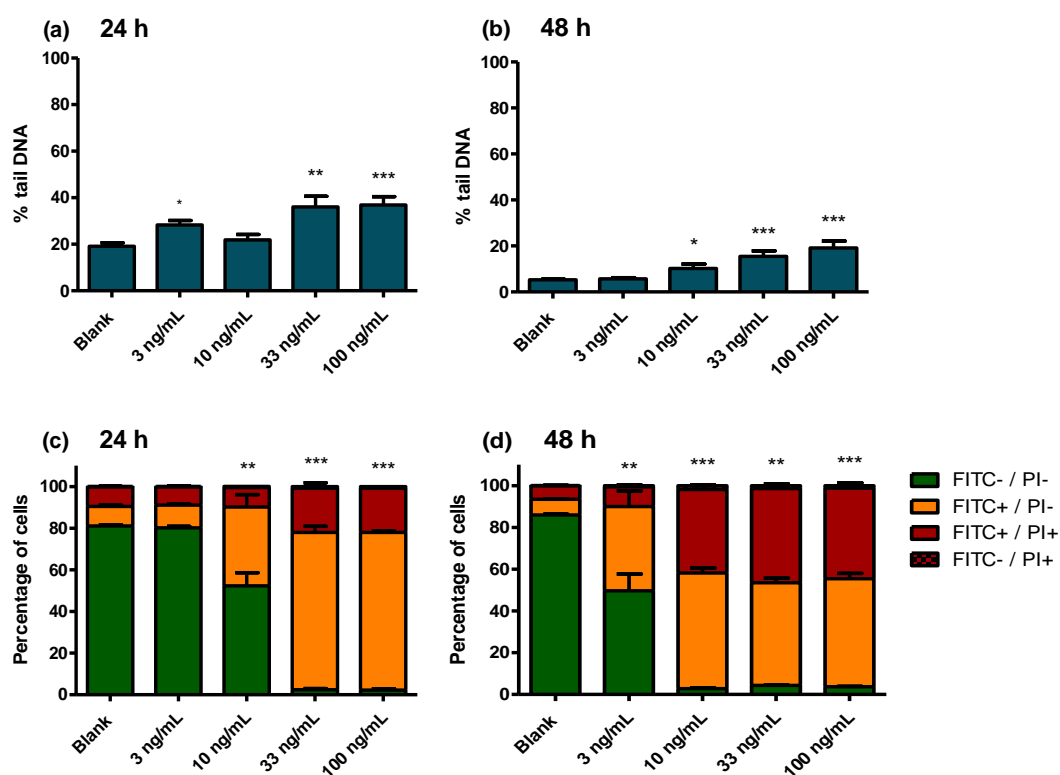
A significant decrease in cell viability could be detected for 33 ng/mL and 100 ng/mL OA at both time points (24h,  $p \leq 0.05$ , 48h  $p \leq 0.01$ ) (**Table 3.2.**). The viabilities, as measured by flow cytometer analysis (the sum of FITC- / PI- and FITC+ / PI- stained cells) correlated well with the viabilities given by the Trypan Blue Exclusion assay with correlation coefficients of  $r = 0.8$  (ns) for 24 hours and  $r = 1$  ( $p \leq 0.05$ ) for 48 hours of exposure. A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be detected for the three highest concentrations at both time points (24h  $p \leq 0.05$  (10 ng/mL) and  $p \leq 0.01$ , 48h  $p \leq 0.001$ ) (**Figure 3.3.c, 3.3.d**). A discrepancy with the reduction in viability observed with the Trypan Blue Exclusion assay (**Table 3.2.**) can be seen at 10 ng/mL of OA, especially at 48 hours.

**Table 3.2.** Viability of Jurkat T cells after exposure to OA at four different concentrations for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Assay	Exposure Time	Blank	Blank Vehicle	OA 3 ng/mL	OA 10 ng/mL	OA 33 ng/mL	OA 100ng/mL
<i>Trypan Blue Exclusion assay</i>	24 hours	98 $\pm$ 1.7	99 $\pm$ 0.8	99 $\pm$ 1.3	100 $\pm$ 1.0	94 $\pm$ 2.0 (*)	87 $\pm$ 7.4 (*)
	48 hours	100 $\pm$ 0.6	99 $\pm$ 0.6	99 $\pm$ 0.5	95 $\pm$ 6.0	57 $\pm$ 11.2 (**)	66 $\pm$ 4.8 (**)

A significant increase in FITC+ / PI- stained cells (early apoptosis) could be identified for the three highest concentrations (10 ng/mL  $p \leq 0.01$ , 33 and 100 ng/mL  $p \leq 0.001$ ) after 24 hours and for all concentrations after 48 hours of exposure to OA (3 and 33 ng/mL  $p \leq 0.01$ , 10 and 100 ng/mL  $p \leq 0.001$ ). A significant increase in DNA fragmentation could be shown for 3 ng/mL ( $p \leq 0.05$ ), 33 ng/mL ( $p \leq 0.01$ ) and 100 ng/mL ( $p \leq 0.001$ ) at 24 hours of exposure and for all concentrations, except the lowest concentration at 48 hours (10 ng/mL  $p \leq 0.05$ , 33 and 100 ng/mL  $p \leq 0.001$ ). The level and

increase in percentage of tail DNA were both lower after 48 hours (**Figure 3.3.a, 3.3.b**).



**Figure 3.3.** The effect of OA on DNA fragmentation in Jurkat T cells after a) 24 hours and b) 48 hours and on apoptosis/necrosis after c) 24 hours and d) 48 hours. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for early apoptosis. Results presented are the mean  $\pm$  SD of 4 independent experiments.

### The effect of okadaic acid on CaCo-2 cells.

A significant decrease in cell number could be detected for the two highest concentrations (33 ng/mL  $p \leq 0.01$ , 100 ng/mL  $p \leq 0.001$ ) at both time points. Additionally a substantial reduction in cell number can be seen at a concentration of 10 ng/mL of OA at 24 hours and 48 hours (**Table 3.3.**).

**Table 3.3.** Cell number of CaCo-2 cells, expressed as % of the blank, after exposure to OA at four different concentrations for 24 hours and 48 hours. Results are the mean of  $\pm$  SD 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*\*).

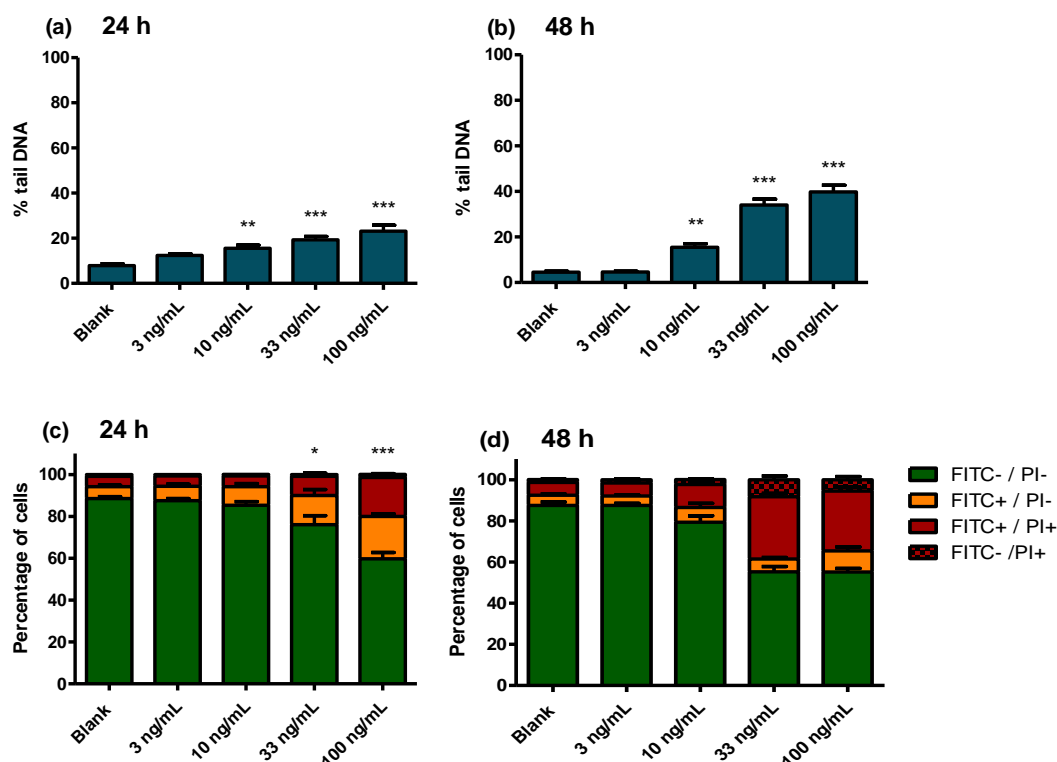
Assay	Exposure Time	Blank	OA 3 ng/mL	OA 10 ng/mL	OA 33 ng/mL	OA 100ng/mL
<i>Trypan Blue Exclusion assay</i>	24 hours	100	82 $\pm$ 15	73 $\pm$ 28	42 $\pm$ 20 (**)	30 $\pm$ 10 (***)
	48 hours	100	102 $\pm$ 9	67 $\pm$ 20	32 $\pm$ 6 (**)	24 $\pm$ 4 (***)

At 24 hours of exposure a significant change in cell viability could only be seen for 33 ng/mL OA ( $p \leq 0.05$ ). At 48 hours a significant, concentration dependent, decrease (10 ng/mL  $p \leq 0.01$ , 33 and 100 ng/mL  $p \leq 0.001$ ) could be seen for the three highest concentrations (**Table 3.4.**). The viabilities, as measured by flow cytometer analysis (the sum of FITC- / PI- and FITC+ / PI- stained cells) correlated well with viability data given by the Trypan Blue Exclusion assay, with correlation coefficients of  $r = 0.9$  ( $p \leq 0.05$ ) at both time points. A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be shown for 100 ng/mL ( $p \leq 0.001$ ) of OA after 24 hours and at the two highest concentrations after 48 hours ( $p \leq 0.001$ ) of exposure (**Figure 3.4.c, 3.4.d**). FITC- / PI+ positive stained cells could be identified at the three highest concentrations at 48 hours (**Figure 3.4.d**).

**Table 3.4.** Viability of CaCo-2 cells after exposure to OA at four different concentrations for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*\*).

Assay	Exposure Time	Blank	Blank Vehicle	OA 3 ng/mL	OA 10 ng/mL	OA 33 ng/mL	OA 100ng/mL
<i>Trypan Blue Exclusion assay</i>	24 hours	99 $\pm$ 0.5	99 $\pm$ 0.5	99 $\pm$ 0.5	99 $\pm$ 1.2	94 $\pm$ 3.0 (*)	95 $\pm$ 1.7
	48 hours	98 $\pm$ 1.5	100 $\pm$ 0.0	96 $\pm$ 2.2	92 $\pm$ 1.8 (**)	72 $\pm$ 2.9 (***)	60 $\pm$ 2.2 (***)

A significant increase in FITC+ / PI- stained cells (early apoptosis) could be detected for the two highest concentrations at 24 hours (33 ng/mL  $p \leq 0.05$ , 100 ng/mL  $p \leq 0.001$ ), no significant increase could be seen after 48 hours (**Figure 3.4.c, 3.4.d**). A significant increase in DNA fragmentation given by the COMET assay could be detected for concentrations of 10 ( $p \leq 0.01$ ), 33 and 100 ng/mL ( $p \leq 0.001$ ) at both time points. The percentage of tail DNA for 33 and 100 ng/mL was substantially higher at 48 hours than at 24 hours (**Figure 3.4.a, 3.4.b**). The increase in tail DNA and the lack of FITC+ / PI- stained cells (early apoptosis) at 48 hours both coincided well with the earlier mentioned increase in FITC+ / PI+ stained cells.



**Figure 3.4.** The effect of OA on DNA fragmentation in CaCo-2 cells after a) 24 hours and b) 48 hours and on apoptosis/necrosis after c) 24 hours and d) 48 hours. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for early apoptosis. Results presented are the mean  $\pm$  SD of 4 independent experiments.

### The effect of okadaic acid on HepG-2 cells.

After 12 hours of exposure to OA a significant reduction in cell number could be identified for the three highest concentrations (10 and 33 ng/mL  $p \leq 0.05$ , 100 ng/mL  $p \leq 0.01$ ). After 24 hours a significant reduction ( $p \leq 0.01$ ) in cell number could be detected for 33 and 100 ng/mL. Although not statistically significant ( $p \geq 0.05$ ) a substantial decrease in cell number could also be seen for the lower two exposure concentrations (**Table 3.5.**)

**Table 3.5.** Cell number of HepG-2 cells, expressed as % of the blank, after exposure to OA at four different concentrations for 12 hours and 24 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*).

Assay	Exposure Time	Blank	OA 3 ng/mL	OA 10 ng/mL	OA 33 ng/mL	OA 100ng/mL
<i>Trypan Blue Exclusion assay</i>	12 hours	100	89 $\pm$ 23	71 $\pm$ 22 (*)	68 $\pm$ 14 (*)	53 $\pm$ 26 (**)
	24 hours	100	75 $\pm$ 28	58 $\pm$ 23	38 $\pm$ 11 (**)	30 $\pm$ 20 (**)

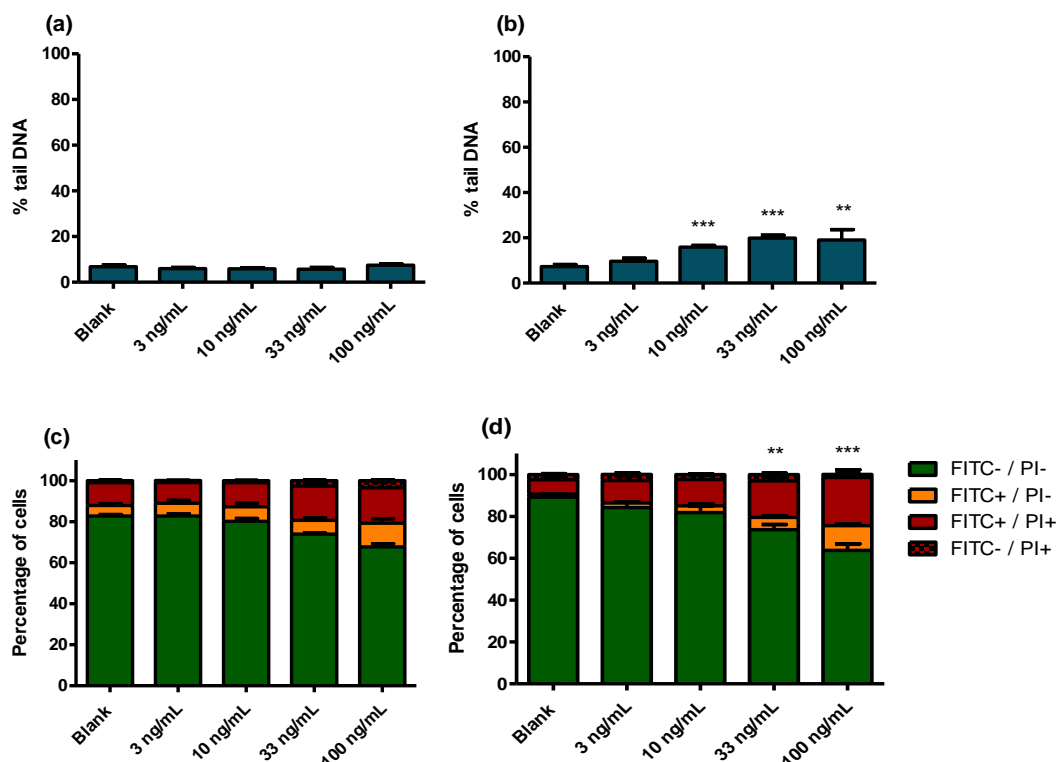
A significant change ( $p \leq 0.01$ ) in cell viability could only be detected at a concentration of 33 ng/mL at both time points (**Table 3.6.**). The viabilities, as measured by flow cytometer analysis (the sum of FITC- / PI- and FITC+ / PI- stained cells) correlated well with the viabilities given by the Trypan Blue Exclusion assay, with correlation coefficients of  $r = 0.9$  at 12 hours (ns) and  $r = 1$  ( $p \leq 0.05$ ) at 24 hours of exposure. A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be detected for the three highest concentrations at 24 hours (10 ng/mL  $p \leq 0.05$ , 33 ng/mL  $p \leq 0.01$ , 100 ng/mL  $p \leq 0.001$ ) (**Figure 3.5.d**). A discrepancy with the results given by the Trypan Blue Exclusion assay (**Table 3.6.**) can be seen for 10 and 100 ng/mL. FITC- / PI+ stained cells could be identified for the two highest concentrations at 12 hours and at all concentrations, except 100 ng/mL after 24 hours, including the blank (**Figure 3.5.c, 3.5.d**).

**Table 3.6.** Viability of HepG-2 cells after exposure to OA at four different concentrations for 12 hours and 24 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value  $< 0.05$  (\*),  $0.01$  (\*\*) and  $0.001$  (\*\*).

Assay	Exposure Time	Blank	Blank Vehicle	OA 3 ng/mL	OA 10 ng/mL	OA 33 ng/mL	OA 100ng/mL
<i>Trypan Blue Exclusion assay</i>	12 hours	98 $\pm$ 1.2	97 $\pm$ 0.6	96 $\pm$ 1.2	94 $\pm$ 2.1	92 $\pm$ 2.4 (**)	95 $\pm$ 2.4
	24 hours	99 $\pm$ 0.0	99 $\pm$ 0.0	99 $\pm$ 1.0	99 $\pm$ 1.3	94 $\pm$ 2.9 (**)	96 $\pm$ 3.4

A significant increase in FITC+ / PI- stained cells (early apoptosis) were only detected at 24 hours for concentrations of 33 ng/mL ( $p \leq 0.01$ ) and 100 ng/mL ( $p \leq 0.001$ ) (**Figure 3.5.d**). A significant increase in DNA fragmentation could be shown for the three highest concentrations (10 and 33 ng/mL  $p \leq 0.001$ , 100 ng/mL  $p \leq 0.01$ ) of OA after 24 hours of exposure (**Figure 3.5.b**). This increase in the percentage of tail DNA coincided with the increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) above described. No significant change ( $p \geq 0.05$ ) for any of the endpoints, except cell viability, could be identified at 12 hours of exposure (**Figure 3.5.a, 3.5.c**)





**Figure 3.5.** The effect of OA on DNA fragmentation in HepG-2 cells after a) 12 hours and b) 24 hours and on apoptosis/necrosis after c) 12 hours and d) 24 hours. The different concentrations are compared to the blank and significance is marked as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). For flow cytometer analysis significances are only marked for early apoptosis. Results presented are the mean  $\pm$  SD of 4 independent experiments.

## Discussion

Okadaic acid (OA) is a polyether fatty acid produced by dinoflagellates of the genus *Dynophysis sp.* and *Prorocentrum sp.*. Together with its analogues, DTX1-3 it forms the group of OA-toxins [3, 23]. Due to their lipophilic nature these toxins can accumulate in shellfish and cause Diarrhoeic Shellfish Poisoning in humans after consumption [3, 7]. Literature regarding OA toxicity is limited; most data available are based on acute toxicity studies. The information available on the genotoxic potential of OA is incomplete and often contradicting. Because of that the main focus of the present study was

to identify the possible genotoxicity of OA in the COMET assay. Previous chapters have outlined the reasoning behind including additional assays.

Attempts were made to ensure that the mixing of cells and the sampling of aliquots for the Trypan Blue Exclusion assay were as constant as possible. Slight variations are likely. While this would not affect the cell viability *per se* it might well account for higher standard deviations (SD) in cell numbers.

Comparison between viability data obtained by the Trypan Blue Exclusion assay and flow cytometer analysis showed good correlation, with coefficients close to 1 for all three cell lines and time points. The absence of statistical power for most coefficients could be due to the sample number. Correlations however, are most likely of biological relevance. Discrepancies between the two assays, for example Jurkat T cells at 10 ng/mL after 48 hours, are probably a result of differences in the handling and preparation processes (centrifugation, re-suspension prior to flow analysis).

Jurkat T cells showed a significant reduction in cell viability for the highest two concentrations at both time points. The cell viabilities after 48 hours are overall much lower than after 24 hours (**Table 3.2.**), demonstrating a dose- and time-dependent effect of OA on cell viability. A significant effect of OA on DNA fragmentation could be detected at 3 ng/mL, 33 ng/mL and 100 ng/mL after 24 hours. The lower tail DNA at 10 ng/mL compared to 3 ng/mL cannot be explained clearly. Most likely experimental errors are responsible rather than biological reasons. A significant increase in tail DNA after 48 hours of exposure to OA could be shown for the three highest concentrations (**Figure 3.3.b**), however the percentage of DNA fragmentation is lower than after 24 hours (**Figure 3.3.a**). This could be an indication for DNA repair mechanisms to have taken place. Looking at the cell numbers (**Table 3.1.**), it can be seen that there is a clear decrease in the cell number from 24 hours to 48 hours of exposure. For instance, at 24 hours, cell cultures exposed to 100 ng/mL had a cell number of 50% compared to the blank. After 48 hours a cell number of 16% was observed compared to the blank. As previously described in Chapter 1 and Chapter 2, this cell loss over the time course points towards

apoptotic mechanisms, rather than repair mechanisms being responsible for the decrease in DNA damage [36]. In the present study, this is supported by data given by flow cytometer analysis. A significant increase in early apoptotic cells (FITC+ / PI-) could be detected for the three highest concentrations of OA after 24 hours and for all four concentrations after 48 hours. The percentage in early apoptosis however was lower after 48 hours. The latter coincides with a significant increase in late apoptosis/necrosis (FITC+ / PI+) at the three highest concentrations after 48 hours (**Figure 3.3.d**). Because of heterogeneity and differences in phases of the cell cycle, individual cells within the same cell population can undergo apoptotic events at different times [37]. This can be seen by the significant increase in late apoptotic/necrotic cells (FITC+ / PI+) at 33 and 100 ng/mL after 24 hours (**Figure 3.3.c**) and is further supported by the cell number. The total number of exposed cells is already significantly lower at 24 hours compared to the blank.

CaCo-2 cells showed a statistically significant reduction in cell viability at 33 ng/mL after 24 hours of exposure to OA. The percentage of viable cells is 94%, hence the decrease is most likely of limited biological relevance. After 48 hours a dose dependent effect could be detected, with significant reductions at 10 ng/mL, 33 ng/mL and 100 ng/mL (**Table 3.4.**). Different methods of detection and time points make a direct comparison of cell viabilities mentioned in the literature and those detected in the current study difficult. This may account for the differences seen. The cell viability in the present study is  $\geq 90\%$  after 24 hours of exposure, while literature reports viabilities between 65% (100 ng/mL) [2] and 85% (8 ng/mL) [9]. In principle it can be said that the reduction in cell viability in the present study is lower than reported in the literature [2, 8, 9, 21, 29, 34]. A significant increase in DNA fragmentation could be detected in the COMET assay for the two highest concentrations at both time points (**Figure 3.4.a, 3.4.b**). However, the percentage of tail DNA after 48 hours was approximately double than after 24 hours. These data indicate a time and dose dependent genotoxic effect of OA on CaCo-2 cells. However, one criterion for genotoxic DNA

damage is that the total cell number remains constant [36]. In the current study, a significant reduction in cell number could be seen already at 24 hours for 33 and 100 ng/mL (**Table 3.3.**), which decreases even further at 48 hours. Together with data from the flow cytometer analysis this indicated an apoptotic rather than genotoxic effect of OA. A significant increase in early apoptosis (FITC+ / PI-) could be seen by flow cytometer analysis for 33 ng/mL and 100 ng/mL after 24 hours. At 48 hours, no significant early apoptosis (FITC+ / PI-) could be seen but a significant increase in late apoptosis (FITC+ / PI+) for the two highest concentrations could be shown. This suggested a progression from early to late apoptosis (**Figure 3.4.c, 3.4.d**).

HepG-2 cells showed a statistically significant reduction in cell viability as determined by the Trypan Blue Exclusion assay at both time points at a concentration of 33 ng/mL. The values (**Table 3.6.**) however, indicate that they might not be of major biological significance at the time points and concentrations tested. Studies conducted by other researchers have shown a significant increase in cytotoxicity of OA on HepG-2 cells at similar concentrations and time points used in this study [2, 22, 23, 28, 29]. This discrepancy could possibly be explained by the different detection methods used. No increase in DNA damage or early and late apoptotic cells could be detected for OA after 12 hours of exposure (**Figure 3.5.a, 3.5.c**). After 24 hours the three highest concentrations of OA showed a significant increase in DNA fragmentation (**Figure 3.5.b**), firstly indicating genotoxic damage. However, a significant increase in late apoptosis (FITC+ / PI+) could also be detected for the three highest concentrations after 24 hours (**Figure 3.5.d**). DNA fragmentation is described in the literature as a late apoptotic event [38]. Therefore the co-occurrence of the two events suggested the DNA damage detected in the present study to be linked to late apoptosis rather than genotoxicity. Additionally early apoptotic cells (FITC+ / PI-) could be seen at concentrations of 33 ng/mL and 100 ng/mL, after 24 hours (**Figure 3.5.d**). This demonstrated apoptotic processes to be involved to an extent in OA toxicity. As mentioned for Jurkat T cells, not all cells within one

population undergo apoptotic processes in the same time frame [37]. Supporting the above hypothesis are the results of the present study showing the change in the total cell numbers found for 12 hours and 24 hours of exposure to OA. After 12 hours the cell number at 10 and 33 ng/mL had already decreased by approximately 30%, compared to the blank. After 24 hours of exposure the cell numbers were below 40% for the three highest concentrations (**Table 3.5.**). The data in the current study indicated an apoptotic effect rather than a genotoxic effect of OA on HepG-2 cells. The loss in cell number due to the completion of the apoptotic process would tie in with the visual observations made during the initial stages of the assays. When performing the assays the exposure medium was transferred to Eppendorf tubes and centrifuged before the attached cells were actively detached using trypsin-EDTA. At both time points a substantial amount of floating cells could be seen in the exposure medium. Although these cells were added to the actively detached cells the total cell numbers were distinctly lower compared to the blank.

The findings in the present study are supported by the limited literature available. In T lymphoma cells and Jurkat T cells OA has been demonstrated to induce apoptosis [39, 40]. For CaCo-2 the reports are more contradictory. Some studies found OA to increase the formation of micronuclei and cause DNA fragmentation in a dose and time dependent manner. The authors therefore concluded OA to be genotoxic [9, 34]. Others inferred OA to execute cell death via necrosis using the Damaged DNA Detection (3D) assay<sup>5</sup> [29]. Another study found OA to induce DNA strand breaks in the COMET assay but also clear indications of apoptosis induction [34]. The findings reported for HepG-2 cells show OA to induce DNA lesions [29], strand breaks [22] and micronuclei [28]. However, observations are in favour of cell death [29] and nuclei fragmentation linked to apoptotic processes [2]. The involvement of apoptosis in OA toxicity has also been widely described as an effect in other cell lines such as human neuroblastoma cells (BE(2)-17,

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<sup>5</sup> The 3D assay quantifies DNA damage through nucleotide excision repair (NER) and base excision repair (BER) [29].

TR14, NT2-N) human lung fibroblasts (NHLF) and rat/human hepatocytes [13, 24, 41, 42]. In general, studies have suggested that OA toxicity is cell line dependent. Rossini et al. [31] found differential activation of caspase isoforms in HeLa S<sub>3</sub> and MCF-7 and concluded the apoptotic effect to be cell line dependent. A similar conclusion was drawn by Valdiglesias et al. [22, 23], Souid-Mensi et al. [29] and Rubiolo et al. [30] for the genotoxic potential of OA. These findings are supported by other studies which show a direct effect on the DNA in BHK-21 and HESV cells [17] but not in CHO-K1 cells [27]. The findings in the present study support the hypothesis that the effect of OA depends on the cell line investigated. The effect on cell viability seems to be similar in Jurkat T cells and CaCo-2 cells; however a higher percentage of tail DNA in the COMET assay could be detected after 24 hours in Jurkat T cells. While the effect after 48 hours is visibly lower in Jurkat T cells again, the amount of DNA fragmentation in CaCo-2 cells has increased. The percentage of early apoptotic cells (FITC+ / PI-) is higher in Jurkat T cells after 24 hours and 48 hours of exposure compared to CaCo-2 cells. HepG-2 cells are stated in the literature to be the most sensitive to OA in comparison to CaCo-2 cells, for example [22]. Neither the viability determined by the Trypan Blue Exclusion assay or the percentage of tail DNA as shown by the COMET assay seems to indicate this sensitivity in the study conducted here. However, genotoxic damage does not lead to a reduction in cell number, while cells that undergo the full apoptotic process are lost [36]. Data given by the Trypan Blue Exclusion assay indicated a severe loss of HepG-2 cells in the time course of 24 hours of exposure to OA. This coincides with early and late apoptotic data given by flow cytometer analysis. Additionally, the DNA fragmentation detected in the COMET assay coincided with late apoptotic cells (FITC+ / PI+) at 24 hours. All data taken together suggests that HepG-2 cells might undergo apoptosis in a faster time course than Jurkat T cells and CaCo-2 cells. Sundquist et al. [37] stated that the induction and time line of apoptosis is very much dependent on cell line, exposure time and compound concentration. Exposure time and the concentrations of OA have been kept constant in the current study indicating a definite cell line dependency. Overall, the data given by the present study implied that the sensitivity of the

cell lines to OA are, in increasing order of sensitivity, CaCo-2 cells  $\approx$  Jurkat T cells  $\rightarrow$  HepG-2 cells.

Technically, no cells should stain FITC- / PI+ only. However, a small percentage could be detected for CaCo-2 cells at the two highest concentrations of OA after 48 hours and in HepG-2 cells after 12 hours. Additionally, FITC- / PI+ stained cells could be seen in HepG-2 cells at all concentrations (including the blank) after 24 hours, except 100 ng/mL. No FITC- / PI+ stained cells could be detected for Jurkat T cells. The FITC- / PI+ stained cells are most likely due to handling and preparation of cells (centrifugation, re-suspension) prior to flow cytometer analysis [43]. Only adherent cell lines seem to be affected, indicating the further possibility of damage due to cell detachment.

## Conclusion

Data obtained in the present study suggests that OA is not *per se* genotoxic. Flow cytometer data gave positive results for early apoptotic cells in all three cell lines. DNA fragmentation is a late event in apoptosis. The detected DNA fragmentation in the COMET assay and late apoptotic cells given by flow cytometer analysis coincided well for all three cell lines at the concentrations and time points used. All data therefore supports the hypothesis that the detected DNA fragmentation is most likely based on apoptotic processes rather than direct genotoxicity. This is in agreement with other literature that showed apoptosis to be involved in the effects of OA on a variety of cell lines [23, 29-31]. The cell line dependency of OA toxicity proposed by other authors can be reinforced in the study conducted here. HepG-2 cells were the most sensitive, while CaCo-2 cells and Jurkat T cells were less sensitive. A clear differentiation between the latter two cell lines is not possible with the data presented in this study.

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## Chapter 4: Azaspiracid

### ***Introduction***

Azaspiracids (AZAs) are a group of lipophilic polyether marine biotoxins with a unique spiral ring assembly. They were first detected in mussels from Ireland in 1995 after consumers in the Netherlands showed symptoms of Diarrhoeic Shellfish Poisoning [1, 2]. However, DSP toxins were below the regulatory limits and the toxin was later identified as a novel marine biotoxin and named azaspiracid [2, 3]. Symptoms of acute AZA poisoning (AZP) occur within 3-18 hours after consumption of contaminated shellfish and included nausea, vomiting, diarrhoea and stomach cramps [2]. A full recovery of the clinical symptoms takes place within 2-5 days. The dinoflagellate *Azadinum spinosum* has recently been recognized as the primary producer of AZA1 and AZA2 [4, 5] and to date over twenty analogues have been identified. Most of these analogues have been either proven or suggested to be biotransformation products in shellfish [6-8] and together with the parent compounds can accumulate in shellfish. This might cause environmental problems throughout the food web. Human consumers are potentially at risk due to the increased presence of AZAs in shellfish meant for the market worldwide [9-13]. Additionally, closures of aquaculture and harvesting sites can impact the local economy [14].

Toxicological studies of AZAs are limited due to the lack of availability of toxins and toxin standards. One of the first studies by Ito et al. [15] identified the gastrointestinal tract (GI) as the main target of AZA. AZA was extracted from mussels and administered to mice at a single dose of 130 or 300 µg per kg body weight by gastric intubation. Mice were sacrificed after 30 minutes, 1, 2, 3 and 4 hours. The study detected shortened villi in the small intestine, degeneration of cells in the large intestine, accumulation of fat droplets in the liver and necrotic lymphocytes in the thymus, spleen and Peyer's patch. In a later study by the same group, an increased weight of several organs and an accumulation of large volumes of gas in the small intestine after chronic exposure to AZA were detected [15]. Mice were dosed

twice a week with 1 µg to 50 µg AZA per kg body weight, up to 40 times in 145 days. Recovery was generally slow and some of the mice developed lung tumours [16]. A recent study by Aune et al. [17] confirmed the findings in the GI tract but was unable to show any further signs of toxicity. 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 [1-3]. Newer 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 [18-20]. In general, molecular effects of AZAs in different cellular systems have been increasingly investigated over the last number of years, also due to the gradually higher availability of standards [21]. Existing data have shown AZAs to have a cytotoxic effect on various cell lines [22-27]. 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, a structure that is involved in cell-cell and cell-surface interactions [20, 23, 26]. Further studies support those findings by showing changes in the E-cadherin pool in epithelial cells [28] and reductions in the level of F-actin [29]. Additionally, AZAs have been shown to increase cellular levels of cAMP [29-31], modulate intracellular pH in lymphocytes [30, 32], modify calcium flux [30, 31, 33] and inhibit cholesterol biosynthesis [34]. Possible implications on heart functions have been investigated recently *in vitro*, showing a blockage of hERG channels [35] and *in vivo*, demonstrating a change in heart physiology of rats [36]. Exposure in the latter study occurred via single intravenous injection at concentrations of 11 µg and 55 µg per kg body weight. However, the exact cellular targets and the mechanism(s) by which AZAs attain their effects are still unknown. Data on long term effects and/or carcinogenicity are limited to the study by Ito et al. [16] with the detection of lung tumours in mice and a study by Colman et al. [37] on Japanese medaka. The latter examined the

teratogenic effect of AZA1 and found effects on the health and development of finfish embryos as well as on their general hatching success.

Due to the lack of information on genotoxicity the purpose of the present study was to investigate the possible DNA damaging effect of AZA1-3. AZA1 and AZA2 are naturally occurring and regularly found in shellfish samples. AZA3 occurs in lower concentrations or is often absent [38]. However, different potencies of AZA1-3 have been detected in *in vivo* and *in vitro* studies and results suggested AZA3 to possibly have a greater effect than AZA1 and AZA2 [24, 39, 40]. The COMET assay was used to detect possible DNA damage. To determine any overt cytotoxicity and/or apoptotic effects of the three analogues, additional assays were included as described in previous chapters.

## ***Material & Methods***

### **Chemicals & Reagents**

Certified reference standards for azaspiracid1-3 were purchased from the NRC, Canada. All chemicals were obtained from Sigma-Aldrich, Ireland unless otherwise indicated. Annexin V and Annexin V detection kit, flow tubes and flow cytometer fluids were purchased from BD Bioscience, UK. Microscope slides and cover slips were ordered from Fisher Scientific, Ireland. All plastic ware was purchased from Sarstedt, Ireland.

### **Cell culture**

Jurkat T cells (human T cell lymphoblast), CaCo-2 cells (human epithelial colorectal adenocarcinoma cells) and HepG-2 cells (human hepatocellular cells) were provided by the European Collection of Cell Cultures (ECACC, operated by Public Health England) and cultured as described in Chapter 2.

### **Cell exposure**

AZAs were supplied at stock concentrations of 1.47  $\mu\text{M}$  for AZA1, 1.50  $\mu\text{M}$  for AZA2 and 1.25  $\mu\text{M}$  for AZA3 dissolved in methanol. Stock concentrations of 1  $\mu\text{M}$  were prepared and kept in a freezer at  $-80^{\circ}\text{C}$ . Working solutions were prepared freshly before use by serial dilution to keep the volume added to the well consistent.

Cells were seeded in 6 well plates at a density of  $2 \times 10^5$  cells/mL. Adherent cells were seeded the night before (to allow re-attachment) while non-adherent cells were seeded 4 hours prior to 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 methanol or being vehicle free. In contrast to previous chapters, the reduction in cell number for HepG-2 cells after 24 hours was less prominent. Therefore, no need was seen to shorten the exposure time to 12 hours. Exposure times for all cell lines, Jurkat T cells, CaCo-2 and HepG-2 cells, were 24 hours and 48 hours.

### **Assay analysis**

The Trypan Blue Exclusion assay, COMET assay and flow cytometer analysis were performed as described in Chapter 2. All cell counts and sample analysis were performed in duplicate and a total of four independent experiments were performed for each analogue, cell line and exposure time.

### **Statistical analysis**

Statistical analysis was carried out using IBM SPSS Statistics 20. Data were expressed as mean  $\pm$  standard deviation (SD) of four independent experiments. Outliers were identified by box plots and where clearly related to an experimental error, removed. Differences between means were established using the non-parametric Kruskal-Wallis-Test with Mann-Whitney U test for pairwise comparison. Results were considered significantly

different with a p-value  $\leq 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*). Correlation between Trypan Blue Exclusion assay data and flow cytometer data (FITC- / PI- plus FITC+ / PI-) was analysed using the Spearman's rank correlation in Graph Pad Prism 5.

## **Results**

### **The effect of azaspiracid1-3 on Jurkat T cells.**

Jurkat T cells were exposed to different concentrations of AZA1-3 for 24 and 48 hours. No significant differences ( $p \leq 0.05$ ) between the blank and vehicle blank could be detected at any stage. Therefore the vehicle blank is used as reference blank when presenting the findings of this study. A significant reduction in cell number could be seen for AZA1-3 at the two highest concentrations (1 nM  $p \leq 0.01$ ; 10 nM  $p \leq 0.001$ ) after 48 hours (**Table 4.1.**). Although not statistically significant ( $p \geq 0.05$ ) a pattern of substantial reduction in cell number could also be seen for AZA2 at the two highest concentrations after 24 hours and at 0.1 nM after 48 hours. A substantial reduction in cell number could also be seen for AZA3 at all concentrations.



**Table 4.1.** Cell number of Jurkat T cells, expressed as % of the blank, after treatment with four different concentrations of AZA1-3 for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value of  $p \leq 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

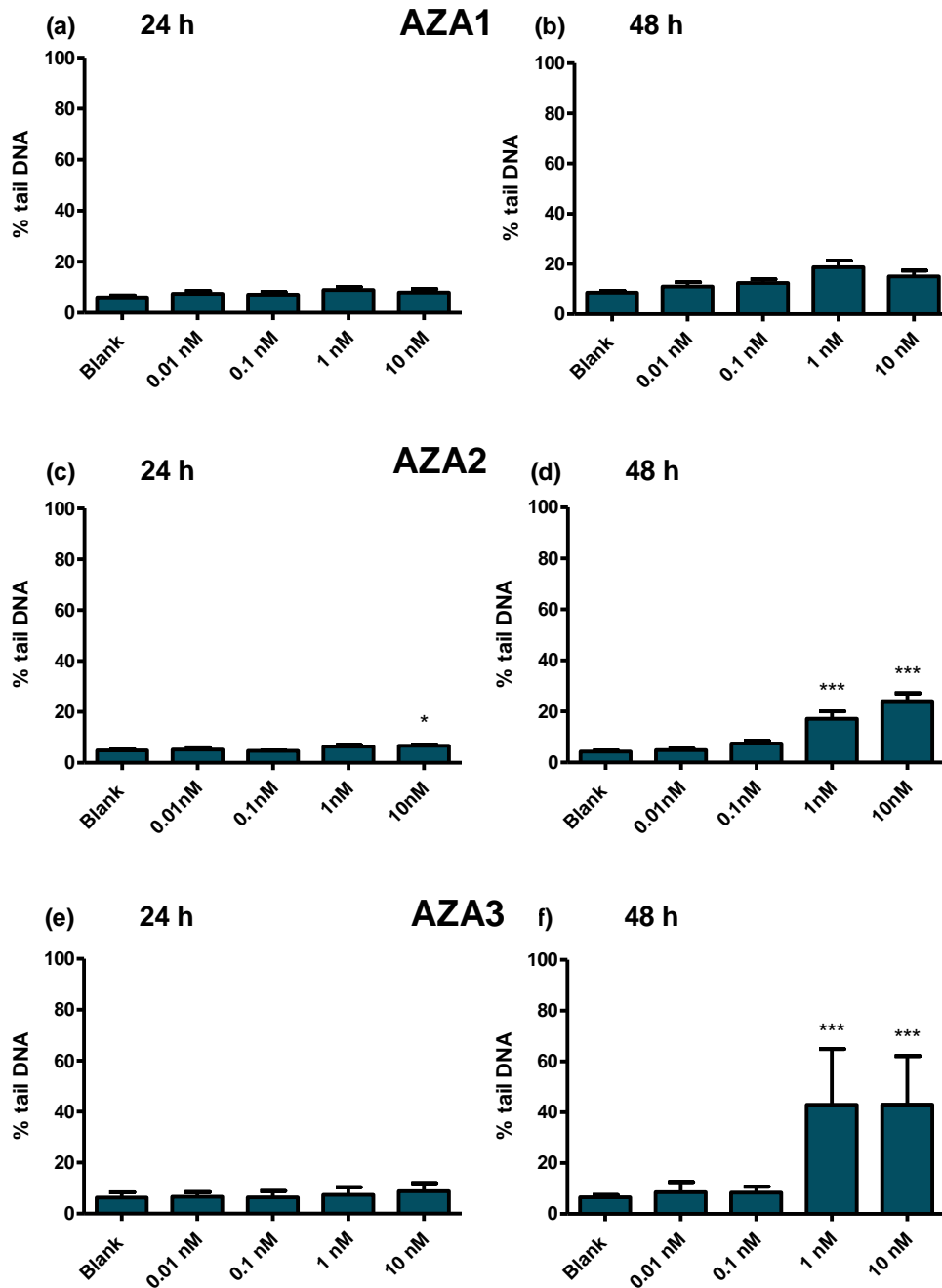
Biotoxin	Exposure Time	Blank	0.01 nM	0.1 nM	1 nM	10 nM
AZA1	24 hours	100	92 $\pm$ 10	89 $\pm$ 8	82 $\pm$ 8	84 $\pm$ 18
	48 hours	100	83 $\pm$ 15	83 $\pm$ 15	61 $\pm$ 13 (**)	64 $\pm$ 7 (**)
AZA2	24 hours	100	104 $\pm$ 9	101 $\pm$ 25	62 $\pm$ 9	72 $\pm$ 21
	48 hours	100	105 $\pm$ 11	52 $\pm$ 9	32 $\pm$ 5 (**)	33 $\pm$ 7 (**)
AZA3	24 hours	100	70 $\pm$ 22	72 $\pm$ 14	63 $\pm$ 16	64 $\pm$ 16
	48 hours	100	92 $\pm$ 29	87 $\pm$ 10	32 $\pm$ 3 (**)	31 $\pm$ 6 (***)

A significant reduction in cell viability could be detected for AZA1 at 10 nM ( $p \leq 0.01$ ) after 24 hours and at 0.1 nM ( $p \leq 0.05$ ), 1 nM ( $p \leq 0.01$ ) and 10 nM ( $p \leq 0.001$ ) after 48 hours. A significant dose and time dependent decrease in cell viability could also be seen for AZA2 and AZA3 at the two highest concentrations at both time points ( $p \leq 0.01$ , except 1 nM (AZA2), 24 h  $p \leq 0.05$ ; 1 nM (AZA3) 48 h  $p \leq 0.001$ ). The percentage of viable cells after 48 hours was considerably lower than after 24 hours of exposure to all three AZAs (**Table 4.2.**).

**Table 4.2.** Cell viability of Jurkat T cells after treatment with four different concentrations of AZA1-3 for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value of  $p \leq 0.05$  (\*), 0.01 (\*\*), and 0.001 (\*\*\*).

Biotoxin	Exposure Time	Blank	Blank Vehicle	0.01 nM	0.1 nM	1 nM	10 nM
AZA1	24 hours	99 $\pm$ 1.2	97 $\pm$ 1.8	95 $\pm$ 4.5	94 $\pm$ 4.7	90 $\pm$ 4.0	87 $\pm$ 2.2 (**)
	48 hours	87 $\pm$ 4.2	85 $\pm$ 6.7	73 $\pm$ 1.7	52 $\pm$ 9.3 (*)	32 $\pm$ 7.3 (**)	26 $\pm$ 8.1 (***)
AZA2	24 hours	97 $\pm$ 0.8	97 $\pm$ 0.8	98 $\pm$ 0.9	96 $\pm$ 0.7	90 $\pm$ 4.1 (*)	88 $\pm$ 2.3 (**)
	48 hours	98 $\pm$ 0.3	97 $\pm$ 1.4	96 $\pm$ 2.3	84 $\pm$ 11.6	61 $\pm$ 5.7 (**)	54 $\pm$ 7.6 (**)
AZA3	24 hours	95 $\pm$ 0.9	95 $\pm$ 0.8	94 $\pm$ 0.7	96 $\pm$ 1.0	85 $\pm$ 3.7 (**)	82 $\pm$ 6.0 (**)
	48 hours	95 $\pm$ 1.1	96 $\pm$ 0.9	93 $\pm$ 2.2	93 $\pm$ 3.5	33 $\pm$ 18.3 (***)	36 $\pm$ 15.6 (**)

AZA1-3 showed different levels of DNA fragmentation in the COMET assay. No significant increase ( $p \geq 0.05$ ) in DNA fragmentation could be detected for AZA1 after 24 hours (**Figure 4.1.a**) or 48 hours (**Figure 4.1.b**), but the percentage of tail DNA at a concentration of 1 nM and 10 nM after 48 hours is fractionally higher than the blank. After 24 hours a significant increase ( $p \leq 0.05$ ) in DNA fragmentation could be identified for AZA2 at 10 nM (**Figure 4.1.c**), no significant change ( $p \geq 0.05$ ) could be seen at any concentration for AZA3 (**Figure 4.1.e**). Both, AZA2 (**Figure 4.1.d**) and AZA3 (**Figure 4.1.f**) however showed a significant increase ( $p \leq 0.001$ ) in the percentage of tail DNA at a concentration of 1 nM and 10 nM after 48 hours of exposure. The increase in DNA fragmentation was highest in AZA3.

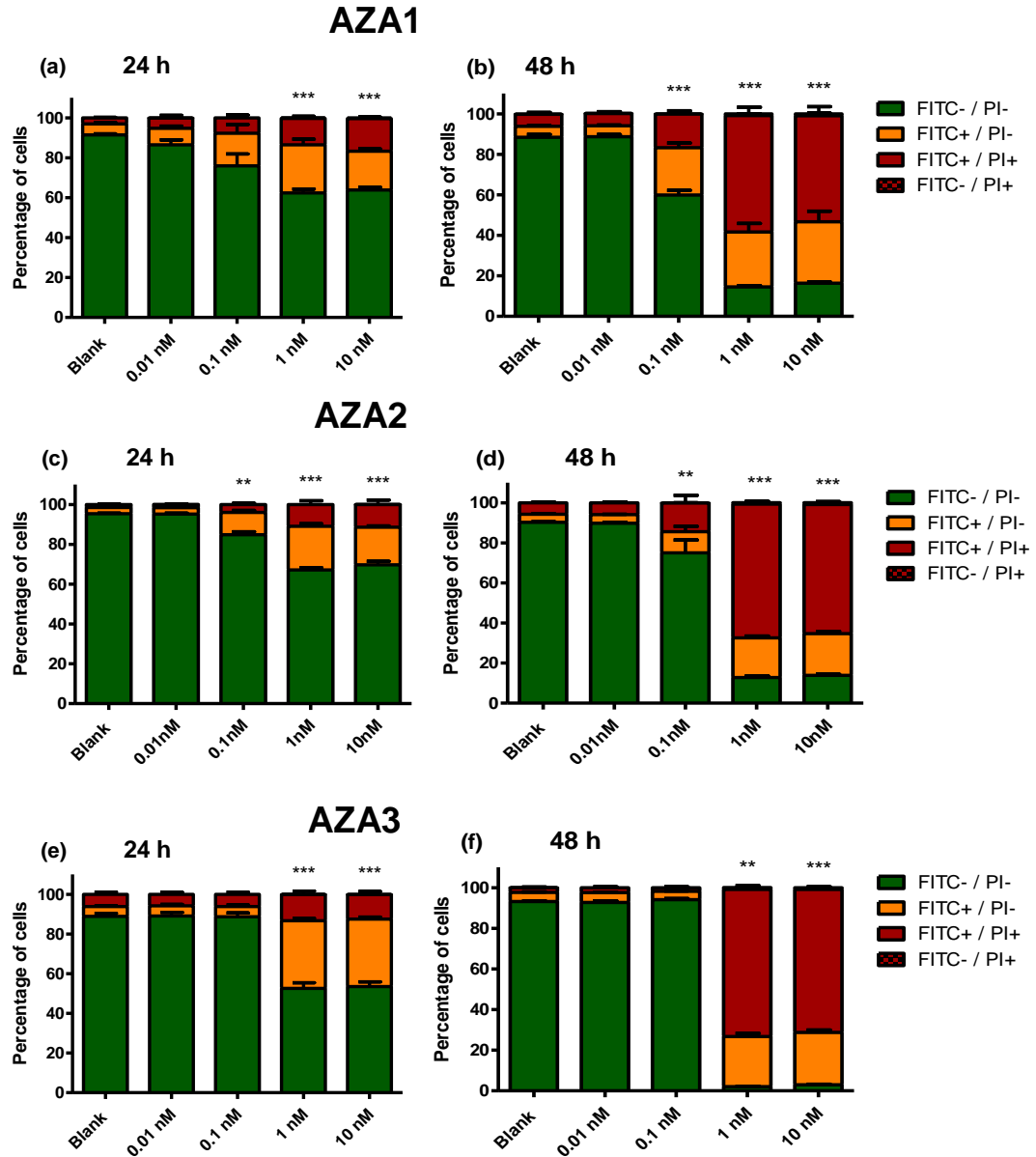


**Figure 4.1.** The effect of AZA1-3 on DNA fragmentation in Jurkat T cells after 24 hours (a, c, e) and 48 hours (b, d, f) of exposure. The different concentrations are compared to the blank and significance is marked as \* ( $p \leq 0.05$ ), \*\* ( $p \leq 0.01$ ) and \*\*\* ( $p \leq 0.001$ ). Results presented are the mean  $\pm$  SD of 4 independent experiments.

Correlation between cell viability obtained by the Trypan Blue Exclusion assay and Flow cytometer analysis (FITC- / PI- plus FITC+ / PI-) gave

coefficients of  $r = 0.9$  ( $p \leq 0.05$ ) for AZA1 and AZA2 at both time points. AZA3 gave coefficients of  $r = 0.4$  (24 hours, ns) and  $0.6$  (48 hours, ns). A significant increase in FITC+ / PI- stained cells (early apoptosis) could be detected by flow cytometer analysis at the two highest concentrations of AZA1 after 24 hours ( $p \leq 0.001$ ). Although not statistically significant ( $p \geq 0.05$ ), an increase at  $0.1$  nM can also be seen, which might be of biological relevance (**Figure 4.2.a**). After 48 hours of exposure a significant increase ( $p \leq 0.001$ ) in FITC+ / PI- stained cells (early apoptosis) could be detected at all concentrations of AZA1, except  $0.01$  nM (**Figure 4.2.b**). Significant amounts of FITC+ / PI+ stained cells (late apoptosis/necrosis) could be identified at the two highest concentrations after 24 hours ( $p \leq 0.001$ ) and at the three highest concentrations ( $0.1$  nM  $p \leq 0.05$ , all others  $p \leq 0.001$ ) after 48 hours of exposure. The percentage of FITC+ / PI+ stained cells (late apoptosis/necrosis) is distinctively higher after 48 hours compared to 24 hours, overall the increase agrees with the reduction in cell viability as observed with the Trypan Blue Exclusion assay. AZA2 showed a significant increase in FITC+ / PI- stained cells (early apoptosis) at the three highest concentrations ( $0.1$  nM  $p \leq 0.01$ , all others  $p \leq 0.001$ ) at both time points (**Figure 4.2.c, 4.2.d**). A significant amount of both FITC+ / PI+ stained cells (late apoptosis/necrosis) could be detected for  $0.1$  nM ( $p \leq 0.05$ ),  $1$  nM ( $p \leq 0.001$ ) and  $10$  nM ( $p \leq 0.001$ ) after 24 hours and  $1$  nM ( $p \leq 0.001$ ) and  $10$  nM ( $p \leq 0.001$ ) after 48 hours. Similar to AZA1, the percentages are considerably higher after 48 hours, which also agrees with the higher reduction in cell viability after 48 hours and the increase in percentage of tail DNA as shown by the COMET assay. Analysis of AZA3 identified a significant increase in FITC+ / PI- stained cells (early apoptosis) for the two highest concentrations at both time points (all  $p \leq 0.001$ , except  $1$  nM  $48$  h  $p \leq 0.01$ ), the values being higher at 24 hours (**Figure 4.2.e, 4.2.f**). FITC+ / PI+ stained cells (late apoptosis/necrosis) could also be detected in significant amounts at the two highest concentrations at both time points (24 h  $1$  nM  $p \leq 0.01$ ,  $10$  nM  $p \leq 0.05$ ; 48 h  $p \leq 0.001$ ) Just as AZA1 and AZA2 the percentage of FITC+ / PI+ stained cells (late apoptosis/necrosis) after 48 hours of exposure to AZA3 is considerable higher than after 24 hours and agrees well with the

increase in DNA fragmentation detected in the COMET assay and the reduction in cell viability shown by the Trypan Blue Exclusion assay.



**Figure 4.2.** The effect of AZA1-3 on apoptosis/necrosis in Jurkat T cells after 24 hours (a, c, e) and 48 hours (b, d, f) of exposure. The different concentrations are compared to the blank and significance is marked as \* ( $p \leq 0.05$ ), \*\* ( $p \leq 0.01$ ) and \*\*\* ( $p \leq 0.001$ ). For flow cytometer analysis significances are only marked for early apoptosis. Results presented are the mean  $\pm$  SD of 4 independent experiments.

**The effect of azaspiracid1-3 on CaCo-2 cells.**

CaCo-2 cells were exposed to different concentrations of AZA1-3 for 24 hours and 48 hours. No significant or otherwise substantial reduction in cell number could be shown for CaCo-2 cells (**Table 4.3.**).

**Table 4.3.** Cell number of CaCo-2 cells, expressed as % of the blank, after treatment with four different concentrations of AZA1-3 for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value of  $p \leq 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Biotoxin	Exposure Time	Blank	0.01 nM	0.1 nM	1 nM	10 nM
AZA1	24 hours	100	81 $\pm$ 19	84 $\pm$ 9	87 $\pm$ 5	92 $\pm$ 28
	48 hours	100	96 $\pm$ 16	109 $\pm$ 20	90 $\pm$ 19	88 $\pm$ 21
AZA2	24 hours	100	100 $\pm$ 10	95 $\pm$ 17	95 $\pm$ 28	99 $\pm$ 37
	48 hours	100	96 $\pm$ 26	88 $\pm$ 8	86 $\pm$ 18	94 $\pm$ 13
AZA3	24 hours	100	108 $\pm$ 41	97 $\pm$ 22	97 $\pm$ 15	93 $\pm$ 10
	48 hours	100	121 $\pm$ 69	107 $\pm$ 39	91 $\pm$ 24	85 $\pm$ 12

A significant decrease in cell viability could be identified for AZA1-3 at 1 nM and 10 nM at both time points (1 nM all  $p \leq 0.01$ , except AZA3 24 h  $p \leq 0.05$ ; 10 nM AZA1 24 h  $p \leq 0.05$  48 h  $p \leq 0.01$ , AZA2 and AZA3  $p \leq 0.001$ ) (**Table 4.4.**). The percentages for viable cells at 100 nM after 48 hours are considerably lower than after 24 hours.

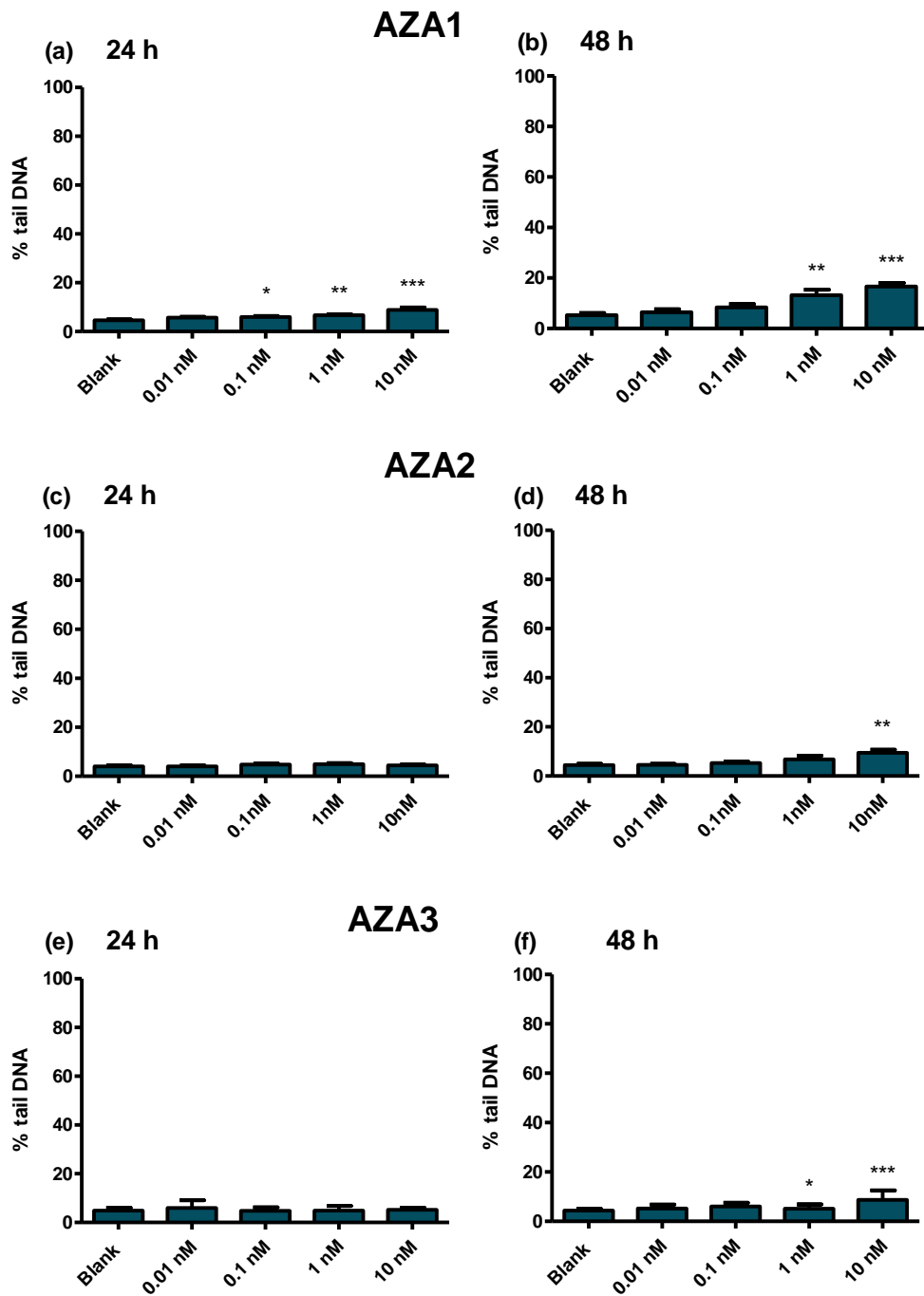
**Table 4.4.** Cell viability of CaCo-2 cells after treatment with AZA1-3 for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value of  $p \leq 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Biotoxin	Exposure Time	Blank	Blank Vehicle	0.01 nM	0.1 nM	1 nM	10 nM
AZA1	24 hours	98 $\pm$ 0.5	98 $\pm$ 0.6	97 $\pm$ 1.2	97 $\pm$ 0.9	92 $\pm$ 2.1 (**)	91 $\pm$ 1.8 (*)
	48 hours	97 $\pm$ 1.1	97 $\pm$ 1.3	95 $\pm$ 2.4	96 $\pm$ 1.8	82 $\pm$ 2.9 (**)	77 $\pm$ 4.6 (**)
AZA2	24 hours	97 $\pm$ 0.6	98 $\pm$ 0.9	96 $\pm$ 1.5	97 $\pm$ 1.5	89 $\pm$ 2.6 (**)	85 $\pm$ 2.6 (***)
	48 hours	98 $\pm$ 0.6	98 $\pm$ 0.9	98 $\pm$ 0.7	97 $\pm$ 1.0	80 $\pm$ 6.4 (**)	67 $\pm$ 1.1 (***)
AZA3	24 hours	98 $\pm$ 1.2	98 $\pm$ 0.4	98 $\pm$ 0.6	97 $\pm$ 0.8	92 $\pm$ 3.7 (*)	86 $\pm$ 7.2 (***)
	48 hours	97 $\pm$ 2.1	98 $\pm$ 0.7	96 $\pm$ 1.4	95 $\pm$ 0.6	82 $\pm$ 13.6 (**)	71 $\pm$ 11.6 (***)

Different effects on DNA fragmentation could be detected in CaCo-2 cells by the COMET assay. Exposure to AZA1 showed a significant increase in DNA fragmentation at concentrations of 0.1 nM ( $p \leq 0.05$ ), 1 nM ( $p \leq 0.01$ ) and 10 nM ( $p \leq 0.001$ ) after 24 hours (**Figure 4.3.a**) and at the two highest concentrations (1 nM  $p \leq 0.01$ , 10 nM  $p \leq 0.001$ ) after 48 hours (**Figure 4.3.b**). The increase in tail DNA is higher after 48 hours than after 24 hours, indicating not only a concentration, but also a time dependent effect. In contrast to AZA1, exposure to AZA2 caused no significant increase ( $p \geq 0.05$ ) in DNA fragmentation after 24 hours (**Figure 4.3.c**) and only at a concentration of 10 nM ( $p \leq 0.01$ ) after 48 hours (**Figure 4.3.d**). The pattern of DNA fragmentation for AZA3 is similar to one observed for AZA2. No significant change ( $p \geq 0.05$ ) in the percentage of tail DNA could be seen

after 24 hours of exposure to AZA3 (**Figure 4.3.e**) but a significant increase in DNA fragmentation could be detected after 48 hours at the two highest concentrations (1 nM  $p \leq 0.05$ , 10 nM  $p \leq 0.001$ ) (**Figure 4.3.f**).

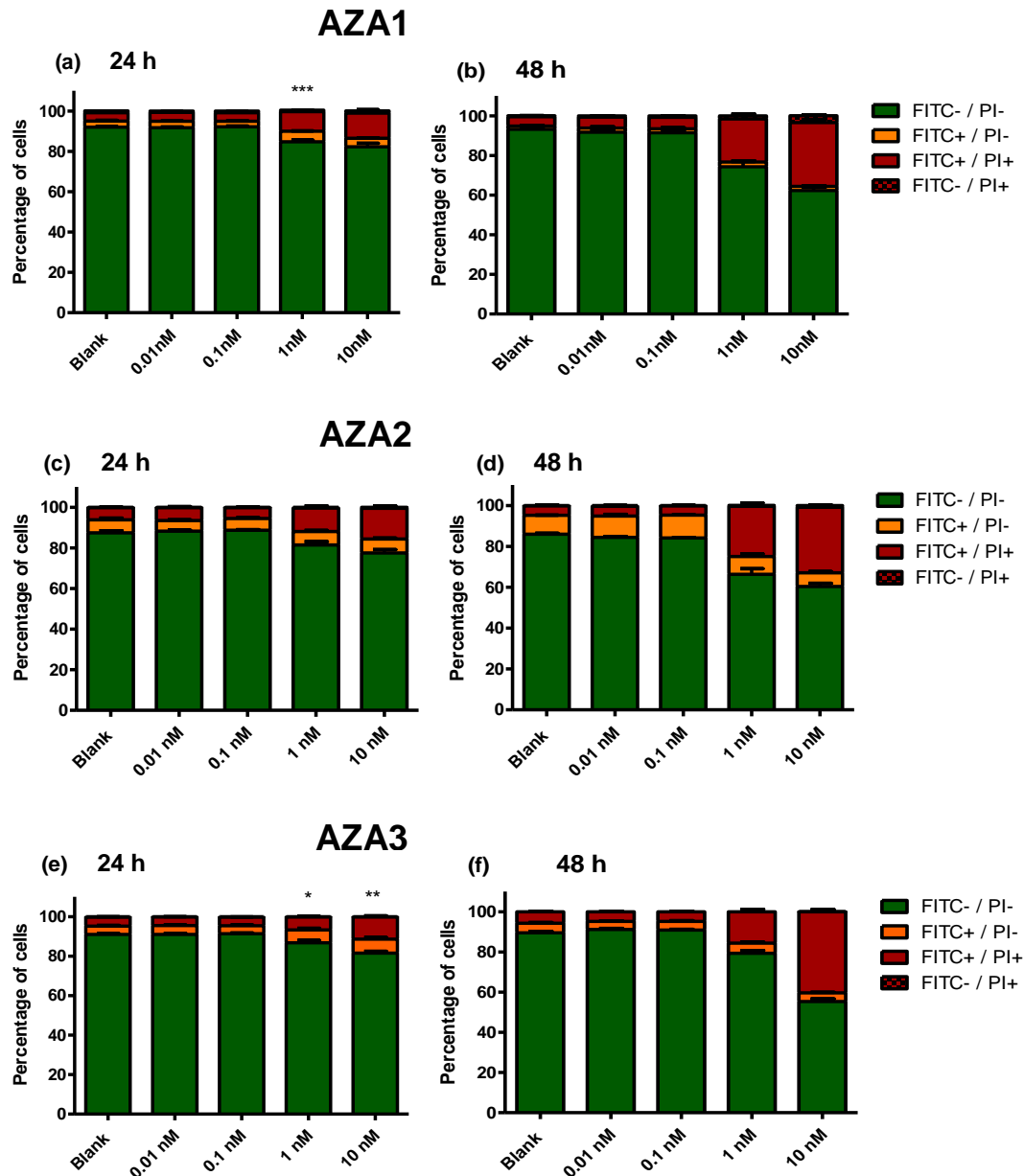




**Figure 4.3.** The effect of AZA1-3 on DNA fragmentation in CaCo-2 cells after 24 hours (a, c, e) and 48 hours (b, d, f) of exposure. The different concentrations are compared to the blank and significance is marked as \* ( $p \leq 0.05$ ), \*\* ( $p \leq 0.01$ ) and \*\*\* ( $p \leq 0.001$ ). Results presented are the mean  $\pm$  SD of 4 independent experiments.

Comparing the viabilities obtained by the Trypan Blue Exclusion assay and flow cytometer analysis (sum of FITC- / PI- and FITC+ / PI- stained cells), correlation coefficients of  $r = 0.5$  (24 hours, ns) and  $r = 0.9$  (48 hours,  $p \leq 0.05$ ) for AZA1,  $r = 0.7$  (24 hours, ns) and  $r = 0.8$  (48 hours, ns) for AZA2 and  $r = 0.9$  (24 hours,  $p \leq 0.05$ ) and  $r = 1$  (48 hours,  $p \leq 0.01$ ) for AZA3 could be calculated. Flow cytometer analysis showed a significant increase ( $p \leq 0.001$ ) in FITC+ / PI- stained cells (early apoptosis) for AZA1 at a concentration of 1 nM after 24 hours (**Figure 4.4.a**), a significant increase for FITC+ / PI+ stained cells (late apoptosis/necrosis) could be shown at both time points for the two highest concentrations ( $p \leq 0.001$ , except 1 nM, 24 h  $p \leq 0.01$ ) (**Figure 4.4.a, 4.4.b**). The considerably higher amount of FITC+ / PI+ stained cells (late apoptosis/ necrosis) after 48 hours compared to 24 hours coincides with the increase in tail DNA as well as the stronger effect of AZA1 on cell viability after 48 hours. FITC- / PI+ stained cells could be identified for 10 nM after 48 hours. No significant increase ( $p \geq 0.05$ ) in FITC+ / PI- stained cells (early apoptosis) could be detected for AZA2. In contrast to AZA1 and AZA3, the blank and the two lowest concentrations at 48 hours show a higher (not statistically significant,  $p \geq 0.05$ ) amount of FITC+ / PI- stained cells (early apoptosis). A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be seen at concentrations of 1 nM ( $p \leq 0.01$ ) and 10 nM ( $p \leq 0.001$ ) at both time points (**Figure 4.4.c, 4.4.d**). Similar to AZA1, the percentage of FITC+ / PI+ stained cells (late apoptosis/necrosis) after 48 hours is considerably higher than after 24 hours and agrees with the decrease in cell viability detected by the Trypan Blue Exclusion assay. AZA3 showed a significant increase in FITC+ / PI- stained cells (early apoptosis) at 1 nM ( $p \leq 0.05$ ) and 10 nM ( $p \leq 0.01$ ) after 24 hours (**Figure 4.4.e**), no significant increase at any concentration could be detected after 48 hours (**Figure 4.4.f**). A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be seen for 1 nM ( $p \leq 0.05$ ) and 10 nM ( $p \leq 0.001$ ) after 24 hours of exposure and for 10 nM ( $p \leq 0.01$ ) after 48 hours. Similar to AZA1 and AZA2 this increase correlates well with the viabilities detected by the Trypan Blue Exclusion assay at both time points and with the increase in tail DNA detected by the COMET assay at 48 hours

of exposure. Although not significant ( $p \geq 0.05$ ), an increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could also be seen at 1 nM after 48 hours, which might be of biological relevance.



**Figure 4.4.** The effect of AZA1-3 on apoptosis/necrosis in CaCo-2 cells after 24 hours (a, c, e) and 48 hours (b, d, f) of exposure. The different concentrations are compared to the blank and significance is marked as \* ( $p \leq 0.05$ ), \*\* ( $p \leq 0.01$ ) and \*\*\* ( $p \leq 0.001$ ). For flow cytometer analysis significances are only marked for early apoptosis. Results presented are the mean  $\pm$  SD of 4 independent experiments.

### The effect of azaspiracid1-3 on HepG-2 cells.

HepG-2 cells were exposed to different concentrations of AZA1-3 for 24 hours and 48 hours. Significant reductions in cell number were detected for AZA1 at the three highest concentrations (0.1 and 1 nM  $p \leq 0.05$ , 10 nM  $p \leq 0.001$ ) after 24 hours and at the two highest concentrations after 48 hours ( $p \leq 0.001$ ). AZA2 only caused a significant reduction in cell number at 1 nM ( $p \leq 0.05$ ) and 10 nM ( $p \leq 0.01$ ) and AZA3 at the highest concentration ( $p \leq 0.05$ ) at 48 hours (**Table 4.5.**). However various substantial but not statistically significant decreases in cell numbers could be seen at 0.01 nM (24 hours) for AZA1, 10 nM and possibly 1 nM (24 hours) for AZA2 and 10 nM (24 hours) and 1 nM (48 hours) for AZA3.

**Table 4.5.** Cell number of HepG-2 cells, expressed as % of the blank, after treatment with four different concentrations of AZA1-3 for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value of  $p \leq 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Biotoxin	Exposure Time	Blank	0.01 nM	0.1 nM	1 nM	10 nM
AZA1	24 hours	100	79 $\pm$ 15	75 $\pm$ 18 (*)	78 $\pm$ 16 (*)	65 $\pm$ 14 (***)
	48 hours	100	88 $\pm$ 13	84 $\pm$ 5	28 $\pm$ 5 (**)	29 $\pm$ 10 (***)
AZA2	24 hours	100	93 $\pm$ 19	98 $\pm$ 44	85 $\pm$ 10	72 $\pm$ 12
	48 hours	100	96 $\pm$ 23	83 $\pm$ 21	30 $\pm$ 15 (*)	21 $\pm$ 11 (**)
AZA3	24 hours	100	118 $\pm$ 25	89 $\pm$ 10	92 $\pm$ 16	71 $\pm$ 31
	48 hours	100	95 $\pm$ 16	105 $\pm$ 24	42 $\pm$ 25	28 $\pm$ 8 (*)

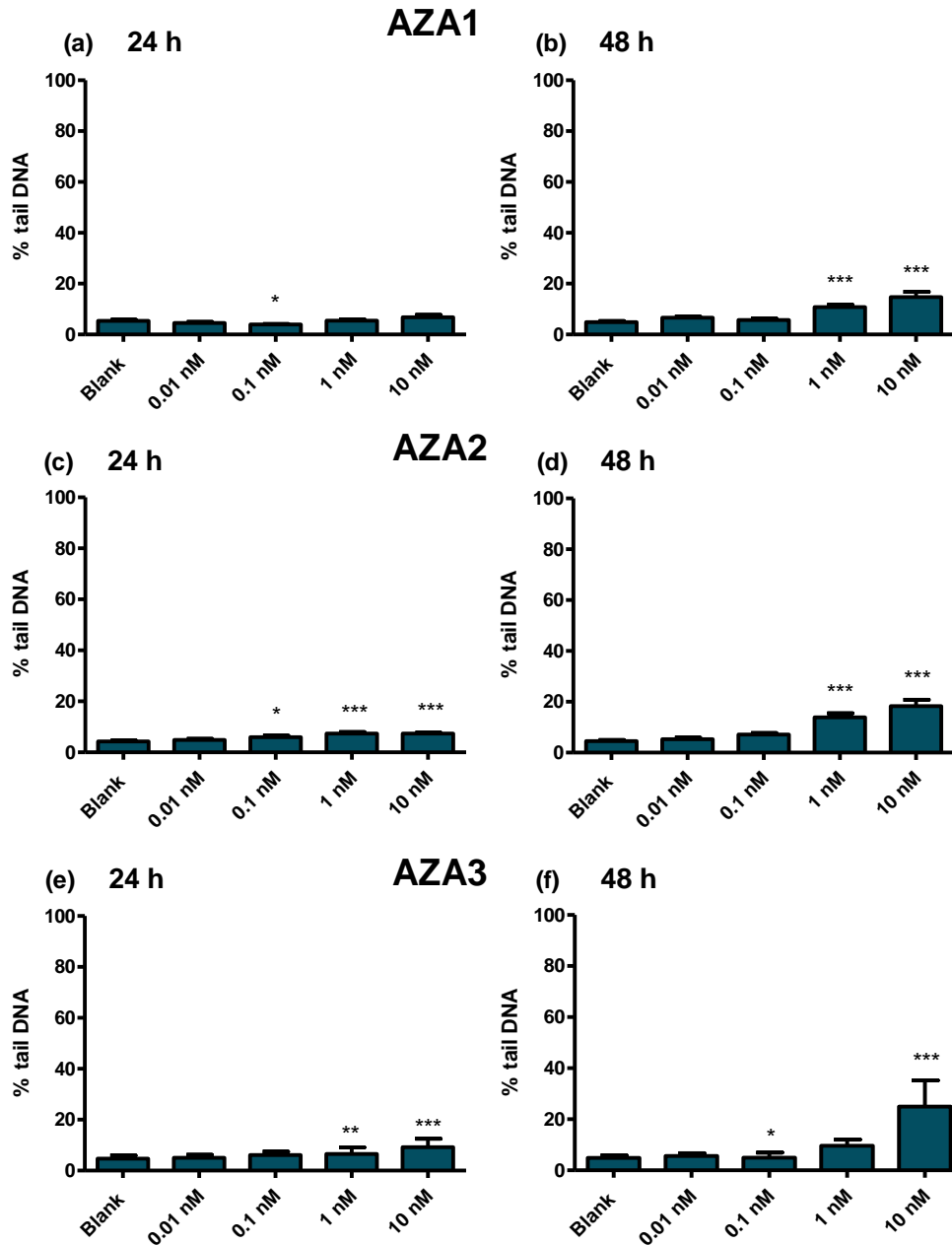
A significant reduction in cell viability could be detected by the Trypan Blue Exclusion assay at 1 nM and 10 nM for AZA1-3 at both time points (1 nM AZA1, AZA2 48 h  $p \leq 0.05$ , AZA2 24h, AZA3  $p \leq 0.01$ ; 10 nM AZA1 24 h  $p \leq 0.05$ , AZA2/AZA3 48 h, AZA3 24 h  $p \leq 0.01$ , AZA2 24 h / AZA3 48 h  $p \leq 0.001$ ) (**Table 4.6.**). The cell viabilities are lower at the two highest concentrations after 48 hours compared to 24 hours, indicating AZAs to have a dose and time dependent effect.

**Table 4.6.** Cell viability of HepG-2 cells after treatment with AZA1-3 for 24 hours and 48 hours. Results are the mean  $\pm$  SD of 4 independent experiments and were considered significantly different with a p-value of  $p \leq 0.05$  (\*), 0.01 (\*\*) and 0.001 (\*\*\*).

Biotoxin	Exposure Time	Blank	Blank Vehicle	0.01 nM	0.1 nM	1 nM	10 nM
AZA1	24 hours	98 $\pm$ 0.7	98 $\pm$ 0.6	98 $\pm$ 0.7	98 $\pm$ 0.6	89 $\pm$ 3.7 (*)	88 $\pm$ 5.1 (*)
	48 hours	98 $\pm$ 0.9	97 $\pm$ 1.8	98 $\pm$ 1.5	97 $\pm$ 1.7	52 $\pm$ 17.3 (*)	23 $\pm$ 6.4 (**)
AZA2	24 hours	97 $\pm$ 2.4	99 $\pm$ 0.3	97 $\pm$ 1.6	96 $\pm$ 1.4	86 $\pm$ 2.9 (**)	84 $\pm$ 2.2 (***)
	48 hours	97 $\pm$ 1.0	96 $\pm$ 2.4	97 $\pm$ 1.8	94 $\pm$ 3.2	74 $\pm$ 6.7 (*)	44 $\pm$ 14.0 (**)
AZA3	24 hours	96 $\pm$ 1.5	96 $\pm$ 1.5	96 $\pm$ 1.7	95 $\pm$ 2.1	82 $\pm$ 5.5 (**)	78 $\pm$ 1.8 (**)
	48 hours	95 $\pm$ 1.8	95 $\pm$ 1.4	93 $\pm$ 0.7	94 $\pm$ 1.1	74 $\pm$ 6.2 (**)	19 $\pm$ 11.9 (***)

Different effects of AZA1-3 on DNA fragmentation could be shown in the COMET assay. A small but significant change in the percentage of tail DNA could be detected for AZA1 at a concentration of 0.1 nM ( $p \leq 0.05$ ) after 24

hours (**Figure 4.5.a**). The value for this DNA fragmentation is lower than the blank, so this change might be of no biological relevance. After 48 hours of exposure a significant increase ( $p \leq 0.001$ ) in DNA fragmentation could be seen at the two highest concentrations (**Figure 4.5.b**). In contrast to AZA1, AZA2 caused a significant increase in DNA fragmentation at concentrations of 0.1 nM ( $p \leq 0.05$ ), 1 nM ( $p \leq 0.001$ ) and 10 nM ( $p \leq 0.001$ ) after 24 hours (**Figure 4.5.c**) and at 1 nM ( $p \leq 0.001$ ) and 10 nM ( $p \leq 0.001$ ) after 48 hours (**Figure 4.5.d**). The values after 48 hours are clearly higher than after 24 hours but are in a similar range as for AZA1. Exposure to AZA3 identified a significant increase in the percentage of tail DNA at the two highest concentrations (1 nM  $p \leq 0.01$ ; 10 nM  $p \leq 0.001$ ) after 24 hours (**Figure 4.5.e**) and for 0.1 nM ( $p \leq 0.05$ ) and 10 nM ( $p \leq 0.001$ ) after 48 hours (**Figure 4.5.f**). The value for 0.1 nM is lower than the blank and probably of no biological relevance. Just as AZA1 and AZA2 the actual increase at 10 nM of AZA3 is higher after 48 hours than after 24 hours. Although not significant ( $p \geq 0.05$ ) the DNA fragmentation at 1  $\mu$ M after 48 hours of exposure is higher than the blank and might be of biological relevance nevertheless.

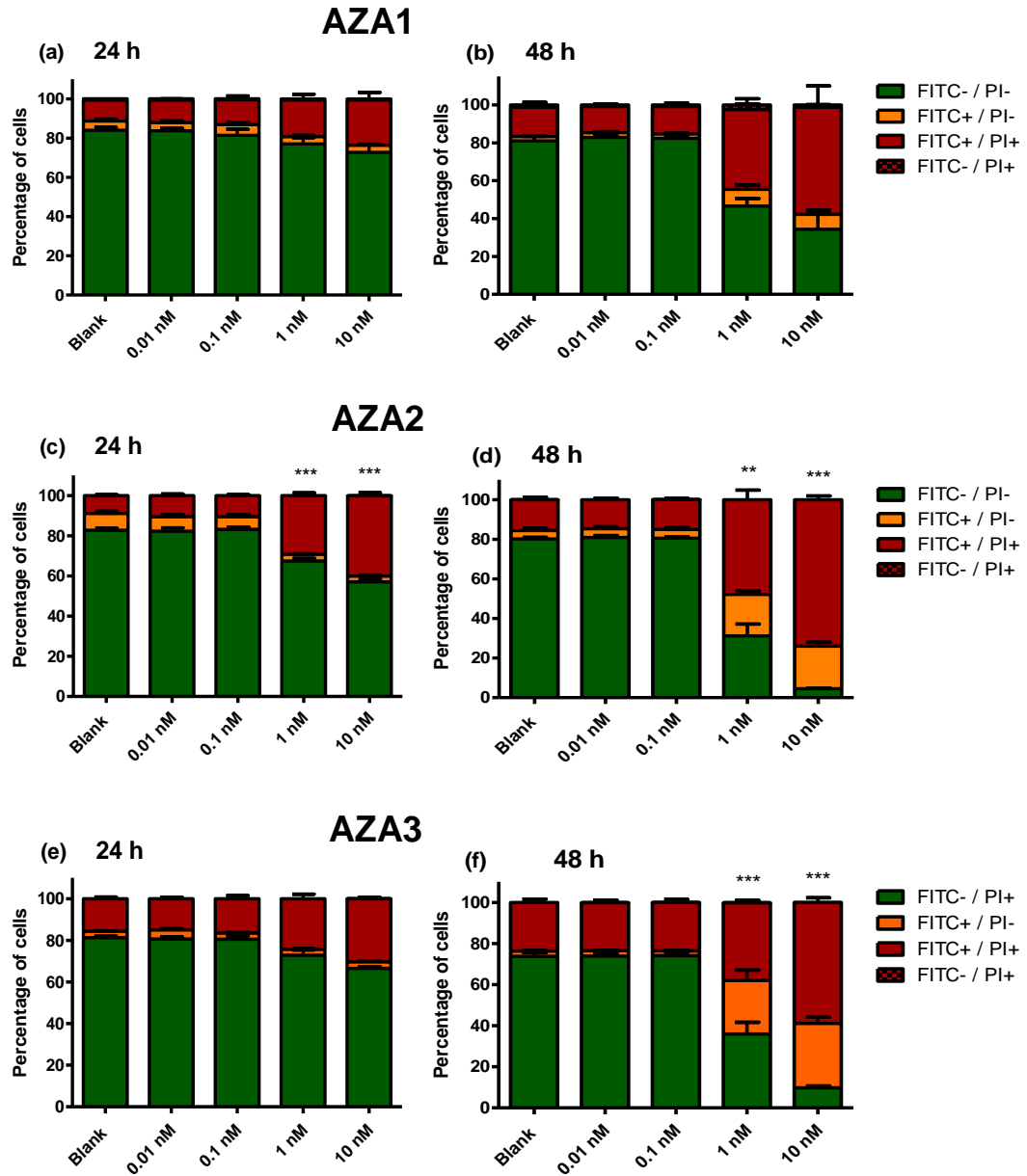


**Figure 4.5.** The effect of AZA1-3 on DNA fragmentation in HepG-2 cells after 24 hours (a, c, e) and 48 hours (b, d, f) of exposure. The different concentrations are compared to the blank and significance is marked as \* ( $p \leq 0.05$ ), \*\* ( $p \leq 0.01$ ) and \*\*\* ( $p \leq 0.001$ ). Results presented are the mean  $\pm$  SD of 4 independent experiments.

Correlation between data obtained by the Trypan Blue Exclusion assay and Flow cytometer analysis (sum of FITC- / PI- and FITC+ / PI- cells) gave correlation coefficients of  $r = 0.2$  (24 hours, ns) and  $r = 0.9$  (48 hours,  $p \leq$

0.05) for AZA1,  $r = 0.8$  (24 hours, ns) and  $r = 0.6$  (48 hours, ns) for AZA2 and  $r = 1$  (24 hours,  $p \leq 0.01$ ) and  $r = 0.9$  (48 hours,  $p \leq 0.05$ ) for AZA3. No significant increase ( $p \geq 0.05$ ) in FITC+ / PI- stained cells (early apoptosis) could be detected at any concentration or time point after exposure to AZA1. A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) however could be seen at the two highest concentrations (1 nM  $p \leq 0.01$ ; 10 nM  $p \leq 0.001$ ) at both time points (**Figure 4.6.a, 4.6.b**). The percentage after 48 hours is higher than after 24 hours. This increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) correlates with the decrease in cell viability as detected by the Trypan Blue Exclusion assay for both time points and coincides with the increase in DNA fragmentation after 48 hours. FITC- / PI+ stained cells could be identified for 1 nM after 48 hours. In contrast to AZA1, AZA2 showed a significant change in FITC+ / PI- stained cells (early apoptosis) at concentrations of 1 nM ( $p \leq 0.001$ ) and 10 nM ( $p \leq 0.001$ ) after 24 hours (**Figure 4.6.c**) and a significant increase (1 nM  $p \leq 0.01$ ; 10 nM  $p \leq 0.001$ ) after 48 hours (**Figure 4.6.d**). The percentage of FITC+ / PI+ stained cells (late apoptosis/necrosis) was significantly increased at the two highest concentrations at both time points (all  $p \leq 0.001$ , except 1 nM, 48 h  $p \leq 0.01$ ), the values are higher after 48 hours. Similar to AZA1 this increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) after exposure to AZA2 coincides with the decrease in cell viability and the increase in DNA fragmentation. AZA3 only shows a significant increase in FITC+ / PI- stained cells (early apoptosis) at 1 nM ( $p \leq 0.001$ ) and 10 nM ( $p \leq 0.001$ ) after 48 hours (**Figure 4.6.f**). A significant increase in FITC+ / PI+ stained cells (late apoptosis/necrosis) could be identified for the same concentrations at both time points (1 nM, 24 h  $p \leq 0.05$ , 48 h  $p \leq 0.01$ ; 10 nM  $p \leq 0.001$ ) (**Figure 4.6.e, 4.6.f**). This coincides with an increase in DNA fragmentation and a decrease in cell viability, similar to what has been shown for AZA1 and AZA2.





**Figure 4.6.** The effect of AZA1-3 on apoptosis/necrosis in HepG-2 cells after 24 hours (a, c, e) and 48 hours (b, d, f) of exposure. The different concentrations are compared to the blank and significance is marked as \* ( $p \leq 0.05$ ), \*\* ( $p \leq 0.01$ ) and \*\*\* ( $p \leq 0.001$ ). For flow cytometer analysis significances are only marked for early apoptosis. Results presented are the mean  $\pm$  SD of 4 independent experiments.

## ***Discussion***

Azaspiracids (AZAs) are a group of polyether marine biotoxins with a unique spiral ring assembly. Because of their lipophilic character they are able to accumulate in shellfish and pose a health risk to consumers as well as a risk to the environment and the shellfish industry [1, 2]. Limited data are available on their acute toxicity and possible long term effects; no data are available on genotoxicity. The main focus of the present study was to identify the possible genotoxic effect of AZA1-3 using the COMET assay. The Trypan Blue Exclusion assay and flow cytometer analysis were included in the present study to determine the possible involvement of overt cytotoxicity and apoptotic processes in the observed DNA fragmentation. All assays were performed on Jurkat T cells, CaCo-2 cells and HepG-2 cells.

Attempts were made to ensure that the mixing of cells and the sampling of aliquots for the Trypan Blue Exclusion assay was as constant as possible. However, slight variations are likely. While this would not affect the cell viability *per se* it might well account for higher standard deviations (SD) in cell numbers.

Technically, no cells should stain FITC- / PI+ only. However, a small percentage could be detected for AZA1 at a concentration of 10 nM (48 hours) in CaCo-2 cells and 1 nM (48 hours) in HepG-2 cells. Most likely this is due to handling and preparation of cells (centrifugation, re-suspension) prior to flow cytometer analysis and this observation is considered of no biological relevance [41]. The same can be expected for the comparison between viability data obtained by the Trypan Blue Exclusion assay and flow cytometer analysis. Correlation coefficients in most cases ranged from  $r = 0.7-0.9$ , for all three cell lines and time points. Although some of the correlations are not statistically significant, they are most likely of biological relevance. The absence of statistical power for some coefficients could be due to the sample number. The few exceptions with low coefficients, for example  $r = 0.2$  are probably a result of the above mentioned handling and preparation processes. Cell samples for the Trypan Blue Exclusion assay are taken straight after detachment, while viability data by flow cytometer

analysis undergo two further washing and re-suspension processes. In some cases the substantial loss in cell number might also contribute to the lower correlation coefficients.

The limited data available on AZAs and especially with focus on AZA1-3 suggests that these analogues have different potencies and may have multiple molecular targets [23, 24, 29, 42]. Twiner et al. [24] found AZA1 to have an effect on the pseudopodia number in Jurkat T cells, while AZA2 and AZA3 showed no effect. Other studies found different effects of AZA1-5 on pH and calcium flux in Jurkat T cells and freshly isolated human lymphocytes. The authors proposed a structure-activity relationship involved in the modulation and coupling of pH and  $\text{Ca}^{2+}$  [43-46]. In the present study, Jurkat T cells showed a time and dose dependent effect of all three AZAs. However, comparing the three different AZAs with each other, AZA1 seems to have the earliest and possibly most potent effect on cell viability. Not only do results show the lowest percentage of viable cells after 48 hours, but also that a lower concentration is needed after 48 hours to cause a significant reduction in cell viability. AZA2 has the least apparent effect of the three AZAs at the concentrations and time points tested. These observations are in slight contradiction with a study by Twiner et al. [24] in which AZA2 and AZA3 showed a stronger effect on cell viability than AZA1. However, a previous study by the same researchers, only on AZA1, detected lower viabilities at the same concentrations which are more in line with the findings here. A possible explanation for the discrepancy between the present study and the above mentioned study by Twiner et al. [24] could be the different methods of detection. Overall, data acquired in the present study are in agreement with published literature. DNA fragmentation in Jurkat T cells, as determined by the COMET assay was mainly detectable after 48 hours. AZA3 and AZA2 showed significant increases in percentage of tail DNA at the two highest concentrations, with AZA3 having the higher values. In contrast to the above mentioned cytotoxicity, AZA3 showed the strongest effect on DNA fragmentation, followed by AZA2. AZA1 showed no significant effect. These data would indicate AZA2 and AZA3 to have a genotoxic effect

on Jurkat T cells. However, one criterion for direct genotoxicity is a consistent cell number [47]. In the current study, all three AZAs induced statistically significant and/or substantial cell losses. This suggests apoptotic processes rather than direct genotoxicity to have taken place. This is supported by flow cytometer results for AZA1-3. After 24 hours of exposure all three analogues showed early apoptotic cells at the two highest concentrations, as well as for 0.1 nM for AZA2 and not significant but still noticeable for AZA1. The significant increase of FITC+ / PI+ stained cells (late apoptosis/necrosis) in combination with significant amounts of early apoptotic cells after 48 hours of exposure suggest a shift from early apoptosis to late apoptosis within the time points tested for all three analogues. In contrast to AZA1 and AZA2, AZA3 only showed negligible amounts of viable cells after 48 hours of exposure for the two highest concentrations. As for the data presented for DNA fragmentation, AZA3 seems to have the most prominent effect on Jurkat T cells. The data presented in the present study suggest the above described DNA fragmentation, as detected by the COMET assay, to be the result of cytotoxicity and/or apoptotic/necrotic processes rather than direct genotoxicity. The fact that DNA fragmentation has been described in the literature as a late apoptotic event [48, 49] and a recent study by Twiner et al. [42] detecting DNA laddering, a late apoptotic hallmark, after treatment of Jurkat T cells with AZA1-3 for 48 hours, support this conclusion. In contrast, a report by Hess et al. [31] mentioned the inability to detect caspase-3, another marker for apoptotic processes, in Jurkat T cells after exposure to AZA1. However, no further information was given in the report on exposure time and concentration, making a direct comparison difficult.

CaCo-2 cells showed a significant time and dose dependent reduction in cell viability for all three analogues. Viabilities identified for CaCo-2 cells by Twiner et al. [42] and Sérandour et al. [27] for AZA1 are well in agreement with the ones found in the study conducted here. No data are available in the literature for the other two analogues. Overall, AZA2 seems to be slightly more potent. The data obtained by the COMET assay on the other hand

showed a dose and time dependent effect of AZA1, while AZA2 and AZA3 seem to have the least or possibly a slower effect on DNA fragmentation. AZA2 only showed a significant increase in percentage tail DNA after 48 hours at 10 nM and AZA3 at 48 hours at 1 nM and 10 nM. However, the values are clearly lower than for AZA1. No reduction in cell number below 80% could be seen for all AZAs at any concentration or time point. This would firstly indicate a genotoxic effect of AZAs on CaCo-2 cells. Flow cytometer analysis identified early apoptotic cells only for AZA1 at 1 nM and for AZA3 at 1 nM and 10 nM after 24 hours; no early apoptosis was detected after 48 hours for any of the three analogues. However, AZA1-3 at the two highest concentrations showed a distinct increase in late apoptotic/necrotic cells after 24 hours and 48 hours of exposure, although not statistically significant. The values are in all cases higher after 48 hours, indicating a time dependent effect. In contrast to cell viability and DNA fragmentation, no clear difference between the three analogues could be observed for flow cytometer data. The previous mentioned study by Twiner et al. [42] also detected DNA laddering as a marker of late apoptotic events in CaCo-2 cells after exposure to AZA1. In the present study, the DNA fragmentation is in agreement with the detection of late apoptotic/necrotic cells but lower than flow cytometer analysis would suggest. The lack of significant amounts of early apoptotic cells and the good correlation of DNA fragmentation with the moderate reduction in cell viability raises the question if the effect of AZA1-3 on CaCo-2 is based on cytotoxic or necrotic rather than apoptotic processes or genotoxicity.

In contrast to previous chapters, initial experiments showed that the reduction in cell number for HepG-2 cells after 24 hours was less prominent than observed with OA and the positive controls (Chapter 2 and 3). Therefore, there was no need to shorten the exposure time to 12 hours for AZAs. This indicates a slower time course of AZA toxicity compared to the positive controls and OA in HepG-2 cells. HepG-2 cells showed a dose and time dependent reduction in cell viability for all three AZAs tested, AZA1 and AZA3 showing a more potent effect than AZA2. Cytotoxicity data in the

literature are only available for AZA1 and results presented in the study by Sérandour et al. [27] are well in agreement with data found here. A time dependent effect can also be seen for the percentage of tail DNA, detected by the COMET assay. In contrast to AZA1, AZA2 and AZA3 already showed significant amounts of DNA fragmentation after 24 hours of exposure. However, the percentages of tail DNA are similar for all three analogues. The DNA fragmentation for AZA1 and AZA2 after 48 hours is similar, higher values were detectable for AZA3. The significant differences for AZA1 at 0.1 nM after 24 hours and AZA3 after 48 hours are lower than the blank and are almost certainly not of biological relevance. The moderate DNA damage given by the COMET assay alone would suggest a genotoxic effect of AZA1-3 on HepG-2 cells. Based on the same consideration as described before, the substantial reduction in cell number detected in the present study indicates apoptotic processes to have taken place. This is supported by flow cytometer analysis. A significant increase in early apoptotic cells could be detected for AZA2 and AZA3 after 48 hours at the two highest concentrations. Late apoptotic/necrotic cells could be shown for AZA1-3 at the two highest concentrations for both time points. In all cases the values after 48 hours of exposure are higher, indicating a time dependent effect. In contrast to data on cell viability, AZA2 caused stronger/earlier effects in the COMET assay and flow cytometer than AZA1, and overall AZA3 seems to be the most potent AZA. All data taken together suggest a cytotoxic or apoptotic/necrotic effect of AZAs on HepG-2 cells rather than direct genotoxicity.

The, at times seemingly, contradicting results of AZAs among the literature available, could be based on differences in the assays, exposure times and concentrations used. Twiner et al. [24] proposed multiple molecular targets for AZAs. Roman et al. [30] found differences in the effect of AZA2 and AZA3 on intracellular  $[Ca^{2+}]$  and pH. The authors suggested a structure-activity relationship as possible explanation. AZA4, which has not been investigated in the present study, showed an opposite effect on cytosolic calcium levels than AZA1-3 [33]. Satake et al. [1] and Ofuji et al. [40] detected different

potencies of AZA1-3 *in vivo*, which have been confirmed in other studies *in vitro* in lymphocytes [24] and neocortical neurons [39]. The order of potency varied among the cell lines, yet AZA1 was in all cases the least potent one of the three analogues. Similar to the study by Cao et al. [39] on neurons, AZA3 has an earlier and possibly more potent effect on Jurkat T cells in the present study. This is in contradiction to the study by Twiner et al. [24] which found AZA2 to be the most potent analogue in Jurkat T cells. Possible explanations are the different approaches. In the current study, all three assays were taken into account while the potency by Twiner et al. [24] is established by cytotoxicity data alone. Also, the methods to determine cell viability in both studies are different. A clear difference in potency of AZA1 and AZA2 in Jurkat T cells and AZA2 and AZA3 in HepG-2 cells as well as all three analogues in CaCo-2 cells cannot be suggested in the present study with the data available. A study by Vilarino et al. [50] found AZA1 and AZA2 to have similar effects on morphological changes in Be(2)-M13 cells. TEFs for AZAs are given in the EFSA report [51], the potency order relative to AZA1 is as follows, AZA2 (TEF = 1.8) > AZA3 (TEF = 1.4) > AZA1 (TEF = 1). These TEFs are based on *in vivo* studies on acute toxicity in mice after *i.p.* administration; these values were adopted by the EFSA panel as an interim measure to provide an estimate of AZAs toxicity and are not considered to be very robust due to the limited data available. Data in the present study do not indicate such clear differences between the three analogues. Direct comparison is difficult because of the differences in models (*in vivo* vs *in vitro*) and endpoints used. The cell lines used in the current study are representing main target organs of AZA toxicity. Limited to no data are available on direct comparison of different cell lines; data available are mostly based on one analogue. Twiner et al. [42] found Jurkat T cells to be the most sensitive to AZA1, followed by CaCo-2 cells and neuroblastoma cells (BE(2)-M17. Sérandour et al. [27] identified HepG-2 cells and Neuro2a cells to be significantly more sensitive to AZA1 than CaCo-2 cells in an inter- and intra-laboratory study. Another study by Ronzitti et al. [28] showed MCF-7 cells to be affected by AZA1 exposure, while no effect on CaCo-2 viability was detectable. All these studies tie in with the results found here. Jurkat T cells

appear the most sensitive to AZAs exposure, followed by HepG-2 cells and the least sensitive being CaCo-2 cells. This descending sensitivity seems to be the same among all three analogues tested. However, within one cell line there seem to be different potencies among AZA1-3.

Although the gastrointestinal tract, lymphatic system and liver are main targets of AZAs [15] it is still unknown how the observed *in vivo* and *in vitro* effects are linked. The disruption of the intestinal barrier is suggested to be a result of morphological changes and alterations in the cytoskeleton, mainly caused by changes in the F-actin levels and E-cadherin system [23, 25, 28, 29, 31]. Experiments with CaCo-2 cells showed severe cell detachment after AZAs exposure (50 nM) [20] and a decrease of TEER<sup>6</sup> in CaCo-2 monolayers [31]. In contrast, no substantial reduction in cell number could be seen in the present study. The most likely explanation for this discrepancy is the lower concentration (max. 10 nM) used, over the same time course. Little is known on the effect of AZAs on the liver, Ito et al. [15, 16, 52] reported increased organ weight, accumulation of fat droplets and sporadic occurrence of necrosis. T and B lymphocytes have been reported to undergo necrosis in the thymus, spleen and Peyers patch [15]. AZAs have also been hypothesised to be tumor initiators/promoters due to the *in vivo* detection of lung tumours in the MBA [16, 52]. Experiments conducted in the current study attempted to increase the general knowledge of AZA toxicity, focusing on genotoxic effects and the potential to cause possible long term effects. Data presented suggest an apoptotic/necrotic effect rather than genotoxicity *per se*, in all three cell lines and analogues tested. Literature on apoptosis induction after AZA exposure is limited and partly contradictory. Román et al. [29] found no induction of apoptosis in BE(2)-M17 after AZA1 exposure, neither did Hess et al. [31] find an increase in caspase-3 in Jurkat T cells. HeLa cells seemed to neither show cytotoxic nor apoptotic effects after exposure to AZA2 [53]. On the other hand, studies by various other groups showed AZAs to have an apoptotic effect. Vilarino et al. [26] detected the

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<sup>6</sup> The transepithelial electrical resistance (TEER) is an indicator of barrier integrity. It is based on ion flux across the paracellular pathway resulting in an electrical resistance [31].



induction of caspase-3 in neuroblastoma cells after AZA1 exposure, but concluded that this activation is not responsible for the disarrangement of the cytoskeleton. Twiner et al. [42] identified many steps, including activation of various caspases involved in apoptotic cell death in Jurkat T cells after exposure to AZA1. Cao et al. [39] confirmed the studies by Vilarino et al. [26] described above, finding AZA1 to produce neuronal apoptosis. However, the authors also described induced neurotoxicity to be apoptotic and necrotic simultaneously. Kellmann et al. [54] exposed human neuroblastoma cells (SH-SY5Y) to AZA1 and investigated protein expressions, identifying increased levels of Annexin AII as well as BAX, an apoptosis regulator. Available literature and results presented here point to apoptotic/necrotic processes being involved to some extent in, or as a result of, AZA toxicity.

## **Conclusion**

Based on data obtained in the present study, AZAs are not genotoxic *per se*. Flow cytometer analysis showed a clear shift from early to late apoptosis in Jurkat T cells and HepG-2 cells; CaCo-2 cells did not show a clear apoptotic profile. In all cases however FITC+ / PI+ stained cells (late apoptosis/necrosis) agreed well with the percentage tail DNA detected in the COMET assay, suggesting this DNA fragmentation to be a result of apoptotic/necrotic processes rather than genotoxicity. Jurkat T cells were the most sensitive to AZAs exposure, followed by HepG-2 cells. CaCo-2 cells were the least sensitive in the study conducted here. While the overall effect on the cell lines seems to be similar for all three analogues, they differ in their potencies within one cell line. AZA3 shows the earliest and possibly strongest effect in Jurkat T cells, the data obtained do not allow for a clear differentiation between AZA1 and AZA2. AZA1 shows the lowest potency in HepG-2 cells, no clear difference in potency can be suggested for AZA2 and AZA3, or all three analogues in CaCo-2 cells. The different potencies of AZA1-3 and sensitivities of cell lines are in agreement with the literature available. Only limited data is available on the involvement of apoptosis in AZA toxicity, the present study contributing to the overall knowledge.

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## Chapter 5

### General Discussion

Harmful algae blooms (HABs) are an increasing global problem [1-4]. The exact reasons for their increasing presence remain unknown but natural changes in the environment as well as human impact have been suggested [5-9]. Of the species involved in HABs only a small percentage are known to be potential toxin producers [1, 10]. They can release toxins either directly into the water or, serving as a food source for filter feeding shellfish, the larvae of some crustaceans and finfish, enter the food web where they can accumulate and/or bio-magnify throughout [1, 11, 12]. Filter feeding shellfish especially are highly tolerant of phycotoxins and can accumulate them up to levels where they can pose a risk to human consumers [1, 2, 13, 14]. It was estimated in the year 2000 that approximately 60,000 individuals suffered intoxication from phycotoxins worldwide [8]. The current legal level of phycotoxins permissible in shellfish meant for the market is controlled by EU Regulation (EC) No 853/2004 [15]. However, these regulations are mostly based on relatively limited acute toxicity data. Concern has been raised that this regulation might not be sufficient to protect all consumers, especially high shellfish consumers<sup>7</sup> [15]. The EFSA Panel on Contaminants in the Food Chain concluded that insufficient evidence is available to establish a tolerable daily intake (TDI) for any of the phycotoxins. For this reason they proposed acute reference doses (ARfDs) [16, 17]. However, these ARfDs are also mostly based on acute toxicity studies on animals by *i.p.* injection which may not fully reflect the human route of oral intoxication [18]. Various *in vivo* and *in vitro* studies have been performed over the years aiming to increase knowledge about phycotoxins and improve the risk assessment and human protection, especially in relation to sub-acute or repeated exposures at sub-clinical doses. One aspect of safety assessments of biotoxins is the investigation of genotoxicity [19]. To date, no information on genotoxicity has

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<sup>7</sup> High consumers = shellfish consumers that eat portion sizes well above the EFSA calculated mean. Data are based on consumption surveys given by various European countries [16, 17].

been published for AZAs [16]. Several studies have investigated the genotoxic potential of OA. While OA has not tested positive in standard genotoxicity tests, such as the Ames test [20], it has tested positive in other assays. In brief, Aonume et al. [20] found OA to test positive in Chinese hamster lung cells (CHL) using diphtheria toxin resistance as a selective marker. A study by Tohda et al. [21] found sister-chromatid exchange, mitotic cells and chromosome/nuclei fragmentation in human lymphoblastoid cells and Chinese hamster ovary cells. The authors concluded OA to be directly genotoxic [21]. This was supported by Fessard et al. [22] identifying chromosome condensation and DNA adduct formation in the absence of cytotoxicity. In contrast, other studies such as Le Hegerat et al. [23] concluded OA to be rather aneugenic. These studies detected premature sister chromatid separation, centromere-positive micronuclei and the loss of whole chromosomes [23-25]. Except for the study by Fessard et al. [22] no information on possible cytotoxicity or other DNA damaging processes have been included in these publications. DNA fragmentation is also one of the effects of cell death by either necrosis or apoptosis. Therefore, assays that investigate primary DNA damage will also detect DNA fragmentation which is due to cytotoxicity or apoptotic processes rather than genotoxicity [26-28]. In the absence of further data, this can lead to misclassification of the genotoxic potential of the test compound. The integrated evaluation of cell death mechanisms as part of genotoxic testing allows the determination of false positive results and for a more precise data interpretation [26, 29]. It has been suggested by the EFSA panel that some of the observations in *in vivo* and *in vitro* genotoxicity studies might reflect cytotoxicity rather than genotoxicity [17].

The present study investigated the genotoxic potential of OA and AZA1-3. In contrast to the above mentioned studies, a more integrated approach was used in this investigation. In addition to determining DNA damage caused by the compounds, the effects on cytotoxicity, cell number and a marker for early apoptosis were included in the study design. Positive controls (Chapter 2) were used to establish in house data for subsequent biotoxin studies and



to illustrate and support the integrated approach. The three positive controls used and their effects are described in greater detail in Chapter 2. In brief, EMS showed a direct genotoxic effect, in the absence of cytotoxicity and apoptosis. In contrast, CdCl<sub>2</sub> a widely used positive control for genotoxic studies, tested positive not only for DNA damage but also for early and late apoptosis. Additionally, Staurosporine, a non-genotoxic apoptosis inducer also showed modest DNA fragmentation. Based on these preliminary studies, data interpretation for all phycotoxins was based on information obtained from all endpoints. OA and AZAs both showed an increase in DNA fragmentation at most time points, concentrations and cell lines investigated in the present study. These data by themselves would suggest a modest genotoxic potential of all four phycotoxins. The modest or strong cytotoxicity observed in the Trypan Blue Exclusion assay for the higher concentrations in Chapter 2, 3 and 4 confirms that the appropriate concentration range was used in these investigations [26, 30, 31]. In cases of a substantial reduction in cell viability, for example with CdCl<sub>2</sub> but also AZAs in Jurkat T cells and HepG-2 cells, the DNA damage detected in the COMET assay coincides with the reduction in cell viability (Chapter 2 and 4). In other cases, such as exposure of all cell lines to the direct genotoxic agent EMS, or Jurkat T cells to OA, the DNA fragmentation occurs in the absence of overt cytotoxicity (Chapter 2 and 3). In general, these data suggest that at least part of the DNA fragmentation detected might be due to other processes than direct genotoxicity. Another criterion for the considered interpretation of findings in genotoxicity assays, which is not frequently included in study designs, is the effect of the test compounds on cell number. If direct genotoxic damage occurs, the cell number should stay substantially constant within the non-cytotoxic concentration range [32]. In this study, all compounds, except Staurosporine, induced a significant reduction in cell number in at least two of the cell lines and one time point (Chapter 2, 3 and 4). It stands to reason that cytotoxic and/or apoptotic processes have taken place rather than direct DNA damage. Cells that have possibly undergone apoptotic or necrotic processes might have been severely damaged and lost within the incubation period and would therefore not be detected by the Trypan Blue Exclusion

assay after the full incubation period. This is most likely an explanation for the reduction in cell number found within the current study, especially after the longer incubation time (Chapter 2, 3 and 4). The possibility that apoptotic processes are responsible for the reduction in cell number, but also the DNA damage detected in the present study, was supported by the findings from the flow cytometrical analysis (Chapter 2, 3 and 4), with the exception of EMS. CdCl<sub>2</sub>, OA and AZAs caused early and late apoptosis in the vast majority of cell lines. Overall, no substantial amount of early or late apoptosis could be seen for EMS. The reduction in cell number for EMS is far more modest than for any other compound and all other endpoints (cell viability, DNA fragmentation, flow cytometer analysis) confirming EMS as a direct genotoxic compound. For CdCl<sub>2</sub> and all phycotoxins investigated, the percentage of late apoptotic cells generally agreed well with the detected cytotoxicity, reduction in cell number and increase in DNA fragmentation. As DNA fragmentation is a late apoptotic event [28, 33] all data in the present study indicate a major involvement of apoptotic processes in the DNA fragmentation observed, rather than direct genotoxicity. The COMET assay workgroup within the 4<sup>th</sup> International Workshop on Genotoxicity Testing [26] came to the consensus that cytotoxicity data should be included in the interpretation of results from COMET analysis. The findings in the present study strongly support this recommendation. Data obtained also stress the need to include other endpoints, such as cell number and markers for apoptosis in genotoxic studies. If the COMET data from the current study were assessed on their own, one might have concluded that OA and AZA1-3 are moderately genotoxic (Chapter 3 and 4). However, taking the additional data into account, including observations made with the positive controls (Chapter 2), allowed for a more considered evaluation. The study design used in this investigation provides a certainty that the right concentration range was applied. Furthermore, cell viability data and cell numbers point towards other processes involved in the detected DNA fragmentation which is also supported by flow cytometer analysis. All aspects together allow for the conclusion that cytotoxicity and apoptosis contribute substantially to the DNA damage detected in the COMET assay.

The time course of biochemical events following compound exposure depends on a variety of factors, such as the compound itself, the cell line, exposure time/concentration and the endpoint investigated [34]. For all endpoints included in the present study, the exposure time, with the exception of OA in HepG-2 cells (Chapter 3), and exposure concentration have been kept constant, leaving the compounds and cell lines as possible factors for differences detected. In the current study, Jurkat T cells, CaCo-2 cells and HepG-2 cells showed different sensitivities to the phycotoxins investigated. For OA (Chapter 3) the order of sensitivity of the cell lines is, in increasing order, CaCo-2 cells < Jurkat T cells < HepG-2 cells whereas for AZAs (Chapter 4) the order of sensitivity is, in increasing order, CaCo-2 cells < HepG-2 cells < Jurkat T cells. The cell line sensitivity seems to be the same for all three AZAs tested. This is in agreement with the limited literature available and has been discussed in greater detail in Chapter 4. The EFSA report [16] gives TEFs for AZAs with potencies relative to AZA1 as follows, AZA2 (1.8) > AZA3 (1.4) > AZA1 (1). Data in the present study do not indicate such clear differences between the three analogues tested. AZA3 for example, seems to be the most potent in Jurkat T cells, while no clear difference can be seen between AZA1 and AZA2. In HepG-2 cells AZA2 and AZA3 are more potent than AZA1; however, no clear distinction between AZA2 and AZA3 can be made. As described in Chapter 4 in more detail, comparison with the literature is difficult. Data available are often limited to a single endpoint [35] and matching endpoints are often detected by different methods (*in vivo* vs *in vitro*) [16].

In contrast to AZAs, the reduction in cell number after initial experiments for HepG-2 cells exposed to OA was substantial. As previously mentioned in Chapter 3, the exposure time therefore had to be reduced to 12 hours and 24 hours. To allow direct comparison of OA and AZAs here, final concentrations of OA have been converted from ng/mL to nM, giving values of 4 nM (3 ng/mL), 12 nM (10 ng/mL), 41 nM (33 ng/mL) and 124 nM (100 ng/mL). The final concentrations of AZA1-3 used in the present study were 0.001, 0.01, 1 and 10 nM. When comparing, for example OA at 12.4 nM and AZAs at 10

nM in HepG-2 cells at 24 hours, the cell number for OA is already substantially lower than for AZAs (**Table 3.5. and 4.5.**). This indicates a higher potency of OA compared to AZAs in HepG-2 cells. Limited data are available on the comparison between OA and AZAs in the literature. Sérandour et al. [36] used the MTT assay to determine the cytotoxic effect of OA and AZA1 on various cell lines, including CaCo-2 cells and HepG-2 cells. Exposure concentrations ranged from 0.001 to 1000 nM at an exposure time of 48 hours. The authors concluded that OA has a significant effect on CaCo-2 cells, but no substantial effects were observed with AZA1. The cell viabilities for OA at 48 hours are slightly lower in the current study compared to the percentages given by Sérandour et al. [36]. However, there is an overall agreement, amongst the findings, of a significant effect of OA on CaCo-2 cells. In contrast, a significant effect of AZA1, after 48 hours of exposure, could be detected on the cell viability of CaCo-2 cells in the present study. Sérandour et al. [36] found HepG-2 cells to be more sensitive to AZA1 than OA [36]. Direct comparison of cytotoxicity data is only possible with data of AZA1. Data obtained in the present study are in agreement with data obtained by Sérandour et al. [36]. Direct comparison of OA data is not possible due to the different time points. A study by Roman et al. [37] investigated the changes in the F-actin pool after exposure of neuroblastoma cells to AZA1 at concentrations of 1 to 10,000 nM for 24 hours ( $IC_{50}$  after 24 hours = 7.5  $\mu$ M) and OA (at  $IC_{50}$  values, data not published). The authors concluded a lower toxicity for AZA1. Although based on different endpoints, data from the present study are in line with the findings by Roman et al. [37]. The data by Roman et al. [37] are furthermore supported by other studies, showing AZA1 to require higher concentrations than OA to cause morphological/cytoskeletal alterations in various cell lines [38, 39]. In the current study, effects on all endpoints can already be shown after 24 hours for OA in all cell lines. Most effects for AZAs are only detectable after 48 hours of exposure. The data obtained in the present study therefore suggest OA to be more potent and faster acting than AZAs.

As can be seen here as well as in previous chapters, the information available on genotoxicity is limited and in parts contradicting. Information on reproductive and developmental effects is scarce and no chronic exposure/carcinogenicity studies on AZAs have been performed using standard tests. The current regulations in place appear to minimize the risk of acute intoxications of humans by contaminated shellfish [18]. However, based on the toxicity data currently available, the EFSA panel concluded that the regulatory limits might not be adequate to fully protect human shellfish consumers from potential long term effects. In view of this, considerations should be given to repeated-dose feeding studies to establish effects of prolonged exposure and robust TDIs [15, 18]. To protect high consumers from acute effects, the EFSA panel proposed 400 g of shellfish meat to be used in risk assessment as a realistic estimate of a large portion [15]. Consumption data are limited and the information available is based on data submitted by various European countries on request from EFSA. These data are based on national food consumption surveys and do not necessarily differentiate between portion size for fish and other seafood and cooked/uncooked shellfish. The EFSA panel in turn used these data to set more conservative, but not unrealistic estimates, of dietary exposure to phycotoxins in the EU. They recommended expanding the database on portion size and frequency of consumption to help with safety assessment [15]. Various aspects of hazard identification are currently missing, such as the previously mentioned genotoxicity, carcinogenicity and long term toxicity data. However, future work should also be considered on the absorption/distribution and metabolism in animals and humans. A study by Aune et al. [40] was unable to identify any synergistic or additive effect when administering OA and AZA1 together to mice. The oral LD<sub>10</sub> and LD<sub>50</sub> were established for both OA and AZA1 by the authors. The doses were then used for the exposure to the toxins at following combinations, OA at LD<sub>10</sub> and AZA1 at LD<sub>10</sub>, OA at LD<sub>50</sub> and AZA1 at LD<sub>10</sub>. Mice were sacrificed after 24-30 hours [40]. Although the time course of effects for OA and AZA appears to be similar in *in vivo studies*, the data given in the present study (Chapters 3 and 4) suggested a later onset of effects for AZA1 compared to OA. While effects

could be seen for OA at 24 hours, most effects of AZAs only became detectable at 48 hours. Therefore, the exposure time investigated by Aune et al. [40] might possibly have been too short to fully identify synergistic/additive effects. In general, attention should focus on the fact that shellfish often contain more than one class of phycotoxins. Further information on combined effects/interactions is needed to fully assess potential risks [15, 18].

In the present study, the genotoxic effect of OA and AZAs were investigated *in vitro* and results might assist in the evaluation of these compounds in view of future risk assessment. Based on data obtained in the current study and in the literature cited here and in previous chapters, OA does not appear to be overtly genotoxic. However, it has been identified as a tumour promoter, as described in more detail in Chapter 1 and Chapter 3 [17, 41-44]. Data on AZAs obtained in the present study also do not indicate overt genotoxicity. Differently to genotoxic compounds, tumour promoters require repeated exposure above a certain threshold to cause an effect. Physical wounding, irritating chemicals and cytotoxic drugs, for example, have been identified as tumour promoters resulting in cell proliferation, altered gene expression as well as inflammation and changes in cell adhesion and cell-cell communications [30]. The available literature has identified occasional tumours after AZAs exposure *in vivo* [45] as well as cytotoxicity and loss of cell-surface and cell-cell interactions [39, 46, 47]. Some of these effects could also be seen in the present study. While no definite answer can be given at this point, data suggest the possibility that AZAs might have the potential to act as tumour promoters.

## Conclusions

The main goal of hazard identification is the realistic assessment of the potential risks a compound might have. In the case of OA and AZAs most information is based on acute toxicity. The limited data available on chronic exposure, carcinogenicity or genotoxicity are often contradicting. This present study used the COMET assay to investigate the genotoxic potential of OA and AZAs. In contrast to most other studies, various endpoints such as cytotoxicity, cell number and possible involvement of apoptosis were included in the data interpretation. The data obtained indicate that OA and AZAs are not genotoxic *per se*. Apoptotic processes make a major contribution to the observed DNA fragmentation. Genotoxic testing is a key component of risk assessment to determine whether a compound can a) cause heritable damage, b) predict genotoxic carcinogenicity if data on carcinogenicity are not available and c) contribute to the knowledge of the mechanisms of action [18, 48]. The data obtained in this study indicates that the risk of genotoxic damage after consumption of shellfish meat containing OA or AZA1-3 is marginal. This suggests no immediate need for more severe regulatory limits, especially for repeated/regular consumption of phycotoxin contaminated food at concentrations below the regulatory limits and/or concentration without acute clinical effects. The information obtained in this study contributes to the overall knowledge of OA and AZAs toxicity. However, further work is necessary to fully assess the potential risk these compounds might have. The lack of a DNA damaging effect but clear contribution of apoptosis to AZAs toxicity might assist in future research on the mechanism of action, which is still unknown. As previously mentioned, shellfish samples often contain more than one toxin group. Hence, information on possible interactions and/or synergistic effects is required. Further research should also include *in vivo* exposure to clinical and sub-clinical levels of phycotoxins, as possible metabolic and/or elimination processes might not be reflected *in vitro*. Much progress has been made but further information is still required to fully assess the potential risk of phycotoxins to human shellfish consumers.

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