Use of rainbow trout cell lines to delineate the roles of p53 in fish and to evaluate the toxicity of emerging environmental contaminants, benzotriazoles and benzothiazoles

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

Epithelial cell lines, RTgill-W1 and RTL-W1 from respectively the gill and liver of rainbow trout, *Onchorhynchus mykiss* (Walbum), were used to investigate the role of p53 in the cellular survival pathways of fish and to evaluate the potential impact on fish of the emerging contaminants, benzotriazoles (BTRs) and benzothiazoles (BTHs). For studying p53, RTgill-W1 was used with two p53 inhibitors, which are termed pifithrins-μ (PFT-μ) or 2-phenylethynesulfonamide (PES) and pifithrin-α (PFT-α). Both agents were developed for cancer chemotherapy but also have been used widely to explore p53 functions in mammals but not in fish. PFT-μ or 2-phenylethynesulfonamide (PES) was identified as an inhibitor of p53 translocation to the mitochondria but subsequently shown to be a HSP70 inhibitor as well. PFT-α was recognized as an inhibitor of p53-mediated transcription. Cellular toxicity was evaluated for seven BTRs: 1H-Benzotriazole (BTR), 4-methyl-1H-benzotriazole (4MBTR), 5-methyl-1H-benzotriazole (5MBTR), tolytriazole (TT), 5,6-Dimethyl-1H-benzotriazole monohydrate (DM), 5-Chlorobenzotriazole (5CBTR) and Hydroxybenzotriazole (OHBTR). The BTHs were Benzothiazole (BTH), 3,3'-diethylthia dicarbocyanine iodide (DTDC), C.I. Sulphur orange 1 (SO), 2-Mercaptobenzothiazole (2MBTH), Zinc 2-Mercaptobenzothiazole (ZincMBTH), Sodium 2-Mercaptobenzothiazole (NaMBTH), 2-Hydroxy-benzothiazole (OHBTH), 2-Aminobenzothiazole (2ABTH), C.I. Vat yellow 2 (VY), N,N-Dicyclohexyl-2-benzothiazolsulfene amide (NNA), 2,2'-Dithiobis (benzothiazole) (DBTH) and 2-(p-aminophenyl)-6-methylbenzothiazole-7-sulfonic acid (MBTHS).

PES had complex actions on RTgill-W1. As judged by three viability assays, cells were killed by 24 h exposures to PES, but cell death was blocked by the anti-oxidant N-acetylcysteine (NAC). Cell death had several hallmarks of apoptosis: DNA laddering, nuclear fragmentation, Annexin V staining, mitochondrial membrane potential decline, and caspase activation. Reactive oxygen species (ROS) production peaked in several hours after the addition of PES and before cell death. HSP70 and BiP levels were higher in cultures treated with PES for 24 h, but this was blocked by NAC. As well, PES treatment caused HSP70, BiP and p53 to aggregate and become detergent-insoluble, and this too was prevented by NAC. Of several possible scenarios to explain the results, the following one is the simplest: PES enhances the generation of ROS, possibly by inhibiting the anti-oxidant actions of p53 and HSP70. ER (Endoplasmic reticulum) stress arises

from the ROS and from PES inhibiting the chaperone activities of HSP70. The ER stress in turn initiates the unfolded protein response (UPR), but this fails to restore ER homeostasis so proteins aggregate and cells die. Despite these multiple actions, PES should be useful for studying fish cellular survival pathways.

PFT-α had unexpected and expected actions on RTgill-W1. When dosed indirectly into RTgill-W1 cultures, PFT-α did not reduce cell viability but caused a transient rise in the mitotic index and a disruption in cytoskeletal microtubules. This suggests for the first time that PFT-α targets the assembly and disassembly of microtubules either directly through an off-target action on tubulin or indirectly through an on-target action on p53-regulated transcription. In cultures with or without FBS, PFT-α at 5.25 μg/ml completely arrested proliferation. When FBS was present, PFT-α increased the number of polyploid cells over 12 days. This suggests that like in mammals, p53 regulates ploidy in fish.

BTRs at high concentrations elicited several toxicological responses in the rainbow trout cell lines. Although DM was not cytotoxic to RTgill-W1, BRT, 4MBRT, 5MBTR, TT, and OHBTR were at concentrations above 15 mg/L, with 5CBTR being the most cytotoxic. Shortly after BTR addition, cultures had elevated reactive oxygen species (ROS) but the antioxidant N-acetyl cysteine (NAC) failed to block cell killing. Cell death was neither accompanied by hallmarks of apoptosis nor blocked by necrosis inhibitor IM-54 or Necrostatin-1, suggesting that cell killing was by neither apoptosis nor necroptosis. The comet assay gave positive results but only at cytotoxic BTR concentrations, suggesting that as judged with this measure of genotoxicity the BTRs were not genotoxicants. Cytochrome P4501A levels in RTL-W1 were elevated slightly by BTR, OHBTR, 4MBRT, and 5MBTR and clearly by 5CBTR and TT. Thus some BTRs might be able to exert toxic actions through the aryl hydrocarbon receptor (AhR).

Like BTRs, BTHs at high concentrations elicited several toxicological responses in the rainbow trout cell lines. NNA, DBTH and MBTHS were not cytotoxic to RTgill-W1, but BTH, DTDC, SO, 2MBTH, ZincMBTH, NaMBTH, OHBTH, 2ABTH, and VY were. The EC_{50} values of the other BTHs in one and twelve day exposures ranged from 0.05 to 396 mg/L, with DTDC being the most cytotoxic. Shortly after addition of cytotoxic BTHs to cultures, reactive oxygen species (ROS) were elevated, but the antioxidant N-acetyl cysteine (NAC) failed to block cell killing. Cell death was neither accompanied by hallmarks of apoptosis nor blocked by IM-54 or

Necrostatin-1, suggesting that cell killing was by neither apoptosis nor necroptosis. At non-cytotoxic BTH concentrations, the comet assay gave positive results only for BTH, suggesting that BTH was a possible genotoxicant. NaMBTH, ZincMBTH, SO, VY, OHBTH, 2ABTH, MBTHS and NNA elevated cytochrome P4501A levels in RTL-W1, weakly for most but strongly for 2ABTH and OHBTH and MBTHS. Thus some BTHs might be able to exert toxic actions through the AhR.

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DEDICATION

This thesis is dedicated to my family.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
2ABTH	2-aminobenzothiazole
2MBTH	2-mercaptobenzothiazole
4D	Tetramerization domain
4MBTR	4-methyl-1H-benzotriazole
5CBTR	5-chlorobenzotriazole
5MBTR	5-methyl-1H-benzotriazole
7-AAD	7-amino-actinomycin
ADAF	Aircraft deicing and anti-icing fluids
AhR	Aryl hydrocarbon receptor
ANOVA	One-way analysis of variance
AP	Alkaline phosphatase
ARNT2	Aryl hydrocarbon receptor nuclear translocator 2
ATG	Autophay-related genes
BCA	Bicinchoninic acid
BTR	Benzotriazole
BTH	Benzothiazole
CEC	Contaminants of emerging concern
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl
CHO	Chinese hamster ovary
CLL	Chronic lymphocytic leukemia
CTD	C-terminal domain
CYP1A	Cytochrome P4501A
DBD	DNA-binding domain
DBTH	2,2'-Dithiobis (benzothiazole)
DISC	Death-inducible signaling complex
DM	5,6-Dimethyl-1H-benzotriazole monohydrate
DMSO	Dimethyl sulfoxide
DTDC	3,3’-diethylthia dicarbocyanine iodide
EC_{50}	Half maximal effective concentration
EPA	Us environmental protection agency
ER	Endoplasmic reticulum
ERα	Estrogen receptor
EROD	Ethoxyresorufin o-deethylase
FBS	Fetal bovine serum
FT-ICR	Fourier transform ion cyclotron resonance
GABAA	Γ-aminobutyric acid type A
GC×GC	Two dimensional gas chromatography
GCLC	Glutamate cysteine ligase catalytic subunit
GC-MS	Gas chromatography-mass spectrometry
G6PDH	Glucose-6-phosphate dehydrogenase
GSTs	Hepatic glutathione S-transferases
HSC70	Heat shock cognate 70
HD_2CFDA	2′,7′-Dichlorofluorescin diacetate
HDAC	Histone deacetylase

HRMS	High resolution mass spectrometry
HSP	Heat shock protein
K_{OC}	Soil organic carbon-water partitioning coefficient
LD_{50}	Lethal dose, 50%
LLE	Liquid-liquid extraction
LMP	Low melting point
LOAEL	Lowest observed adverse effect level
MAP	Microtubule-associated protein
MBTHS	2-(p-aminophenyl)-6-methylbenzothiazole-7-sulfonic acid
MLA	Mouse lymphoma assay
MOMP	Mitochondrial outer membrane permeabilization
MS	Mass spectrometry
MSDS	Material safety data sheets
MTT	Tetrazolium salt (3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide)
NAC	N-acetylcysteine
NaMBTH	Sodium 2-mercaptobenzothiazole
NER	Nucleotide excision repair
NMP	Normal melting point
NMR	Nuclear magnetic resonance spectroscopy
NNA	N,N-Dicyclohexyl-2-benzothiazolsulfene amide
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PES	2-phenylethynesulfonamide
PER	Photoreactivation repair
PFT-α	Pifithrin-α
PFT-μ	Pifithrins-μ
PPP	Pentose phosphate pathway
PRD	Proline-rich domain
PS	Phospholipid phosphatidylserine
PTP	Mitochondrial permeability transition pore
OHBTH	2-Hydroxy-benzothiazole
OHBTR	Hydroxybenzotriazole
ROS	Reactive oxygen species
RYA	Recombinant yeast assays
S9	S9 liver extracts
SBSE	Stir bar sorptive extraction
SO	C.I. Sulphur orange 1
SPE	Solid-phase extraction
TAD	N-terminal transactivation domain
TCA	Tricarboxylic acid cycle
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCMTB	2-thiocyanomethylthiobenzothiazole
TDS	Thermal desorption system
TFT	Trifluorthymidine
tk	Thymidine kinase

TNF	Tumor necrosis factor
TT	Tolytriazole
UPLC	Ultraperformance liquid chromatography
UPR	Unfolded protein response
UV	Ultra-violet
WWTP	Wastewater treatment plant
VY	C.I. Vat yellow 2
ZincMBTH	Zinc 2-mercaptobenzothiazole

CHAPTER 1

General introduction

1.1 Introduction

Environmental toxicology is an interdisciplinary science dealing with the fate and effect of toxicants in the environment (Bols et al., 2005). Toxicants are substances discharged through human activities into the natural world with the potential to impact ecosystems. These substances have been termed ecotoxicants and are diverse. They arise from industries, with heavy metals and polycyclic aromatic hydrocarbons (PAHs) being examples, but also from medical, personal care, and farming practices, with pharmaceuticals, fragrances, and antibiotics.

All organisms are subjects of environmental toxicology but fish or teleosts are commonly examined and have some important features (Bols et al., 2001). Teleosts are the most diverse group of vertebrates, with approximately 20,000 different species occupying all aquatic niches. Thus evaluating the toxicity of ecotoxicants to fish can help in understanding their impact on the aquatic environment in general. Additionally, effects on fish are important for what they say about potential impacts on human health (Zelikoff, 1998). Although ecotoxicants are often released first into aquatic environments, humans ultimately can be exposed (Adams & Greeley, 1999). Many biological processes have been conserved through evolution and so effects on the fish can provide insights into how ecotoxicants might act on humans. Finally, understanding the impact of ecotoxicants on teleosts can be of economic importance because ecotoxicants, such as mercury, can impact commercial fisheries.

Environmental toxicology can be considered as encompassing three disciplines that study the ecotoxicants problem at different levels of organization: ecotoxicology, toxicology, and cellular toxicology. The discipline of toxicology developed first and examines the adverse effects of chemical, physical or biological agents on individual organisms. Among the teleosts, rainbow trout have been used frequently and at one time were described as the ‘white rat’ of fish toxicology (Wolf & Rumsey, 1985). Ecotoxicology attempts to bring ecology and toxicology together and has been defined in various ways. One common definition is that ecotoxicology studies the impact of environmental contaminants on populations and ecosystems. Cellular toxicology might be defined as studying the effects of substances on the basic processes of cell homeostasis, death, proliferation, and differentiation and on the genetic systems and signal

transduction pathways that coordinate them. These three disciplines integrate the toxicological responses but at the levels of populations, individuals, and cells.

Cellular toxicology might be considered the most fundamental of the environmental toxicology disciplines because cells probably respond first to toxicants, and depending on the response, result in disruption at higher levels of organization. However, where fish are considered, this is perhaps the most under developed of the disciplines. This thesis has two overarching goals.

1. To improve the use of rainbow trout cell lines as tools in environmental toxicology by using inhibitors of p53 to understand functions of this master regulatory protein in fish.
2. To use rainbow trout cell lines to study the cell biology of two related classes of emerging contaminants, benzotriazoles (BTRs) and benzothiazoles (BTHs).

1.2 Cellular toxicology

For multicellular animals, such as fish and mammals, cell cultures are used frequently as the subjects of cellular toxicology (Bols et al., 2005; O'Brien, 2014). This is commonly referred to as in vitro toxicology. Cell cultures offer several advantages over whole animals as experimental tools in toxicology. Dosing of cell cultures is done more easily and reproducibly and produces less toxic waste. Results are obtained more rapidly than with intact animals and with less cost. Experiments with cell cultures satisfy a societal desire to reduce the use of animals in toxicology testing. Cell cultures allow cellular phenomena to be studied in a controlled and in some cases a completely defined environment, independent of the complexities and variability of systemic or larger physiological controls and allow mechanisms of toxicity to be delineated at the molecular and cellular level.

The cell cultures from vertebrates can be either primary cultures or cell lines. Primary cultures are originated directly from the cells, tissues or organs of fish or mammal and typically last for only a few days. The primary cultures can be from either a tumor or normal tissue. Cell lines arise from primary cultures and can be propagated indefinitely. Many mammalian cell lines arise from cancers but nearly all fish cell lines arise normal tissues. Most often they grow attached to a plastic growth surface and maintain either a fibroblastic or epithelial shape. A

famous human epithelial cell line that is often used in in vitro toxicology is HepG2 from a hepatocellular carcinoma (Powell et al., 2011). Some rainbow trout epithelial cell lines are RTgill-W1 from the gill (Bols et al., 1994; Lee et al., 2009) and RTL-W1 from the liver (Lee et al., 1993).

For in vitro toxicology, putative toxicants or ecotoxicants have been evaluated for their ability to elicit changes in many cellular processes. Prominent among the cellular processes are the maintenance of genome stability, the balance between cell death and survival, and signal transduction. Each of these processes has been evaluated in multiple ways and led to several sub-disciplines. One sub-discipline is genotoxicology, which has developed many different end points, with DNA damage being one. The balance between cell death and survival involves investigating the cell death mechanisms, with apoptosis perhaps being most studied. Many signal transduction pathways coordinate these and other processes and several of the most important pathways are mediated through the tumor suppressor protein, p53. Genotoxicants, cell death mechanisms, and p53 in vitro are briefly reviewed.

1.2.1 Identifying genotoxicants in vitro

Genotoxicity tests are undertaken to determine whether putative ecotoxicants have the potential to cause mutations or chromosomal damage (Garcia-Canton et al., 2012). If they do, exposure of humans or animals to them may lead to adverse consequences, including cancer, developmental anomalies or genetic diseases. From an ecotoxicological perspective, mutations or chromosomal damage could lead to reproductive impairment and population declines (Jha, 2008). A battery of tests has received regulatory approval for determining whether substances are genotoxic (Garcia-Canton et al., 2012; Kirkland, 2011). One of the most famous is the Ames test, which uses *Salmonella typhimurium* and *Escherichia coli* and can be run with or without a vertebrate liver extract (S9 fraction). The Ames test measures bacterial reverse mutations.

Assays with mammalian cell lines are part of the battery of in vitro tests that have received regulatory approval. One of the most commonly used mammalian cell line test is the mouse lymphoma assay (MLA). The MLA is a mammalian gene mutation assay and uses the mouse leukaemia cell line, L5178Y tk+/- (Lloyd & Kidd, 2012). These cells have thymidine kinase (tk)

activity and are susceptible to the nucleoside analogue, trifluorthymidine (TFT). If exposure to a chemical causes a forward mutation so that a cell becomes tk-/-, the cells will survive and form colonies in the presence of TFT. The number of colonies provides a measure of the genotoxicity for that chemical. The exposure to the chemical can be done in the absence or presence of an S9 extract in order to test whether metabolic activation of the chemical is needed for it to act as a mutagen.

Genetic tests with fish cell lines have yet to become common in environmental toxicology despite considerable interest (Kienzler et al., 2013). One likely reason for this is the lack of drug resistant fish cell lines, such as tk-. Another cause for this could be the lack of sophisticated information on the karyotypes of fish cells that allow chromosomal rearrangements and other aberrations to be detected easily. However, one assay that is being considered for regulatory approval (Garcia-Canton et al., 2012) and can be done with fish cell lines is the comet assay (Bokan et al., 2013).

Single cell gel electrophoresis or comet assay is a well established, sensitive and inexpensive tool for measuring visual evidence of DNA damage and repair on the level of individual eukaryotic cells. The neutral comet assay developed by Ostling and Johanson (1984) detects only the double strand breaks, whereas the alkaline comet assay developed by Singh et al (1988) detects double and single strand DNA breaks as well as alkali labile sites. Increased DNA migration in comparison to the negative control may indicate the induction of DNA damage. Modified comet assay using lesion specific enzymes can also detect DNA lesions such as DNA cross-links and oxidative DNA damage (Tice et al., 2000; Jha, 2008; Dhawan et al., 2009). The comet assay with fish cell lines is a suitable tool for in vitro screening of environmental contaminants. There are a number of studies using fish gill cell lines for comet assay. For instance, RTgill-W1 has been used in comet assay to study UV-induced Nucleotide Excision Repair (NER) and Photoreactivation Repair (PER) (Kienzler et al., 2013). In an acute toxicity study, a statistically significant increase in DNA damage as judged with the comet assay was observed in RTgill-W1 following the exposure to $CuSO_4$ (Bopp et al., 2008).

1.2.2. Cell death mechanisms

One of the most fundamental cellular responses is death and can be conveniently studied in vitro. The first and simplest way that cell death has been, and continues to be, studied in vitro is measuring cell viability (Bols et al., 2005). Substances that elicit a loss of cell viability are termed cytotoxic and the concentrations at which they are cytotoxic can be used as part of risk assessment. However, over the years the loss of cell viability has been found to occur by different underlying cell death mechanisms. Having knowledge of these mechanism can provide insight into how toxicants might cause tissue damage in vivo and how they might interact with each other, improving risk assessment (Orrenius et al., 2011). For example, a specific chemical class might kill cells in a characteristic way and can be used as a signature or biomarker for exposure to these chemicals. Currently, many different cell death mechanisms have been proposed and named (Kroemer et al., 2009; Maltese & Overmeyer, 2014; Smith & Yellon, 2011), but usually three main categories of cell death are considered. These are apoptosis, necrosis, and autophagy.

1.2.2.1 Apoptosis

The term apoptosis was first introduced by Kerr and colleagues in 1972 to describe the form of cellular suicide accompanied by cytoplasmic shrinkage, nuclear chromatin condensation, nuclear fragmentation and blebbing of the plasma membrane (Kerr et al., 1972; Delhalle et al., 2003; Orrenius et al., 2011). This word is derived from ancient Greek words ‘apo’ and ‘ptosis’, which together means petals falling off from flowers. Apoptosis is a programmed event that plays an important role in maintaining bodily homeostasis and occurs throughout normal ontogenesis. Apoptosis also acts as a protective mechanism that eliminates damaged cells upon physiological and pathological stimuli. Repression of this programmed cell death will lead to the accumulation of virtually immortal cells such as tumor cells (Kerr et al., 1972; Delhalle et al., 2003; Elmore, 2007). Apoptosis can be induced by extra- and intracellular signals. The extrinsic or death receptor pathway involves the ligation of the surface tumor necrosis factor (TNF) receptors and formation of the death-inducible signaling complex (DISC). Once DISC is formed, it activates caspase-8 and initiates the execution phase of apoptosis. The intrinsic or mitochondrial pathway is regulated by the adversarial interaction between the pro- and anti-

apoptotic proteins of the Bcl-2 family (Green and Evan, 2002). Following pro-apoptotic stress, such as ER stress and DNA damage, the effector proteins of the pro-apoptotic Bcl-2 family, BAK and BAX homo-oligomerize within the outer membrane of mitochondria and initiate mitochondrial outer membrane permeabilization (MOMP) with the release of apoptotic activators such as cytochrome c and endonuclease G (Nemajerova et al., 2005). Recently, a third pathway has been discovered which involves the regulation of granzyme A and B (Mader et al., 2011).

1.2.2.2 Necrosis

The term necrosis is derived from ancient Greek words ‘nekros’, which means dead body. Necrosis is usually defined as a passive and un-regulated form of cell death with distinct morphological characteristics such as cell and organelle swelling, plasma membrane rupture, intracellular contents spillage with subsequent cell lysis and inflammatory responses (Galluzzi et al., 2007). Due to a lack of specific discriminative biomarkers of necrosis to date, the determination of necrotic cell death is usually limited to electron microscopy. A combination of different methods is highly recommended in the detection of necrosis. In contrast to the energy requiring apoptosis, ATP depletion favors the necrotic cell death. Necrosis may be triggered by various external stresses that cause metabolic failure, such as hypoxia, mechanical force, ischemia and membrane-permeablilizing toxins (Hotchkiss et al., 2009). Although necrosis has been long regarded as an accidental process resulting from excessive stress, recent studies have suggested an alternative death pathway, termed necroptosis. This was considered a form of programmed or regulated necrosis (Kroemer et al., 2009; Smith & Yellon, 2011).

1.2.2.3 Autophagy

Autophagy is a relatively slow self-degeneration process that accompanied by massive autophagic vacuolization of content of cytoplasm (Galluzzi et al., 2007). Autophagy is mainly a survival mechanism that recycles redundant organelles and macromolecules during periods of nutrient shortage. The cytoplasmic particles packed in double-membraned vacuoles (autophagosomes) are delivered to lysosomes and digested by lysosomal hydrolases (Hotchkiss et al., 2009). Autophagy is also essential in the removal of damaged organelles and misfolded

proteins in response to cellular stress. Autophagic cell death has been observed in Drosophila melanogaster larvae and some mammalian cells in vitro (Maiuri et al., 2007, Scott et al., 2007). However, cell death executed by autophagy has never been found in a mammalian model in vivo and no chemical has been found to induce autophagic cell death in tumor cells. Whether cell death can be mediated through autophagy or simply occurs with autophagy is still under debate (Galluzzi et al., 2007; Kroemer and Levine, 2008). More and more evidences indicate that autophagy is a protective process and suppression of autophay-related genes (ATG) in mammilian cells often results in an acceleration of cell death (Kroemer and Levine, 2008; Kroemer et al., 2009; Hotchkiss et al., 2009)

1.2.2.4 Cell death mechanisms in fish cell lines exposed to ecotoxicants

The response of fish cell lines to ecotoxicants has more commonly been evaluated only as a loss of cell viability rather than a mode of cell death (Bols et al., 2005), and relative to mammalian cells little is known about the death mechanisms of piscine cells (Krumschnabel and Podrabsky, 2009). However, a few studies have been done on the mode of cell death in cultures of fish cell lines exposed to heavy metal ecotoxicants. RTgill-W1 was shown to die in response to cadmium by a mixture of apoptosis and necroptosis (Krumbschnabel et al., 2010). This conclusion was reached because the necroptosis inhibitor, necrostatin-1, and the pan-caspase inhibitor zVAD-fmk reduced signs of cell deterioration. The topminnow hepatoma cell line, PLHC-1, died by both apoptosis and necrosis in response to arsenic trioxide (Selvaraj et al., 2013). Clearly more information is needed on how fish cell lines die in response to different ecotoxicants. Such knowledge would improve the utility of fish cell lines as tools in fish (eco)toxicology as well as provide insights into the toxic actions of the ecotoxicant.

1.2.3 Tumor suppressor p53

Tumor suppressor protein p53 is a master regulator protein (Toledo & Wahl, 2006). p53 contains several conserved domains such as N-terminal transactivation domain (TAD), proline-rich domain (PRD) adjacent, DNA-binding domain (DBD), tetramerization domain (4D) and C-terminal domain (CTD). Post-translational modifications at conserved residues in these domains regulate p53 activity. Phosphorylation at TAD and PRD stabilizes p53 by blocking the binding

site of p53 negative regulator MDM2 and enhances the association with its co-activators p300 and CBP. Phosphorylation at CTD up regulates the transcriptional activity of p53 by facilitating a conformational change, whereas neddylation of CTD seems to inhibit the transcriptional activity of p53 (Toledo & Wahl, 2006).

In unstressed cells, the majority of p53 is localized in the nucleus and total p53 is usually maintained at a low level by ubiquitination of its pivotal negative regulator, MDM2 and other ubiquitin-protein isopeptide ligases (Kruse & Gu, 2008). Interestingly, as a pivotal negative regulator of p53, MDM2 is transactivated by p53. Thus, they form a negative feed-back loop that keeps a low level of p53 in normal cells (Lu et al., 2000). Upon stress, p53 is activated by a wide variety of protein kinases (Albrechtsen et al., 1999). Phosphorylation and other post-translational modifications stabilize p53 in the nucleus and enhance formation of p53 tetramers (Dhar & Clair, 2009). p53 tetramer binds to its DNA binding site and trans-activates a spectrum of genes involved in many processes.

1.2.3.1 Cellular processes regulated by p53

The activities of p53 regulate a network of interconnected cellular processes. The tumour suppressive function of p53 is achieved through p53 mediating apoptosis, cell cycle arrest and senescence (Liu et al., 2014). The response depends on the magnitude of the stress. With mild stress, activation of p53 induces cell cycle arrest; with harsh stress, p53 activation leads to apoptosis. The stresses include ionizing radiation causing DNA damage, chemotherapeutic drugs, and aberrant growth signals (Ellias et al., 2014). Other broad cellular processes that activated p53 regulates are autophagy (Maiuri et al., 2010) and endoreplication (Aylon & Oren, 2011). Besides stressful conditions, p53 functions to regulate the normal or constitutive activities of the cell necessary for cellular homeostasis (Zheltukhin & Chumakov, 2014). These include energy metabolism (Wang & Gu, 2013; Zheltukhin & Chumakov, 2014) and antioxidant defenses (Zheltukhin & Chumakov, 2014).

1.2.3.2 Transcription-dependent and independent p53 mechanisms

Usually p53 regulates cellular processes by acting as a transcription factor (Elias et al., 2014) but recent work has discovered transcription-independent functions for p53 as well (Comel et al. 2014). This is illustrated in cell death mechanisms and in energy metabolism.

Most often p53 appears to regulate apoptosis by a transcription-dependent mechanism, but p53 can also initiate apoptosis in a transcription-independent manner. With severe stress, such as DNA damage, p53 activation leads to apoptosis (Ellias et al., 2014). Activated p53 regulates the transcription of genes important to apoptosis, such as PUMA, Bax and Noxa. These pro-apoptotic factors lead to the permeabilization of the mitochondrial outer membrane (MOMP). However p53 can cause MOMP and apoptosis in a transcription-independent manner. During stress p53 accumulates in the cytoplasm and mitochondria. The p53 acts directly in the outer mitochondrial membrane to displace pro-apoptotic proteins from their negative regulators and leads to oligomerization and activation of Bak and cytochrome c release.

A transcription-independent action of p53 might occur with another mode of cell death, necrosis (Vaseva et al., 2012). In mouse embryo fibroblasts, oxidative stress caused p53 to accumulate in the mitochondrial matrix. The p53 then interacted with cyclophilin D and triggered mitochondrial permeability transition pore (PTP) opening and necrosis.

The involvement of p53 in energy metabolism appears to be through transcription-dependent and transcription-independent actions (Comel et al., 2014). Examples are seen in glutamine metabolism and the pentose phosphate pathway (PPP). Mitochondrial glutaminase converts glutamine to glutamate, leading to the formation of α-ketoglutarate, an intermediate in the tricarboxylic acid cycle (TCA). Expression of the glutaminase gene is activated by p53. Cytoplasmic p53 can transiently interact with the rate-limiting enzyme of PPP, glucose-6-phosphate dehydrogenase (G6PDH), and inhibit its activity.

1.2.3.3 p53 in fish

Mammalian p53 homologs have been characterized in several fish species. These include rainbow trout (Caron de Fromentel et al., 1992; Liu et al., 2011), zebrafish (Cheng et al., 1997), channel catfish (Luft et al., 1998), flounder (Cachot et al., 1998), medaka (Krause et al., 1997), orange-spotted grouper (Qi et al., 2013); tilapia (Mai et al., 2012), and whitefish (Brzuzan et al., 2009). Generally the studies reveal a high degree of functional homology with mammalian p53 (Krumschnabel & Podrabsky 2009).

From the few studies that have been done with fish, the regulation of p53 levels in teleosts appears to differ from the regulation in mammals. This has been seen most clearly with in vitro studies on cell lines. Several chemotherapeutic agents that are known to cause DNA damage increase p53 levels in mammalian but not piscine cell lines (Embry et al., 2006; Liu et al., 2011).

Few studies have focused on p53-mediated signal transduction pathways and the processes that they control in fish. Most studies have been done with zebrafish, with p53 being shown to be involved in several classic activities. These include up-regulating gene expression, inducing apoptosis after UV irradiation, and suppressing tumor formation (Storer & Zon, 2010). Knock down of Mdm2, the main negative regulator of p53, in zebrafish embryos was shown to cause excessive apoptosis and early growth arrest (Langheinrich et al. 2002). In contrast, overexpression of Mdm2 in zebrafish liver did not lead to hyperplastic livers or liver cancer but caused liver atrophy (Chen et al. 2008). Whether p53 will work similarly in other teleosts is unknown. Interestingly UV failed to induce p53 in the medaka, suggesting that p53 behaves differently in this species (Chen et al., 2001). Even among mammals the possibility that p53-mediated tumor suppression acts differently between mouse and humans has been raised (Aylon and Oren, 2011). Thus extending p53 work to other fish species is needed. One way to do this is to use cell lines, which are available from many species (Bols et al., 2005), and to use p53 inhibitors to attempt to understand the functions in fish cells.

1.2.3.4 p53 activators and inhibitors

Activators and inhibitors of p53 are being identified and explored as new pharmaceuticals for a variety of human diseases (Gudkov & Komarova, 2005; Selivanova, 2014). The involvement of p53 has been shown in diseases such as atherosclerosis, diabetes, Alzheimer's, Parkinson's, and Huntington's (Nayak et al., 2009). However, most of the attention has been for cancer treatments because p53 is the most frequently mutated gene in human cancers (Liu et al., 2014). Often the p53 mutations in human tumours are missense mutations and result in the expression of full-length mutant p53 proteins (Liu et al., 2014). Normally p53 mediates tumour suppression through cell cycle arrest, senescence and apoptosis (Eischen & Lozano, 2014). Frequently these tumour suppressive actions are lost in the mutant p53. Additionally, some mutant p53 proteins gain oncogenic functions, such as promoting tumour cell proliferation and blocking apoptosis, which are defined as mutant p53 gain-of-function (Freed-Pastor & Prives, 2012).

Two strategies are being undertaken to target mutant p53s. Inhibitors might prevent the gain-of-functions that promote tumour development. However, the most promising approach appears to be to identify compounds that reactivate or restore the wild type p53 functions (Selivanova, 2014). For example, compound NSC-319725 restores the structure and function of the R175H mutant p53 and causes apoptosis in tumours of mice (Liu et al., 2014). Another molecule under investigation is PRIMA-1, which converts mutant p53 conformation to wild type and sensitizes tumour cells to chemotherapy (Liu et al., 2014).

As well, drugs that target wild type p53 are being developed to improve cancer treatments (Gudkov & Komarova, 2005). In many cases irradiation or chemotherapy eliminates tumour cells by activating p53 and triggering apoptosis. Therefore activators of wild type p53, such as nutlin, have been developed to enhance the process. However, during these therapies the normal cells surrounding the tumour need to be protected. Therefore inhibitors of wild type p53 are being developed for use with cancer treatments to protect healthy cells around tumours. One group of inhibitors is referred to as pifithrins for protein fifty three inhibitor. The two main types are pifithrin-α 2-(2-imino-4,5,6,7-tetrahydrobenzothiazole-3-yl)-1-p-tolyethanone hydrobromide),

which is abbreviated PFT-α, and pifithrin-μ (2-phenylacetylenesulfonamide), which is sometimes abbreviated PFT-μ or PES.

1.2.3.5 p53 inhibitor pifithrin-α (PFT-α)

PFT-α was identified initially as a compound that blocked p53-mediated apoptosis (Komarov et al., 1999). Subsequently PFT-α was demonstrated to inhibit the transcriptional activity of p53 by impeding the binding of p53 to its DNA sites (Charlot et al., 2006). PFT-α reduced the activation of p53-regulated genes, including cyclin G, p21WAF1, 14-3-3-σ and MDM2 without changing the amount of p53 protein itself (Beretta et al., 2008). Other data with adult rats suggested that PFT-α did not affect the synthesis of p53 but acted by inhibiting p53 nuclear translocation and preventing its binding to its specific DNA sites (Leker et al., 2004). In many studies PFT-α acted as designed and protected mammalian cells from apoptosis (Liu et al., 2005; Shao et al., 2010; Sinn et al., 2010). For example, PFT-α has been shown to inhibit apoptosis in HCT116 cells after gamma irradiation (Sohn et al., 2009) and in neurons after treatment with amyloid β-peptide (Culmsee et al., 2001). Therefore, PFT-α is sold and generally used as an inhibitor of p53 that blocks p53-dependent transcriptional activation and apoptosis.

However, some paradoxical or off target actions of PFT-α have been reported. PFT-α was cytotoxic to the mouse epidermal JBC C1 41 cell line (Kaji et al., 2003), two wild type p53 human tumor cell lines (Walton et al., 2005), and murine myoblast cell line, C2C12 (Waters et al., 2010).

1.2.3.6 p53 inhibitor PES

PES was identified in a screen for compounds that would impair the localization of p53 to the mitochondria and block apoptosis (Strom et al., 2006). Normally, p53 inactivates anti-apoptotic protein Bcl-xL and Bcl-2 on the mitochondrial outer membrane by forming complexes with them, which will lead to MOMP and release of apoptotic activator proteins (Nemajerova et al., 2005). PES inhibited apoptosis by strongly inhibiting the translocation of p53 to mitochondria and reducing the affinity of p53 to anti-apoptotic protein Bcl-xL and Bcl-2. PES did not affect the

transcriptional activity of p53 but only p53 mediated apoptosis acting through mitochondria (Strom et al., 2006).

Subsequent to its initial discovery PES was shown to have effects that might not be mediated through p53. PES was cytotoxic to B-chronic lymphocytic leukemia (CLL) cells from human patients in a p53 independent way (Steele et al., 2009). Additionally, PES was identified as an inhibitor of HSP70. In human osteosarcoma and melanoma cells, PES was able to bind HSP70 at its C-terminus and disrupt the interaction between HSP70 and its co-chaperones. The disruption of HSP70 caused protein aggregation and lysosome membrane de-stabilization which finally led to cell death with impaired autophagy (Leu et al., 2009).

1.2.3.7 p53 inhibitors and fish

Inhibitors of p53 appear not to have been used in fish systems. However, use of the inhibitors might give insights into the actions of p53 in fish. Knowledge of how fish cells respond to p53 inhibitors has an additional purpose. Drugs that target p53 are likely to be more widely used in the future and pharmaceuticals that have broad usage have the potential to be released into the aquatic environment. Therefore understanding how p53 drugs might act on fish cells might help assess the risk of such a release.

1.3 Emerging contaminants

The human health based guideline values and potential ecotoxicological effects of many ecotoxicants remain largely unknown and so they have been described as emerging contaminants. Emerging contaminants are not necessarily new compounds but compounds in the environment without regulatory status and whose behaviour, fate and ecotoxicological effects are poorly understood and are thought to be potential threats to the receiving environment and human health. The phrase ‘emerging contaminants’ has been described as fashionable but hard to define (Sauve & Desrosiers, 2014).

One line of thought has been to change ‘emerging contaminants (ECs)’ to ‘contaminants of emerging concern (CEC)’. CECs are defined as “naturally occurring, manufactured or manmade

chemicals or materials" that have now been discovered or suspected to have a presence in various environmental compartments and "whose toxicity or persistence are likely to significantly alter the metabolism of a living being" (Sauve & Desrosiers, 2014). CEC can be considered a moving target because the production and the science of them will continuously change. However, CEC have several essential features. CEC cannot be properly evaluated for their risk to human health and the environment because toxicological and ecotoxicoloigcal data on them is inadequate. Two groups of chemicals that currently are being described as emerging contaminants or contaminants of emerging concern are benzotriazoles and benzothiazoles (Deblonde et al., 2011; Dummer, 2014; Jana et al., 2011) and benzothiazoles (Deblonde et al., 2011; Richardson & Ternes, 2014).

1.4 Benzotriazoles (BTRs)

Benzotriazoles (BTRs) are bicyclic, heterocyclic compounds that consist of a benzene ring in which two adjacent carbon atoms are covalently bonded to three nitrogen atoms in a five membered ring. Perhaps the most commonly studied BTRs are 1H-benzotriazole (BTR) and tolytrizazole (TT). BTR is an odourless, white crystalline solid with a low vapour pressure (0.04 mmHg at 20 ºC) and a weak acid (pKa=8.2) as well as a weak base (pKb=1.6). The compound is fairly soluble in water (20 g/L, log Kow=1.27) and in several organic solvents. Generally, TT is considered as a mixture of methyl isomers, 4-methyl-1-H-benzotriazole (4MBTR) and 5-methyl-1-H-benzotriazole (5MBTR). TT is a light brown powder with a low vapor pressure (0.03 mmHg at 20 ºC) and moderate solubility in water (7 g/L, log Kow=1.89) (Giger et al., 2006). Other BTRs that have drawn the interest of several scientific disciplines are hydroxybenzotriazole (OHBRT), 5-chlorobenzotriazole (5CBTR), and 5,6-dimethyl-1H-benzotriazole monohydrate (DM). Hydroxybenzotriazole (OHBTR) is a white crystalline powder and a weak acid (pKa=4.60) with moderate solubility in water (5 g/L at 30 ºC) (Subirós-Funosas et al., 2009). 5CBTR is an off-white powder with moderate solubility in water. DM is more hydrophobic than most BTRs and is nearly insoluble in water (log Kow=3.05). BTRs are manufactured on an industrial scale and used for a variety of purposes in many commercial products.

1.4.1 Production and uses of BTRs

The amount of BTRs synthesized around the world is large. For example, in 1999 at least 9,000 tons of BTRs were produced in the US (Wang et al., 2013). The annual global production is thought to be much higher (Herzog et al., 2014).

BTRs have a variety of important commercial uses. The largest one is likely as chemical corrosion inhibitors in consumer goods and in industrial products. Among consumer goods, dishwashing detergents contain BTRs to diminish corrosion of nonferrous metals (Janna et al., 2011). For industrial products, BTRs are used as an anticorrosive agent in aircraft deicing and anti-icing fluids (ADAF), in motor vehicle antifreeze and in brake fluids (Breedveld et al., 2003). BTRs are also used as UV light stabilizers in plastic and as antifogging agents in photography (Wang et al., 2013).

Some BTRs are used as the starting material in the synthesis of new pharmaceuticals (Kale et al., 2010). For example a series of BTRs were evaluated for their ability to inhibit histone deacetylase (HDAC) and cell proliferation (Li et al., 2013). OHBTR has been used as an additive in oligonucleotide couplings and in racemization-free peptide coupling (König and Geiger, 1970).

1.4.2 Release and detection of BTRs in the environment

BTRs enter the environment because they are in “down-the-drain” products (Janna et al., 2011). Estimates of possible inputs of BTR and TT from different dishwasher formulations to a sewage treatment plant in England have been calculated at 7.72 g of BTR and 148 g of TT per day (Janna et al., 2011). BTRs also enter the environment through their presence in aircraft deicing and anti-icing fluids (ADAF). Every year in the US more than 80 million litres of sewage waters that are contaminated with ADAFs are released into the environment (Cancilla et al., 2003a).

A number of analytical techniques have been used to detect BTRs in the environment, most commonly in water. The most widely used extraction and enrichment step of chemicals in aqueous samples are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). The

application of new extraction methods using ionic liquids, which are organic salts with a low melting point, is increasing. Most popular analytical methods to identity unknown aquatic contaminants and their transformation products are high resolution mass spectrometry (HRMS) with ultraperformance liquid chromatography (UPLC) or two dimensional gas chromatography (GC×GC) using Triple quadruple, time-of-flight or fourier transform ion cyclotron resonance (FT-ICR) MS systems (Fischer et al., 2012). Nuclear magnetic resonance spectroscopy (NMR) is often used as a complementary analytical technique to confirm tentative structures proposed by LC/HRMS and LC/MS/MS (Jover et al., 2009; Richardson and Ternes, 2014).

1.4.2.1 BTRs in aquatic environments

BTRs have been detected in different aquatic environments, including wastewater, rivers, ground water, and drinking water. In several countries the waters of wastewater treatment plants (WWTPs) have been found consistently to contain BTRs (Voutsa et al., 2006; Reemtsa et al., 2010; Liu et al., 2012). WWTPs appear to be a point source for BTRs in the natural environment (Herzog et al., 2014). BTRs have been identified in rivers of Switzerland, Germany and England (Vousta et al., 2006; Giger et al., 2006; Kiss & Fries, 2009, 2012; Reemsta et al., 2006, 2010; Janna et al., 2011). The Rhine River had BTR concentrations ranging from 130 to 3500 ng/L (Reemsta et al., 2010) and might be the source of BTRs found in the North Sea (Wolschke et al., 2011). BTRs were detected in groundwater around airports where ADAF had been used (Breedveld et al., 2003; Cancilla et al., 1998, 2003). Finally BTRs have been detected in tap water. The US Geological Survey found 5.4% of untreated drinking water that was sampled had 5MBTR (Focazio et al., 2008). In England, methyl-substituted BTR was found in all sampled drinking water, with concentrations ranging from 0.5-69.8 ng/L (Janna et al., 2011).

1.4.2.2 BTRs in soil

Although soil appears to have been examined infrequently for BTRs, several studies have focused on 4-methyl-1H-benzotriazole (4MBTR). In soil from near airports, 4MBTR was detected at concentrations as high as 249 mg/kg soil (Cornell, 2001). For soil samples from around Fairchild Air Force Base, 4MBTR and 5MBTR were detected at concentrations up to 3.93 mg/kg wet weight (Cancilla et al., 2003). Two years after the closing of an airport, 4MBTR

was still found in soil (Breedveld et al., 2003). The half-lives for some BTRs in soil ranged from 217 to 345 days (Lai et al., 2014). BTRs have also been detected in sludge from sewage treatment plants in Spain (Herrero et al., 2014). Some BTRs are expected to have high mobility in soil. Some studies have been done on the sorption and portioning parameters of benzotriazole compounds in different top spoils (Hansch et al., 1995; Hart et al 2004). For example, based on an estimated soil organic carbon-water partitioning coefficient (K_{OC}) value of 145, BTR is expected to be partially protonated in most environmental matrices and its cation is expected to bind to soil organic matter and clays (Hansch et al., 1995).

1.4.2.3 BTRs in air

BTRs in the air have been investigated in at least one situation. BTR was detected in the working zone of what was described as a copper rod shop in Russia (Pervova et al., 2010).

1.4.2.4 Degradation of BTRs in the environment

A considerable amount of research has focussed on how BTRs might be removed from the environment, especially wastewaters. Biodegradation has received the most attention, but other methods such as ozonation, flocculation, photo-degradation, and phyto-degradation have been explored (Dummer, 2014). In the initial studies with aerobic and anaerobic digesters, biodegradation of 4MBTR was found to be very slow (Gruden et al., 2001; Cornell 2001). WWTP were found to remove a variable proportion of the BTRs but the removal was always incomplete (Voutsa et al., 2006; Liu et al., 2012; Sahar et al., 2011). In fact WWTPs are considered major sources for these compounds in the aquatic environment (Herzog et al., 2014a). The removal of BTRs in WWTP was found to be due to aerobic biodegradation (Herzog et al. 2014b), and methods for optimizing this have been developed (Yuan et al., 2014). In general, the major BTR removal mechanism in aquatic systems might be aerobic biodegradation (Herzog et al. 2014b). However, other mechanisms might contribute to BTR degradation. In two constructed wetlands, 80-90% of TT was removed (Matamoros et al., 2010). In this case, as well as biodegradation, additional mechanisms might have contributed to BTR removal, including sorption, and plant uptake.

1.4.3 Toxicology of BTRs

The toxicology of BTRs has been examined at different levels but is not comprehensive in any one area. Some of the first toxicology information on BTRs was obtained with plants (Davis, 1954). However, in this section of the thesis the focus is on the toxicology of BTRs to humans, laboratory rodents and aquatic organisms, especially fish.

1.4.3.1 Toxicology of BTRs to humans

Limited information is available on the toxicity of BTRs to humans. Material Safety Data Sheets (MSDS) for most BTRs lists them as eye and skin irritants. In the Netherlands, the Health Council classified BTR as a suspect human genotoxic carcinogen (Dummer, 2014). Primary literature on the toxicology of BTRs to humans appears to be slight to nonexistent.

Some information on the exposure of humans to BTRs is available. Human urine samples and indoor dust have been monitored to understand human exposure. BTRs were detected in the urine of people living in Japan, India, Vietnam, China, Greece, and US (Asimakopoulos et al., 2013). The overall urine concentrations varied significantly among the seven countries. India had the highest (2.8 ng/ml); Korea, the lowest (0.2 ng/ml). In the Asian countries TT predominated. Indoor dust samples from households and offices in the USA, China, Japan and Korea were found to contain BTRs (Wang et al., 2013). However the concentrations and compounds varied among the four countries. China had the highest concentrations, with a maximum of ~2000 ng/g. The most abundant one in the USA was TT. The major BTRs in the dust from China was BTR (1H-benzotriazole). Office dust had higher concentrations than the dust from homes. One source of BTRs in offices could be printing ink. BTRs are added to ink in order to protect the metal parts of printers and copiers. The daily intake of BTRs through indoor dust ingestion was estimated. For Korean children, this was calculated as 0.19 ng/kg-bw/day (Wang et al., 2013).

1.4.3.2 Toxicology of BTRs to laboratory rodents

In the primary literature, few papers on the toxicology of BTRs to rats and mice can be found but several review articles cite anonymous reports by the industry and by government scientific

agencies. The US Environmental Protection Agency (EPA) gives a lethal dose 50 (LD_{50}) of 600-675 mg/kg for TT in the rat and another report lists the LD_{50} for OHBTR in the rat at 5000 mg/kg (Anonymous, 1992). BTR was transformed to 4- and 5-hydroxy-benzotriazole at a relatively low rate (<5%) after one-hour incubation with a phenobarbital-induced rat liver microsomal suspension (Hoffman & Pooth, 1982). In rats BTR increased the mRNA levels of hepatic glutathione S-transferases (GSTs) Ya, Yb1, Yc1 and Yc2 by 2 to 3 fold (200 mg/kg, 3 days) but not the expression of GST Yb2 (Kim & Cho, 1996). OHBTR had a low toxicity to rats: the oral lethal dose was 5000 mg/kg (Anonymous, 1992). The oral carcinogenicity studies in rats and mice failed to find evidence that BTR was carcinogenic. In a chronic rat study (78 weeks), The LOAEL (lowest observed adverse effect level) of BTR was 295 mg/kg/bw/d for histological changes in the liver, decreased body weight gain and inflammation for the prostate/uterus (Schriks et al., 2009). An 18 months oral study of BTR in Fishcer 344 rats (up to 12.100 ppm) and a 24 months oral study of BTR in B6C3F1 mice (up to 23500 ppm) showed no evidence of pathology in the reproductive organs (prostate/testis/epididymis of males and uterus/ovaries of females) (Benzotriazoles Coalition, 2001).

1.4.3.3 Toxicology of BTRs to fish

In the few studies on fish, BTRs have been found not to be very toxic but might act as an endocrine disruptor. For fathead minnow (*Pimephales promelas*), the concentration of 5MBTR that killed 50 % of the fish (LC_{50}) in a 96 h exposure was 22 mg/L (Cancilla et al 2003a). For BTR, the LC_{50} was 65 mg/L (Pillard et al., 2001). The LC_{50}s for bluegill and rainbow trout were found within the range of these values (Hartwell et al., 1995; Milanova et al., 2001).

The first studies on possible endocrine actions of BTRs compared the responses in a yeast assay with the responses of fathead minnows (Harris et al., 2007). When the yeasts that had been genetically engineered to express the human estrogen receptor (ERα) were challenged with estradiol or BTR, estradiol activated the receptor but BTR did not. Similar results were observed with 5MBTR (Seeland et al., 2012). Yet, Harris et al. (2007) found that in their yeast assay that BTR did act as an ERα antagonist. The authors pointed out that potentially any anti-estrogenic properties of BTR in vivo might also come through inhibition of aromatase, the enzyme that

converts androgens to estrogens. Yet, when fathead minnows were exposed to 0.01, 0.10, and 1.00 mg/L of BTR, no evidence of estrogenic and anti-estrogenic activities were seen. However, more recent studies with the marine medaka (*Oryzias melastigma*) and Chinese rare minnows (*Gobiocypris rarus*) do suggest endocrine effects (He et al, 2012; Liang et al., 2014). When medaka were exposed to BTR (0.01-1.00 mg/L), vitellogenin mRNA increased in the liver and CYP1A1 mRNA decreased (He et al., 2012). When exposed to BTR at 0.05, 0.5 and 5 mg/L for 28 days, Chinese rare minnows showed no mortality but fish in the 5 mg/L group did undergo several significant changes in a sex-dependent manner (Liang et al., 2014). In the livers, histological damage was seen but vitellogenin mRNA was still up regulated. Also in the 5 mg/L group, females had degenerated ovaries but in males spermatogenesis was stimulated. The plasma levels of 17β-estradiol were increased in males but decreased in females. The authors concluded BTR has the potential to cause endocrine disruption but the mechanism is not yet clear.

BTRs have been detected in fish. When fathead minnows were placed for several weeks near an outfall that received ADAF contaminated runoff from an airport, 4MBTR and 5MBTR were detected in total fish extracts (Cancilla et al., 2003a). Whether bioaccumulation of these compounds would occur over the long term would be interesting to determine.

1.4.3.4 Toxicology of BTRs to other aquatic multi-cellular organisms

Other multicellular organisms of aquatic environments have been investigated for their sensitivity to BTRs. Exposure of the vascular plant, duckweed (*Lemna minor)*, which grows on the surface of ponds, to BTR and 5MBTR caused slight changes in frond structure and inhibited the rate of growth (Seeland et al., 2012). BTR, 4MBTR, and 5MBTR were toxic to the water flea, *Ceriodaphnia dubia* (Pillard et al., 2001). The most toxic was 5MBTR, with a 48 h LC_{50} of 79 mg/L. BTR was assessed for developmental effects on the marine invertebrate *Ciona intestinalis* (Chordata, Ascidiae): malformed embryos were seen upon exposure to 32 mg/L (Kadar et al., 2010). Some triazoles and BTRs were predicted through quantitative structure-activity relationships (QSARs) but without experimental data to be toxic to daphnia (Cassani et al., 2012). Indeed experiments have shown that that BTR and 5MBTR were both acutely (48 h) and

chronically (21 days) toxic to two daphnia species (*Daphnia magna, D. galeata*) (Seeland et al., 2012). For acute toxicity, EC_{50} values ranged from 8.13 mg/L for 5MBTR to 107 mg/L for BTR. In the chronic tests, *D. galeata* was the most sensitive with EC_{10} values of 0.97 mg/L for BTR and 0.40 mg/L for 5MBTR (Seeland et al., 2012).

1.4.3.5 Cytotoxicity of BTRs

BTRs have been shown to be toxic to single cell organisms but few, if any, cytotoxic studies have been done with primary cell cultures and cell lines from vertebrates. The toxicity of several BTRs to the bacteria, *Vibrio fischeri*, was evaluated in the Microtox test (Pillard et al., 2001). The EC_{50}s for 4MBTR, 5MBTR, and TT were respectively 21, 8.7 and 7.3 mg/L. BTR concentrations higher than 0.05 mg/L were toxic to yeast (Seeland et al., 2012). At up to 5 mM, OHBTR was not toxic to the white rot fungus (*Phanerochaete chrysosporium*) or the green algae (*Desmodesmus subsicaptus*) (Papinutti & Forchiassin, 2003). However, BTR and 5MBTR did inhibit the growth of the green algae (*P. chrysporium*) with EC_{10}s of 1.18 mg/L and 2.86 mg/L respectively (Seeland et al., 2012). The authors speculated that the because of their chemical structure BTRs may inhibit the electron transport chain that supports oxidative phosphorylation in the mitochondria, robbing cells of their main energy supply process. Studies devoted directly to investigating the toxicity of BTRs to animal cells are needed.

1.4.3.6 Genotoxicity of BTRs

Only two BTRs, BTR and OHBTR, appear to have been examined for their potential to cause genetic damage. For BTR, the information is conflicting (Dummer, 2014). BTR was genotoxic in bacterial mutagenic assays with *Salmonella typhimurium* and *Escherichia coli*. However, in the *E. coli* SOS Chromotest, which complements mutagenic assays like the Ames test, BTR was negative. Additionally, in mammalian tests, BTR was negative in an in vitro cell mutation assay with Chinese hamster ovary (CHO) cells and in vivo in a mouse bone marrow micronucleus assay. In the Ames test, OHBTR was negative (Allen & Panfili, 1986). Clearly more research is needed on the genotoxicity of BTRs.

Table 1.1 Applications and environmental occurrences of benzotriazoles (BTRs) in this thesis

Benzotriazoles	Structure	Applications	Environmental occurrence
1H-Benzotriazole CAS 95-14-7		corrosion inhibitor; UV stabilizer; silver protection; antifogging agent	Glatt River, Switzerland, 0.64-3.7 µg/L; Landwehr Canal, Germany, 0.9 µg/L (Weiss et al., 2006); Rhine River, Europe, 0.13-0.35 µg/L (Reemtsma et al., 2009); Human urine, up to 11 ng/ml (Alexandros et al., 2013)
4-Methy-1H-benzotriazole CAS 29878-31-7		corrosion inhibitor; UV stabilizer; silver protection; antifogging agent	Rhine River, Europe, 0.2-0.5 µg/L; Havel River, Germany 2.1µg/L (Reemtsma et al., 2009); Hengstbach River, Germany 0.467 ng/L (Kiss & Fries, 2009)
5-Methly-1H-benzotriazole CAS 136-85-6		corrosion inhibitor; UV stabilizer; silver protection; antifogging agent	Danube River, Europe, 0.05-0.24 µg/L (Muller et al., 2012); Main, Hengstbach, and Hegbach River, Germany, 25-281 ng/L (Kiss & Fries, 2009)
Tolytriazole CAS 29385-43-1		corrosion inhibitor; UV stabilizer; silver protection; antifogging agent	Glatt River, Switzerland, 0.12-0.63 µg/L; Lake Tegel, Germany, 0.2 µg/L (Weiss et al., 2006); Streams, USA, 0.1-2.4 µg/L (Kolpin et al., 2002); Human urine, up to 4.4 ng/ml (Alexandros et al., 2013)
Hydroxybenzotriazole CAS 2592-95-2		additive used in oligonucleotide couplings and in racemization-free peptide coupling	Not reported
5-Chlorobenzotriazole CAS 94-97-3		corrosion inhibitor; UV stabilizer; silver protection; antifogging agent	Drinking water, Netherland, 0.01-0.2 µg/L (Pena et al., 2011)
5,6-Dimethyl-1H-benzotriazole monohydrate CAS 4184-79-6		chemical synthesis; heterocyclic building blocks	Human urine, up to 20.1 ng/ml (Alexandros et al., 2013); Drinking water, Netherland, 0.01-0.2 µg/L (Pena et al., 2011)

***Citations are in the reference list**

Table 1.2 Background information on benzotriazoles (BTRs) to be tested on fish cells

Benzotriazoles (Abbreviations)	Molecular weight (g/mol)	$LogK_{ow}$	WS* (mg/L)	Toxicity reported
1H-Benzotriazole (BTR)	119.13	1.17	>500	107 mg/L (48h EC50) in *D. magna*; 14.7 mg/L (48h EC50) in *D. gakeata* (Seeland et al., 2011); 35 mg/L (96 h LC50) in rainbow trout (Milanova et al., 2001)
4-Methy-1H-benzotriazole (4MBTR)	133.15	1.71	>500	118 mg/L (48h LC50) in *C. dubia*; 63 mg/L (96h LC50) in *P.promelas* (Pillard et al., 2000)
5-Methly-1H-benzotriazole (5MBTR)	133.15	1.71	>1000	51.6 mg/L (48h EC50) in *D. magna*; 8.13 mg/L (48h EC50) in *D. gakeata* (Seeland et al., 2011); 79 mg/L (48h LC50) in *C. dubia* (Pillard et al., 2000)
Tolytriazole (TT)	133.15	1.71	>500	1.73-2.15 mg/L (LC50 inhalation) in rat; 23.5-37.6 mg/L (21d EC50) in *D. magna* (Dummer, 2013); 31 mg/L (LC50) in bluegill; 600-675 mg/kg (LD50) in rat (EPA, 2012)
Hydroxybenzotriazole (OHBTR)	135.13	0.69	>1000	5000 mg/kg (LDLo oral) in rat (Acute toxicity data, 1992)
5-Chlorobenzotriazole (5CBTR)	153.57	1.81	>500	Not reported
5,6-Dimethyl-1H-benzotriazole monohydrate (DM)	165.19	3.05	Nearly Insoluble	0.8 mg/L (15 min EC_{50}) Microtox tests (Cancilla., 1997)

*WS=solubility in water with 0.5% DMSO

$LogK_{ow}$ values were calculated with EPISuite (version 4.1)

1.5 Benzothiazoles (BTHs)

BTHs are substances that contain a 1,3-benzothiazole skeleton. The skeleton is made up of a 5-membered 1, 3- thiazole ring fused to a benzene ring. A thiazole is a heterocyclic compound that contains both sulphur and nitrogen. Benzothiazole (BTH) can be considered the parent compound for BTHs and perhaps one of the most important derivatives is 2-Mercaptobenzothiazole (2MBTH) and its zinc and sodium salts, ZincMBTH and NaMBTH. Other important BTHs are 2-hydroxybenzothiazole (OHBTH) and 2-aminobenzothiazole (2ABTH). Chemists synthesize many derivatives of BTH. Several derivatives of concern are dyes, 3-3'diethylthiadicarbocyanine (DTDC), C.I. Sulphur orange I (SO), and C.I. vat yellow 2 (VY). Less studied BTHs are N,N-dichlohexyl-2-benzothiazolesulfene amide (NNA), 2,2'-dithiobis (benzothiazole) (DBTH), and 2-(p-aminophenyl)-6-methylbenzothiazole-7-sulfonic acid (MBTHS). Although a great variety of BTH derivatives are synthesized for commercial uses, in nature BTHs are rare.

1.5.1 BTHs in nature

BTHs have been found in only a few natural products (Le Bozec & Moody, 2009). The light-emitting compound, luciferin, from the firefly is partly made up of BTH (De Wever & Verachtert, 1997; White et al., 1961). Several BTHs are aroma or flavour compounds, which are compounds with a smell or odour. The first identified was 2MBTH in cranberries (Anjou & von Sydow, 1967). Subsequently, BTH and 2MBTH were found to be aroma constituents of tea leaves (Vitzthum et al., 1975). Tobacco smoke contains BTH (Schmeltz & Hoffman, 1977; Seo et al., 2000). BTH is found in some odour producing fungi, such as Polyporus frondosus and Aspergillus clavatus (Seiftert & King, 1982; Gallois et al., 1990). BTHs are likely in the marine biosphere. Several BTHs, including 2MBTH, were identified in culture extracts of a marine bacterium from the sponge *Tedania ignis* (Stierle et al., 1991).

1.5.2 Industrial production and uses of BTHs

BTHs are synthesized for industrial purposes around the world in large quantities. In the United States in 1980s, the annual production of BTHs was reported as over 163,000 tons (U.S.

International Trade Commission, 1982). In 1993 the manufacture of just BTH alone was reported to be between 4.5-450 tons in the US (Technical Resources International Inc, 1997). Although occurring naturally, 2MBTH is synthesized on an industrial scale. The annual production of 2MBTH has been listed at approximately 40,000 tons in Europe and more than 1 million pounds in the US (Chipinda et al., 2007).

The commercial uses of BTHs are wide ranging. Their main use is in rubber and latex manufacturing processes. In the rubber industry BTHs are vulcanization accelerators (Stolcova, & Hronec, 1996; DeWever & Verachtert, 1997; Nawrocki et al., 2005). By catalysing the formation of sulfide linkage between unsaturated elastomeric polymers, they allow the production of a flexible and elastic cross-linked material. BTHs, such as 2MBTH, are also used as antioxidants and stabilizers in the production of rubber and plastics (Chipinda et al., 2007). Another general use of BTHs is as biocides in a variety of industrial settings. For example, they are used as fungicides in lumber and leather industry and slimicides in paper and pulp industry (DeWever & Verachtert, 1997). They have also been used as herbicides, with one being the active ingredient of Tribunil (Cheng et al, 1978), and as algicides (DeWever & Verachtert, 1997). Another general application of BTHs is as corrosion inhibitors. For this purpose, they have been added to cooling water towers, car antifreeze, greases, and cutting oils (Brownlee et al., 1992; Reddy & Quinn, 1997). BTHs are used as photosensitizers in photography and are constituents of azo dyes (Reddy & Quinn, 1997; Asimakopoulos et al., 2013).

1.5.3 BTHs in medicine and in food

Some BTHs are prepared for humans either as pharmaceuticals for different medical conditions or as flavourings for foods. Being a heterocyclic compound, BTH is the starting material for the synthesis of larger structures. These often have biological activities, such as antitumor, antimicrobial, antidiabetic and anti-inflammatory (Ali and Siddiqui, 2013). Some 2-substituted benzothiazoles that have pharmaceutical uses include the immunosuppressive drug frentizole and the calcium channel blocker fostedil (Kumar et al., 2014). One BTH, 2-sulfinylbenzoathiazole (XS238), was a promising inhibitor of vacuolar H+-ATPase inhibitor and might have value in treating osteoporosis and other bone diseases (Corbett et al., 1997). A series of BTH derivatives have been explored for their potential to be p53 inhibitors (Christodoulou et

al., 2011). These include the commercially available pifithrin-α (PFT-α) and pifithrin-β (PFT-β). PFT-β is also known as cyclic PFT-α. A few BTHs provide flavour to food and BTH has been found in wine (Bellavia et al., 2000).

1.5.4 Release and detection of BTHs in the environment

BTHs enter the environment in several ways. They leach from rubber products, especially fine particles from automobile tires (Reddy & Quinn, 1997; De Wever & Verachtert, 1997). Water runoff from paved roads has been shown to be a diffuse source for the entry of BTHs into streams (Fries et al., 2011). WWTPs also release BTHs. BTHs have been measured in both influent and effluent of WWTPs (Fries et al, 2011). Some BTHs, like 2MBTH, seem resistant to breakdown by activated sludge (De Wever & Verachtert, 1997). Therefore, WWTPs can be a point source for the entry of BTHs into the aquatic environment. Another source is leaching from landfills because many products that contain 2MBTH are put into landfills. The annual flux of BTHs into Pearl River delta in China has been estimated at 79 tons per year (Ni et al., 2008).

The measurement of BTHs in environmental samples is done by methods similar to those used for BTRs (see section 1.6.2), but some methods have been developed more specifically for BTHs. One new method that has been developed for detecting BTH in untreated wastewater is stir bar sorptive extraction (SBSE) (Fries, 2011). SBSE is a solvent-free extraction technique that allows the extraction and enrichment of organic compounds from aqueous matrices. A novel polyacrylate (PA)-coated stir bar (PA Twister®) was used for untreated wastewater in Germany. After extraction, desorption was done in a thermal desorption system (TDS) and analysis done by gas chromatography-mass spectrometry (GC-MS). This method performed better than all previously used extraction techniques for measuring BTHs in wastewater (Fries, 2011).

1.5.4.1 BTHs in aquatic environments

BTHs have been found in natural and man-made water bodies. When BTHs were analyzed in samples from 15 rivers in the Schwarzbach watershed of Germany, they were detected in all rivers (Fries et al., 2013). The concentrations ranged from 58 to 856 ng/L. In the Pearl River Delta of China the concentrations of BTHs ranged from 220 to 611 ng/ml (Ni et al., 2008). The

most prominent derivative was BTH. BTHs have also been detected in marine water samples from a coastal-industrial zone in the northern Aegean Sea (Grigoriadou et al., 2008) and from the German Bight of the North Sea (Bester et l., 1997). BTHs have also been reported in groundwater and drinking water (Fries et al., 2013; Van Leerdam et al., 2009). Wastewaters from Australia, China, and Europe have been found to contain BTHs (Fiehn et al., 1994; Kloepfer et al., 2005; Asimakopoulos et al., 2013; Loi et al., 2013). For untreated wastewater from Beijing China, the mean BTH concentrations were 2.629 μg/L in industrial wastewater, 2.26 μg/L in municipal wastewater, and 0.131 μg/L in domestic wastewater (Kloepfer et al., 2005). Overall the results from various countries suggest that BTHs are a ubiquitous occurrence in the aquatic environment.

1.5.4.2 BTHs in soil

Only a limited amount of work has been done on the subject of BTHs in soil. Sewage sludge was found to contain 2-hydroxybenzothiazole (Herrero et al., 2014) but soil appears not to have been examined. However, the mobility of BTHs in soil has been considered. Based on an estimated KOC value of 295, BTH is expected to have moderate mobility in soil (Hansch et al., 1995). Based on an estimated soil organic carbon-water partitioning coefficient (KOC) value of 1600, 2MBTH is expected to have medium to low mobility in soil, depending on the pH. If released on land, leaching is more likely to occur in alkaline soil (Albaiges, 1982). Based on an estimated KOC value of 1600, 2ABTH is expected to adsorb to suspended soil and sediment in water (Rouchaud et al., 1988).

1.5.4.3 BTHs in air

Little research has been done on BTHs in air. The potential of BTH to volatize has been noted (Reddy & Quinn, 1997). BTH was among a series of potential organic emissions that were looked for in the air around waste incineration plants in Germany (Jay & Stieglizt, 1995). BTH was found at 0.3 $\mu g/m^3$.

1.5.4.4 Degradation of BTHs in the environment

The stability of BTHs in the environment and how BTHs might be removed from wastewaters have been subjects of considerable research efforts. In natural sunlight, 2MBTH undergoes photolysis to BTH (Brownlee et al., 1992). However, 2MBTH might also undergo biomethylation to form 2-(methyl-thio) benzothiazole, which is resistant to sunlight photolysis (Brownlee et al., 1992). Gamma irradiation has been shown to have some potential as a method for 2MBTH removal from industrial wastewater (Bao et al., 2014). Another approach being explored is destruction through ozonation (Derco et al., 2014). However by far, biodegradation has received the most attention (De Wever & Verachter, 1997). Yet the extent to which BTHs are removed during transit through WWTP varies from report to report and with different BTHs (Derco et al., 2014; De Vos et al., 1993). BTH resisted anaerobic degradation, but degraded rapidly under aerobic conditions into OHBTH and dihydrogenobenzothiazole (De Wever et al., 1998). Yet some BHTs are not readily biodegraded. 2MBTH appears to be one of the more stable BTHs and generally inhibits the degradation of organic compounds in the wastewater treatment process (De Wever & Verachter, 1997). BTHs are not expected to bioaccumulate in aquatic organisms because of their water solubility (Chudoba et al., 1977; Lyman et al., 1990; Reddy & Quinn 1997).

1.5.5 Toxicology of BTHs

The toxicology of BTHs is reviewed in this section of the thesis, with the focus on the toxicology of BTHs to humans, laboratory rodents and aquatic organisms, especially fish.

1.5.5.1 Toxicology of BTHs to humans

Very limited information is available on the toxicity of BTHs to humans. Generally BTHs are listed on Material Safety Data Sheets (MSDS) as eye and skin irritants. The cause of allergic contact dermatitis from various types of rubber footwear was identified as 2MBTH and DBTH (Kaniwa et al., 1994). Other studies have found 2MBTH to be a common cause of contact allergy in rubber items (Zina et al., 1987; Wilkinson et al., 1990; Ikarashi et al., 1993). Epidemiology studies on workers at a chemical factory suggested that 2MBTH was a possible

human carcinogen (Sorhan, 2009). In recombinant yeast assays (RYA), BTH was found to activate the human aryl hydrocarbon receptor (AhR) and estrogen receptor, with EC50s respectively of 10.2 mg/L and 5.5 mg/L (Noguerol et al., 2006).

Information on the exposure of humans to BTHs is limited. Perhaps their first detection in humans was the measurement of BTH in human atherosclerotic aortas, presumably from tobacco smoke (Ferrario et al., 1985). BTHs were detected in the urine of people living in Japan, India, Vietnam, China, Greece, and US (Asimakopoulos et al., 2013). The detection rate was 100 % in Vietnam and BTH was the major derivative. BTHs have also been looked for in dust samples from households and offices in the USA, China, Japan and Korea (Wang et al., 2013). Indoor dust samples from all countries had BTHs, but the concentrations and compounds varied among the four countries, being especially high in Korea (2000 ng/g) and in the US (1290 ng/g). The most abundant one was OHBTH. Proportionally, dust from urban homes had more OHBTH than dust from rural homes. Possibly this contaminant came from rubber tires from high traffic areas in urban locations. The daily intake of BTHs through indoor dust ingestion was estimated. For Korean children, this was calculated as 4.22 ng/kg/bw/day (Wang et al., 2013).

1.5.5.2 Toxicology of BTHs to laboratory rodents

The lethal doses for some BTHs in rodents are available from anonymous reports by industry and government agencies. The US Environmental Protection Agency (EPA) gives for NNA an oral lethal dose 50 (LD_{50}) in rats of more than 5000 mg/kg/bw and a dermal LD_{50} for rabbits at more than 2000 mg/kg/bw (ITC/USEPA). The oral LD_{50}s of DTDC for rat and mouse were 165-192 mg/kg and 4-16 mg/kg, respectively (Booth & McDonald, 1982). DBTH has low toxicity to lab rodents. The oral LD_{50} of DBTH for mouse and rat were 7000 mg/kg and more than 12000 mg/kg, respectively (International Polymer Science and Technology, 1976; National Technical Information Service) and had no adverse effects on pre- and postnatal development of the rat (Ema et al., 1989). In an acute oral study, the LD_{50} of 2ABTH was more than 1000 mg/kg for mouse (Vigorita et al., 1990).

A few papers on the developmental and biochemical effects of BTHs in mammals have appeared. In gestation day 9.5 rat embryos, yolk sac circulatory system and tail development was inhibited by 2ABTH and optic and tail development by BTH (Han et al., 1999). BTH increased the mRNA levels of some hepatic glutathione S-transferases (GSTs) in adult rats (Kim & Cho, 1996) and was metabolized through thiazole ring scission in guinea pigs (Wilson et al., 1991). BTH was found to inhibit rabbit liver aldehyde oxidase but not to act as a substrate (Gristwood & Wilson, 1988). The authors attributed the inhibition to chelation of iron or molybdenum atom in the enzyme by the sulfur atom in the thiazole nucleus.

Among the BTHs, the most information is available for 2MBTH. 2MBTH was found to be an in vivo and in vitro inhibitor of the murine dopamine β-hydroxylase, which catalyzes the formation of the neurotransmitter noradrenaline (Johnson et al., 1970). The intravenous LD_{50} of 2MBTH in mice was 178 mg/kg (U.S. Army Armament Research & Development Command). The mice oral LD_{50}s of 2MBTH were 1558 and 1490 mg/kg for male and female, respectively (Ogawa et al., 1989). In an acute oral study, the LD_{50} of NaMBTH were 1615 and 1337 mg/kg for male and female Sprague-Dawley rats, respectively. In a 13-week dermal toxicity study, the LOEL of NaMBTH was 1000 mg/kg/d for Sprague-Dawley rats (RED, 1994). The LD_{50}s of ZincMBTH were 5735 and 5221 mg/kg for male and female Sprague-Dawley rats, respectively (RED, 1994). In a two year neoplastic study, 2MBTH increased incidences of mononuclear cell leukemia, pancreatic acinar cell adenomas, adrenal gland pheochromocytomas, and preputial gland adenomas or carcinomas (combined) in male F334/N rats and adrenal gland pheochromocytomas and pituitary gland adenomas in female F334/N rats (NTP, 1998). By contrast, 2MBTH showed no reproductive effects in a two-generation study on Sprague-Dawley rats (Mercieca et al., 1991).

1.5.5.3 Toxicology of BTHs to fish

The toxicity of BTHs to fish has been explored with just a few BTHs and fish species. The BTHs have been BTH, VY and 2MBTH. For in vivo studies the species were medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carasssium auratus*), bluegill (*Lepomis macrochirus*), and sheepshead (*Cyprinodon variegatus*).

For exposure of medaka to BTH, the 48 h LC_{50} was 110 mg/L (Yoshitada et al., 1986). The LD_{50} of VY for fathead minnows was higher than 180 mg/L (Little & Lamb, 1972). For 2MBTH, the 96 hours LC_{50} for rainbow trout was 1.3-6.2 mg/L and the approximate 48 hours fatal concentration for goldfish was 2 mg/L (Verschueren, 1983; Milanova et al., 2001). The 24 hours LD_{50} of NaMBTH were 2.0 and 5.6 mg/kg for rainbow trout and bluegill, respectively (ITC/USEPA, 1983). When sheepshead larvae were exposed to 3.75, 7.5, 15, 30 and 60 mg/L of BTH, mortality occurred after 5 days exposure to 60 mg/L ($LC_{50} = 41.9$) (Evans et al., 2000). Larval growth was decreased at all concentrations. Histology revealed that the morphology of the gills had been altered but the histology of the central nervous system appeared normal. When brain cells in primary culture and two epithelial cell lines were exposed to BTH, the epithelial cell lines were more sensitive and significant cytotoxicity was observed at 30 and 60 mg/L of BTH. The authors concluded that the BTH is a gill toxicant and not a neurotoxicant.

1.5.5.4 Toxicology of BTHs to other aquatic multicellular organisms

The toxicology of BTHs to a few other aquatic animals has been examined. These are the water fleas, *Daphnia magna* and *Ceriodaphnia dubia*, and the African clawed toad, *Xenopus laevis*. For D. magna, the BTH EC_{50} to was 50 mg/mL (Hendriks et al., 1994). The acute and chronic toxicities of 2-thiocyanomethylthiobenzothiazole (TCMTB), BTH, 2MBTH, and OHBTH to C. dubia were studied (Nawrocki et al., 2005). TCMTB was the most toxic with acute and chronic EC_{50}s of 15.3 and 9.6 µg/L. In Xenopus, BTH promoted the binding affinity of γ-aminobutyric acid type A (GABAA) to receptors responsible for neurotransmission in the central nervous system (Hossain et al., 2003). The toxicological consequences of this were unclear. Also in Xenopus, 2MBTH was found to cause thyroid disruption (Tietge et al., 2013). The chemical inhibited in vitro activity of thyroid peroxidase, which is a key enzyme in the synthesis of thyroid hormone. When Xenopus larvae were exposed to 2MBTH for 21 days, the level of circulating T4 was reduced, the histology of the thyroid was altered, and metamorphosis was delayed.

1.5.5.5 Cytotoxicity of BTHs

A considerable amount of work has been done on the antibacterial effects of BTHs. 2MBTH and BTH impaired luminescence processes in *Vibrio fischeri* (Reemtsma et al., 1995). Growth of several bacteria was inhibited about 50 % at 42 mg/L and 100 % at about 135 mg/L (Folinova et al., 1978). Some studies suggested that 2MBTH was bacteriostatic rather than bactericidal (De Wever & Verachtert, 1997). The actions of BTHs on complex mixtures of bacteria also have been studied. At 7 mg/L BTH inhibited activated sludge respiration (Walker, 1989). 2MBTH also inhibited the nitrifying activity of activated sludge (Tomlinson et al., 1966).

Several possible mechanisms that might underlie the toxicity of BTHs to bacteria have been investigated (De Wever & Verachtert, 1997). 2MBTH inhibited bacterial tryptophan synthetase, membrane-bound lactate dehydrogenase, and several enzymes of the glycolytic pathway. However, because at physiological pHs 2MBTH was hydrophobic, the primary target was thought to be the cell membrane (De Wever & Verachtert, 1997). This was supported by several experimental results. 2MBTH caused potassium leakage. In preparations of bacterial cell membrane fragments, 2MBTH inhibited the electron transport chain. This was attributed to a general effect on cell membrane linked processes.

The antifungal properties of BTHs have been evaluated on yeast and several other fungi. For 15 Candida strains, growth was inhibited by 50 % at concentrations between 1 and 78 mg/L of 2MBTH (Bujdakova et al., 1993). 2MBTH strongly inhibited the growth of *Trichophyton rubrum, Microsporum gypseum*, and *Epidermophyton floccosum* (Foltinova et al., 1978). The thiol group of 2MBTH appeared to be essential for toxicity because BTH was a less effective fungicide (De Wever & Verachtert, 1997).

To date, the only study of BTHs on protozoans appears to have been done with the ciliated protozoan, *Tetrahymena pyriformis*. The concentration of BTH that caused a 50 % inhibition (EC_{50}) in the growth over 24 h was 160 mg/L (Yoshioka et al., 1986).

For animal cells in culture, work on the cellular effects of BTHs has been restricted to studies on a few cell lines from mammals and on two cell lines and primary cell cultures from fish. When a recombinant mouse hepatoma cell line, Hepa1c1c7, was used in a chemical-activated luciferase gene expression assay (CALUX), 2MBTH was identified as an Ah receptor agonist (He et al., 2011). In cultures of the human cell line, HaCT, several parameters were monitored upon the addition of 2MBTH (McKim et al., 2010). The expression of mRNA for cytochrome P450 1A1 (CYP 1A1) and for glutamate cysteine ligase catalytic subunit (GCLC) was increased in a dose-dependent manner. 2MBTH did not alter expression of several other genes or deplete cells of glutathione. HaCT cell viability was monitored with the tetrazolium salt (3-(4,5-dimethylthiazoyl)-2,5-diphenyltetrazolium bromide) (MTT). The EC_{50} was >2500 μM 2MBTH. MTT also was used to compare the viability of primary brain cell cultures from sheepshead minnow and tilapia with cultures of two fish epithelial cell lines, FHM from fathead minnow and CCO from catfish, upon exposure to BTH (Evans et al., 2000). The epithelial cells were more sensitive than the brain cells to BTH, supporting the contention that BTH is a gill toxicant and not a neurotoxicant. Clearly more research is needed on the effects of BTHs on the cells of vertebrates.

1.5.5.6 Genotoxicity of BTHs

Studies have been done on the possible genotoxicity of several BTHs, BTH, 2MBTH, VY, DBTH and NNA. BTH was not mutagenic in the bacterial Ames test or in a mammalian gene mutation test, the mouse lymphoma assay (MLA) with the leukaemia cell line, L5178Y tk+/- (Seifried et al., 2006). 2MBTH was examined in bacterial Ames test with and without S9 extract and found not to be mutagenic in either form of the test (Zeiger et al., 1987; Yamaguchi et al., 1991). In rats no significant covalent binding of 2MBTH to DNA was observed (Brewster et al., 1989). However, 2MBTH might be genotoxic to mammals. In the MLA with a rat liver S9, 2MBTH was mutagenic (NTP, 1998). As well, 2MBTH (351.8-400.8 µg/ml) increased the frequency of chromosomal aberrations and sister chromatid exchanges in Chinese hamster ovary (CHO) cells (Anderson et al., 1990). Similar results were obtained with VY and MBTHS. VY was negative in the Ames test without or with an S9 extract but positive when tested in the MLA with an S9 extract (National Cancer Institute). DBTH was mutagenic in the bacterial Ames test

for some workers (Zeiger et al., 1988). Yet others found that DBTH was not mutagenic in the Ames test but did induce genetic damage to mammalian cells (Crebelli et al., 1984; Hinderer et al., 1983). NNA was not mutagenic in either bacterial or mammalian cell tests (B G Chemie, 1994). Overall the genotoxicity of BTHs needs more research.

Table 1.3 Applications and environmental occurrences of benzothiazoles (BTHs) in this thesis

Benzothiazoles	Structure	Applications	Environmental occurrence*
3,3'-diethylthiadicarbocyanine iodide CAS 514-73-8		fluorescent indicators and probes; laser dyes; photographic emulsions; veterinary anthelmintic; heartworm microfilaricide	Not reported
C.I. Sulphur orange 1 CAS 1326-49-4		dyeing cotton, linen, viscose fibers	Not reported
2-Mercapto-benzothiazole CAS 149-30-4		vulcanization accelerator; antifungal drug; coating agent; corrosion inhibitor	Estimated release amount: United States, 3962-12871 lbs (TOXMAP, 2012); Catalonian Rivers, Spain, 0.01-12.8 μg/L (Céspedes et al., 2006)
Zinc 2-Mercapto-benzothiazole CAS 155-04-4		rubber accelerator; antioxidant; bacteriostat; fungicide; paints; latex, oil, varnish; adhesives	United States production: 927 tons in 1979 (IARC Monographs, 1982)
Sodium 2-Mercapto-benzothiazole CAS 2492-26-4		bacteriostat; fungicide; emulsions, resin, latex, polymer metal working cutting fluids	United States production: 6639 tons in 1979 (IARC Monographs, 1982) United States production: 20000 tons in 1982 (Monsanto)
2-Hydroxy-benzothiazole CAS 934-34-9		organic Intermediates; plant growth regulator; vulcanization accelerator	Human urine, up to 14.7 ng/ml (Alexandros et al., 2013); Catalonian Rivers, Spain, 0.01-0.17 μg/L (Céspedes et al., 2006)
2-Amino-benzothiazole CAS 136-95-8		reactants or reaction intermediates; photographic chemicals	Human urine, up to 2.7 ng/ml (Alexandros et al., 2013)
Benzothiazole CAS 95-16-9		vulcanization accelerator; fungicide; herbicide; corrosion inhibitor; flavoring substance	German bight of the North Sea, 0.03-2.74 ng/L (Bester et al., 1997); Pearl River Delta, China, 162-476 ng/L (Ni, et al., 2008); Human urine, up to 181 ng/ml (Alexandros et al., 2013); Drinking water, Alberta, Canada, 0.1-1 μg/L (Headley, 1987)
C.I. Vat yellow 2 CAS 129-09-9		ink; leather; paint dyestuffs	Not reported
N,N-Dicyclohexyl-2-benzothiazolsulfene amide CAS 4979-32-2		rubber chemicals	Not reported
2,2'-Dithiobis (benzothiazole) CAS 120-78-5		heterocyclic buiding blocks; rubber chemicals	Not reported
2-(p-aminophenyl)-6-methylbenzothiazole-7-sulfonic acid CAS 130-17-6		intermediates of dyes and pigments	Not reported

***Citations are in the reference list**

Table 1.4 Background information on benzothiazoles (BTHs) to be tested on fish cells

Benzothiazoles (Abbreviations)	Molecular weight (g/mol)	$LogK_{ow}$	WS* (mg/L)	Toxicity reported
3,3'-diethylthia dicarbocyanine iodide (DTDC)	518.48	1.96	>100	4-16 mg/kg (LD_{50} oral) in mouse (Booth& Mcdonald., 1982); 1 mg/kg (LD_{50} intravenous) in mouse (U.S. Army Armament Research & Development Command)
C.I. Sulphur orange 1 (SO)	N/A	N/A	>20	Not reported
2-Mercaptobenzothiazole (2MBTH)	167.25	2.42	>100	0.73 ppm (LC_{50}) in rainbow trout; 2.9 ppm (EC_{50}) in daphnia (RED, 1994); 100 mg/L is toxic to several types of bacteria (De Wever et al., 1994); 1.3-6.2 mg/L (96 h LC_{50}) in rainbow trout (Milanova et al., 2001)
Zinc 2-Mercaptobenzothiazole (ZincMBTH)	397.88	5.02	>50	5505 mg/kg oral lethal dose in rat (RED, 1994); 200 mg/kg (LD_{50} intraperitoneal) mouse (National Technical Information Service)
Sodium 2-Mercaptobenzothiazole (NaMBTH)	189.23	-0.48	>100	1476 mg/kg oral lethal dose in rat (RED, 1994); 0.5-5 g/kg oral lethal dose in human (Gosselin et al., 1976); 1.6-2.4 mg/L (24 h LC_{50}) in rainbow trout; 4.6-7.1 mg/L (24 h LC_{50}) in blue gill (ITC/USEPA, 1983)
2-Hydroxybenzothiazole (OHBTH)	151.19	1.76	>300	15.1 mg/L (48 h EC_{50}) in C. dubia (Nawrocki et al., 2005)
2-Aminobenzothiazole (2ABTH)	150.20	2.00	>500	When heated to decomposition, it emits highly toxic fumes (Sax, 1979); >1000 mg/kg (LD_{50} oral) in mouse (Virgorita et al., 1990)
Benzothiazole (BTH)	135.19	2.01	>1000	50 mg/L (EC_{50}) in D.magna (Hendriks et al., 1994); 900 mg/kg (LD_{50} oral) in mouse (Moran et al., 1980); 95 mg/kg (LD_{50} intravenous) in mouse (Clayton & Clayton., 1981)
C.I. Vat yellow 2 (VY)	474.55	7.22	>100	>180 mg/L (96 h LD_{50}) in fathead minnow (Little & Lamb., 1972)
N,N-Dicyclohexyl-2-benzothiazolsulfene amide (NNA)	346.55	4.80	>250	>5000 mg/kg/bw (LD_{50} oral) in rat; >2000 mg/kg/bw (LD_{50} dermal) in rabbit (BG Chemie, 1994)
2,2'-Dithiobis (benzothiazole) (DBTH)	332.49	4.66	10<	100 mg/kg (LD_{50} intraperitoneal) in mouse; >12000 mg/kg (LD_{50} oral) in rat (National Technical Information Service)
2-(p-aminophenyl)-6-methylbenzothiazole-7-sulfonic acid (MBTHS)	320.39	0.41	>200	178 mg/kg (LD_{50} intraperitoneal) in mouse (U.S. Army Armament Research & Development Command)

***WS=solubility in water with 0.5% DMSO**

$LogK_{ow}$ values were calculated with EPISuite (version 4.1)

1.6 Thesis objectives

The rainbow trout cell lines, RTgill-W1 from the gill and to a lesser extent RTL-W1 from the liver are used in this thesis to study two p53 inhibitors and nineteen emerging contaminants. Phosphoprotein p53 is a master regulatory protein in mammals and the inhibitor, pifithrin-μ or PES, impairs the movement of p53 to the mitochondria and the inhibitor, pifithrin-α or PFT-α, prevents p53 from acting as a transcription factor in the nucleus. The emerging contaminants are seven benzotriazoles (BTR) and twelve benzothiazoles (BTH). The four thesis objectives are:

1. To evaluate the responses of fish cells to the p53 inhibitor, pifithrin-μ or PES (Chapter 2).

2. To evaluate the responses of the fish cells to the p53 inhibitor, pifithrin-α (Chapter 3).

3. To evaluate the cytotoxicity and genotoxicity of benzotriazoles to fish cells (Chapter 4).

4. To evaluate the cytotoxicity and genotoxicity of benzothiazoles to fish cells (Chapter 5).

CHAPTER 2

An inhibitor of p53 and/or HSP70, 2-phenylethyne-sulfonamide, causes oxidative stress, unfolded protein response and apoptosis in the rainbow trout gill cell line, RTgill-W1*

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2.1 Introduction

Understanding the key regulators of fundamental cellular events in fish cells is essential in order to effectively use fish as sentinels of environmental and human health (Sherry, 2003; Van der Schalie et al., 1999). However, most research has focused on rodent and human cells, and regulation in fish cells is poorly understood and might not always proceed exactly as in higher vertebrates (Lu and Abrams, 2006). For mammalian cells p53 is a central regulatory protein (Galluzzi et al., 2011; Ryan, 2011) and the heat shock protein (HSP) 70 family is a key to maintaining protein homeostasis and signalling pathways (Mayer and Bukau, 2005). Both p53 and the HSP70 family regulate a fundamental cellular event, cell death, especially by apoptosis (Dudeja et al., 2009). One approach for dissecting the linkages between regulatory proteins and cellular actions has been the development of specific inhibitors. 2-phenylethynesulfonamide (also known as pifithrin-μ or PES) was identified as an inhibitor of p53 (Strom et al., 2006), often acting in an anti-apoptotic manner (Hagn et al., 2010) or acting in a pro-apoptotic fashion in some cases (Steele et al., 2009). Subsequently PES was found to inhibit two members of the HSP70 family, HSP70 and heat shock cognate 70 (HSC70) (Leu et al., 2009, 2011). Such inhibitors are being intensively studied with mammalian cells in order to develop chemotherapeutic strategies (Leu et al., 2011). Yet, they also could be used as basic research tools to understand the regulation of key processes in fish and fish cells, but first more information is needed on how fish cells might respond.

For fish, exposure to environmental contaminants is expected to begin at the gills. Therefore, the effect of PES on the rainbow trout gill epithelial cell line, RTgill-W1 (Bols et al., 1994), has been studied. RTgill-W1 express p53 (Liu et al., 2011) and have been used for a variety of research purposes (Lee et al., 2009), including investigations of apoptosis (Krumschnabe et al., 2007). PES killed RTgill-W1 in a process with the characteristics of apoptosis and caused the accumulation in the detergent-insoluble fraction of several proteins, including HSP70 and p53. Although these actions were reminiscent of reports with some mammalian cell lines, a unique feature with the fish cells was the blocking of killing and insoluble-protein accumulation with the reactive oxygen species (ROS) scavenger, N-acetylcysteine (NAC). In RTgill-W1, PES appears to be acting as an inhibitor of HSP70 but p53 inhibition might still have a role.

2.2 Materials and Methods

2.2.1 Cell culture

RTgill-W1, a rainbow trout gill epithelial cell line developed in our laboratory (Bols et al. 1994), was routinely cultured in 75 cm² culture flasks (Nunc, Kamstrupvej, Denmark) at room temperature in Leibovitz's L-15 culture medium (Sigma-Aldrich, Ltd., Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin solution (10000 units/ml penicillin, 10 mg/ml streptomycin, Sigma-Aldrich)

2.2.2 Cytotoxicity assay

2.2.2.1 Plating and dosing

Cells were seeded in 96 well plates (Becton and Dickinson Company, Franklin Lakes, NJ. USA) at a density of $4x10^4$ cells per well in 200 µl of L-15 growing medium with 10% FBS supplement. Cells were allowed to settle and reattach for 24 h at room temperature before being exposed to any compounds. The cells were then dosed with varying concentrations of 2-phenylethynesulfonamide (PES) (Calbiochem, La Jolla, CA) in L-15 with or without 10% FBS. For co-exposure, cell cultures were pre-treated with pan caspase inhibitor (z-VAD-fmk) (Calbiochem, La Jolla, CA) or N-acetylcysteine (NAC, Sigma) 1 h before adding PES. Application of chemicals to cell cultures was done by adding culture medium mixed with chemical solution to the culture well. The final concentration of the solvents (such as DMSO or water) in each well was the same as for the control wells, which were only dosed with solvent. After 24 h, cultures were evaluated for cytotoxicity. In no cases was the solvent used at a concentration that was cytotoxic.

2.2.2.2 Measuring cell viability

Three fluorescent indicator dyes were used to evaluate cell viability. Metabolic activity was measured by Alamar Blue (Medicorp, Montreal, PQ). Cell membrane integrity was evaluated with 5-carboxyfluorescein diacetate (CFDA-AM) (Molecular Probes, Eugene, OR). Lysosome integrity was monitored with Neutral Red (Sigma-Aldrich). Alamar Blue, CFDA-AM and

Neutral Red were prepared in Dulbecco's phosphate buffered saline (DPBS, Lonza, Walkersville, MD USA) to give final concentrations of 5% (v/v), 4 µM and 1.5% (v/v) respectively. Cells were incubated with dyes for 1 h in dark then quantified by fluorescence plate reader (Spectra-max Gemini XS microplate spectrofluorometer; Molecular Devices, Sunnyvale, CA). The excitation and emission wave-lengths used were 530 and 590 nm for Alamar Blue, 485 and 530 nm for CFDA-AM, 530 and 640 nm for Neutral Red. Results were calculated as a percent of the control culture

2.2.3 Determining apoptosis

In order to test for apoptosis, cultures were evaluated for nuclear fragmentation, genomic DNA laddering, membrane phospholipid phosphatidylserine (PS) translocation, caspases activity and mitochondrial membrane potential change. RTgill-W1 cells were seeded at a density of $4x10^5$ cells in $9cm^2$ slide flasks (Nunc, Kamstrupvej, Denmark) for H33258 staining and at a density of $1x10^6$ cells in 25 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) for DNA gel electrophoresis, PE Annexin V assay, caspase activity assay and $DiOC_2(3)$ assay. Cells were incubated at room temperature for 24 h in L-15 growth medium with 10% FBS and then exposed to varying concentrations of PES in L-15 without 10% FBS.

2.2.3.1 Hoechst 33258 Stain

24 h after treatment, RTgill-W1 cultures were fixed by adding an equal volume of Carnoy’s fixative (methanol:glacial acetic acid, 3:1), which was prepared fresh with each use, to existing media, exposing the cells for 2 min. The media and fixative were then removed and fresh fixative was added to the cells twice for 5 min. Following fixation, the cells were stained with 0.5 µg/ml Hoechst 33258 for 10 min in dark. After several washes with deionized water and the final wash with Mcllvaine’s buffer, a coverslip was mounted onto the slide with Mcllvaine’s buffer and glycerol (1:1). The fluorescent nuclei were visualized using a fluorescent microscope with an ultra-violet (UV) filter (Nikon Optishot).

2.2.3.2 DNA fragmentation ladder

24 h after treatment, cells were collected and genomic DNA was extracted using a GenElute™ mammalian genomic DNA miniprep kit according to manufacturer's instructions (Sigma-Aldrich). 25 µl of DNA was resolved by electrophoresis on a 2% (w/v) agarose gel mixed with gel red (1 in 10000) (Biotium, CA) for 3 h at 60 V. The DNA ladders were visualized under UV transillumination.

2.2.3.3 PE Annexin V

Early stage apoptosis was examined using a PE Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions. PE -Annexin V detects early stage apoptosis by binding phospholipid phosphatidylserine (PS) which is externalized to the outer leaflet of the plasma membrane in apoptotic cells. 7-Amino-Actinomycin (7-AAD), a DNA intercalating dye, was added along with PE-Annexin V to detect compromised membrane integrity. After treatment, cells were harvested by trypsinization at different time periods (6, 12, 18 and 24 h) and washed twice with cold DPBS. The cells were then resuspended in 1 ml of 1X binding buffer ($1x10^5$ cells per assay). The suspended cells were incubated with PE Annexin V and 7-AAD for 15 min at room temperature in the dark. Then, 400 µl of 1X binding buffer was added to the cells for flowcytometric analysis (10000 events/sample) and data were analyzed by the Flowjo software (Treestar, Inc., San Carlos, CA).

2.2.3.4 Caspase activity assay

24 h after treatment, the activities of caspase-3, -8 and -9 were measured using a colorimetric assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. Cells were lysed in chilled cell lysis buffer and 100 µg protein was incubated at 37°C for 1 h with 200 µM DEVD-pNA (caspase-3 substrate), IETD-pNA (caspase-8 substrate) or LEHD-pNA (caspase-9 substrate). Caspase-3, -8 and -9 activities were measured by spectrophotometric detection of the chromophore pNA at 405 nm.

2.2.3.5 Mitochondrial membrane permeability changes

Changes in mitochondrial membrane permeability were assessed by using the MitoProbe™ $DiOC_2(3)$ assay kit (Invitrogen). $DiOC_2(3)$ (3′,3′-diethyloxacarbocyanine iodide) at concentrations below 100 nM, accumulates in mitochondria with active membrane potentials. The stain intensity decreases with reagents such as CCCP (carbonyl cyanide 3-chlorophenylhydrazone) that disrupt mitochondrial membrane potential.$DiOC_2(3)$ is visualized by flow cytometry with excitation at 488 nm and a green emission filter (FL-1: 530 nm) according to the manufacture's instructions. This method allows quantification of cells with depolarized mitochondria. Briefly, PES treated cells were harvested by trypsinization at different time periods (6, 12, 18 and 24 h). After trypsinization, cells were washed once in pre-warmed DPBS and incubated with 5 µl of $DiOC_2(3)$ (50 nM) at 37°C for 30 min. As a positive control, cells were incubated with 1 µl of CCCP (50 µM) for 5 min at 37°C before $DiOC_2(3)$ staining. After washing, cells were resuspended in PBS for flow cytometric analysis (10000 events/sample) and histograms were analyzed by the Flowjo software (Treestar, Inc., San Carlos, CA).

2.2.4 Intracellular ROS measurement

Intracellular ROS were determined by using 2′,7′-Dichlorofluorescin diacetate (H2DCFDA) (Sigma- Aldrich). RTgill-W1 cells were seeded in 96 well plates at a density of $4x10^4$ cells per well in L-15 growth medium with 10% FBS. After 24 h incubation at room temperature to allow reattachment, the cells were treated with different concentrations of PES with or without 5mM NAC for 1-24 h. Cells were then incubated with H_2DCFDA working solution of 10 µM in PBS at RT for 40 min. Afterwards, cells were washed twice with pre-warmed PBS and incubated with pre-warmed PBS for 10 min. ROS were measured using a fluorescent plate reader (Spectra-max Gemini XS microplate spectrofluorometer; Molecular Devices, Sunnyvale, CA) at an excitation and emission wave-lengths of 485 and 530 nm. ROS level was expressed as percentage of the fluorescence over control samples.

2.2.5 Western blot

RTgill-W1 cells were seeded in 25 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) at $1x10^6$ cells per flask in L-15 growth medium with 10% FBS. After 24 h incubation at room temperature to allow reattachment, the cells were treated with different concentrations of PES with or without 5mM NAC, and incubated again at room temperature for 24 h. Whole-cell protein extracts were prepared as described by Leu et al. (2009) and protein concentrations were determined with bicinchoninic acid (BCA) protein assay according to manufacturer's instructions (Pierce, Rockford, IL, USA). Protein was separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5% milk for 1 h, incubated with primary rabbit anti-rainbow trout p53 antibody (1:200) (Liu et al. 2011), rabbit anti-Xenopus HSP70 antibody (1:200) (Gauley et al. 2008) or rabbit anti-GRP78/BiP antibody (Sigma-Aldrich) (1:1000) for 2 h, then incubated with secondary goat anti-rabbit AP antibody (1:30000) (Bio-Rad, Hercules, CA) for 1 h. AP substrates were then added to the blot (33 µl 5-bromo-4-chloro-3-indolyl phosphate p-Toluidine Salt and 66 µl Nitro BT mixed with 10 ml PH9.5 AP buffer) (Fisher Scientific, Fair Lawn, N.J.). Rabbit anti-actin antibody (1:200) (Sigma-Aldrich) was used to demonstrate equal loading of protein in each lane.

2.2.6 Data analysis

All graphs and statistical analyses were done using GraphPad InStat (version 4.01 for Windows XP, GraphPad Software, San Diego, CA, www.graphpad.com). One-sample t test was used for analysis of differences between treated groups and control group, and was considered significant at the 95% confidence level.

2.3 Results

2.3.1 Cytotoxic action of PES on RTgill-W1

PES reduced RTgill-W1 viability in a dose-dependent manner after 24 h treatment. This occurred whether RTgill-W1 were in L-15 alone (Fig.2.1A) or in L-15 with FBS (data not shown). As judged by light microscopy, cells treated with 3.0 µg/ml (16.55 µM) (data not shown) or 5.25 µg/ml (28.96 µM) (Fig.2.1C) PES retracted from neighbouring cells but remained loosely attached to the growth surface after 24 h, and cells exhibited a rounded morphology in absence of plasma membrane blebbing or autophagic vacuoles. In L-15 without FBS, the EC_{50} values for PES were 2.68±0.32 µg/ml (14.78±1.77 µM) (n=3) as evaluated by Alamar Blue, 5.54±0.66 µg/ml (30.55±3.63 µM) (n=3) as evaluated by CFDA-AM and 2.20±0.24 µg/ml (12.13±1.32 µM) (n=3) as evaluated by Neutral Red.

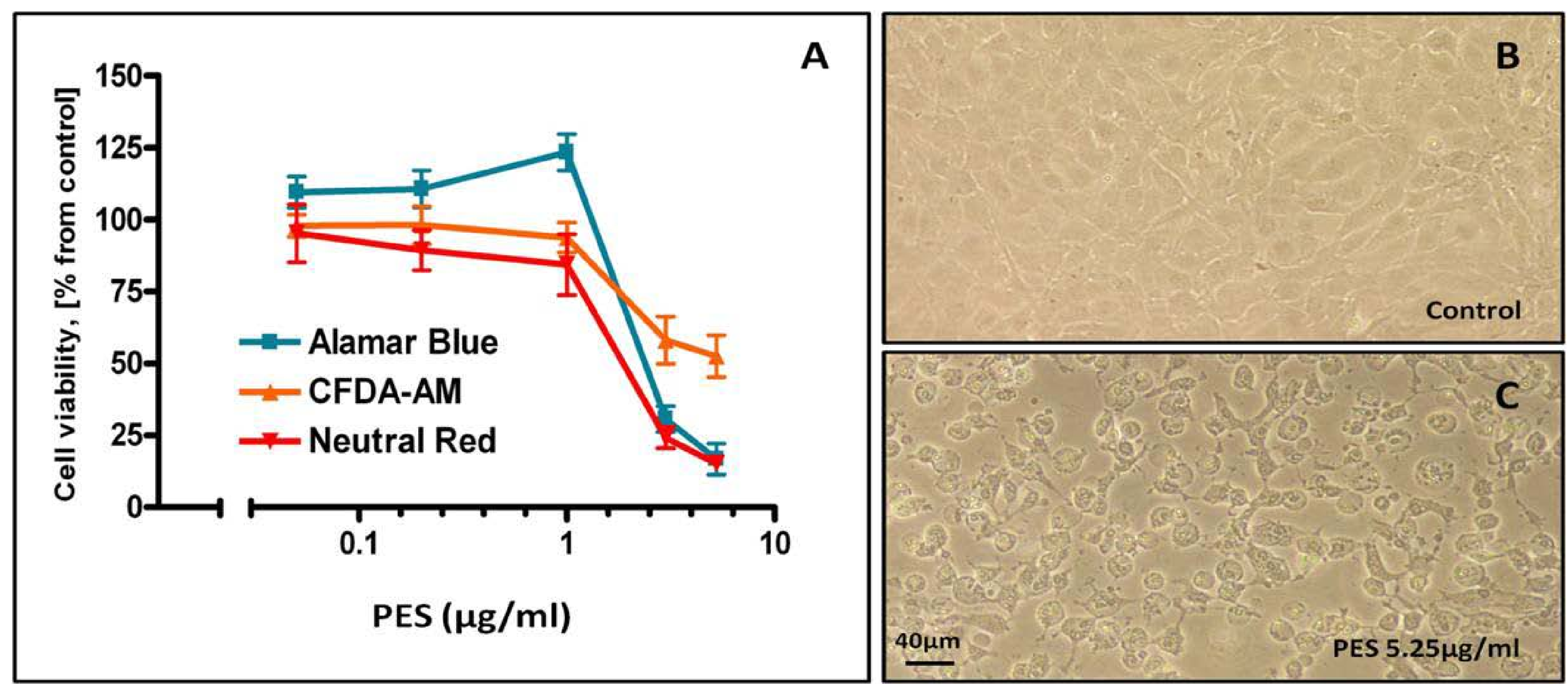


Figure 2.1 Cytotoxicity of PES on RTgill-W1 cells.

Cells at a density of $4x10^4$ per well were exposed to 5 concentrations of PES for 24 h in L-15 without FBS. Cytotoxicity was measured by Alamar Blue, CFDA-AM and Neutral Red. The y-axis represents the percentage of cell viability compared to control cells treated with DMSO. The x-axis represents the concentration of PES in µg/ml (A). The remaining panels are cell morphologies in cultures dosed with DMSO only for 24 h (B) or dosed with 5.25 µg/ml of PES for 24 h (C). Pictures were taken at 200X magnification.

2.3.2 PES induced apoptosis in RTgill-W1

After 24 h treatment with 5 different concentrations of PES (0.01, 0.05, 0.2, 1.0, 3.0 or 5.25 µg/ml), genomic DNA samples from treated cultures were run on a 2% agarose gel. RTgill-W1 cells dosed with 3.0 µg/ml or 5.25 µg/ml PES showed a clear degradation of chromosomal DNA in to 180 bp oligomers, which is a characteristic of apoptosis (Fig.2.2A). Moreover, the cells treated with PES (3.0 or 5.25 µg/ml) showed clear nuclear condensation and fragmentation from Hoechst 33258 staining (Fig.2.2C&D). The DNA laddering and nuclear fragmentation induced by PES, occurred whether RTgill-W1 were in L-15 alone (Fig.2.2) or in L-15 with FBS (data not shown).

The induction of apoptosis by PES was also quantified by assessing the proportion of cell with externalized PS at the plasma membrane. Following treatment of PES for different time periods, cells were stained with both PE-Annexin V and 7-AAD. Control samples were viable and largely negative for both PE-Annexin V and 7-AAD. The cells incubated with 3.0 or 5.25 µg/ml PES for 12 or 18 h showed a significant increase in the percentage of early apoptotic cells (PE-Annexin V positive and 7-AAD negative) (Fig.2.3B,C,E&F). Furthermore, cells treated with 5.25 µg/ml PES for 18 h showed an increase (about 20%) in the percentage of cells that were already dead or in late stage apoptosis (PE-Annexin V positive and 7-AAD positive) (Fig.2.3F).

Caspase -3, -8 and -9 activities were analysed by measuring the cleavage of their synthetic peptide substrates, DEVD-pNA, IETD-pNA and LEHD-pNA, respectively. As detected by colorimetric method, significant increases of caspase-3, -8 and -9 activities were seen in RTgill-W1 cells 24 h following PES (3.0 or 5.25 µg/ml) treatment (Fig.2.3H).

We next carried out an analysis of mitochondrial membrane potential ($\Delta\Psi m$) changes using flow cytometry after staining RTgill-W1 cells with $DiOC_2(3)$. This cationic cyanine dye accumulates in mitochondria with active membrane potentials, a reduction of green fluorescence indicates loss of $\Delta\Psi m$. As judged by the scale set by positive and negative controls, a significant increase in the percentage of cells with impaired $\Delta\Psi m$ was seen after treatment with PES (3.0 or 5.25 µg/ml) for 12 or 18 h (Fig.2.4A-G).

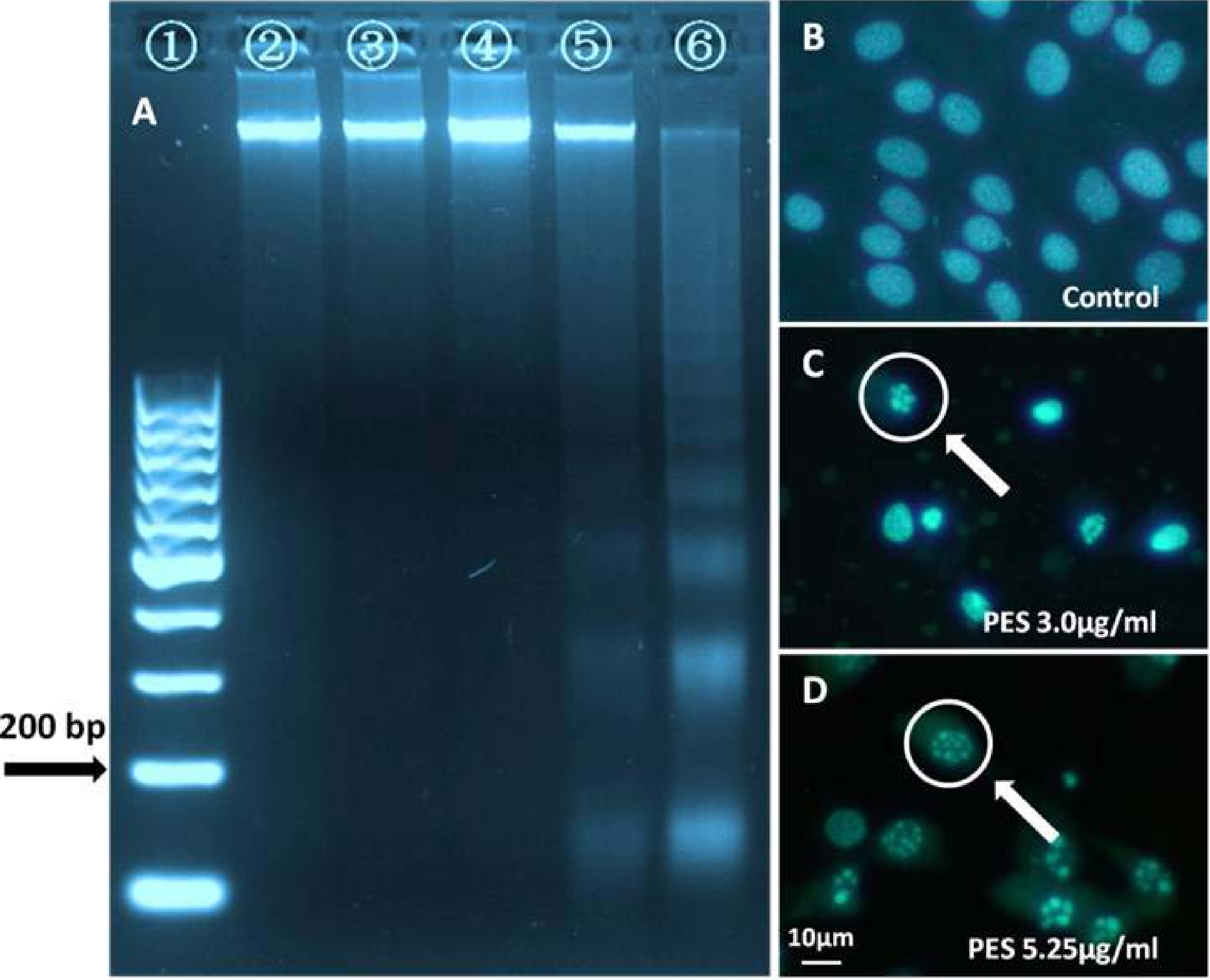


Figure 2.2 PES induced DNA laddering and nuclear fragmentation in RTgill-W1 cells.

Cultures in L-15 without FBS were exposed to different concentrations of PES for 24 h and then analyzed for hallmarks of apoptosis. In panel A, genomic DNA was extracted from 0.05, 0.2, 1.0, 3.0 and 5.25 µg/ml PES treated cultures and run on a 2 % agarose gel (lanes 2-6). Lane 1 is the 100 bp DNA ladder with the black arrow identifying 200 bp. The remaining panels reveal nuclear morphologies by Hoechst 33258 staining and fluorescence microscopy in cultures treated with DMSO only (B), PES (3.0 µg/ml) (C), or PES (5.25 µg/ml) (D).White arrows indicate examples of nuclear fragmentation. All pictures were taken at 400X magnification.

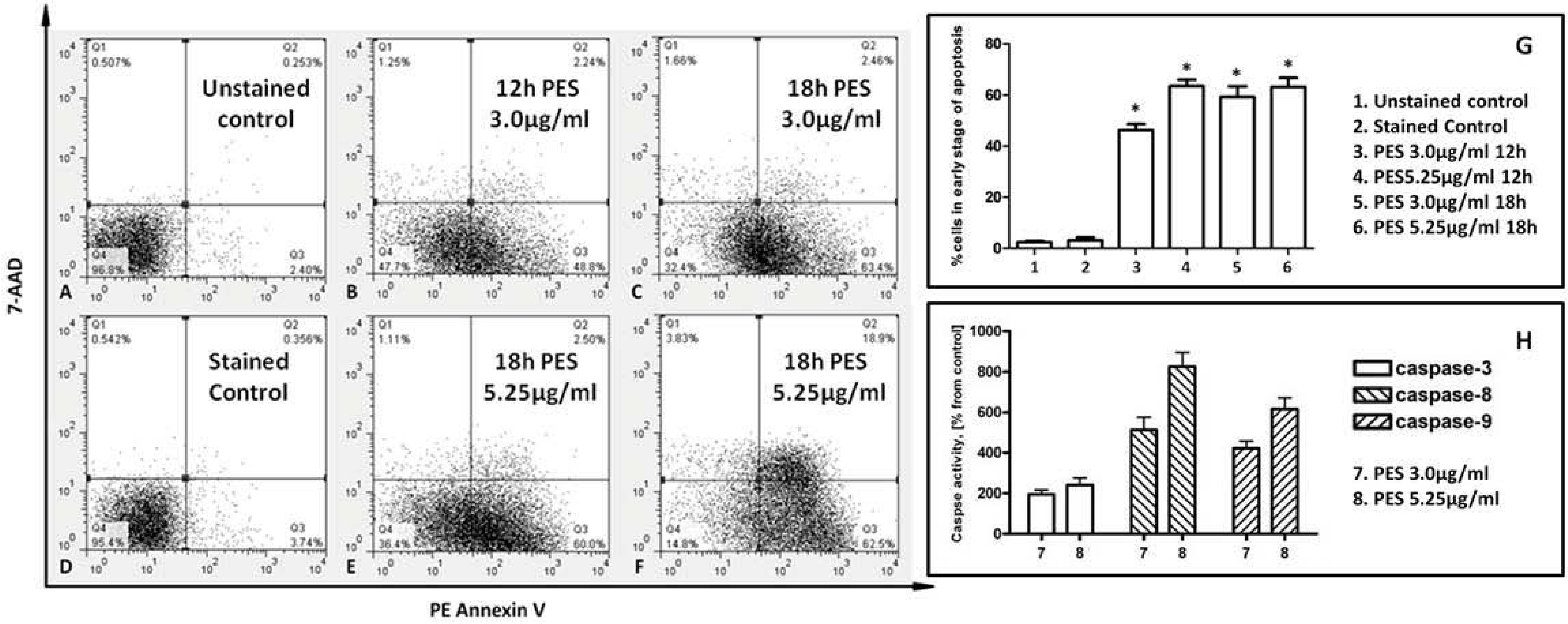


Figure 2.3 PES induces the activation of caspases and apoptosis.

The flow cytometry dotplots shown in A-F are representative of one of the three separate experiments (the other two repeats are not shown). Negative controls without and with stain (A & D) are viable (PE Annexin V and 7-AAD negative) and D was used to set the quadrant. Cultures treated with PES (3.0 µg/ml) 12 h (B), PES (5.25 µg/ml) 12 h (E), PES (3.0 µg/ml) 18 h (C) and PES (5.25 µg/ml) 18 h (F) (other treatments are not shown) were positive for early stage apoptosis (PE Annexin V positive and 7-AAD negative). A portion of cells in F are in late stage apoptosis or already dead (PE Annexin V and 7-AAD positive). Panel G shows the percentage of PE Annexin V positive and 7-AAD negative cells after the indicated treatments (n=3). Caspase-3, -8 and -9 activities were determined by colorimetric protease assay. Samples treated with 3.0 or 5.25 µg/ml PES for 24 h were read at 405 nm and enzyme activities were expressed as percentage of control samples (n=3). Data were presented as mean±SE of three separate replicates. * indicates a significant difference between the treated and control samples ($P<0.05$).

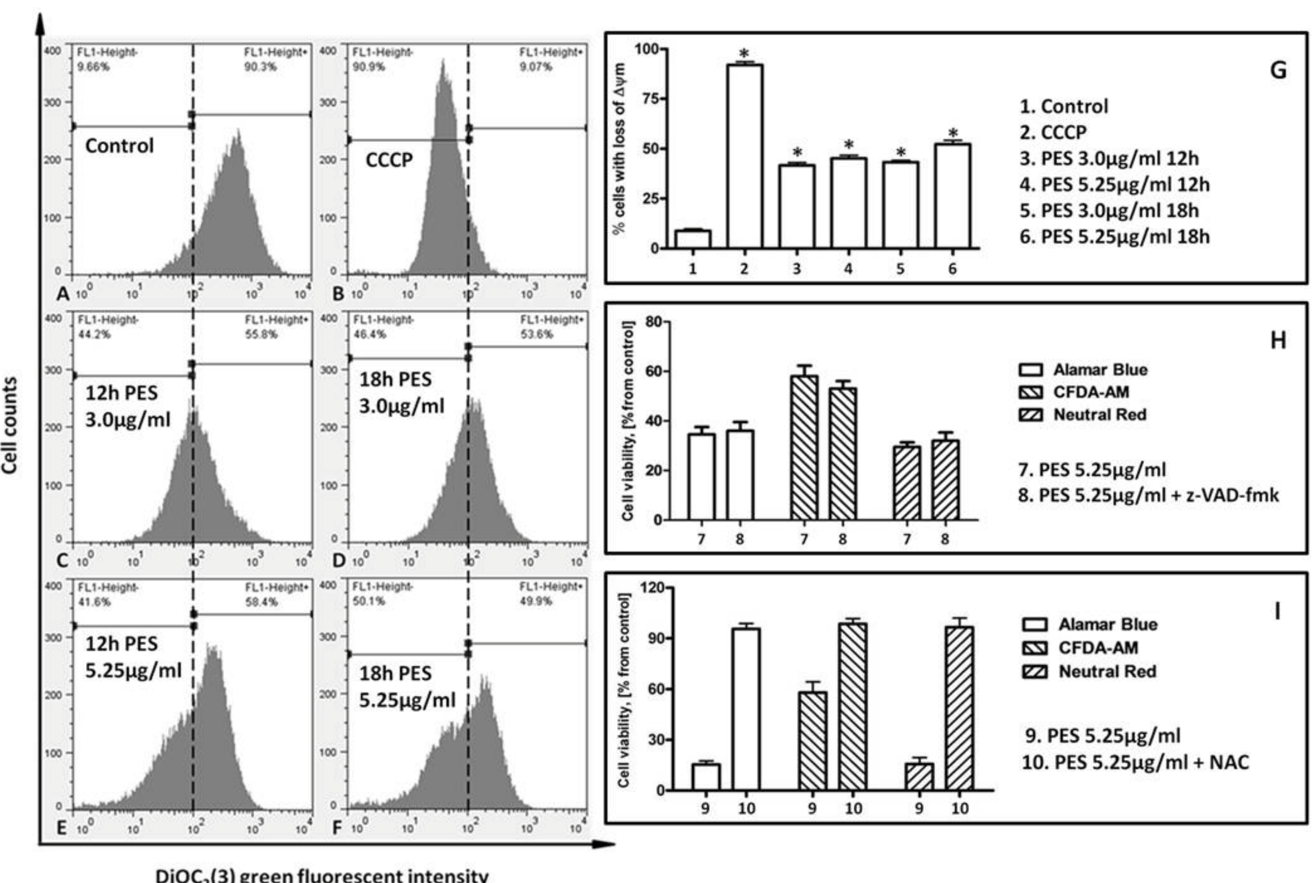


Figure 2.4 PES disrupts mitochondrial membrane potential in RTgill-W1 cells.

The flow cytometry analysis of ΔΨm shown in A-F are representative of one of the three separate experiments (the other two repeat are not shown). Cells treated with different concentrations of PES were harvested and stained with $DiOC_2(3)$. No treatment (A) was used as negative control and CCCP (B) was used as positive control. Cultures treated with 3.0 µg/ml PES for 12 h (C), 5.25 µg/ml PES for 12 h (E), 3.0 µg/ml PES for 18 h (D) and 5.25 µg/ml PES for 18 h (F) showed reduction of green fluorescence corresponds to the loss of ΔΨm. Panel G represents the percentage of cells with loss of ΔΨm (n=3). The remaining panels represent the effect of z-VAD-fmk (H) or NAC (I) on the cytotoxicity of PES (5.25 µg/ml). Cytotoxicity was measured by Alamar Blue, CFDA-AM and Neutral Red and presented in percentage of cell viability from control cultures (n=3). Data were presented as mean±SE of three separate replicates. * indicates a significant difference between the treated and control samples (P<0.05).

2.3.3 PES induced apoptosis mediated by oxidative stress in RTgill-W1

To further elucidate the mechanism of PES-induced apoptosis, pan caspase inhibitor, z-VAD-fmk or antioxidant, N-acetylcysteine (NAC) was added to RTgill-W1 cultures in order to inhibit cell death. Neither z-VAD-fmk nor NAC was cytotoxic in RTgill-W1 cultures after 24 h treatment. The cultures treated with PES plus varying concentrations of z-VAD-fmk did not show significant increases in cell viability relative to PES treatment alone, suggesting the activation of caspases is not the central pathway of the apoptosis induced by PES (Fig.4H). PES was also found to increase the intracellular ROS level in RTgill-W1 (Fig.2.5). The pre-treatment with 5 mM NAC, an oxidant scavenger, completely blocked cell death in cultures exposed to PES (Fig.2.4I). The cultures treated with PES plus 5 mM NAC exhibited a normal morphology and were negative for apoptosis assays (data not shown).

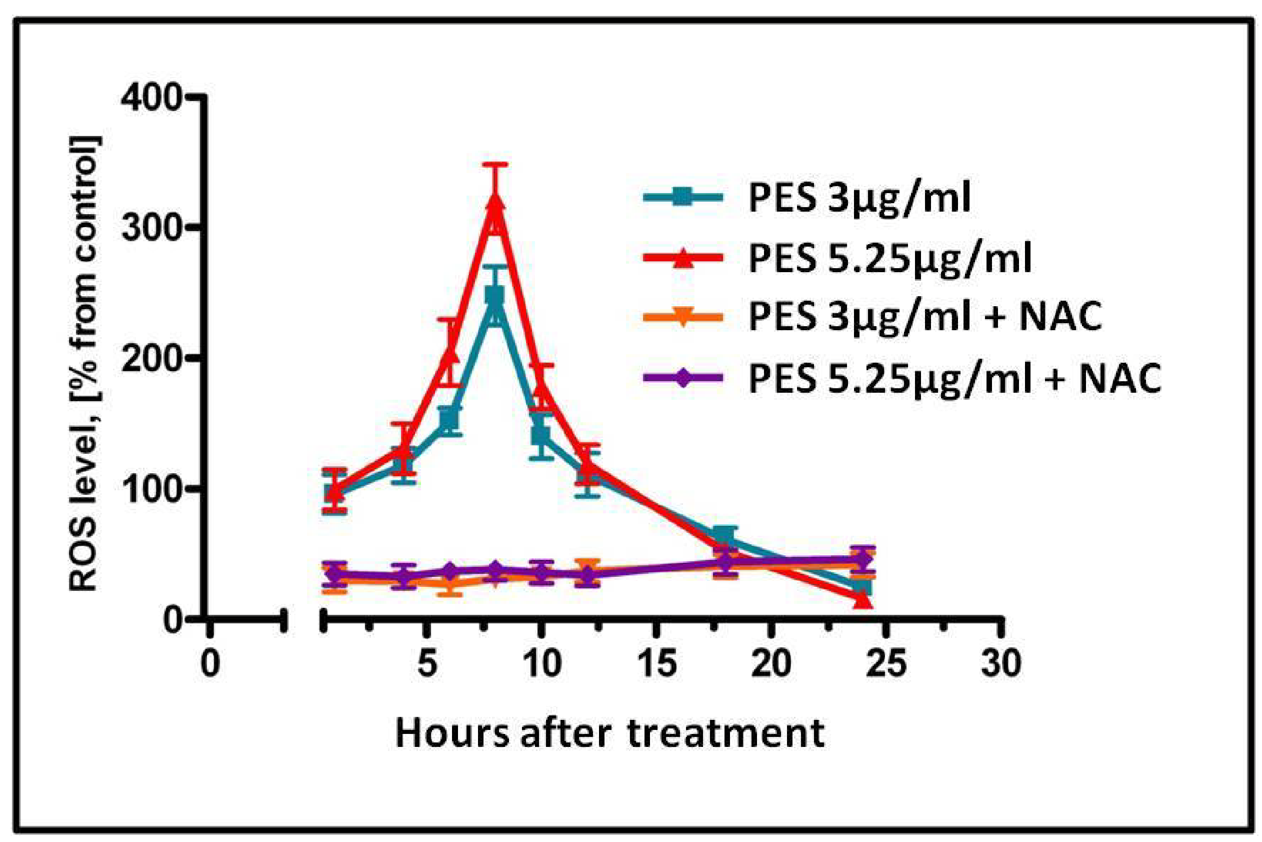


Figure 2.5 PES increased the ROS level in RTgill-W1.

Cells were exposed to 3.0 or 5.25 µg/ml of PES with or without 5mM NAC for different time periods. ROS level was measured by H_2DCFDA. The y-axis represents the percentage of ROS level compared to control samples treated with DMSO. The x-axis represents the time after treatment.

2.3.4 PES accumulated p53, BiP and HSP70 in detergent insoluble fractions

We finally determined the effects of PES on p53 in RTgill-W1 cells. PES was shown to induce an accumulation of p53 in the detergent insoluble fraction, even under conditions in which there was no significant decrease in protein abundance in the detergent-soluble fraction. In cultures treated with 5.25 μg/ml PES, p53 was almost completely lost in the detergent soluble fraction. PES also increased the abundance of BiP and HSP70 both in the detergent-soluble and insoluble fraction. Preventing the cell death by pre-treatment of 5 mM NAC almost completely blocked the increased abundance of p53, BiP and HSP70 in the detergent insoluble fraction and restored the total abundance of these proteins (Fig.2.6).

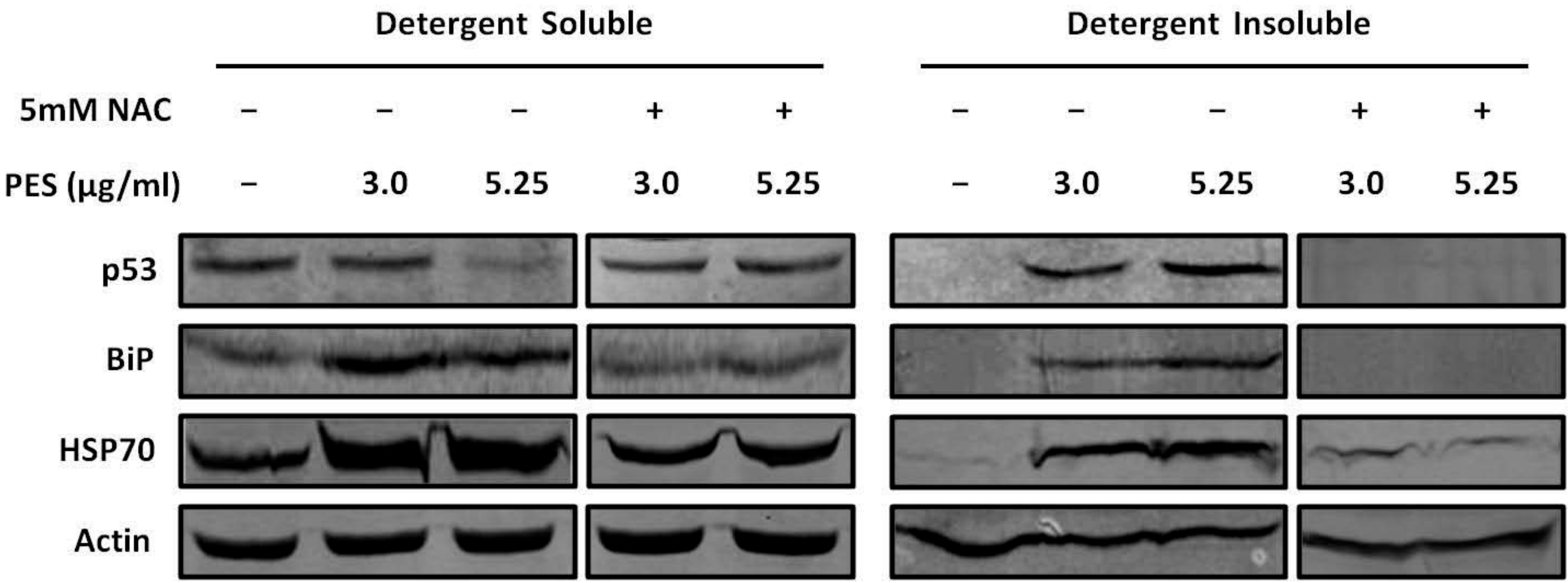


Figure 2.6 Effect of PES on p53, BiP and HSP70 levels in RTgill-W1.

Cells were exposed to 3.0 or 5.25 μg/ml of PES with or without 5 mM NAC for 24 h. Detergent soluble and insoluble proteins were extracted and separated by a 12 % SDS-PAGE gel and then transferred to a nitrocellulose membrane. Detection was carried out with primary rabbit anti-rainbow trout p53 antibody (1:200), rabbit anti-Xenopus HSP70 antibody (1:200), rabbit anti-GRP78/BiP antibody (1:1000) and secondary goat anti-rabbit AP antibody (1:30000). Anti-actin antibody was used as a loading control.

2.4 Discussion

Although often not observed to be cytotoxic when used in mammalian systems (Strom et al., 2006), PES has been found in this first study with fish cells to kill the rainbow trout gill epithelial cell line, RTgill-W1. Exposures of RTgill-W1 cultures to increasing concentrations of PES brought about a progressive loss of cell viability as judged by three indicator dyes. Alamar Blue, CFDA-AM, and Neutral Red revealed a profound impairment in respectively cellular metabolism, plasma membrane integrity, and lysosomal activity. The cytotoxicity of PES is not unique to piscine cells because PES has been reported to kill some but not all mammalian cells, with tumor cells generally being more sensitive than their normal counter parts (Kaiser et al., 2011; Leu et al., 2009; 2011; Steele et al., 2009). The loss of mammalian cell viability usually was detected with the MTT assay and reported for leukemic cells growing in suspension as well as for adherent cell lines with fibroblast- and epithelial-like morphologies (Leu et al., 2011; Steele et al., 2009). The EC_{50}s or IC_{50}s varied with the cell source. For a 48 h exposure of human leukemic cells and cell lines, the range was from 2.5 to 37.2 μM (Kaiser et al., 2011). For 24 h exposed RTgill-W1 cultures, the three viability assays gave EC_{50}s within this range. The killing curves for individual leukemia cell lines were noted to be remarkably steep (Kaiser et al., 2011). Likewise, PES killed RTgill-W1 over a very narrow range (3 to 10 μg/ml). This is the first report of cell killing by PES being blocked with the antioxidant, N-aetylcysteine (NAC).

The protection of RTgill-W1 by NAC suggests that reactive oxygen species (ROS) are involved in the cytotoxicity of PES. NAC has long been used to protect cells against ROS-induced cytotoxicity and possibly does so by directly scavenging ROS (Zhang et al., 2011). In RTgill-W1 cultures PES enhanced ROS production as judged with the fluorescent dye, H_2DCFDA. Additionally the evaluation of cultures with the indicator of cellular metabolism, Alamar Blue, hinted at a possible reason for the elevation in ROS. At a non-lethal PES concentration (1 μg/ml) the Alamar Blue readings were increased by approximately 25 %. These results suggest that PES stimulated cellular metabolism and concurrently ROS production, leading to cell death. The stimulation could be brought about by inhibitory actions of PES on p53 and/or HSP70. As well as being known as the guardian of the genome under stressful conditions, p53 is increasingly being seen to act under basal (non-stress) conditions (Hafsi and Hainaut, 2011) and energy and ROS production are some of the processes regulated (Hafsi and Hainaut,

2011; Maillet et al, 2012). Additionally ROS levels might be elevated through PES inhibiting the contribution of HSP70 to antioxidant defences (Azad et al., 2011; Guo et al., 2007).

The ROS generated from PES exposure, as well PES itself, likely caused endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) in RTgill-W1. In mammalian cells misfolded or unfolded proteins trigger an adaptive response known as ER stress. Among the triggers for ER stress is oxidant stress (Jung et al., 2007; Hernandes-Gea et al., 2013). Also contributing to the accumulation of misfolded and unfolded proteins in RTgill-W1 would be the inhibition by PES of HSP70/HSC70 activities. ER stress initiates the UPR. UPR is a complex signalling program mediated by three ER transmembrane receptors that work to restore folding homeostasis (Chakrabarti et al., 2011). Early on UPR does this by inducing the expression of chaperones such as HSP 70 and BiP to increase protein folding. For RTgill-W1, a 24 h of exposure to PES increased the levels of HSP70 and BiP but NAC blocked this increase. The results suggest that the enhanced generation of ROS in cultures exposed to PES initiated ER stress, which led to the UPR and an increase in chaperone levels. If the UPR fails to restore ER homeostatsis in mammalian cells, cell death often ensues and usually this is by apoptosis (Logue et al., 2013; Chakrabarti et al., 2011).

The killing of RTgill-W1 by PES had many apoptotic hallmarks, suggesting that apoptosis had a role in the fish cell death. As in several mammalian cell lines (Steele et al., 2009; Kaiser et al., 2011), PES treatment of RTgill-W1 resulted in Annexin V staining, indicating the externalization of PS and apoptosis. Additionally for RTgill-W1 PES caused nuclear fragmentation and DNA laddering. As well PES led to MOMP and the activation of caspase-3,-8 and -9, consistent with a mitochondrial apoptotic pathway with sequential activation of cytochrome c, Apaf-1 and caspase-9-containing apoptosome. Caspase-8 is apical in the extrinsic pathway. In the intrinsic pathway, caspase-8 is downstream of caspase-3 and cleaved by caspase-6 (Fulda and Debatin 2006; Inoue et al. 2009). However, the caspase inhibitor z-VAD-fmk failed to block the killing of RTgill-W1 by PES. Possibly the involvement of caspases in PES-induced cell death varies with the cell type. For human cells treated with PES, caspase 3 was activated in some cancer cell lines, but not in others, and caspase inhibitors blocked the death of some cell lines but not others (Kaiser et al., 2011; Leu et al., 2009). Possibly PES could induce an alternative apoptotic pathway besides the mitochondrial death pathway. Alternative modes of ER

stress-induced cell death have been proposed, including the involvement of autophagy (Logue et al., 2013).

A feature of treating RTgill-W1 with PES was the accumulation of proteins in the detergent-insoluble cell fraction. Previously protein accumulation in the detergent-insoluble fraction has been observed after PES treatment of human tumor cell lines and of a tumor model in vivo (Leu et al., 2009, 2011). In these studies two proteins that are prominently involved in autophagy were affected, the microtubule-associated protein-1 light-chain 3 (LC3-II) and the adaptor/scaffold protein, sequestersome-1 (SQSTM1 or p62). PES increased LC3-II and SQSTM1 levels and their accumulation in the detergent-insoluble fraction, as well as SQSTM1 oligmerization (Leu et al., 2009, 2011). Other proteins accumulating in the detergent insoluble fraction of human cells included HSP70 and p53 (Leu et al., 2011). In the case of human cells the accumulation of proteins in the detergent-insoluble fraction was attributed to PES inhibiting HSP70/HSC70 activities, which ultimately impaired the two major protein degradation pathways, the autophagy-lysosome system and the proteasome (Leu et al., 2011). Impairing degradation in this manner was thought to contribute to the cytotoxicity of PES. For RTgill-W1, only a few proteins were examined but upon PES treatment, the detergent-insoluble fraction had relative to control cultures increased amounts of HSP70, BiP and p53. NAC blocked this aggregation of p53, BiP and HSP70. As NAC also promoted the survival of cells in PES-treated cultures, the accumulation of detergent insoluble proteins appears to contribute to the killing of RTgill-W1 by PES

In summary the sequence of cellular events that transpires upon exposure of RTgill-W1 to PES is complex and interconnected but the following is one possible synopsis. Likely by PES inhibiting p53, cellular metabolism and ROS generation is stimulated, and by PES inhibiting HSP70, ROS levels are increased further and become more damaging. The elevated ROS initiates ER stress that likely is further exacerbated by PES inhibiting the chaperone functions of HSP70, causing misfolded proteins in the ER.   This leads to yet more ROS and ER stress. The ER stress triggers the unfolded protein response (UPR) and apoptosis. The amelioration of ER stress by the UPR fails to be resolved because the inhibition of HSP70 by PES interferes with protein degradation pathways (Leu et al., 2011). Inhibiting protein degradation causes misfolded

proteins to aggregate and accumulate in the detergent insoluble fraction, inactivating the proteins and contributing to the death of cells.

2.5 Conclusion

Exposing RTgill-W1 cultures to the p53 and hsp70 inhibitor, 2-phenylethynesulfonamide (PES or pifithrin-μ), led to an increase in ROS generation, HSP70 and BiP levels, and accumulation of HSP70, BiP and p53 in the detergent-insoluble fraction, and to cell death by apoptosis. Although likely acting on several targets in fish cells, PES should still be a valuable tool in fish toxicology for exploring cellular survival pathways under normal conditions and upon exposure to toxicants.

CHAPTER 3

The p53 inhibitor, pifithrin-α, disrupts microtubule organization, arrests growth, and induces polyploidy in the rainbow trout gill cell line, RTgill-W1

3.1 Introduction

Delineating the cell death and survival pathways in fish cells is important for understanding the fundamental mechanisms behind the effects of environmental contaminants on fish. One of the key switches between death and survival in mammalian cells is the tumour suppressor protein, p53 (Lanni et al., 2012). This protein has been identified in fish (Caron de Frommentel et al., 1992; Chen et al., 2001; Cheng et al., 1997; Storer & Zon, 2010) and in fish cell lines (Embry et al., 2006; Liu et al., 2011). Yet most investigations of p53 functions in fish have been done in vivo (Chen et al., 2001; Chen et al., 2009) and how p53 acts at the cellular level is poorly understood. One approach for examining how p53 works in cells is to monitor cellular responses after the addition of a p53 inhibitor. Many inhibitors have been developed to block different p53 functions, as part of efforts to improve cancer chemotherapy, (Beretta et al., 2008; Selivano, 2014). One inhibitor is 2-phenylethynesulfonamide (PES) or pifithrin-μ (Strom et al., 2006). PES blocks the translocation of p53 to the mitochondria. When PES was added to cultures of the rainbow trout gill cell line, RTgill-W1, the cells underwent oxidative stress, the unfolded protein response, and apoptosis (Zeng et al., 2014). These results suggested that in rainbow trout cells p53 might constitutively regulate energy metabolism and coordinate antioxidant defenses.

In the current work, an inhibitor that targets another activity of p53 was studied in RTgill-W1. This was pifithrin-α (PFT-α), which was identified as blocking p53-dependent apoptosis and transcription in mammals (Komarov et al., 1999). The rainbow trout cell cultures remained viable upon the addition of PFT-α but responded in ways rarely, if ever, seen with mammalian cells. In RTgill-W1 cultures PFT-α caused a transient rise in the mitotic index, a disruption of cytoskeletal microtubules, an arrest of cell proliferation, and an accumulation of tetraploid and polyploid cells. These results suggest that in RTgill-W1 cells p53 might be more involved in regulating microtubule-associated proteins than in mammalian cells but like in mammalian cells regulates ploidy.

3.2 Materials and Methods

3.2.1 Cell culture

RTgill-W1, a rainbow trout gill cell line developed in our laboratory (Bols et al., 1994), was routinely cultured in 75 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) at room temperature in Leibovitz's L-15 culture medium (Sigma-Aldrich, Ltd., Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin solution (10000 units/ml penicillin, 10mg/ml streptomycin, Sigma-Aldrich).

3.2.2 Cytotoxicity assay

3.2.2.1 Plating and dosing

Cells were seeded in 96 well plates (Becton and Dickinson Company, Franklin Lakes, NJ. USA) at a density of $4x10^4$ cells per well in 200µl of L-15 growth medium with 10% FBS supplement. Cells were allowed to settle and reattach for 24 h at room temperature before being exposed to any compounds. The cells were then dosed with varying concentrations of PFT-α (Calbiochem, La Jolla, CA) in L-15 with or without 10% FBS. Application of PFT-α to cell cultures was done by two different methods, direct dosing and indirect dosing. Direct dosing involved directly adding a small volume (1µl) of toxicant solution to the culture well. Indirect dosing involved adding culture medium mixed with toxicant solution to the culture well. The final concentration of the toxicant solvent, DMSO (0.5% v/v) in each well was the same as for the control wells, which were only dosed with DMSO. After 24 h, cultures were evaluated for cytotoxicity. In no cases was the solvent used at a concentration that was cytotoxic.

3.2.2.2 Measuring cell viability

Three fluorescent indicator dyes were used to evaluate cell viability. Metabolic activity was measured by Alamar Blue (Medicorp, Montreal, PQ). Cell membrane integrity was evaluated with 5-carboxyfluorescein diacetate (CFDA-AM) (Molecular Probes, Eugene, OR). Lysosome integrity was monitored with Neutral Red (Sigma-Aldrich). Alamar Blue, CFDA-AM and

Neutral Red were prepared in Dulbecco's phosphate buffered saline (DPBS, Lonza, Walkersville, MD USA) to give final concentrations of 5 % (v/v), 4 µM and 1.5 % (v/v), respectively. Cells were incubated with dyes for 1 h in the dark then quantified by fluorescence plate reader (Spectra-max Gemini XS microplate spectrofluorometer; Molecular Devices, Sunnyvale, CA). The excitation and emission wave-lengths used were 530 and 590 nm for Alamar Blue, 485 and 530 nm for CFDA-AM, 530 and 640 nm for Neutral Red. Results were calculated as a percent of the DMSO-treated control culture

3.2.3 Hoechst 33258 Stain

For evidence of apoptosis, cultures were evaluated for nuclear fragmentation. RTgill-W1 cells were seeded at a density of $4x10^5$ cells in 9 cm^2 slide flask (Nunc, Kamstrupvej, Denmark) for H33258 staining. After treatment, RTgill-W1 cultures were fixed by adding an equal volume of Carnoy's fixative (methanol:glacial acetic acid, 3:1), which was prepared fresh with each use, to existing media, exposing the cells for 2 min. The media and fixative were then removed and fresh fixative was added to the cells twice for 5min. Following the fixation, the cells were stained with 0.5 µg/ml Hoechst 33258 for 10 min in dark. After several washes with deionized water and the final wash with Mcllvaine's buffer, a coverslip was mounted onto the slide with Mcllvaine's buffer and glycerol (1:1). The fluorescent nuclei were visualized using a fluorescent microscope with ultra-violet (UV) filter (Nikon Optishot). The areas of nuclei were measured using ImageJ (http://imagej.nih.gov/ij/).

3.2.4 Intracellular ROS measurement

Intracellular ROS were determined by using 2′,7′ -Dichlorofluorescin diacetate (H_2DCFDA) (Sigma- Aldrich). RTgill-W1 cells were seeded in 96 well plates at a density of $4x10^4$ cells per well in L-15 growth medium with 10% FBS. After 24 h incubation at room temperature to allow reattachment, the cells were indirectly dosed with different concentrations of PFT-α for 1-24 h. Cells were then incubated with H_2DCFDA working solution of 10 µM in PBS at RT for 40 min. Afterwards, cells were washed twice with pre-warmed PBS and incubated with pre-warmed PBS

for 10 min. ROS were measured using a fluorescent plate reader (Spectra-max Gemini XS microplate spectrofluorometer; Molecular Devices, Sunnyvale, CA) at an excitation and emission wave-lengths of 485 and 530 nm. ROS level was expressed as percentage of the fluorescence over control samples.

3.2.5 Monitoring cell proliferation

The effect of PFT-α (indirect dosing) on the proliferation of RTgill-W1 cells was evaluated in 12-well tissue culture plates (Becton and Dickinson) in L-15 with or without 10% FBS. Cells were plated in 12 well plates at a density of $6\text{x}10^4$ cells per well in 2 ml medium. Before treatment, cells were allowed to attach to the well for 24 h at room temperature. Cells were indirectly dosed with 3 concentrations of PFT-α with a final DMSO concentration of 0.5% (v/v) and the control wells were indirectly dosed with only DMSO to this level. Cells were counted with a coulter counter (Coulter Electronics of Canada, Burlington, ON) according to the manufacturer's instructions immediately upon treatment and subsequently every three days over a period of 12 days. In order to test whether cells arrested by PFT-α can re-enter the cell cycle after removal of the chemical, RTgill-W1 cells in L-15 with or without 10% FBS were exposed to 5.25 µg/ml (14.29 µM) of PFT-α (indirect dosing) for three days and then the medium with PFT-α was removed, the culture vessels were rinsed with DPBS, and fresh L-15 with or without 10% FBS (no PFT-α) was added to the cultures. The cells were counted with a coulter counter every three days over the next 9 days.

3.2.6 May-Grunwald-Giemsa stain

May-Grunwald Giemsa staining (EMD4Biosciences, NJ, USA) was used to reveal nuclei and cell morphology. Cells treated with PFT-α were fixed in a mixture of ethanol and acetone (1:1) for 20 min. After fixation, cells were covered with May Grunwald for 3 min and counterstained with Giemsa (1:50 in tap water) for 5 min.

3.2.7 Cell cycle analysis

The cell cycle stage of PFT-α treated (indirect dosing) cells was measured by using a DNA fluorochrome, propidium iodide (PI) (Sigma). After removal of the old media, cells were harvested by trypsinization and suspended in 70% ethanol. After 2 h incubation at 4 ℃, cells were washed once in DPBS and incubated with 1 ml of PI staining solution (0.1 % (v/v) Triton X-100, 10 μg/mL PI and 100 μg/mL DNase-free RNase) at 37 ℃ for 10 min in dark. The samples were then measured (10000 events/sample) using FACScan laser flow cytometer analysis system (Becton-Dickinson, San Jose, CA). DNA content distribution was analyzed by FlowJo software (Treestar, Inc., San Carlos, CA).

3.2.8 Immunofluorescence analysis

Cells were seeded in 4 chamber polystyrene vessel tissue culture treated glass slides (BD Falcon, Discovery Labware, Bedford, MA, USA) at $2x10^5$ cells per chamber in L-15 growth medium with or without 10 % FBS. After 24 h incubation at room temperature to allow reattachment, the cells were indirectly dosed with 5.25 μg/ml PFT-α and incubated again at room temperature for 3, 6, 9 or 12 days. After treatment, cells were rinsed twice with DPBS, fixed with 4 % paraformaldehyde (Sigma-Aldrich) for 20 min and permeabilized with 0.5 % Triton X-100 (Sigma-Aldrich) in DPBS for 10 min. Cells were rinsed twice with DPBS and blocked with 1 % BSA (Sigma-Aldrich) containing 0.5 % Triton X-100 in DPBS at room temperature for 30 min. Cells were then incubated with mouse monoclonal anti-β-tubulin antibody (1:100) (Sigma-Aldrich) for 1 h. After two washes of 5 min each in DPBS, the cells were incubated with goat anti-mouse IgG FITC-conjugated antibody (1:100) (Sigma-Aldrich) for 30 min in the dark. DAPI (Sigma-Aldrich) was added to a final concentration of 10 μg/ml for 15 min to stain the nuclei and then cells were rinsed twice with DPBS. Slides were examined using a Zeiss Axiovert 200 confocal microscope with a ×63 oil immersion objective and ZEN 2011 softwaere (Carl Zeiss, MicroImaging GmbH, Germany) according to the manufacturer's instructions.

3.2.9 Western blot

RTgill-W1 cells were seeded in 25 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) at $1x10^6$ cells per flask in L-15 growth medium with or without 10 % FBS. After 24 h incubation at room temperature to allow reattachment, the cells were treated with 5.25 μg/ml PFT-α (indirect dosing), and incubated again at room temperature for 3, 6, 9 or 12 days. Whole-cell protein extracts were prepared as described by Liu et al. (2011) and protein concentrations were determined by bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions (Pierce, Rockford, IL). Protein was separated by 12 % SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5 % milk for 1 h, incubated with primary rabbit anti-rainbow trout p53 antibody (1:200) (Liu et al., 2011) for 2 h, then incubated with secondary goat anti-rabbit AP antibody (1:30000) (Bio-Rad, Hercules, CA) for 1 h. AP substrates were then added to the blot (33 µl of 5-bromo-4-chloro-3-indolyl phosphate p-Toluidine Salt and 66 µl of Nitro BT mixed with 10 ml of PH 9.5 AP buffer) (Fisher Scientific Fair Lawn, N.J.). Rabbit anti-actin antibody (1:200) (Sigma-Aldrich) was used to demonstrate equal loading of protein in each lane.

3.2.10 Caspase activity assay

The activities of caspase-3, -8 and -9 were measured using a colorimetric assay kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. RTgill-W1 cells were indirectly dosed with 5.25 µg/ml of PFT-α for 3, 6, 9 or 12 days. Cells were lysed in chilled cell lysis buffer and 100 µg protein was incubated at 37 ℃ for 1 h in 200 µM DEVD-pNA (caspase-3 substrate), IETD-pNA (caspase-8 substrate) or LEHD-pNA (caspase-9 substrate). Caspase-3, -8 and -9 activities were measured by spectrophotometric detection of the chromophore pNA at 405 nm.

3.2.11 Data analysis

All graphs and statistical analyses were done using GraphPad InStat (version 4.01 for Windows XP, GraphPad Software, San Diego, CA, www.graphpad.com). Student's t-test and ANOVA were used to identify statistically significant differences. P-values <0.05 were considered to be statistically significant.

3.3 Results

3.3.1 Cells lost viability in cultures dosed directly with PFT-α

Directly dosing PFT-α into RTgill-W1 cultures in L-15 or in L-15 with 10 % FBS caused a loss of cell viability at the highest concentrations (Fig.3.1). When cultures were viewed under the phase contrast microscope after 24 h in PFT-α, cultures appeared the same in PFT-α at ≤ 1.0 μg/ml as in DMSO (control) (Fig.3.1A), whereas in PFTα at 3 μg/ml (8.17 μM) and 5.25 μg/ml (14.29 μM) cells appeared dark, shriveled and disrupted (Fig.3.1B). In PFT-α at ≤ 1.0 μg/ml, the viability of cultures was unchanged as evaluated with the indicator dyes, Alamar Blue (AB) for metabolism, CFDA-AM for cell membrane integrity, and Neutral Red (NR) for lysosomal function (Fig.3.1C). However, with PFT-α at 3 and 5.25 μg/ml, cultures were judged as dead in each of the three cell viability assays (Fig.3.1B), and nuclei had shrunk as revealed by H33258 staining for nuclear DNA (Fig.3.2). Therefore, direct dosing was not done in further experiments.

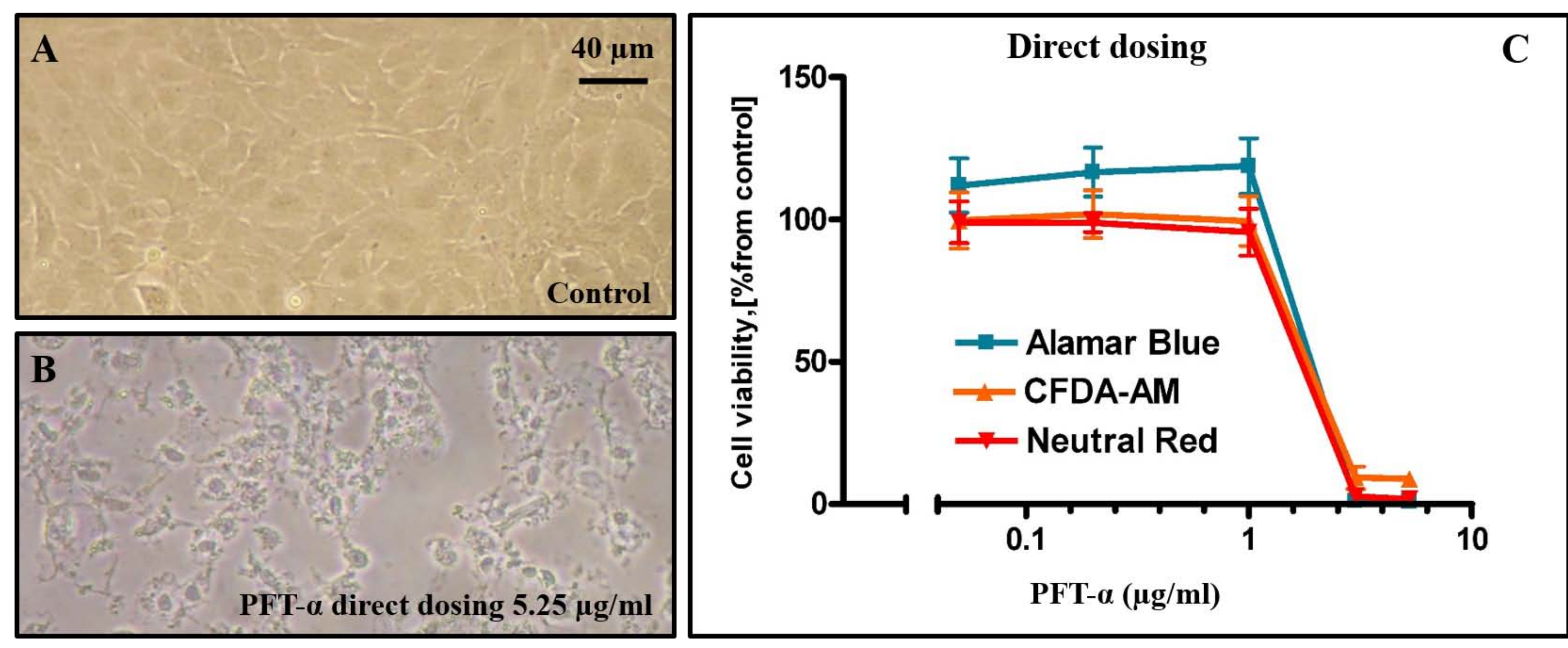


Figure 3.1 Effect of directly dosing PFT-α into RTgill-W1 cultures on cell appearance and viability.

Panels A and B show the phase-contrast microscopy appearance of cultures 24 h after being directly dosed with either DMSO (A, control) or PFT-α (B, 5.25 µg/ml in DMSO) (100X magnification). Cell viability was evaluated with three fluorescent indicator dyes, Alamar Blue for metabolism, CFDA-AM for plasma membrane integrity and Neutral Red for lysosome function, and expressed as a percentage of the relative fluorescent units (RFUs) recorded for each assay in control cultures. Panel C shows cell viability (y-axis) in cultures 24 h after being directly dosed with increasing PFT-α concentrations (x-axis).

3.3.2 Cells remained viable in cultures dosed indirectly with PFT-α

Cells in cultures indirectly dosed with PFT-α remained attached to the growth surface and were viable by several measures over at least 12 days. As revealed after H33258 staining, the nuclear area was significantly larger for cells in PFT-α treated culture than for cells in control cultures (Fig.3.2). The increase was observed whether the culture medium had FBS or not after 24 h in PFT-α. How long this difference was maintained was difficult to evaluate because after several days with PFT-α, the cultures acquired multinucleated cells, often with irregularly shaped nuclei (Fig.3.3). Even at the highest PFT-α concentrations, 3 μg/ml (8.17 μM) and 5 μg/ml (14.29 μM) cell viability was the same as in control cultures as assessed with CFDA-AM and NR (Fig.3.4). AB readings were about 15 % higher in the PFT-α treated cultures, indicating perhaps a slight stimulation of metabolism (Fig.3.4). ROS levels in RTgill-W1 cultures treated with PFT-α were not significantly different from that of control (data not shown). The p53 level declined slightly in PFT-α treated cultures without FBS but was slightly elevated in cultures with FBS (Fig.3.5A). PFT-α significantly increased the caspase-8 activity of RTgill-W1 cultures with or without FBS (Fig.3.5B & C). However, PFT-α did not alter the activities of caspase-3 and -9 (data not shown).

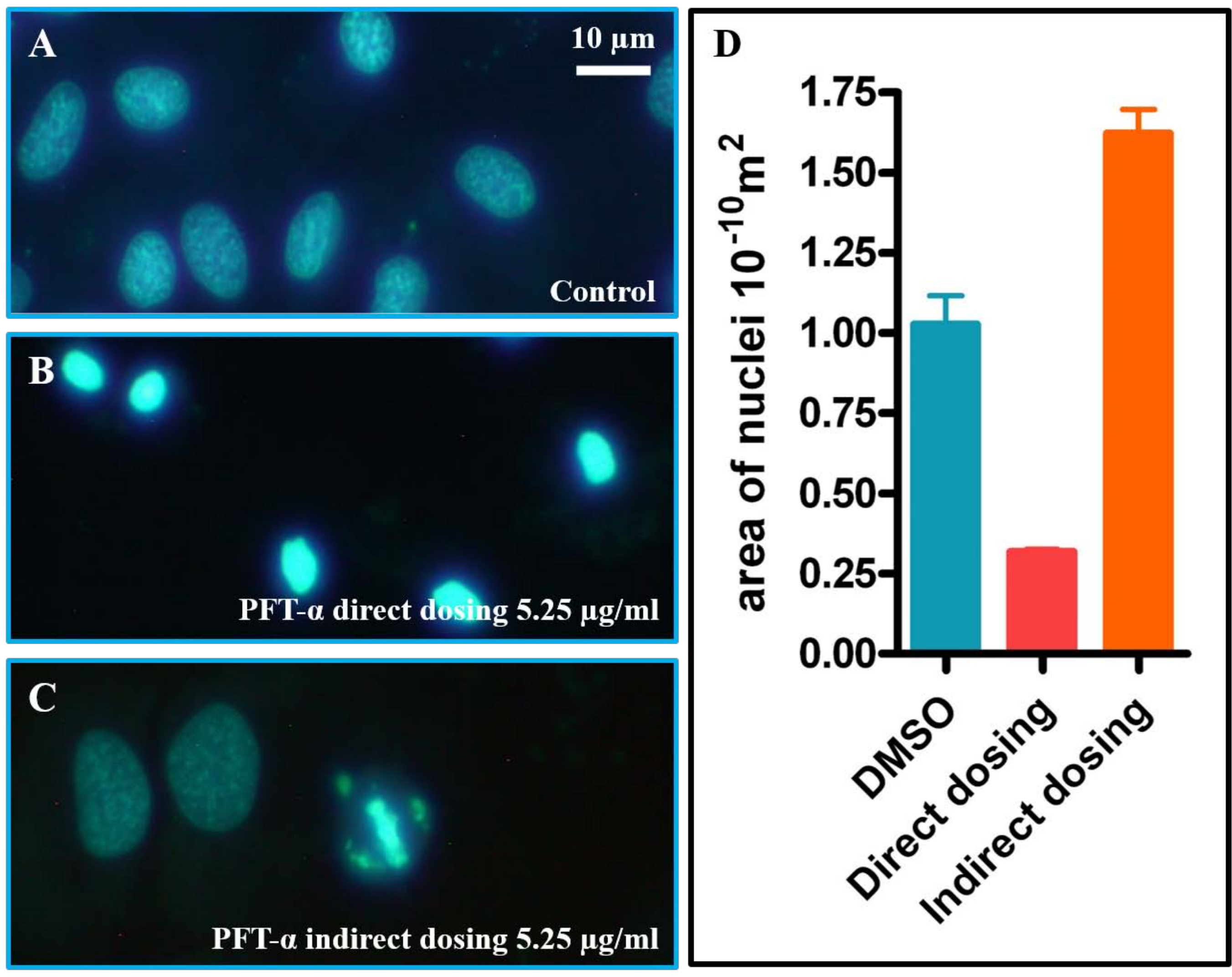


Figure 3.2 Effect of directly and indirectly dosing PFT-α into RTgill-W1 cultures on nuclear size.

Nuclei were visualized by fluorescence microscopy after H33258 staining for nuclear DNA. Panels A, B and C show nuclei in cultures 24 h after being directly dosed with either DMSO (A, control) or PFT-α (B, 5.25 μg/ml) or indirectly dosed with PFT-α (400X magnification). Panel C shows for cells in the three cultures the mean nuclear area, which were calculated with ImageJ. A comparison of the means by one-way analysis of variance (ANOVA) was significant ($p < 0.05$) and a Tukey-Kramer Multiple Comparisons test found all pair wise comparisons were significantly different ($p < 0.05$).

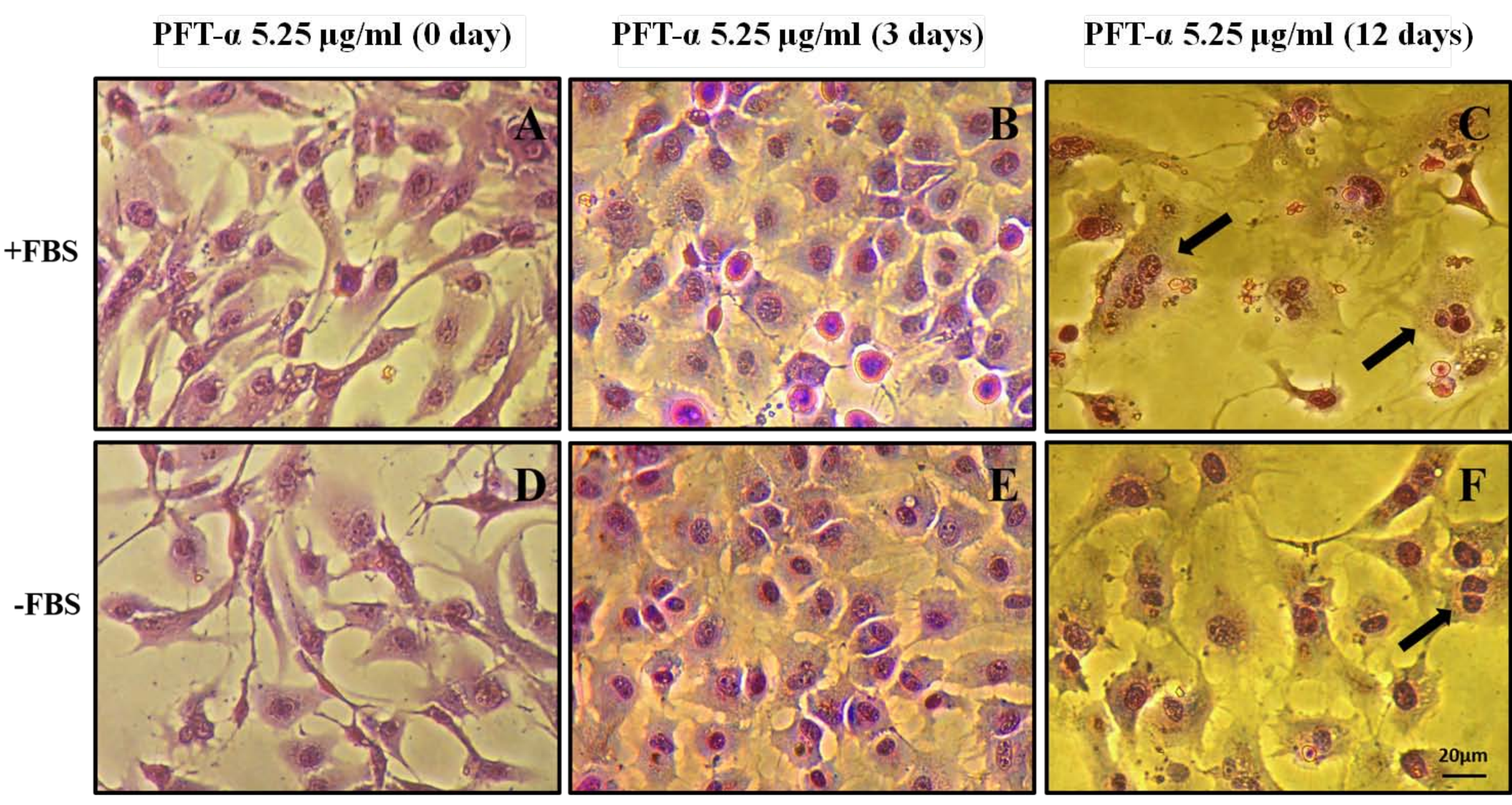


Figure 3.3 Effect of long-term exposure to PFT-α on the appearance of RTgill-W1 cultures as visualized by May-Grunwald-Giemsa staining.

After being indirectly dosed with 5.25 µg/ml PFT-α, cultures with (A, B & C) and without FBS (D, E & F) were stained immediately (A & D) or 3 (B & E) and 12 days (C & F) afterwards. The black arrows indicate multinucleated cells (C & F) (200 X magnification).

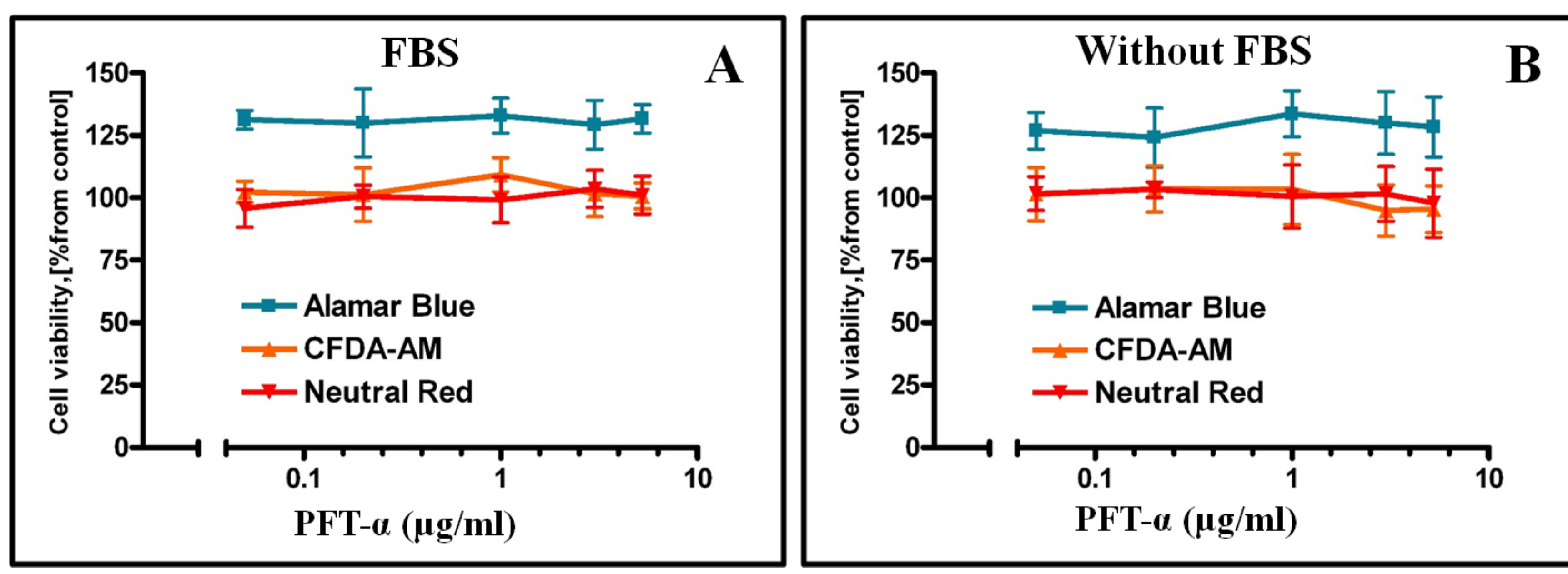


Figure 3.4 Effect of indirectly dosing PFT-α into RTgill-W1 cultures on cell viability.

After 24 h of exposure with (A) or without FBS (B), cultures were examined for cell viability with three indicator dyes as described in Figure 1. Cell viability is plotted on the y-axis against increasing PFT-α concentrations on the x-axis.

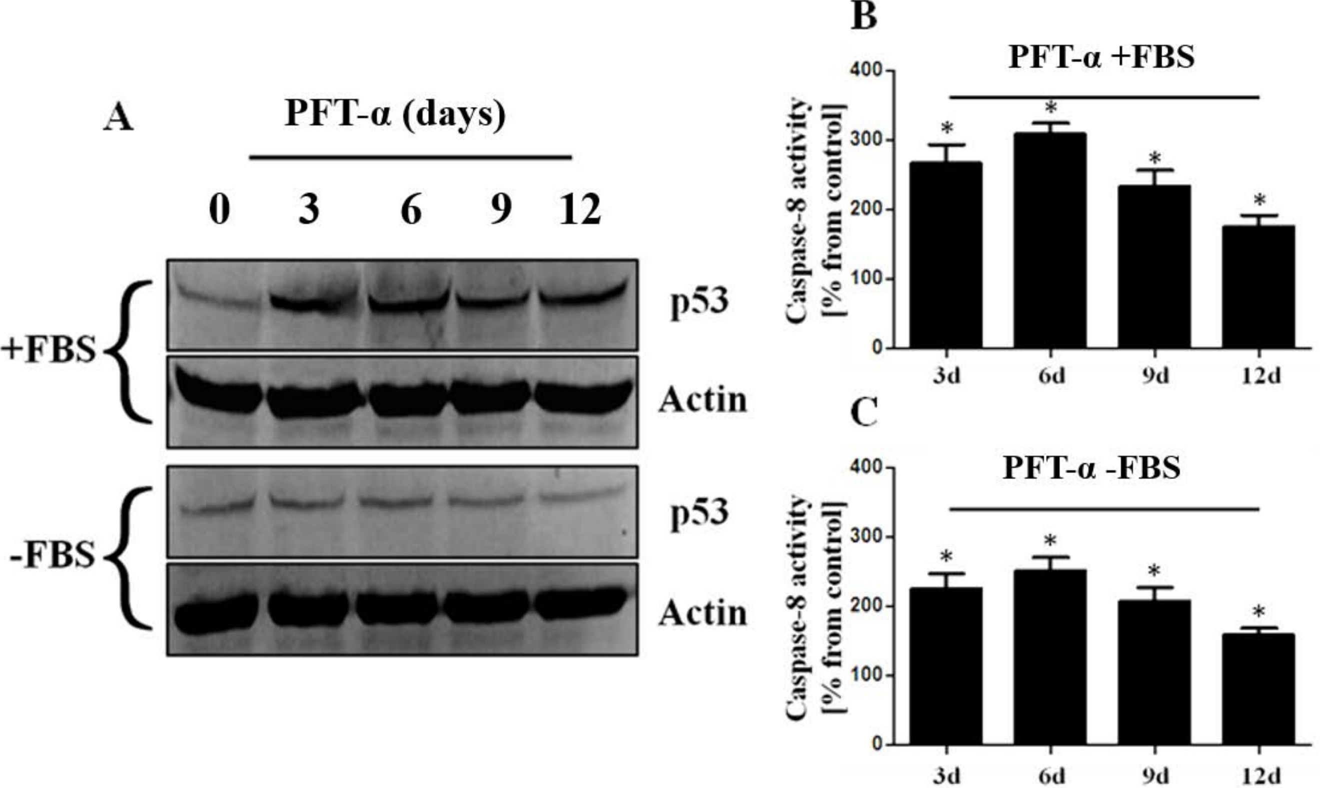


Figure 3.5 Effect of long-term exposure to PFT-α on p53 levels and caspase-8 activity in RTgill-W1 cultures.

In panel A, cultures with and without FBS were indirectly dosed with 5.25 μg/ml of PFT-α and incubated for 0, 3, 6, 9, and 12 days at which times cell extracts were prepared. SDS PAGE was used to separate polypeptides in 50 μg of cell extract followed by western blotting to identify p53 and actin. For caspase-8 activity, cultures with (B) and without FBS (C) were dosed indirectly with either DMSO (control) or 5.25 μg/ml of PFT-α. Caspase-8 activities were measured with a commercial colorimetric assay (BioVisions) at 0, 3, 6, 9, and 12 days in control and PFT-α cultures. For each time point the activities were compared with a t-test and found to be significantly different ($p<0.05$). Caspase-8 activity in PFT-α cultures is plotted as a percentage of the activity in control cultures of the same age.

3.3.3 PFT-α increased the mitotic index and disrupted microtubules

As viewed by phase contrast microscopy and after H33258 staining, cultures that had been incubated for 24 h with PFT-α at 5.25 μg/ml had more cells undergoing mitosis than cultures with DMSO (control) (Fig.3.6). In PFT-α cultures the mitotic index was approximately 20 fold higher in control cultures (Fig.3.6). However this difference had disappeared by 72 h and mitotic figures were hard to find in PFT-α cultures. Thus the increase in mitotic cells was transitory.

As microtubule-disrupting compounds are often reported to increase the mitotic index, cultures with or without FBS were immuno-fluorescently stained for microtubules and examined by confocal microscopy (Fig.3.7). The staining revealed a cytoplasmic network of microtubules in control cultures. By contrast in cultures with PFT-α 5.25 μg/ml, the network of microtubules had collapsed around the nuclei after 3 days and was completely disrupted by 12 days.

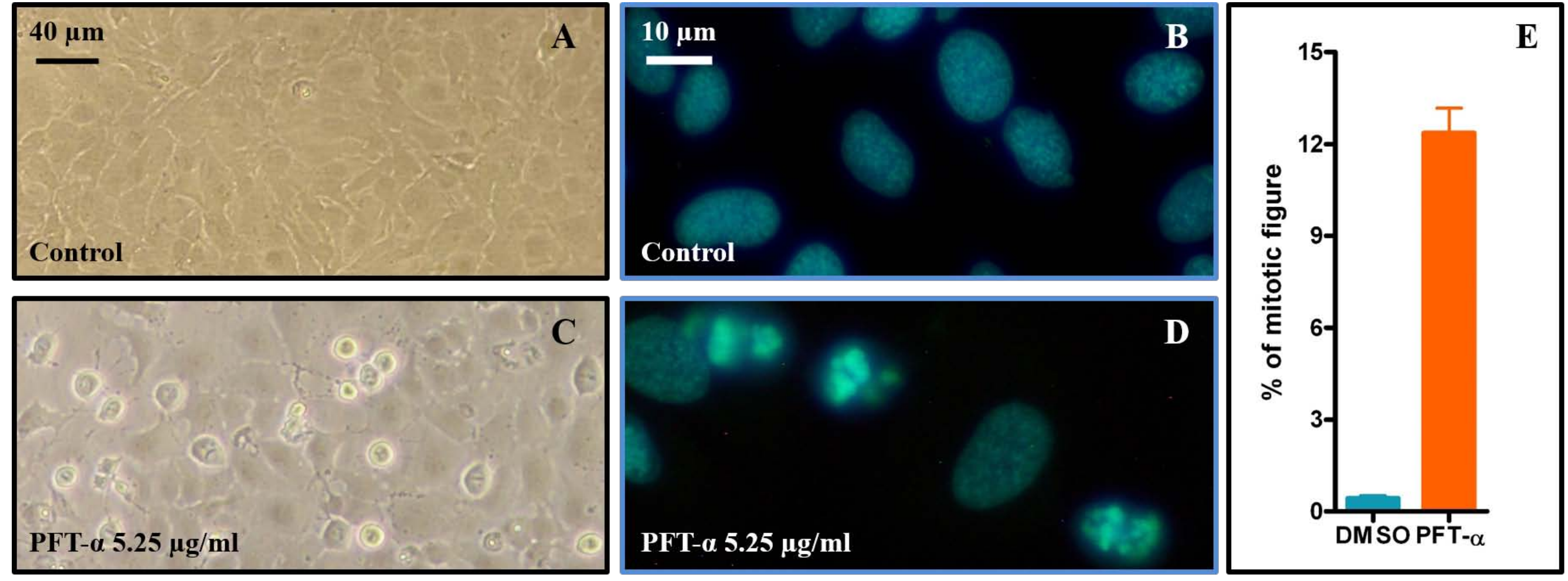


Figure 3.6 Effect of PFT-α on the appearance of mitotic figures in RTgill-W1 cultures.

Cultures with FBS were indirectly dosed with 0.5 % DMSO (A & B) or 5.25 µg/ml of PFT-α (C & D) and examined 24 h later by phase contrast microscopy (A & C) or H33258 staining (B & D) (400 X magnification). The proportion of mitotic figures in the control and PFT-α treated cultures is plotted in E.

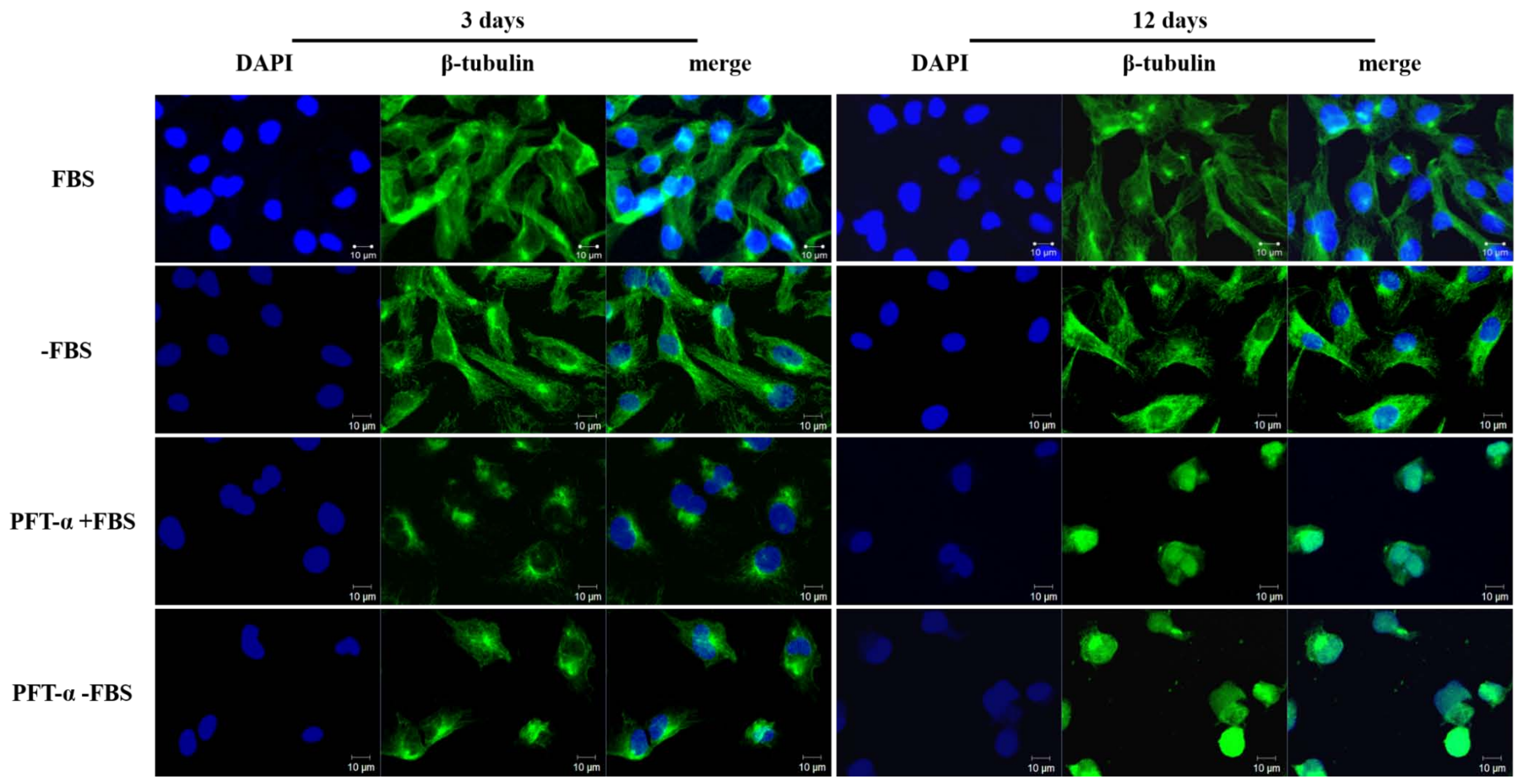


Figure 3.7 Effect of PFT-α on the immunofluorescent staining of microtubule networks in RTgill-W1 cells.

Cultures in L-15 were indirectly dosed with either DMSO (top 2 rows) or 5.25 μg/ml of PFT-α (bottom 2 rows). At 3 and 12 days after dosing, cultures were fixed and stained for microtubules with primary anti-tubulin antibody followed by secondary FITC-conjugated antibody (green) and for nuclei with DAPI (blue).

3.3.4 PFT-α inhibited cell proliferation

PFT-α inhibited RTgill-W1 cell proliferation. In cultures with or without FBS, PFT-α at 1.0, 3.0 and 5.25 μg/ml inhibited the accumulation of cells over 12 days (Fig.3.8A & B). Complete inhibition was seen at 5.25 μg/ml PFT-α in L-15 with FBS and 3.0 μg/ml PFT-α in L-15 alone. As senescence can occur upon the arrest of cell proliferation, cultures were stained for senescence-associated β-galactosidase (SAβGal), but the number of cells staining was less than 1 %, and unchanged by PFT-α.

3.3.5 PFT-α caused the accumulation of tetraploid and polyploid cells

When cultures in L-15 alone or L-15 with FBS were analyzed by Flow cytometry, the distribution of cell cycle stages differed between control and PFT-α (5.25 μg/ml)-treated cultures, with differences in tetraploid and polyploid cells being most notable (Fig.3.9). In the program that was used to analyze and present the data, tetraploid cells were identified as G2/M and polyploid cells as super G2.

PFT-α increased the proportion of tetraploid cells in both types of cultures, with the increase being most notable 3 days after the addition of PFT-α. After 3 days in L-15 alone, 18.4 ± 0.9 % (n= 3) of the cells were tetraploid or G2/M in control cultures, and 38.2 ± 1.6 % (n= 3) were in G2/M in PFT-α treated cultures. After 3 days in L-15 with FBS, 17.6 ± 1.7 % (n= 3) of the cells were in G2/M in control cultures, and 35.2 ± 2.6 % (n= 3) were in G2/M in PFT-α treated cultures. Thus with or without FBS, PFT-α approximately doubled the number of cells in G2/M. PFT-α increased the proportion of polyploid cells most noticeably in cultures with FBS after 12 days of treatment. After 12 days in L-15 alone, 6.7 ± 0.9 % (n= 3) of the cells were polyploid or super G2, and 10.5 ± 4.1 % (n= 3) were polyploid in PFT-α treated cultures. By contrast, after 12 days in L-15 with FBS, 4.2 ± 0.4 % (n= 3) of the cells were polyploid in control cultures, and 25.9 ± 3.7 % (n= 3) were polyploid in PFT-α treated cultures. Thus in cultures with FBS, PFT-α caused approximately a six fold increase in the number of polyploid cells.

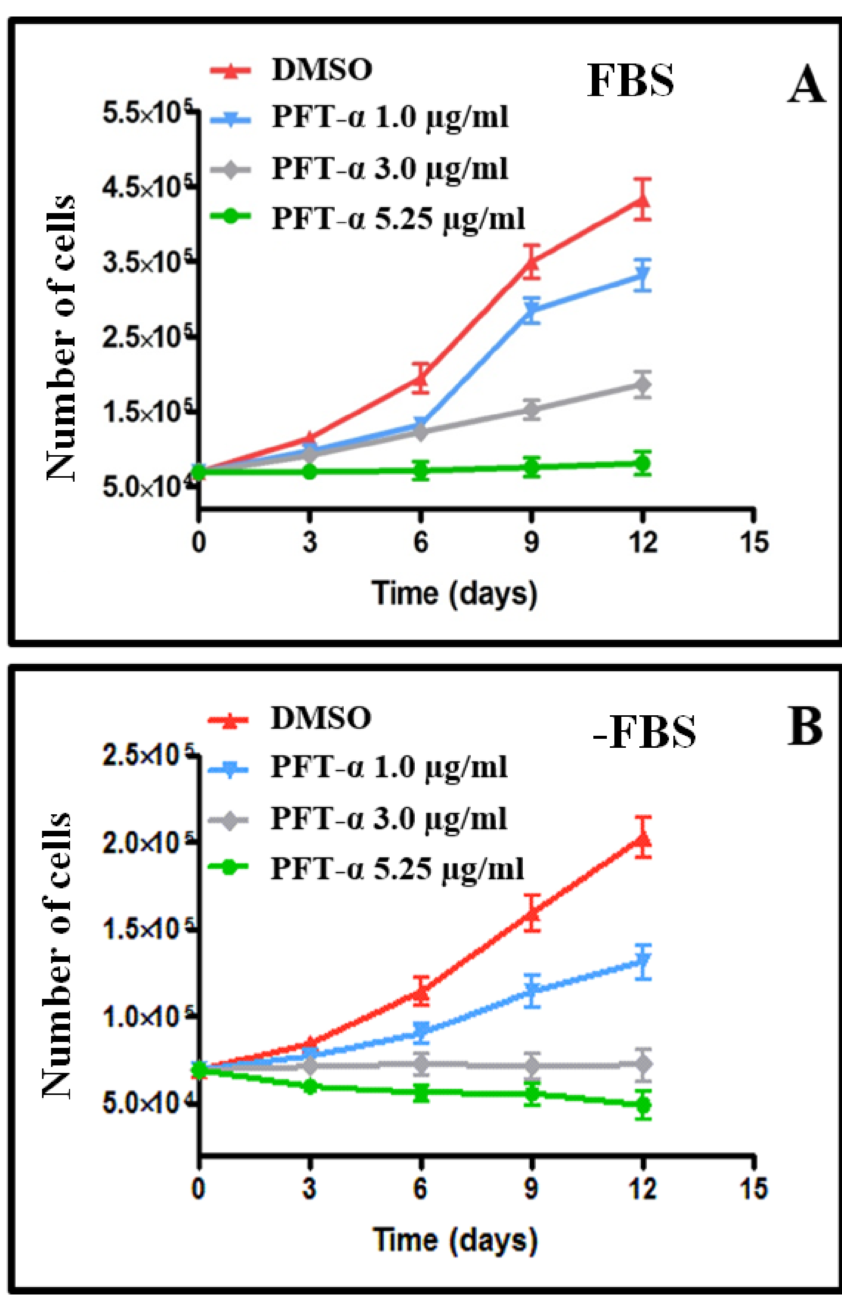


Figure 3.8 Effect of PFT-α on proliferation in RTgill-W1 cultures.

Cultures with (A) and without FBS (B) were indirectly dosed with different PFT-α concentrations and incubated for 0, 3, 6, 9, and 12 days at which times the cells were enumerated in replicate cultures with a Coulter counter. The mean cell numbers with standard deviations (n=3) are plotted against culture time.

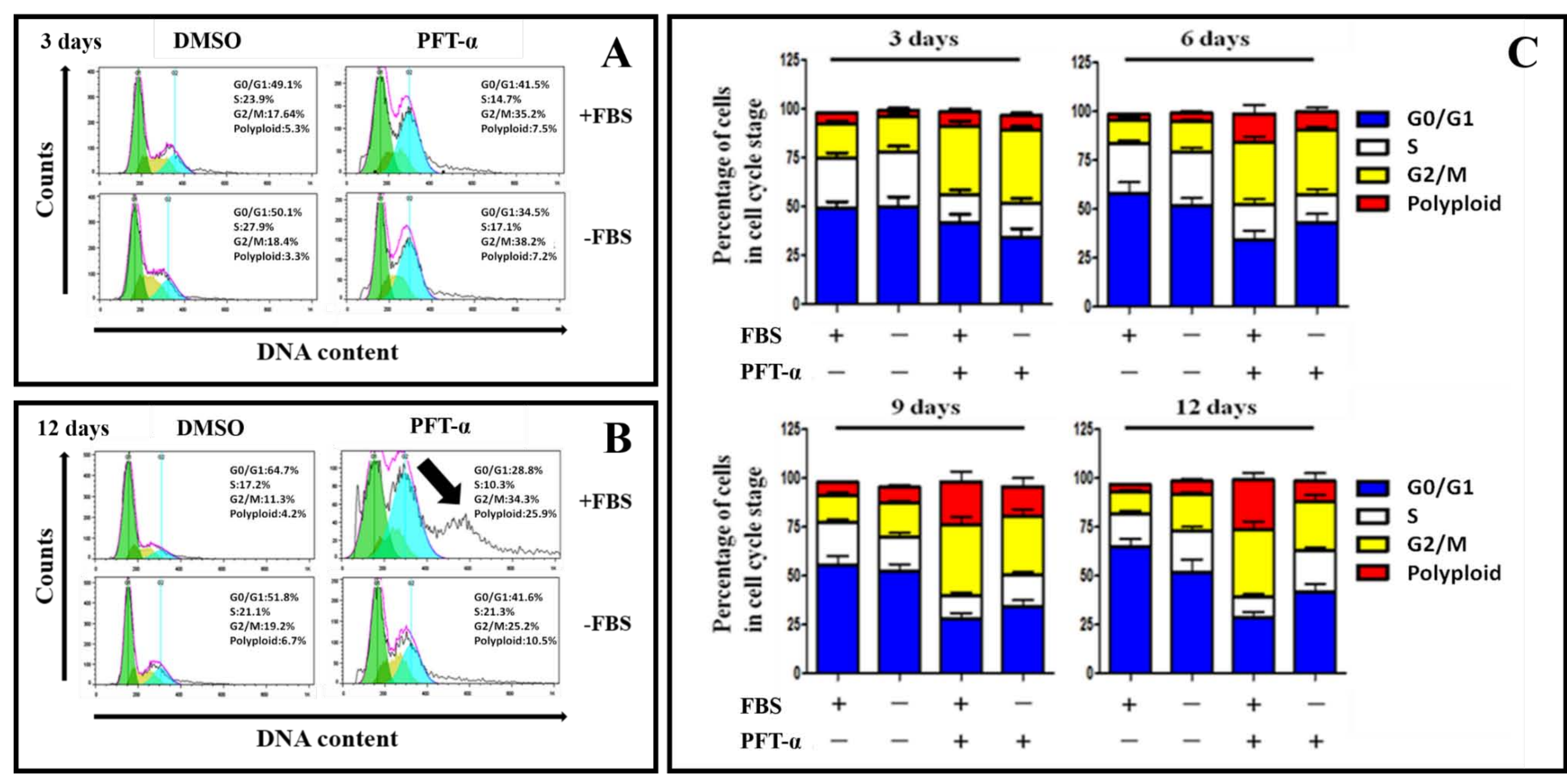
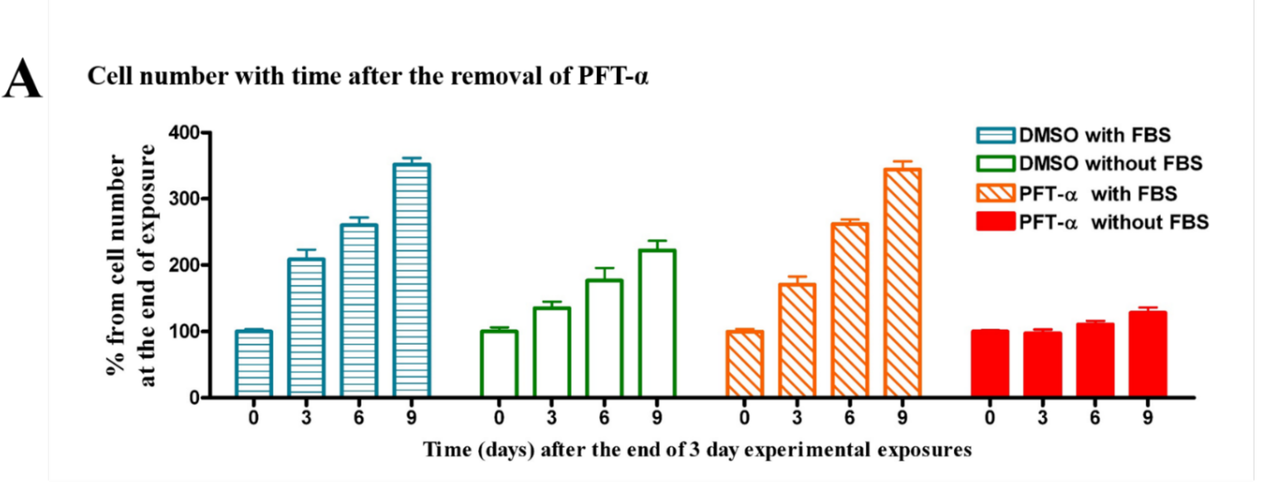


Figure 3.9 Effect of PFT-α on the cell cycle distribution in RTgill-W1 cultures.

Cultures with or without FBS were indirectly dosed with DMSO or with 5.25 µg/ml of PFT-α. Cell cycle stages were determined in these cultures 3 (A), 6, 9 and 12 days (B) afterwards by Flow cytometry of PI stained cells and analysis of the data by Flow Jo. The bar graphs in panel C summarize the data of three independent experiments.

3.3.6 Cells partially recovered and proliferated upon PFT-α removal

Recovery from the growth arrest induced by PFT-α depended on whether FBS was present (Fig.3.10). When PFT-α (5.25 μg/ml) was added to cultures in L-15 with 10% FBS for 3 days to arrest growth and then removed, RTgill-W1 cells proliferated over the next 9 days but not as much as in control cultures which indicates PFT-α induced a reversible cell cycle arrest instead of senescence. Nine days after the removal of PFT-α, cell number had increased 324 % (n=3), whereas in control cultures the increase was 354 % (n=3). At 9 days after the removal of PFT-α, the cultures still had some large, flattened cells and the proportion of G2/M cells and polyploidy cells was still high. Thus when FBS was present, RTgill-W1 had some limited potential to recover from PFT-α or 9 days was not long enough for RTgill-W1 to get fully recovered. When PFT-α (5.25 μg/ml) was added to cultures in L-15 alone for 3 days and then removed, the cell number and morphology in RTgill-W1 cultures remained largely unchanged over the next 9 days. The proportion of G2/M cells and polyploidy cells continued to be high. When a confluent flask culture in L-15 was treated for 3 days with pifithrin-α (5.25 μg/ml), allowed to recover for 9 days in L-15 without any additions, subcultivated with trypsin, and split into two flasks, the cells attached to the growth surface and grew to confluency if in these new flasks the L-15 had FBS. Thus in the absence of FBS, cells continued to remain viable 9 days after the removal of PFT-α. However, for these cells to proliferate again FBS had to be present.



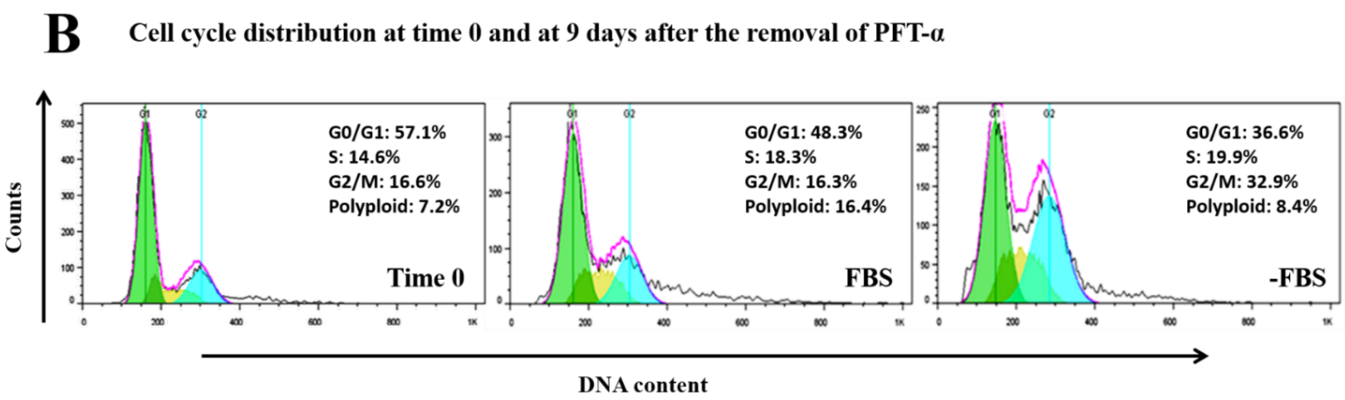


Figure 3.10 Recovery of cell proliferation in RTgill-W1 cultures after 3 day exposures to PFT-α.

Cultures in L-15 without or with FBS were indirectly dosed with either 5.25 µg/ml PFT-α or DMSO (control) and incubated for 3 days at which time the medium was removed and replaced with either L-15 or L-15 with FBS. In (A) cell number was determined immediately after the change of medium in 3 replicate cultures for each of the 4 culture conditions, which were recorded as time 0 and set at 100 %, and 3, 6 and 9 days later. The cell numbers for these later times were expressed as a percentage of their respective values at time zero. In B the cell cycle distribution was determined by flow cytometry at time zero and at 9 days after the removal of PFT-α.

3.4 Discussion

PFT-α was dosed directly and indirectly into RTgill-W1 cultures but dosing directly at high concentrations rapidly and completely killed cells making this an unsuitable way of studying the inhibitor. Directly dosing animal cell cultures with chemicals that are dissolved in dimethyl sulfoxide (DMSO) has been noted before to cause cell death at concentrations that when dosed indirectly has no effect (Schnell et al., 2008; Tanneberger et al., 2010). This has been attributed to the DMSO with the drug mixing heterogeneously into the culture medium causing cells to be transiently exposed to exceptionally high concentrations of the drug before the drug distributes evenly through the culture (Schnell et al., 2008; Tanneberger et al., 2010). Therefore, the effects of indirectly dosing PFT-α on cell viability and cell proliferation in RTgill-W1 cultures were studied and are discussed.

3.4.1 Effect of PFT-α on RTgill-W1 cell viability

When indirectly dosed into RTgill-W1 cultures, PFT-α did not induce cell killing. Previously, PES was found to induce reactive oxygen species (ROS) and apoptosis in indirectly dosed RTgill-W1 cultures (Zeng et al., 2014). By contrast, PFT-α failed to reduce three measures of cell viability (alamar Blue for metabolism, CFDA-AM for plasma membrane integrity and NR for lysosomal activity), induce reactive oxygen species (ROS), or activate caspases 3 and 9. PFT-α did slightly elevate caspase-8 activity but recently caspase 8 has been shown to be involved in more cellular processes than just cell death (Salvesen & Walsh, 2014). For mammalian cells, PFT-α most commonly protected against death processes (Beretta et al., 2008; Hashimoto et al, 2005, 2009; Sohn et al., 2009) but has to be used with caution because the drug killed some cells (Mullign et al. 2012; Walton et al, 2005). For fish cells as represented by RTgill-W1, PFT-α likely could be used to study the role of p53 in mediating cytotoxic responses after experimental treatments. However interpreting any change in growth would be difficult because as discussed below PFT-α affected RTgill-W1 proliferation.

3.4.2 Effects of PFT-α on RTgill-W1 cell proliferation

The addition of PFT-α to RTgill-W1 cultures by indirect dosing elicited several effects on the cell cycle. At 5.25 µg/ml, these teleost cell cultures had a transitory rise in the mitotic index, a block in cell proliferation, and an induction of polyploidy. Most of these effects have not been observed in studies of PFT-α on mammalian cells, although recently PFT-α was found to suppress murine embryonic stem cell renewal (Abdelalim & Tooyama, 2012). In RTgill-W1 cells, PFT-α also affected microtubules. Microtubules have not been noted as a target in mammals (Meng et al., 2014; Qi et al., 2014), and might be the underlying mechanism behind the effects of PFT-α on RTgill-W1 cell proliferation.

In RTgill-W1, PFT-α appears to target microtubules because the drug disrupted the network of cytoskeletal microtubules and transitorily increased the mitotic index. From studies with mammalian cells, these two observations would suggest a common target, microtubules. Drugs that target microtubules often temporarily interfere with mitosis and cause a transient rise in the mitotic index (Blajeski et al., 2002). Indeed, hundreds of structurally diverse compounds have been reported to arrest mitosis through their actions on microtubules in the mitotic spindle (Dumontet & Jordan, 2010). Some examples are colchicine, nocodazole, and taxol. These compounds bind different sites on tubulin, which assembles into microtubules, and block mitosis by impairing different steps in the formation and function of the mitotic spindle (Dumontet & Jordan, 2010).

PFT-α might be acting in RTgill-W1 directly through an off-target action on microtubules and/or indirectly through an on-target inhibition of p53. The tubulins of teleosts and mammals are similar but their colchicine-binding sites can differ (Skoufias et al., 1992) so perhaps PFT-α is better able to bind and disrupt teleost microtubules. Alternatively, microtubule-associated proteins, which are involved in the assembly and disassembly of microtubules (Dumontet & Jordan, 201), might be more tightly regulated by p53 in teleosts. In mammals, p53 regulates the expression of several microtubule-associated proteins by acting either as a transcriptional inducer or repressor (Ahn et al., 1999; Galmarini et al., 2003; Johnsen et al., 2000; Murphy et al., 1996; Utrera et al., 1998). Thus by blocking the transcriptional activity of p53 in RTgill-W1, PFT-α

might alter the expression of proteins involved in the assembly and disassembly of microtubules, leading to microtubule disruption and a transitory mitotic arrest.

An example of just one of several proteins that might be invoked to explain the current results is stathmin (STMN1 or Op18). Either over- or under-expression of stathmin impedes cell proliferation (Rubin & Athweh, 2004). In some cell lines, over expression of stathmin destabilizes microtubules and causes the accumulation of cells in mitosis (Marklund et al., 1996; Rubin & Atweh, 2004). Normally p53 suppresses the expression of stathmin (Ahn et al., 1999; Johnsen 2000). Perhaps in RTgill-W1 cultures, PFT-α might release the p53 suppression of the stathmin gene, elevating the level of stathmin and causing microtubule disruption and a mitotic index increase.

The action of PFT-α on RTgill-W1 microtubules might have triggered a mitotic spindle checkpoint. In mammalian cells, microtubule-depolymerizing agents, such as nocodazole or colchicine, arrest cells in mitosis by triggering the mitotic checkpoint (Blajeski et al., 2002). This checkpoint cells does not require p53 (Lanni & Jacks, 1998; Minn et al., 1996). Thus having a possibly impaired p53 as a result of PFT-α would not prevent a mitotic spindle checkpoint from functioning in RTgill-W1. In mammalian cells, the mitotic checkpoint is temporary. Upon prolonged treatment with microtubule-depolymerizing agents, cells escape the spindle checkpoint through mitotic slippage. Without completing anaphase and cytokinesis, the cells return to interphase with a tetraploid DNA content (Lanne & Jacks, 1998; Andreassen et al., 2001). Therefore, if PFT-α at 5.25 μg/ml were to trigger the mitotic spindle checkpoint in RTgill-W1 cultures, this would explain the transitory rise in mitotic index, the increase in the number of cells with a G2 DNA content, and the arrest of cell proliferation.

During long exposures, PFT-α might also be acting on a G1 checkpoint to arrest proliferation and cause polyploidization. After escaping the mitotic spindle checkpoint, mammalian cells with a functional p53 arrest in G1, but without p53 they enter S phase and endoreduplicate their DNA, resulting in polyploidization (Meek, 2000; Margolis et al., 2003; Vogel et al., 2004). Thus the increased number of polyploid cells in RTgill-W1 cultures after 12 days with PFT-α (5.25 μg/ml) might have been due to two actions of the drug. Firstly, as discussed in the previous paragraph, PFT-α might have triggered the mitotic spindle checkpoint. Secondly as cells escaped the mitotic checkpoint overtime, PFT-α might have blocked the p53-mediated G1 checkpoint that would

normally have come into play. This would have allowed cells in complete growth medium (L-15 with FBS) to enter S phase and become polyploid. Therefore like in mammalian cells (Aylon & Oren, 2011; Vogel et al., 2004) p53 appears to regulate polyploidy in teleost cells.

When microtubule-depolymerizing agents trigger sequentially the mitotic spindle and G1 checkpoints in mammalian cell cultures, p53 levels have been found to increase (Vogel et al., 2004; Orth et al., 2012) and cells to senesce (Margolis et al., 2003; Meek 2000; Pitto et al., 2011) but PFT-α appeared not to elicit these responses in RTgill-W1 cultures. The p53 protein level was slightly up regulated by PFT-α but only in cultures with FBS. RTgill-W1 senescence was evaluated by staining for senescence associated β-galactosidase (SA-βGal) activity, but the activity was low in control RTgill-W1 cultures and was unchanged by PFT-α. Possibly SA-βGal is a poor marker for fish cell senescence in vitro (Vo et al., 2015). Alternatively, a p53 that is unimpaired by PFT-α might be required for fish cellular senescence.

An increase in nuclear size was one of the early responses to PFT-α by RTgill-W1. Although nuclear size changes have not be been noted previously with this drug, several observations suggest that PFT-α might be increasing nuclear size by acting through either microtubules and/or p53. The microtubule-depolymerizing agent, nocodazole, caused nuclei in mouse embryo cells in culture to enlarge (Mazumer & Shivashankar, 2010). Nuclear proteins are required for nuclear growth in some experimental systems (Webster et al., 2009) and p53 has been postulated to act as a suppressor of nuclear import (Feldherr et al., 1994). When mouse 3T3 cells were treated for 3 h with PFT-α, nuclear pore size increased, although the nuclear import rate for a specific protein, BSA, was unchanged (Feldherr et al., 2001). How the increase in nuclear size relates to the transitory rise in mitotic index and arrest of cell proliferation will be interesting to explore in the future.

3.5 Conclusions

The response of RTgill-W1 to PFT-α raised the difficulty of interpreting experiments with p53 inhibitors due to their possible off-target actions but possibly revealed the importance of p53 in regulating microtubules in fish cells. PFT-α caused a transient rise in the mitotic index and the disruption of cytoskeletal microtubules in RTgill-W1, suggesting that p53 might be regulating

the assembly and disassembly of microtubules in the fish cells. The results reinforce the view that experiments with inhibitors must be interpreted with caution and other approaches should be used as well.

CHAPTER 4

Use of rainbow trout cells to investigate the toxicity of the emerging contaminants, benzotriazoles

4.1 Introduction

Benzotriazoles (BTRs) are emerging contaminants (EC) (Deblonde et al., 2011; Dummer, 2014; Jana et al., 2011). From 1H-benzotriazole (1HBT or BTR), the parent compound, hundreds of BTRs have been synthesized. BTRs are heterocyclic compounds, consisting of a benzene ring in which two adjacent carbon atoms are covalently bonded to three nitrogen atoms in a five membered ring. The BTRs of this study are illustrated in Table 1. The annual global production is large, perhaps much greater than 9,000 tons (Wang et al., 20013; Herzog et al., 2014a), because BTRs have several useful properties that are exploited in diverse commercial products. For example, some BRTs are added as corrosion inhibitors to detergents and aircraft deicing fluids (Breedveld et al., 2003; Janna et al., 2011). Many of the uses are in “down-the-drain” products (Janna et al., 2011). As a result, BTRs enter the environment through sewage and industrial wastewater and have been detected in several European rivers (Vousta et al., 2006; Giger et al., 2006; Kiss & Fries, 2009, 2012; Reemsta et al., 2006, 2010; Janna et al., 2011). The Rhine River had BTR concentrations ranging from 130 to 3500 ng/L (Reemsta et al., 2010) and was a likely source of BTRs in the North Sea (Wolschke et al., 2011).

Limited information is available about the toxicology of BTRs, especially their impact on fish. The acute toxicity of BTR has been determined in a few species, fathead minnow, bluegill and rainbow trout, and found not to be very toxic (Hartwell et al., 1995; Milanova et al., 2001; Pillard et al., 2001). The concentration that killed 50 % of the fish (LC_{50}) in a 96 h was approximately 50 mg/L. Studies on sub-lethal effects have focused on their potential to cause endocrine disruption (Harris et al., 2007; Kent et al., 2014, Liang et al., 2014). The outcomes are hard to generalize but BTR did seem to have the potential to impact the male reproductive system (Kent et al., 2014; Liang et al, 2014). Recently transcriptional profiles have been examined in zebrafish eleuthero-embryos after exposure to BTR or to BTRs that are used in products to prevent UV damage and referred to as BUVs such as UV-P and UV-326 (Kent et al, 2014). UV-P and UV-326 but not BTR activated the aryl hydrocarbon receptor (AhR)-pathway, as indicated by the increased expression of several genes in the pathway (Kent et al., 2014). The largest increase was in transcripts for CYP1A1, which codes for cytochrome P4501A1 (CYP1A1). P4501A is involved in xenobiotic metabolism and the AhR pathway mediates many toxic actions of dioxin and dioxin-like compounds (Furness and Whelan, 2009).

One approach for investigating the potential of environmental toxicants to impact fish is to use fish cell cultures (Bols et al., 2005) but this has yet to be done for BTRs. Cell cultures, most often of cell lines, can be used to compare the relative capacity of compounds to be genotoxic and cytotoxic and to trigger reactive oxygen species (ROS) production and the AhR pathway. For genotoxicity, several endpoints can be studied and the comet assay, which can measure single and double- strand DNA breaks (Dhawan et al., 2009), has been applied successfully to fish cell lines (Bopp et al., 2008; Kienzler et al., 2013). For cytotoxicity, cellular functions can be evaluated with indicator dyes such as Alamar Blue (AB) for metabolism, with 5-carboxyfluorescein diacetate acetoxymethyl (CFDA AM) for cell membrane integrity, and Neutral Red (NR) for lysosomal activity (Dayeh et al., 2014). The fluorescent indicator dye 2'7'-dichlorofluorescein, can be used to measure ROS (Chen et al., 2010; Zeng et al., 2014). An increase in the amount of P4501A1 as measured through western blots can be used to assess the ability to activate the AhR pathway (Hahn et al., 1993).

In this study, two rainbow trout epithelial cell lines, RTgill-W1 from the gill and RTL-W1 from the liver, have been used to evaluate the toxicity of seven BTRs (Table 1.1&1.2). RTgill-W1 was used to study the capacity of the BTRs to be cytotoxic, genotoxic, and stimulate ROS production, whereas P4501A induction was studied with RTL-W1. Of the 7 BTRs, 5,6-dimethyl-1H-benzotriazole (DM) elicited no responses. By contrast, 5-chlorebenzotriazole (5CBTR) caused changes in all endpoints and most often did so at the lowest dose, suggesting that this compound has the most potential to have a toxic impact on fish.

4.2 Materials and Methods

4.2.1 Cell culture

The rainbow trout gill cell line (RTgill-W1) established by Bols et al. (1994) and the rainbow trout liver cell line (RTL-W1) established by Lee et al. (1993) were routinely cultured in 75 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) at room temperature in Leibovitz's L-15 culture medium (Sigma-Aldrich, Ltd., Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin solution (10000 units/ml penicillin, 10 mg/ml streptomycin, Sigma-Aldrich).

4.2.2 Cytotoxicity assay

4.2.2.1 Plating and dosing

Cells were seeded in 96 well plates (Becton and Dickinson Company, Franklin Lakes, NJ. USA) at a density of $4x10^4$ cells per well in 200 µl of L-15 growth medium with 10% FBS supplement. Cells were allowed to settle and reattach for 24 h at room temperature before being exposed to any compounds. The cells were then dosed with varying concentrations of BTR, 4MBTR, 5MBTR, 5CBTR, DM (Sigma-Aldrich), TT, OHBTR (AK Scientific, Inc) in L-15 without FBS. For co-exposure, cell cultures were pre-treated with N-acetylcysteine (NAC, Sigma-Aldrich), Necrosis Inhibitor IM-54, Necrostatin-1 (Santa Cruz Biotechnology) 1 h before adding BTRs. Application of chemicals to cell cultures was done by adding culture medium mixed with chemical solution to the culture well. The final concentration of the solvents (such as DMSO or water) in each well was the same as for the control wells, which were only dosed with solvent. After 24 h or 12 d, cultures were evaluated for cytotoxicity. In no cases was the solvent, NAC, IM-54 or Necrostain-1 used at a concentration that was cytotoxic.

4.2.2.2 Measuring cell viability

Three fluorescent indicator dyes were used to evaluate cell viability. Metabolic activity was measured by Alamar Blue (Medicorp, Montreal, PQ). Cell membrane integrity was evaluated

with 5-carboxyfluorescein diacetate (CFDA-AM) (Molecular Probes, Eugene, OR). Lysosome integrity was monitored with Neutral Red (Sigma-Aldrich). Alamar Blue, CFDA-AM and Neutral Red were prepared in Dulbecco's phosphate buffered saline (DPBS, Lonza, Walkersville, MD USA) to give final concentrations of 5 % (v/v), 4 μM and 1.5 % (v/v) respectively. Cells were incubated with dyes for 1 h in dark then quantified by fluorescence plate reader (Spectra-max Gemini XS microplate spectrofluorometer; Molecular Devices, Sunnyvale, CA). The excitation and emission wave-lengths used were 530 and 590 nm for Alamar Blue, 485 and 530 nm for CFDA-AM, 530 and 640 nm for Neutral Red, respectively. Results were calculated as a percent of the control culture

4.2.3 Determining apoptosis

In order to test for apoptosis, cultures were evaluated for nuclear fragmentation, genomic DNA laddering and membrane phospholipid phosphatidylserine (PS) translocation. RTgill-W1 cells were seeded at a density of $4x10^5$ cells in 9 cm^2 slide flasks (Nunc, Kamstrupvej, Denmark) for H33258 staining and at a density of $1x10^6$ cells in 25 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) for DNA gel electrophoresis and PE Annexin V assay. Cells were incubated at room temperature for 24 h in L-15 growth medium with 10 % FBS and then exposed to varying concentrations of BTRs in L-15 without 10 % FBS.

4.2.3.1 Hoechst 33258 Stain

24 h or 12 d after treatment, RTgill-W1 cultures were fixed by adding an equal volume of Carnoy’s fixative (methanol:glacial acetic acid, 3:1), which was prepared fresh with each use, to existing media, exposing the cells for 2 min. The media and fixative were then removed and fresh fixative was added to the cells twice for 5 min. Following fixation, the cells were stained with 0.5 µg/ml Hoechst 33258 for 10 min in dark. After several washes with deionized water and the final wash with Mcllvaine’s buffer, a coverslip was mounted onto the slide with Mcllvaine’s buffer and glycerol (1:1). The fluorescent nuclei were visualized using a fluorescent microscope with an ultra-violet (UV) filter (Nikon Optishot).

4.2.3.2 DNA fragmentation ladder

At 24 h or 12 d after treatment, cells were collected and genomic DNA was extracted using a GenElute™ mammalian genomic DNA miniprep kit according to manufacturer's instructions (Sigma-Aldrich). 25 µl of DNA was resolved by electrophoresis on a 2 % (w/v) agarose gel mixed with gel red (1 in 10000) (Biotium, CA) for 3 h at 60 V. The DNA ladders were visualized under UV transillumination.

4.2.3.3 PE Annexin V

Early stage apoptosis was examined using a PE Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's instructions. PE -Annexin V detects early stage apoptosis by binding phospholipid phosphatidylserine (PS) which is externalized to the outer leaflet of the plasma membrane in apoptotic cells. 7-Amino-Actinomycin (7-AAD), a DNA intercalating dye, was added along with PE-Annexin V to detect compromised membrane integrity. After treatment, cells were harvested by trypsinization at different time periods (6 and 24 h) and washed twice with cold DPBS. The cells were then resuspended in 1 ml of 1X binding buffer ($1x10^5$ cells per assay). The suspended cells were incubated with PE Annexin V and 7-AAD for 15 min at room temperature in the dark. Then, 400 µl of 1X binding buffer was added to the cells for flowcytometric analysis (10000 events/sample) and data were analyzed by the Flowjo software (Treestar, Inc., San Carlos, CA).

4.2.4 Intracellular ROS measurement

Intracellular ROS were determined by using 2′,7′ -Dichlorofluorescin diacetate (H_2DCFDA) (Sigma-Aldrich). RTgill-W1 cells were seeded in 96 well plates at a density of $4x10^4$ cells per well in L-15 growth medium with 10 % FBS. After 24 h incubation at room temperature to allow reattachment, the cells were treated with different concentrations of BTRs for 0-24 h. Cells were then incubated with H_2DCFDA working solution of 10 µM in PBS at RT for 40 min. Afterwards, cells were washed twice with pre-warmed PBS and incubated with pre-warmed PBS for 10 min. ROS were measured using a fluorescent plate reader (Spectra-max Gemini XS microplate

spectrofluorometer; Molecular Devices, Sunnyvale, CA) at an excitation and emission wavelengths of 485 and 530 nm. ROS level was expressed as percentage of the fluorescence over control samples.

4.2.5 Alkaline comet assay

To evaluate the level of DNA damage in RTgill-W1 cultures treated with BTRs the comet assay was carried out under alkaline conditions, basically as described by Singh et al (1988). RTgill-W1 cells were seeded in 12 well plates (Becton and Dickinson Company, Franklin Lakes, NJ. USA) at a density of $4x10^5$ cells per well in 2 ml of L-15 growth medium with 10 % FBS supplement. Cells were allowed to settle and reattach for 24 h at room temperature before being exposed to any compounds. The cells were then dosed with varying concentrations of BTRs in L-15 without FBS for 24 h or 12 d. Cultures treated with hydrogen peroxide (100 µM) were used as positive control. After treatment, cells were harvested by trypsinization (0.05 % trypsin in DPBS) and 10 µl of cell suspension ($2x10^6$ cells/ml) was mixed with 120 µl of 0.5% low melting point (LMP) agarose (Sigma-Aldrich) at 37 ºC. The mixture was then placed onto a frosted microscope slide precoated with 1.5 % normal melting point (NMP) agarose. The slides were covered with cover slips and allowed to set for at least 10 min at 4 ºC. The coverslips were removed and the slides were immersed in ice-cold, freshly prepared lysis solution (1 % Triton X-100, 2.5 M NaCl, 10 mM Tris base, 0.1 M EDTA, 10 % DMSO, pH 10). After at least 1 h, the slides were placed in a horizontal gel electrophoresis tank (CSL-COM10, Cleaver Scientific) and DNA was allowed to unwind for 10 min in freshly made alkaline solution (300 mM NaOH and 1 mM EDTA; pH > 13) before the electrophoresis was carried out for 10 min at 300 mA and 25 V at 4 ºC. Then, the slides were neutralised in 0.4 M Tris base (pH 7.5) with three washes of 5 min each, rinsed in distilled water and dehydrated in absolute methanol. Lysis, unwinding and electrophoresis were conducted under dim light to prevent additional DNA damage. Before visualization, each slides was stained with ethidium bromide (20 µg/ml) and covered with a coverslip.

A minimum of 150 randomly captured comets (50 from each replicate slide) per samples were examined by fluorescent microscopy (Nikon Optishot). Manual scoring was performed on

the basis of the type of comet visualized on the slide. The comets were counted and classified into scores of ‘0’, ‘1’,‘2’, ‘3’ and ‘4’ according to DNA damage and head/tail migration (from undamaged, class 0, to maximally damaged, class 4) (Fig.4.2). Each single comet was scored visually and assigned into a unit from 0 to 4 depending on the relative intensity of DNA fluorescence in the tail. Three independent experiments were conducted in each treatment.

4.2.6 Western blotting for CYP1A protein

RTL-W1 cells were seeded in 25 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) at $1x10^6$ cells per flask in L-15 growth medium. After 24 h incubation at room temperature to allow reattachment, the cells were treated with BTRs at varying concentrations, and incubated again at room temperature for 48 h. Cells treated with 97.6 pM of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was used as positive control. Whole-cell protein extracts were prepared as described by Liu et al. (2011) and protein concentrations were determined by bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions (Pierce, Rockford, IL). Protein was separated by 12 % SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked with 5 % milk for 1 h, incubated with primary mouse anti-fish CYP1A antibody (1:3000) (Cedarlane, Burlintong, Canada) for 2 h, then incubated with secondary goat anti-mouse AP antibody (1:20000) (Sigma-Aldrich) for 1 h. AP substrates were then added to the blot (33 µl of 5-bromo-4-chloro-3-indolyl phosphate p-Toluidine Salt and 66 µl of Nitro BT mixed with 10 ml of PH 9.5 AP buffer) (Fisher Scientific). Ponceau staining was used as loading control.

4.2.7 Data analysis

All graphs and statistical analyses were done using GraphPad InStat (version 4.01 for Windows XP, GraphPad Software, San Diego, CA, www.graphpad.com). Statistical comparison was done using ANOVA test followed by Tukey-Kramer Multiple Comparisons Test.

4.3 Results

4.3.1 Acute and Sub-chronic cytotoxicity of BTRs

DM was not soluble in L-15 and was not able to reduce the viability of RTgill-W1 up to 12 days. BTR, 4MBTR, 5MBTR, TT, OHBTR and 5CBTR reduced RTgill-W1 viability in a dose-dependent manner after 24 h and 12 d treatment. As judged by light microscopy, cells treated with BTRs retracted from neighbouring cells but remained loosely attached to the growth surface. Most cell deaths were accompanied by cytoplasmic swelling (Fig.4.1) and nuclei condensation (revealed by Hoechst 33258 staining, data not shown), some dead cells appeared ruptured (Fig.4.1). EC_{50} values of cells treated with BTRs were evaluated by Alamar Blue, CFDA-AM and Neutral Red (Table 4.1 & 4.2).

For 24 h exposure of BTRs, metabolic activity overall responded most sensitively. The EC_{50} values for cell membrane integrity are about 1.5 to 4.2 fold higher compared to metabolic activity. The difference between metabolic activity and lysosomal integrity is less distinct, ranging from 0.6 to 1.2 fold. 5CBTR has the highest acute cytotoxicity among the seven BTRs tested. The EC_{50} values for 5CBTR were 44.8 ± 5.6 mg/L (0.29 ± 0.04 mM) (n=3) as evaluated by Alamar Blue, 81.7 ± 24.8 mg/L (0.53 ± 0.16 mM) (n=3) as evaluated by CFDA-AM and 70.2 ± 7.9 mg/L (0.46 ± 0.05 mM) (n=3) as evaluated by Neutral Red. BTR has the lowest acute cytotoxicity among the six soluble BTRs tested. The EC_{50} values for BTR were 360.2 ± 37.7 mg/L (3.02 ± 0.32 mM) (n=3) as evaluated by Alamar Blue, 544.5 ± 29.9 mg/L (4.57 ± 0.25 mM) (n=3) as evaluated by CFDA-AM and 565.7 ± 27.6 mg/L (4.74 ± 0.23 mM) (n=3) as evaluated by Neutral Red.

For 12 d exposure of BTRs, the three measures of cell viability gave broadly similar EC_{50} values. 5CBTR has the highest sub-chronic cytotoxicity among the seven BTRs tested. BTR has the lowest acute cytotoxicity among the six soluble BTRs tested.

The treatment with inhibitor of the necroptosis, Necrostatin-1 (up to 100 μM) or a selective inhibitor of oxidative stress-induced necrotic cell death, IM-54 (up to 20 μM), failed to inhibit cell death in cultures exposed to BTRs (data not shown).

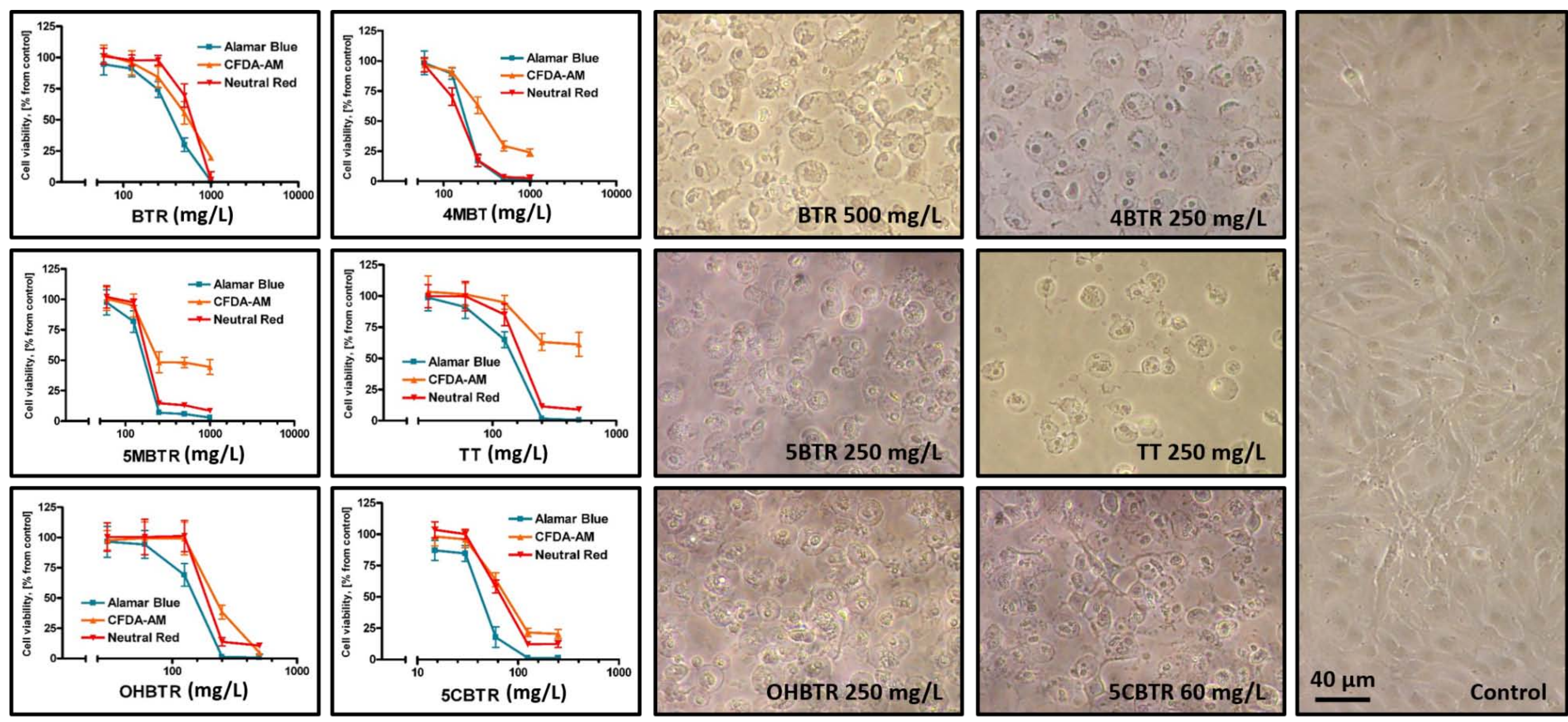


Figure 4.1 Acute cytotoxicity of benzotriazoles on RTgill-W1.

RTgill-W1 cells were treated with 5 concentrations of BTR, 4MBTR, 5MBTR, TT, OHBTR or 5CBTR for 24 h in L-15 without FBS. Cytotoxicity was measured by Alamar Blue, CFDA-AM and Neutral Red. Y-axis represents the percentage of cell viability from control treated with 0.5 % DMSO. X-axis represents the concentration of BTRs in mg/L. Pictures were taken at 200X magnification.

Table 4.1 Effect of 24 hours benzotriazoles exposures on RTgill-W1 cell viability

	Mean EC50 ± SEM (n=3) measured for three viability assays*			
	Alamar Blue♦ (metabolism)	CFDA-AM□ (membrane integrity)	Neutral Red# (lysosomal activity)	
Benzotriazoles (most to least toxic according to AB assays)	**mg/L (mM)**	**mg/L (mM)**	**mg/L (mM)**	**Statistical comparison of viability assays**
5CBTR	**44.8 ± 5.6 (0.29 ± 0.04)**	**81.7 ± 24.8 (0.53 ± 0.16)**	**70.2 ± 7.9 (0.46 ± 0.05)**	**AB=CF=NR**
TT	**139.1 ± 14.2 (0.87 ± 0.09)**	**591.4 ± 58.7 (3.71 ± 0.37)**	**172.5 ± 16.5 (1.08 ± 0.10)**	**CF>AB=NR**
5MBTR	**140.7 ± 18.3 (1.05 ± 0.14)**	**511.9 ± 61.2 (3.84 ± 0.46)**	**183.0± 24.3 (1.37 ± 0.18)**	**CF>AB=NR**
OHBTR	**141.1 ± 11.6 (1.19 ± 0.10)**	**231.5 ± 20.3 (1.95 ± 0.17)**	**222.1 ± 19.7 (1.87 ± 0.16)**	**CF=NR>AB**
4MBTR	**168.7 ± 3.3 (1.27 ± 0.02)**	**348.1 ± 59.5 (2.62 ± 0.44)**	**139.8 ± 12.4 (1.05 ± 0.09)**	**CF>AB=NR**
BTR	**360.2 ± 37.7 (3.02 ± 0.32)**	**544.5 ± 29.9 (4.57 ± 0.25)**	**565.7 ± 27.6 (4.74 ± 0.23)**	**CF=NR>AB**
DM	**Not cytotoxic**	**Not cytotoxic**	**Not cytotoxic**	**Not applicable**

*** ANOVA across rows and down columns were significant ($p<0.05$), except for the 5CBTR row, and were followed by Tukey-Kramer Multiple Comparisons Test ($p<0.05$).**

♦ Mean for BTR was significantly different from the means for the other BTRs and the mean for 5CBTR was different from the means for the other BTRs.

□ Mean for TT was significantly different from the means for the other BTRs, except for 5MBTR and BTR and the mean for 5CBTR was significantly different from the means for the other BTRs except for OHBTR.

Mean for BTR was significantly different from the means for the other BTRs and the mean for 5CBTR was different from the means for the other BTRs, except for 4MBTR.

Table 4.2 Effect of 12 days benzotriazoles exposures on RTgill-W1 cell viability

	Mean EC50 ± SEM (n=3)measured for three viability assays*			
	Alamar Blue♦ (metabolism)	**CFDA-AM□ (membrane integrity)**	**Neutral Red# (lysosomal activity)**	
Benzotriazoles (most to least toxic according to AB assays)	**mg/L (mM)**	**mg/L (mM)**	**mg/L (mM)**	**Statistical comparison of viability assays**
5CBTR	**15.6 ± 2.7 (0.10 ± 0.02)**	**18.4 ± 3.2 (0.12 ± 0.02)**	**17.4 ± 3.6 (0.11 ± 0.02)**	**AB=CF=NR**
4MBTR	**58.6 ± 4.2 (0.44 ± 0.03)**	**77.8 ± 7.9 (0.59 ± 0.06)**	**69.2 ± 8.2 (0.52 ± 0.06)**	**AB=CF=NR**
TT	**66.2 ± 5.9 (0.41 ± 0.04)**	**82.7 ± 10.3 (0.51 ± 0.06)**	**87.3 ± 6.9 (0.55 ± 0.04)**	**AB=CF=NR**
5MBTR	**75.2 ± 6.1 (0.56 ± 0.05)**	**87.7 ± 5.6 (0.65 ± 0.04)**	**77.8 ± 4.5 (0.58 ± 0.03)**	**AB=CF=NR**
OHBTR	**121.1 ± 8.8 (1.02 ± 0.07)**	**149.8 ± 9.5 (1.26 ± 0.08)**	**114.8 ± 10.2 (0.97 ± 0.09)**	**AB=CF=NR**
BTR	**131.6 ± 11.3 (0.89 ± 0.08)**	**165.6 ± 12.6 (1.12 ± 0.09)**	**142.2 ± 11.8 (0.96 ± 0.08)**	**AB=CF=NR**
DM	**Not cytotoxic**	**Not cytotoxic**	**Not cytotoxic**	**Not applicable**

*** ANOVA across rows were not significant (p >0.05), and ANOVA down columns were (p<0.05) and were followed by Tukey-Kramer Multiple Comparisons Test (p<0.05).**

♦ Mean for BTR was significantly different from the other BTRs, except for OHBTR and mean for 5CBTR was significantly different from the means for the other BTRs.

□ Mean for BTR was significantly different from the other BTRs, except for OHBTR and mean for 5CBTR was significantly different from the means for the other BTRs.

Mean for BTR was significantly different from the other BTRs, except for OHBTR and mean for 5CBTR was significantly different from the means for the other BTRs.

4.3.2 BTRs induced non-apoptotic cell death in RTgill-W1

After 24 h or 12 d treatment with different cytotoxic, sub-cytotoxic or non-cytotoxic concentrations of BTRs, genomic DNA from treated cultures were run on a 2 % agarose gel. RTgill-W1 cells dosed with BTRs were negative for nucleosomal-size DNA fragmentation or DNA laddering. RTgill-W1 cells treated with BTRs were negative for nuclear fragmentation revealed by Hoechst 33258 staining (data not shown). Moreover, BTR induced early stage apoptosis was measured by assessing the proportion of cell with externalized PS at the outer leaflet of plasma membrane. RTgill-W1 cells were treated with BTRs at one cytotoxic concentration for 6 hours or one cytotoxic or sub-cytotoxic concentration for 24 hours. Following treatment of BTRs for different time periods, cells were stained with both PE-Annexin V and 7-AAD. Control samples were viable and largely negative for both PE-Annexin V and 7-AAD. After 6 hours incubation, RTgill-W1 cultures treated with BTRs were viable, dying or died (both PE-Annexin V and 7-AAD positive) without a big increase in early stage apoptosis (PE-Annexin V positive and 7-AAD negative) (Fig.4.2). After 24 hours incubation, none of the cultures had more than 10 % cells in early stage apoptosis which indicates apoptosis might not be the type of cell death induced by BTRs (Fig. 4.3).

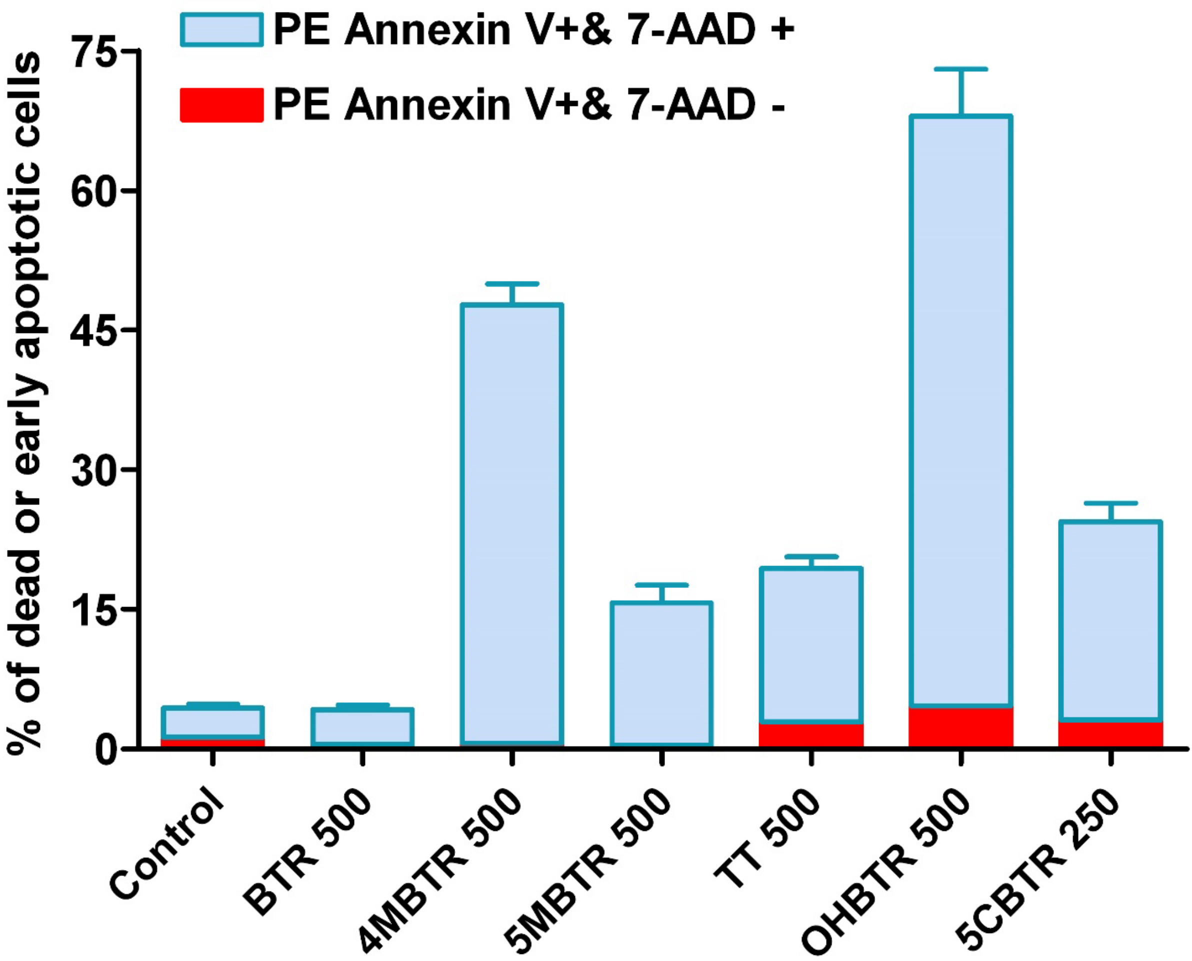


Figure 4.2 BTRs failed to induce early apoptosis in RTgill-W1 in 6 hours.

RTgill-W1 cells were treated with BTR (500 mg/L), 4MBTR (500 mg/L), 5MBTR (500 mg/L), TT (500 mg/L), OHBTR (500 mg/L) or 5CBTR (250 mg/L) for 6 h in L-15 without FBS. The percentage of dead cells (both PE-Annexin V and 7-AAD positive) and early apoptotic cells (PE-Annexin V positive and 7-AAD negative) were measured by flow cytometry.

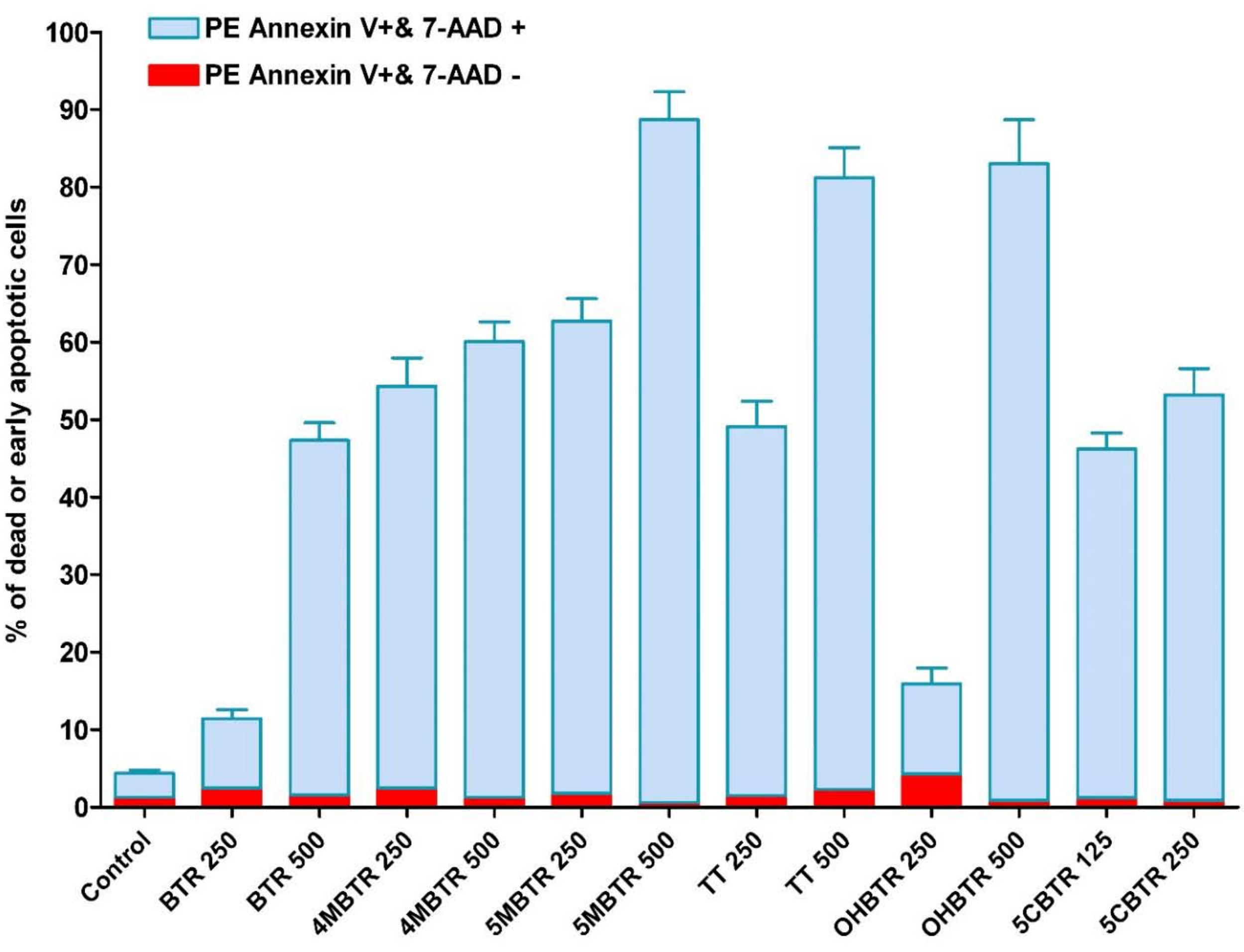


Figure 4.3 BTRs failed to induce early apoptosis in RTgill-W1 in 24 hours.

RTgill-W1 cells were treated with BTR (250, 500 mg/L), 4MBTR (250, 500 mg/L), 5MBTR (250, 500 mg/L), TT (250, 500 mg/L), OHBTR (250, 500 mg/L) or 5CBTR (125, 250 mg/L) for 24 h in L-15 without FBS. The percentage of dead cells (both PE-Annexin V and 7-AAD positive) and early apoptotic cells (PE-Annexin V positive and 7-AAD negative) were measured by flow cytometry.

4.3.3 BTRs increased the ROS level in RTgill-W1

RTgill-W1 cells were exposed to BTRs at cytotoxic, sub-cytotoxic or non-cytotoxic concentrations for 0-24 h. The intracellular ROS levels of RTgill-W1 were then measured by H_2DCFDA (Fig.4.4). BTR at cytotoxic (500 mg/L) and sub-cytotoxic (250 mg/L) concentrations induced an up to 500 % ROS level increase in RTgill-W1 (compared with control culture) after 2 h incubation and then the ROS level dropped. At non-cytotoxic concentration (125 mg/L), BTR was still able to increase the ROS but at a lower level. 4MBTR, 5MBTR, TT and OHBTR induced a similar increase in ROS level in RTgill-W1. At non-cytotoxic (30 mg/L) and sub-cytotoxic (60 mg/L) concentrations, 5CBTR was able to induce an over 200 % increase in ROS level. However, at cytotoxic concentration (125 mg/L), 5CBTR did not significantly increase the generation of ROS in RTgill-W1. The pre-treatment with oxidant scavenger NAC (up to20 mM) failed to inhibit cell death in cultures exposed to BTHs (data not shown).

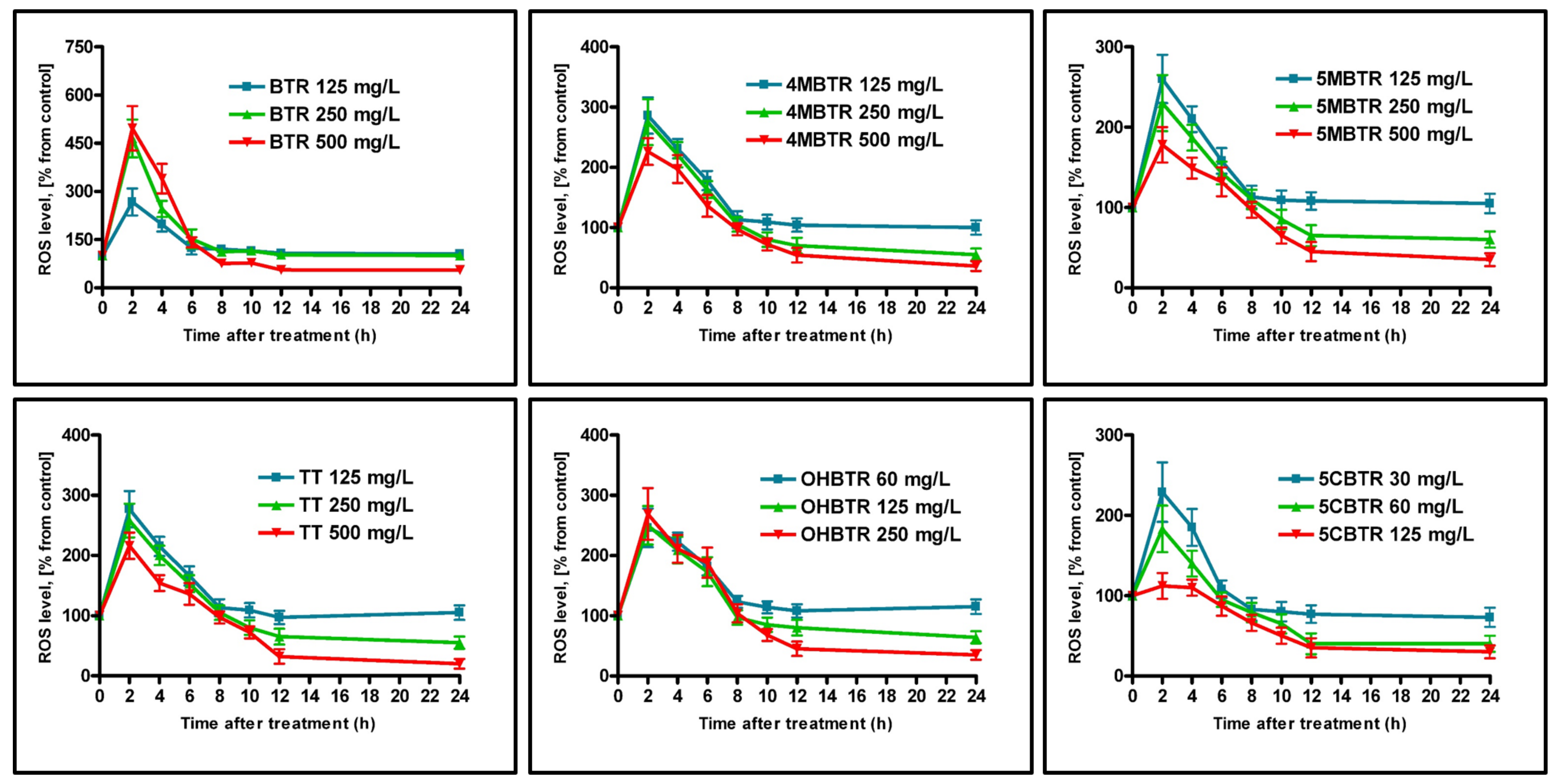


Figure 4.4 BTRs increased the ROS level in RTgill-W1

RTgill-W1 Cells were exposed to BTRs at non-cytotoxic, sub- cytotoxic or cytotoxic concentrations for 0-24h. ROS level was measured by H_2DCFDA. The y-axis represents the percentage of ROS level compared to control samples only treated with 0.5% DMSO. The x-axis represents the time after treatment (hours).

4.3.4 BTRs only induced DNA damage at cytotoxic concentrations

Manual comet assay scoring was done by following the methods of Singh et al. (1988) and Collins et al. (1997). In the negative control cultures only treated with 0.5 % DMSO, almost all the nuclei were of type 0, the typical condensed, round nuclei, indicating intact DNA. While in positive cultures treated with 200 μM of H_2O_2, almost all the cells had a comet assay score of 4. Results of this study clearly showed that 24 h treatment with BTR, 4MBTR, 5MBTR, TT and 5CBTR caused significantly elevated DNA damage mainly at cytotoxic concentrations in RTgill-W1 cell, as evidenced by the increase in the number of cells with a comet assay score of 1, 2, 3 and 4 (Fig.4.6). In contrast, 24 h treatment with OHBTR was not able to induce significant DNA damage at any concentrations tested. After 12 d incubation with BTRs at non-cytotoxic concentrations (up to the highest non-cytotoxic concentration), none of the RTgill-W1 cultures showed any significant DNA damage compared with negative control (Fig.4.7).

4.3.5 BTRs slightly increased the CYP1A level in RTL-W1.

RTL-W1 was less sensitive to BTRs compared to RTgill-W1. The BTRs at their median effective concentrations to RTgill-W1 did not impair the cell viability of RTl-W1 (data not shown). BTRs slightly increase the level of CYP1A in RTL-W1 at concentrations that are sub-cytotoxic to RTgill-W1, especially 5CBTR and TT (Fig.4.8).

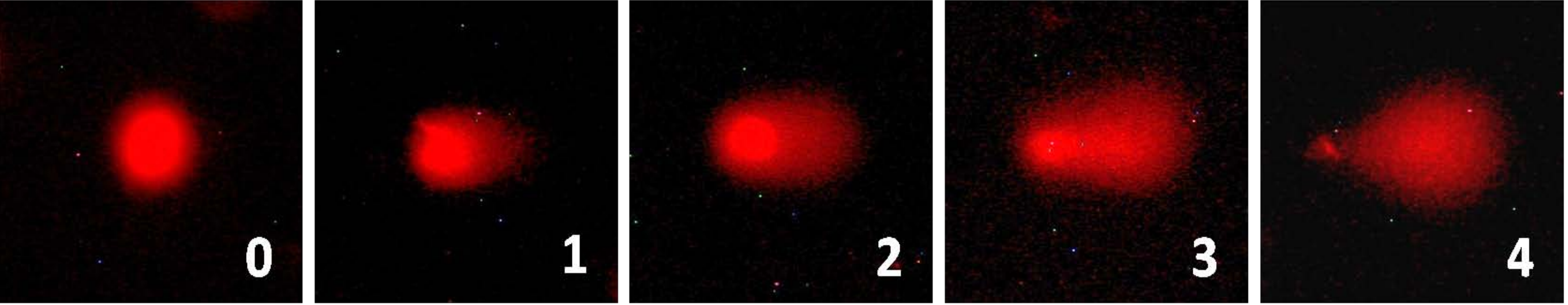


Figure 4.5 Images of comets showing different levels of DNA damage.

Images of comets from RTgill-W1 stained with ethidium bromide showing different levels of DNA damage. They represent classes 0 to 4 as used for visual scoring.

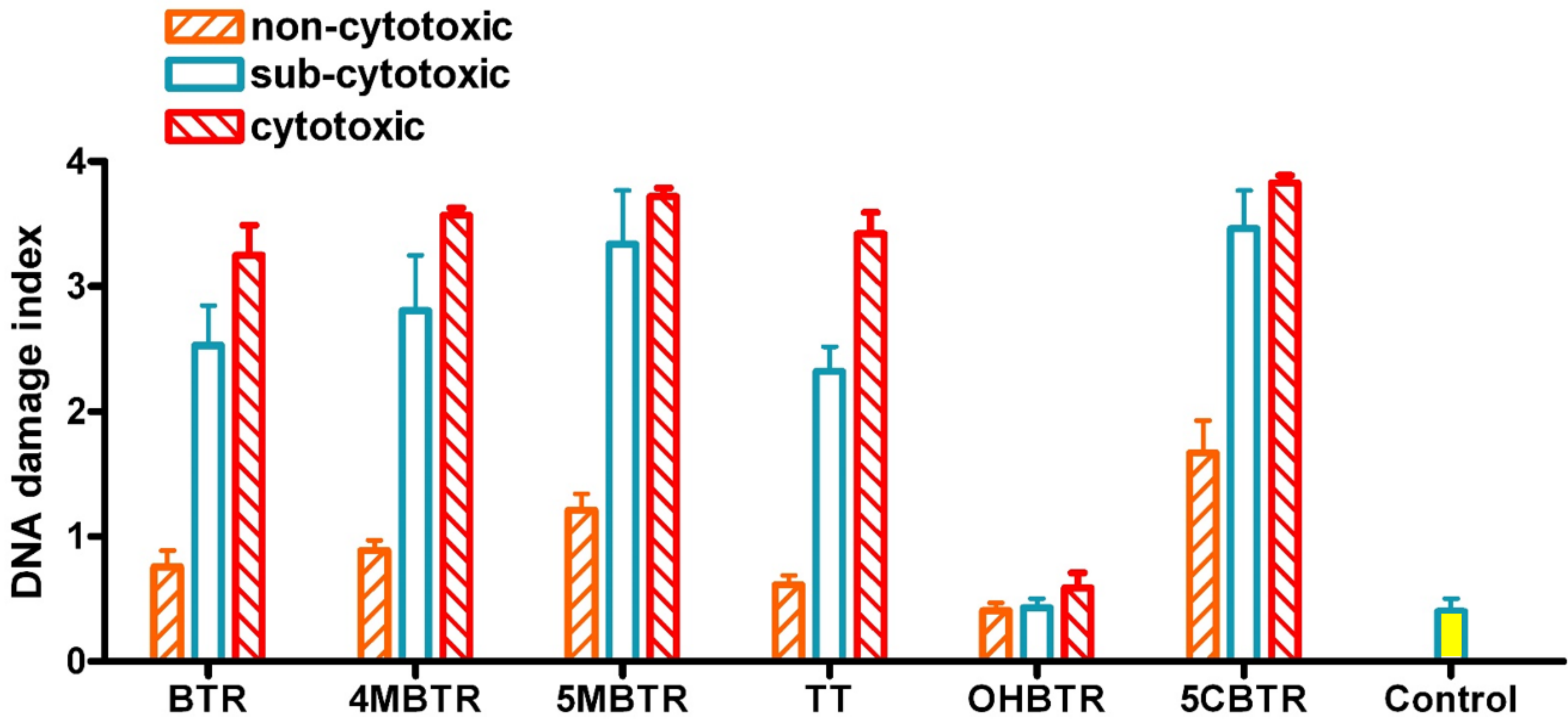


Figure 4.6 DNA damage index observed in RTgill-W1 24 h after treatment of BTRs.

RTgill-W1 were treated with BTRs at non-cytotoxic, sub-cytotoxic or cytotoxic concentrations for 24 h. Concentrations of BTRs (from high to low) are: BTR (1000, 500, 250 mg/L), 4MBTR (500, 250, 125 mg/L), 5MBTR (500, 250, 125 mg/L), TT (500, 250, 125 mg/L), OHBTR (500, 250, 125 mg/L) and 5CBTR (125, 60, 30 mg/L). Control culture was only treated with 0.5 % DMSO.

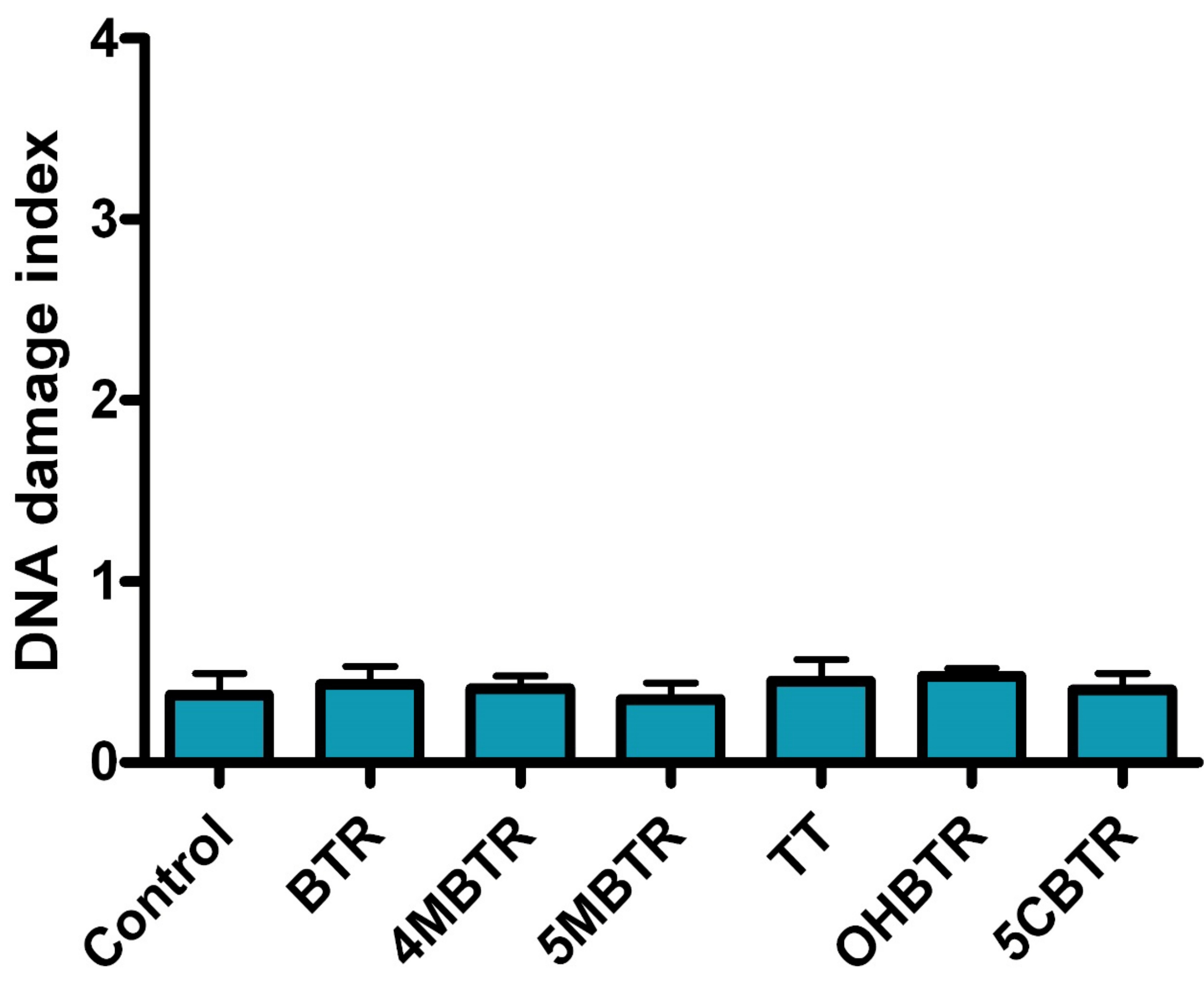


Figure 4.7 DNA damage index observed in RTgill-W1 12 d after treatment of BTRs

RTgill-W1 were treated with BTR (60 mg/L), 4MBTR (30 mg/L), 5MBTR (30 mg/L), TT (30 mg/L), OHBTR (30 mg/L) and 5CBTR (7.5 mg/L) for 12 days. Control culture was only treated with 0.5 % DMSO.

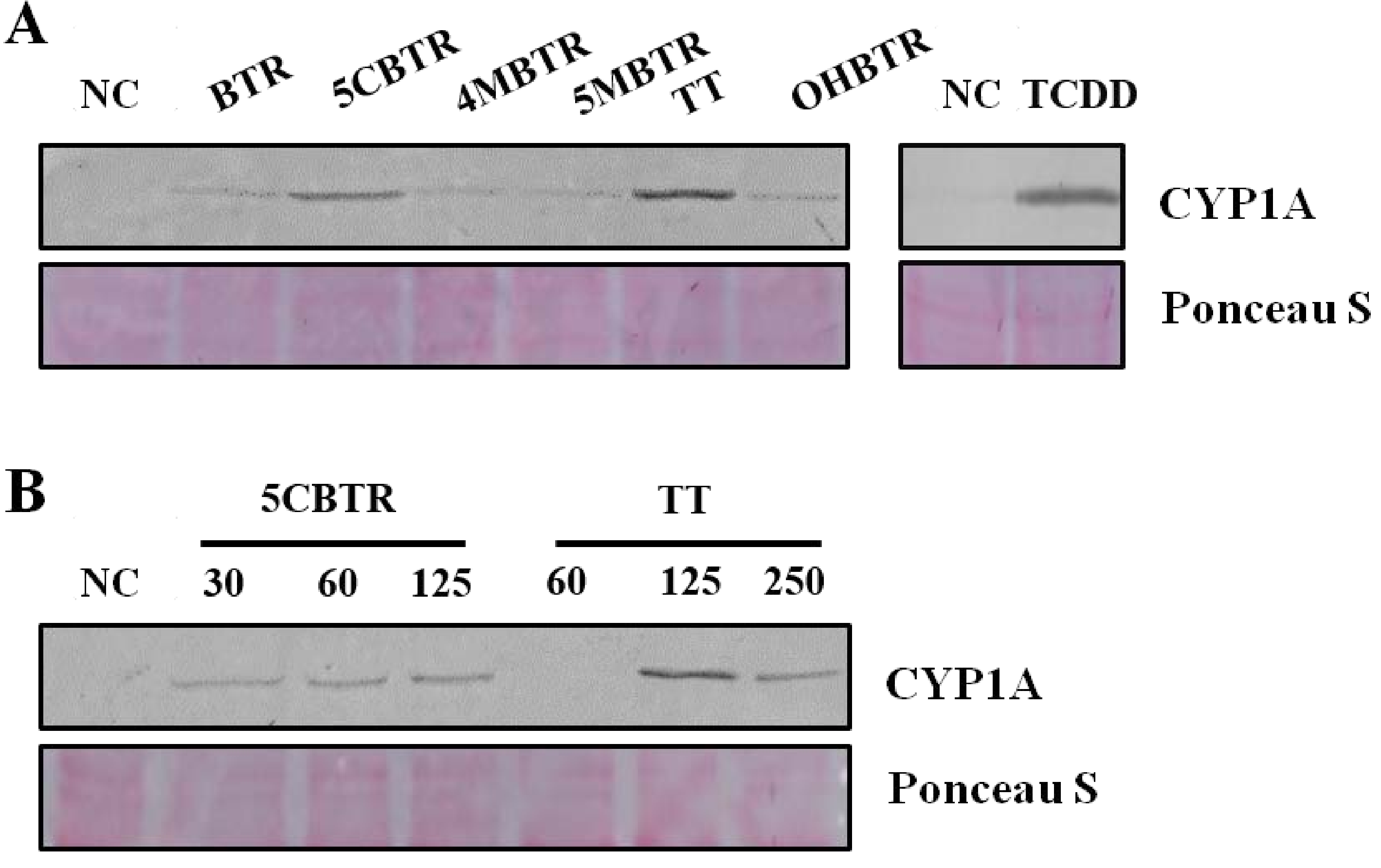


Figure 4.8 Effect of BTRs on CYP1A levels in RTL-W1 cultures.

RTL-W1 cultures were treated with 0.5 % DMSO (negative control), 97.6 pM TCDD (positive control), BTR (250 mg/L), 5CBTR (60 mg//L), 4MBTR (125 mg/L), 5MBTR (125 mg/L), TT (125 mg/L) or OHBTR (125 mg/L) (A) or 5CBTR (30, 60, 125 mg/L) or TT (60, 125, 250 mg/L) (B) for 48 h at which times cell extracts were prepared. SDS PAGE was used to separate polypeptides in 25 µg of cell extract followed by western blotting to identify CYP1A. Ponceau staining was used as loading control.

4.4 Discussion

In this study, the in vitro acute and sub-chronic cytotoxicities of seven BTRs were assessed in the rainbow trout gill cell line RTgill-W1 in order to provide a more cogent toxicity estimate for risk assessment. The cytotoxic endpoints were a diminishment in energy metabolism as evaluated by the capacity of cells to reduce resazurin (Alamar Blue), a decrease in membrane integrity as measured by the conversion of CFDA-AM to CF (5'-carboxyfluorescein) and a loss of lysosome integrity as assessed by the ability of cells to incorporate Neutral Red. In general, BTRs had low cytotoxicity in RTgill-W1 after 24 h treatment and the EC_{50}s of BTRs were 2-3-fold lower after 12 d treatment. 5CBTR exhibited the highest acute and sub-chronic cytotoxicities in RTgill-W1, whereas DM was nearly insoluble in the culture media with or without the aid of a carrier solvent and it showed no cytotoxicity in RTgill-W1 at its maximum soluble concentration. These acute cytotoxicity results are in line with those obtained for BTRs in other models (Milanova et al., 2001; Pillard et al., 2001; Seeland et al., 2012). The 48 h LC_{50} values of 102 mg/L (BTR), 79 mg/L (5MBTR) for *C.dubia* (Pillard et al., 2001), the 48 h EC_{50} value of 107 mg/L (BTR) and the 21 d EC_{50} value of 23.5-37.6 mg/L (TT) for *D.magna* (Seeland et al., 2012; Dummer, 2014) are in the same range as in the present study. Unfortunately, there are almost no chronic or sub-chronic toxicity data available for BTRs.

The mechanism by which BTRs caused cell death was investigated and might be best described by a new term, "uncontrolled necrosis" (Feoktistova & Leverkus, 2015). The cell death induced by BTRs lacked the hallmarks of apoptosis, such as nuclear fragmentation, DNA laddering and externalization of phospholipid phosphatidyl serine. In contrast, BTRs induced cell swelling, nuclei condensation and plasma membrane rupture, suggesting that BTRs may induce necrotic cell death in RTgill-W1. A prior exposure to specific necroptosis inhibitor, Necrostatin-1 did not protect RTgill-W1 from BTRs, suggesting that the BTRs were not killing cells by necroptosis.

Possibly narcosis contributes to BTR cytotoxicity because this is the most common mode of toxic action by industrial organic chemicals. Narcosis toxicity is generally related to accumulation of the organic compounds in the lipid bi-layer region of the cell membrane and disruption of cell membrane functions. Studies of quantitative structure-activity relationships (QSAR) have shown that the toxic potency of narcotic toxicants is associated with their physical

nature such as the Octanol-Water Partition Coefficient (Kow). As shown by Tanneberger et al (2013), the baseline toxicity of organic compounds in RTgill-W1 can be predicted from the QSAR model for metabolic activity: log EC_{50} (mM) = -0.96 (± 0.09) log Kow + 1.57 (± 0.28). The 24 h EC_{50} values of BTR, 4MBTR, 5MBTR, TT, OHBTR and 5CBTR obtained from RTgill-W1 are generally similar or higher than the predicted baseline EC_{50}s, suggesting that these BTRs might act as narcotics or baseline toxicants, which elicit minimal toxicity. Therefore, the cell membrane swelling and disruption was probably due to the partitioning of BTRs into the membrane (van Wezel & Opperhuizen, 1995). The 24 h EC_{50} value of OHBTR was 3-17 fold lower than the predicted baseline EC_{50}, suggesting that OHBTR might elicit specific toxicity in RTgill-W1. However, the 6 BTRs are all polar benzotriazolic compounds (Casado et al., 2014), so the toxicity of OHBTR is probably due to its polar character which involved the hydrogen bond interactions with polar residues at the membrane-water phase and uncoupling of electron transport over mitochondrial membranes (Argese et al., 2001).

Several studies have demonstrated that BTR and TT decreased the growth rate of protozoa and vascular water plants, probably due to inhibition of the electron flow during oxidative phosphorylation in the mitochondria and cell respiration (Cornell et al., 2000; Castro et al., 2004; Seeland et al., 2012). Mitochondria dysregulation might alter the production of ROS. However, the mechanism of the mild ROS induction in BTRs treated culture is less clear. The oxidative stress might be responsible for the cellular toxicity of BTRs and might contribute to the potential genotoxicity of BTRs by damaging nucleic acids (Gill and Tuteja, 2010). However, treatment with antioxidant NAC and IM-54 failed to inhibit the cell death induced by BTRs, suggesting that the generation of ROS might not be the cause of cell death, but just a side effect.

The current study would appear to be the first in which the comet assay has been used to study BTRs for their possible genotoxicty but whether the BTRs are DNA-damaging agents is still open to interpretation. The results clearly showed that 24 h exposures to BTR, 4MBTR, 5MBTR, TT and 5CBTR caused a high level of DNA damage in RTgill-W1, but only at cytotoxic concentrations. The number of damaged nuclei of type 1, 2, 3 and 4 increased in a dosed dependent manner resulting in a many fold increase in the DNA damage index with exposure with these 5 BTRs. A similar level of DNA damage was observed in RTgill-W1 treated with BTR, 4MBTR, 5MBTR, TT and 5CBTR. However, the generally accepted guideline for in

vitro genotoxicity testing cautions that when the putative genotoxicant decreases the viability of cells by more than 30 %, false positives might arise (Tice et al., 2000). For most BTRs, the DNA damage was detected at concentrations that caused the viability, as judged with AB and NR, to be diminished to this level or lower. On the other hand, as illustrated with OHBTR, this magnitude of cell death did not lead to the comet assay being positive in all cases. Few reports are available on the genotoxicity BTRs, but BTR has been shown to be mutagenic in the Ames tests and TT has been shown to cause mutation in L5178Y mouse lymphoma cell mutation assays (Dutch Expert Committee on Occupational Standards, 2000; Benzotriazoles Coalition, 2001; Seifried et al., 2006).

The elevation of cytochrome P4501A1 levels suggests that BTRs might be able to activate the AhR pathway. This should be studied further by examining whether an increase occurs in a measure of the catalytic activity of P4501A1, such ethoxyresorufin o-deethylase (EROD). Only one other study appears to have examined the possibility that BTRs might act through the AhR pathway. When zebrafish eleuthro-embryos were exposed to benzotriazole UV-stabilizers (BUVs), UV-P and UV-326 caused changes in the expression of several genes in the AhR-pathway (Kent et al, 2014). Both BTRs increased CYP1A1 transcript levels. Additionally, UV-P enhanced transcript levels for aryl hydrocarbon receptor 1 (AhR1) and aryl hydrocarbon receptor nuclear translocator 2 (ARNT2).

4.5 Conclusion

In this study, a ranking of BTRs from the least to the most cytotoxic to a fish gill cell line has been established. 5CBTR was the most cytotoxic and DM was the least cytotoxic of the tested compounds. BTRs induced necrotic cell death in RTgill-W1, accompanied with a generation of ROS. In addition, acute exposure of some BTRs at cytotoxic concentrations caused DNA damage in RTgill-W1. Our results showed inadequate evidence that BTRs are genotoxic to RTgill-W1. The effective concentrations for BTRs in RTgill-W1 were much higher than reported environmental concentrations, which are usually in µg/L or ng/L (Weiss et al., 2006; Kiss & Fries, 2009; Reemtsma et al., 2010). However, considering that BTRs may occur at cytotoxic concentrations to RTgill-W1 cells at certain location or during certain time of the year. For

example, BTR and its derivatives have been reported in ground water at a major North American airport at concentrations >100 mg/L (Cancilla et al., 1998). It is important to assess the toxicity of BTRs in aquatic organisms.

CHAPTER 5

Use of rainbow trout cells to investigate the toxicity of the emerging contaminants, benzothiazoles

5.1 Introduction

Benzothiazoles (BTHs) and benzotriazoles (BTRs) are emerging contaminants (EC) (Deblonde et al., 2011; Richardson & Ternes, 2014), with only a little information on their toxicity to fish (Little & Lamb, 1972; Milanova et al., 2001; Verschueren, 1983; Yoshitada et al., 1986). However, recently two rainbow trout epithelial cell lines, RTgill-W1 from the gill and RTL-W1 from the liver, were used to evaluate the toxicology of seven BTRs (Chapter 4). Six BTRs were cytotoxic and stimulated reactive oxygen species (ROS) production and all induced cytochrome P4501A. Whether BTHs behave similarly is of interest because they are produced in large quantities but often for different purposes, meaning that they are sometimes released into different environments (Deblonde et al., 2011; Richardson & Ternes, 2014), and they have a slightly different core structure. BTHs contain a 5-membered 1, 3- thiazole ring fused to a benzene ring.

Several BTHs are especially commercially important and produced on a large scale. These include benzothiazole (BTH), 2-mercaptobenzothiazole (2MBTH), and its zinc and sodium salts, ZincMBTH and NaMBTH, 2-hydroxybenzothiazole (OHBTH), and 2-aminobenzothiazole (2ABTH). BTHs are used as accelerators and stabilizers in the rubber industry (Stolcova, & Hronec, 1996; Nawrocki et al., 2005; Chipinda et al., 2007), as corrosion inhibitors in greases and cutting oils (Brownlee et al., 1992; Reddy & Quinn, 1997), and as biocides in diverse industrial processes (DeWever & Verachtert, 1997). The annual production of 2MBTH has been estimated at approximately 40,000 tons in Europe and more than 1 million pounds in the US (Chipinda et al., 2007).

In this chapter RTgill-W1 and RTL-W1 have been used to evaluate the toxicity of twelve BTHs (Table 1.3 & 1.4). Most BTHs were cytotoxic, caused oxidative stress, and induced cytochrome P4501A, suggesting that they have the potential to be toxic but like the BTRs, only at very high concentrations.

5.2 Materials and Methods

5.2.1 Cell culture

The rainbow trout gill cell line (RTgill-W1) established by Bols et al. (1994) and the rainbow trout liver cell line (RTL-W1) established by Lee et al. (1993) were routinely cultured in 75 cm^2 culture flasks (Nunc, Kamstrupvej, Denmark) at room temperature in Leibovitz's L-15 culture medium (Sigma-Aldrich, Ltd., Oakville, ON, Canada) supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich) and 1 % penicillin-streptomycin solution (10000 units/ml penicillin, 10 mg/ml streptomycin, Sigma-Aldrich).

5.2.2 Cytotoxicity assay

5.2.2.1 Plating and dosing

The cell lines, RTgill-W1 and RTL-W, were grown routinely and used to evaluate the cytotoxicity of benzothiazoles (BTHs) as described in detail in Chapter 4 for the benzotriazoles (BTRs). Briefly the cells were grown at room temperature in the basal medium, L-15, supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Cytotoxicity testing was done in 96 well plates, with cell viability being evaluated with three indicator dyes: Alamar Blue, 5-carboxyfluorescein diacetate (CFDA-AM), and Neutral Red. The protocols for these procedures were as described in Chapter 4 and in point by point form in Dayeh et al., 2003; 2103. AK Scientific (California, USA) was the source of ZincMBTH (P629); 2MBTH (L287); SO (T891); NaMBTH (M906); VY (T949); NNA (P958) and Sigma-Aldrich was the source of DTDC (173754); OHBTH (407607); 2AMBTH (108812); BTH (101338); DBTH (D218154); MBTHS (S405299).

5.2.2.2 Measuring cell viability

Three fluorescent indicator dyes were used to evaluate cell viability. Metabolic activity was measured by Alamar Blue (Medicorp, Montreal, PQ). Cell membrane integrity was evaluated with 5-carboxyfluorescein diacetate (CFDA-AM) (Molecular Probes, Eugene, OR). Lysosome integrity was monitored with Neutral Red (Sigma-Aldrich). Alamar Blue, CFDA-AM and

Neutral Red were prepared in Dulbecco's phosphate buffered saline (DPBS, Lonza, Walkersville, MD USA) to give final concentrations of 5 % (v/v), 4 μM and 1.5 % (v/v) respectively. Cells were incubated with dyes for 1 h in dark then quantified by fluorescence plate reader (Spectra-max Gemini XS microplate spectrofluorometer; Molecular Devices, Sunnyvale, CA). The excitation and emission wave-lengths used were 530 and 590 nm for Alamar Blue, 485 and 530 nm for CFDA-AM, 530 and 640 nm for Neutral Red, respectively. Results were calculated as a percent of the control culture

5.2.3 Cell death mechanisms

The mechanisms by which BTHs caused cell death were investigated in several ways, using methods that have been described in detail in Chapters 2 and 4. RTgill-W1 cultures were examined for nuclear and DNA fragmentation and phosphatidylserine (PS) externalization in order to determine whether these compounds killed cells through the process of apoptosis. After being exposed for 6 and 24 h, cultures were stained with H33258 and examined by fluorescence microscopy to see whether nuclei appeared intact or had fragmented. For the biochemical detection of DNA fragmentation, a GenElute™ genomic DNA miniprep kit (Sigma-Aldrich) was used to isolate DNA from cultures that had been exposed for 12 and 24 h. The DNA was subjected to electrophoresis on a 2% (w/v) agarose gel mixed with gel red (1 in 10000) (Biotium, CA) for 3 h at 60 V to determine whether laddering had occurred. PS externalization to the outer leaflet of the plasma membrane is a marker of early stage apoptosis and was measured with the PE Annexin V Apoptosis Detection Kit I (BD Biosciences). This was done 6 and 24 h after cultures had been exposed to BTHs at concentrations that had been defined as cytotoxic from the cell viability assays of section 5.3.1. PE-Annexin V binds externalized PS and was added to cells along with actinomycin (7-AAD), which is excluded from living cells but enters dead cells and stains their nuclei. Flow cytometry was used to enumerate the proportion of RTgill-W1 cells staining with both reagents, which are the dead cells, and with PE-annexin V only, which are the early apoptotic cells. Flow cytometric data (10000 events/sample) were analyzed by the Flowjo software (Treestar, Inc., San Carlos, CA). As a positive control, cultures were exposed to 2-phenylehtyensulfonamide (PES), which in chapter 2 (Zeng et al., 2014) was found to cause RTgill-W1 to die by apoptosis.

The cell death mechanisms were also investigated through the use of inhibitors for oxidative stress, N-acetylcysteine (NAC, Sigma-Aldrich), and for cell death pathways, IM-54 and Necrostatin-1 (Santa Cruz Biotechnology). NAC protects cells against oxidative stress, possibly by directly scavenging reactive oxygen species (ROS) (Zhang et al., 2011). IM-54 is an inhibitor of necrosis caused by oxidative stress (Sodeoka & Dodo, 2010). Necrostatin-1 inhibits the controlled necrotic process, necroptosis (Xie et al., 2013). These inhibitors were used at up to 20 mM for NAC, 100 μM for necrostanin-1, and 20 μM for IM-54 and were added 1 h before the addition of the BTHs. Cell viability was monitored 1 and 12 days later as described in sections 5.3.1.

5.2.4 Oxidative stress

The fluorescent dye, 2′, 7′ -dichlorofluorescin diacetate (H_2DCFDA) is generally agreed to provide an overall index of oxidative stress (Chen et al., 2010) and was used to measure reactive oxygen species (ROS) in RTgill-W1 cultures. After the cultures were set up in 96 well plates as described previously in chapters 2 and 4, they were dosed with BTHs. In each experiment, a specific BTH concentration and incubation time was done in 5 identical wells. The BTHs that were identified as cytototoxic in section 5.2.2.2 were added to RTgill-W1 cultures at three doses: one that was not cytotoxic; one that caused approximately a 50 % loss of cell viability; and one that was very cytotoxic, causing greater than a 85 % loss in cell viability. The BTHs that were not identified as cytotoxic in section 5.2.2.2 were used at one concentration. The concentration was just a little below the concentration where the BTHs were observed to come out of solution and leave crystals in the culture medium. After the addition of a test compound, ROS were evaluated in cultures with H2DCFDA immediately, 2, 4, 6, 8 10, 12 and 24 h later. The results recorded as relative fluorescent units (RFUs). The mean RFUs were subjected to a one way analysis of variance (ANOVA). If for a particular compound and concentration the p value was less than 0.05, the compound was concluded to cause oxidative stress and the Dunnett multiple comparison test was applied. Zero time was the control and conditions that led to values significantly greater than the control ($p < 0.05$) were judged as having caused oxidative stress. For presentation in graphs and tables, the RFUs for each test condition were presented as a

percentage of zero time. Each compound at each concentration was tested in three independent experiments.

5.2.5 Alkaline comet assay

The alkaline comet assay was performed and scored as documented in Chapter 4 for BTRs, and, basically as described by Singh et al (1988). Briefly RTgill-W1 cells were seeded in 12 well plates (Becton and Dickinson Company, Franklin Lakes, NJ. USA) at a density of $4x10^5$ cells per well in 2 ml of L-15 with 10 % FBS and allowed to settle and reattach for 24 h at room temperature before the exposures began for either 24 h or 12 days. For 24 h, BTHs were added at three different concentrations: very cytotoxic (<15 % cell viability), cytotoxic (~50 % cell viability) or non-cytotoxic (100 % cell viability). These concentrations were defined by the cell viability assays. The concentrations from most cytotoxic to non-cytotoxic vary with each test compound and as follows: VY (250, 125, 60 mg/L), 2ABTH (500, 250, 125 mg/L), BTH (600, 300, 150 mg/L), OHBTH (300, 150, 75 mg/L), NaMBTH (100, 50, 25 mg/L), ZincMBTH (125, 60, 30 mg/L), 2MBTH (100, 50, 25 mg/L), SO (30, 15, 7.5 mg/L) and DTDC (0.25, 0.1, 0.05 mg/L). For 12 day exposures, a range of only non cytotoxic concentrations were used. The highest non cytotoxic concentration that was tested for each compounds were as follows: DBTH (5 mg/L), NNA (250 mg/L), MBTHS (250 mg/L), VY (15 mg/L), 2ABTH (30 mg/L), BTH (30 mg/L), OHBTH (15 mg/L), NaMBTH (12.5 mg/L), ZincMBTH (5 mg/L), 2MBTH (12.5 mg/L), SO (3 mg/L) and DTDC (0.025 mg/L) for 12 d. Manual scoring of the comet assay was performed as described previously in Chapter 4. The comets were counted and classified into scores of ‘0’, ‘1’,‘2’, ‘3’ and ‘4’ according to DNA damage and head/tail migration (from undamaged, class 0, to maximally damaged, class 4). Three independent experiments were conducted for each treatment.

5.2.6 Cytochrome P4501A induction

Western blotting was used to monitor the potential of BTHs to induce P4501A or CYP1A. This was done with RTL-W1 as described in Chapter 4 for BTRs. The exposures to BTHs were for 48 h. The primary antibody was mouse monoclonal anti-CYP1A fish (C10-7) (1:3000)

(#173132, Cayman Chemical, Ann Arbor MI, US) from the Canadian distributor Cedar Lane, Burlington, ON.

5.2.7 Data analysis

All graphs and statistical analyses were done using GraphPad InStat (version 4.01 for Windows XP, GraphPad Software, San Diego, CA, www.graphpad.com). Statistical comparison was done using ANOVA test followed by Tukey-Kramer Multiple Comparisons Test.

5.3 Results

5.3.1 Comparing the cytotoxicity of the BTHs

Most BTHs were cytotoxic to RTgill-W1 and RTL-W1. Alamar Blue, CFDA-AM and Neutral Red were used to assay cell viability and showed dose-dependent declines in assay values, allowing EC_{50}s to be calculated for each compound with each fluorescent dye (Fig 5.1 Tables 5.1 & 5.2). Fig 5.1 illustrates the loss of cell viability in RTgill-W1 cultures after 24 h exposure to 2MBTH. Table 5.1 summarizes the EC_{50} values for 2MBTH and 8 other BTHs that were cytotoxic to RTgill-1 and RTL-W1. When the EC_{50}s for the nine cytotoxic BTHs after 24 h exposures were subjected to ANOVA, they were different ($p < 0.05$) with both cell lines. Yet when Tukey-Kramer Multiple Comparison tests were done, the EC_{50}s for only a few BTHs were consistently different from the others ($p < 0.05$). For both cell lines, the EC_{50}s for DTDC were the lowest and significantly different from the EC_{50}s for the other BTHs. For both cell lines, the EC_{50}s for 2ABTH were the highest or second highest and significantly different from the EC_{50}s for the other BTHs. Thus the most cytotoxic was DTDC and the least cytotoxic was 2ABTH. The rank order for the other cytotoxic BTHs was hard to establish because their EC50s were broadly similar when all three assays were considered.

For 24 h exposures to a specific compound, the assay with CFDA-AM, which measures cell membrane integrity, was less sensitive for many BTHs than those with alamar Blue and neutral red, which respectively monitor energy metabolism and lysosomal activity (Table 5.1). However, after 12 day exposures, the EC_{50}s for the three dyes gave for a particular compound very similar EC_{50}s (Table 5.2).

RTgill-W1 and RTL-W1 gave comparable but not identical results for the 9 cytotoxic BTHs. When an unpaired t test was done to compare EC_{50}s between the two cell lines, they were different for each compound ($p<0.05$). RTL-W1 was much more sensitive to BTH. The EC_{50}s for BTH in RTgill-W1 were approximately 8 fold higher than in RTL-W1 (Table 5.1). RTL-W1 was less sensitive to VY, 2ABTH, OHBTH, NaMBTH, ZincMBTH, 2MBTH, SO and DTDC compared to RTgill-W1. In general the 24 h EC_{50}s for these compounds were about 2 fold higher in RTL-W1 than in RTgill-W1 (Table 5.1).

MBTHS, DBTH, and NNA were not cytotoxic to either cell line, even when tested at the highest concentrations possible without the compounds becoming insoluble in the culture medium. Fig 5.1 illustrates the results of exposing RTgill-W1 for 24 h to MBTHS. MBTHS, DBTH, and NNA were not cytotoxic even after 12 day exposures (Table 5.2).

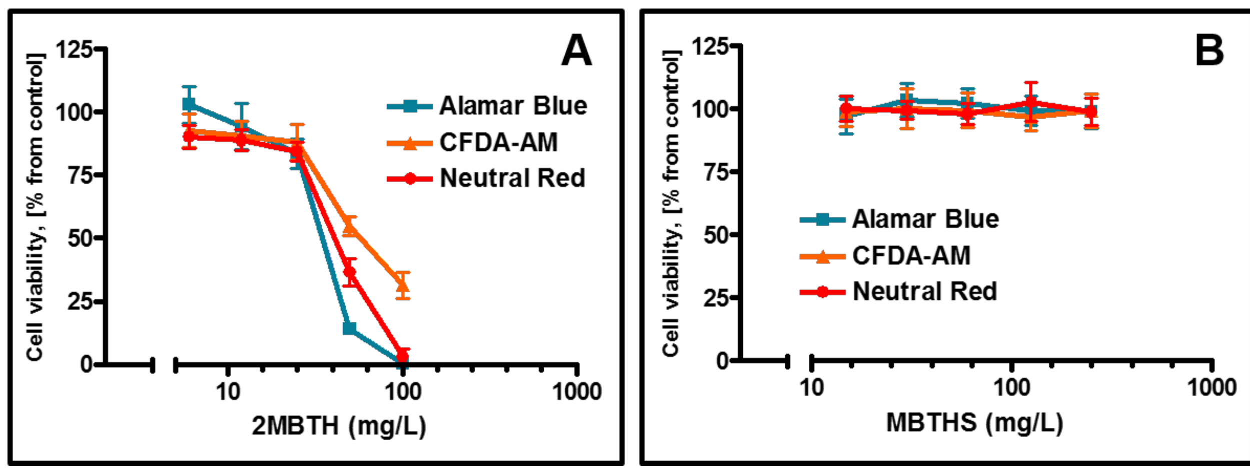


Figure 5.1 Effect of 24 h exposures to BTHs on viability RTgill-W1 cells.

After being exposed for 24 h to five concentrations (X axis) of 2MBTH (A) and MBTHS (B), RTgill-W1 cultures were monitored for cell viability with three fluorescent indicator dyes: Alamar Blue (-square-), CDFD-AM (-triangle-) and neutral red (-circle-). The viability assays were recorded as relative fluorescent units (RFUs), which were used to calculate EC_{50}s. For each assay the RFUs were expressed as a percentage (Y axis) of the RFUs in control cultures (100%) and the results of one experiment shown.

Table 5.1 Effect of 24 hours benzothiazoles (BTHs) exposures on RTgill-W1 and RTL-W1 cell viability

		Mean EC50 ± SEM (n=3) measured for three viability assays*			
		Alamar Blue	CFDA-AM	Neutral Red	
Benzothiazoles	Cell line	mg/L	mg/L	mg/L	Statistical comparison of viability assays
DTDC	RTgill-W1 RTL-W1	0.14 ± 0.03 0.35 ± 0.07	0.36 ± 0.06 0.62 ± 0.09	0.14 ± 0.01 0.31 ± 0.05	CF>AB=NR CF>AB=NR
SO	RTgill-W1 RTL-W1	11.2 ± 2.7 25.4 ± 4.2	16.1 ± 1.9 42.3 ± 4.6	19.3 ± 2.1 27.8 ± 2.5	AB=CF=NR CF>AB; AB=NR;CF=NR
2MBTH	RTgill-W1 RTL-W1	34.7 ± 1.9 63.7 ± 5.9	61.2 ± 7.3 104.6 ± 9.8	41.5 ± 5.4 73.7 ± 6.3	CF>AB; AB=NR;CF=NR CF>AB=NR
NaMBTH	RTgill-W1 RTL-W1	38.2 ± 2.8 85.2 ± 7.9	88.6 ± 8.2 143.9 ± 16.3	51.1 ± 4.5 97.8 ± 8.6	CF>AB=NR CF>AB=NR
ZincMBTH	RTgill-W1 RTL-W1	39.8 ± 3.1 91.6 ± 12.4	119.1 ± 8.2 206.3 ± 17.9	44.2 ± 3.7 90.6 ± 10.2	CF>AB=NR CF>AB=NR
OHBTH	RTgill-W1 RTL-W1	116.9 ± 2.8 266.7 ± 32.5	368.2 ± 53.4 735.7 ± 95.2	154.7 ± 7.3 352.6 ± 41.6	CF>AB=NR CF>AB=NR
BTH	RTgill-W1 RTL-W1	183.0 ± 14.8 23.8 ± 3.1	210.1 ± 23.9 31.6 ± 4.9	176.6 ± 10.9 20.3 ± 3.5	CF>AB=NR AB=CF=NR
2ABTH	RTgill-W1 RTL-W1	209.1 ± 15.1 463.2 ± 35.5	396.6 ± 19.4 692.7 ± 75.4	271.3 ± 6.5 523.5 ± 47.3	AB=CF=NR CF>AB; AB=NR;CF=NR
VY	RTgill-W1 RTL-W1	219.4 ± 16.8 342.5 ± 42.6	227.6 ± 13.2 409.6 ± 11.7	192.7 ± 11.1 395.3 ± 14.9	CF>AB; AB=NR;CF=NR AB=CF=NR
NNA	RTgill-W1 RTL-W1	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not applicable
DBTH	RTgill-W1 RTL-W1	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not applicable
MBTHS	RTgill-W1 RTL-W1	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not applicable

* Where the ANOVA across rows was significant ($p<0.05$), the Tukey-Kramer Multiple Comparisons Test ($p<0.05$) was done and results are shown in the column on the right.

Table 5.2 Effect of 12 days benzothiazoles (BTHs) exposures on RTgill-W1 cell viability

	Mean EC_{50} ± SEM (n=3) measured for three viability assays*			
	Alamar Blue (metabolism)	CFDA-AM (membrane integrity)	Neutral Red (lysosomal activity)	
Benzothiazoles (most to least toxic according to AB assays)	mg/L (% of 24 h EC_{50})	mg/L (% of 24 h EC_{50})	mg/L (% of 24 h EC_{50})	Statistical comparison of viability assays
DTDC	0.05 ± 0.01 (35.7 % ± 7.1 %)	0.07 ± 0.01 (19.4 % ± 2.7 %)	0.06 ± 0.01 (42.8 % ± 7.1 %)	AB=CF=NR
SO	4.12 ± 0.39 (36.8 % ± 3.5 %)	4.97 ± 0.53 (30.9 % ± 3.3 %)	4.82 ± 0.28 (24.9 % ± 1.5 %)	AB=CF=NR
ZincMBTH	10.8 ± 1.8 (27.1 % ± 4.5 %)	13.2 ± 2.3 (11.1 % ± 1.9 %)	10.5 ± 0.9 (23.8 % ± 2.0 %)	AB=CF=NR
2MBTH	17.9 ± 4.2 (51.6 % ± 12.1 %)	18.5 ± 2.9 (30.2 % ± 4.7 %)	16.7 ± 2.1 (40.2 % ± 5.0 %)	AB=CF=NR
NaMBTH	21.3 ± 1.7 (55.8 % ± 4.5 %)	22.8 ± 2.1 (25.7 % ± 2.4 %)	21.5 ± 2.8 (42.1 % ± 5.4 %)	AB=CF=NR
VY	34.6 ± 6.2 (15.8 % ± 2.8 %)	45.2 ± 4.6 (19.9 % ± 2.0 %)	49.8 ± 7.7 (25.8 % ± 4.0 %)	AB=CF=NR
OHBTH	46.5 ± 3.3 (39.8 % ± 2.8 %)	70.5 ± 6.7 (19.1 % ± 1.8 %)	46.3 ± 2.5 (29.9 % ± 1.6 %)	CF>AB=NR
2ABTH	64.6 ± 5.3 (30.9 % ± 2.5 %)	86.7 ± 4.4 (21.9 % ± 1.1 %)	62.9 ± 8.3 (23.2 % ± 3.1 %)	AB=CF=NR
BTH	76.2 ± 10.9 (41.6 % ± 5.9 %)	106.9 ± 10.5 (50.8 % ± 5.0 %)	91.9 ± 10.1 (52.0 % ± 5.7 %)	AB=CF=NR
NNA	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not applicable
DBTH	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not applicable
MBTHS	Not cytotoxic	Not cytotoxic	Not cytotoxic	Not applicable

*ANOVA across rows were not significant (p >0.05), except for OHBTH and were followed by Tukey-Kramer Multiple Comparisons Test (p<0.05). ANOVA down columns were significant (p<0.05) and were followed by Tukey-Kramer Multiple Comparisons Test (p<0.05).

5.3.2 Oxidative stress

Most BTHs caused a transitory oxidative stress. This was seen after the addition of BTHs to RTgill-W1 cultures as a rapid rise and then decline in ROS levels as measured with H_2DCFDA. Fig 5.2 illustrates this for OHBTH, along with an example of one that did not change ROS levels. Table 5.4 summarizes the results for all twelve BTHs. ROS levels were consistently elevated 2 h after the dosing with ten BTHs at concentrations that were not cytotoxic as defined in section 5.3.1 and Table 5.1. When cultures had been dosed at very cytotoxic concentrations, VY, 2ABTB, OBTH, NaMBTH, ZnMBTH, 2MBTH and SO but not BTH and DTDC also caused oxidative stress (Table 5.3). The increase in ROS was always transitory. Oxidative stress lasted only for 2 h in the case of DTDC and up to 8 h in the case of VY and for 4 to 6 h for the other BTHs (Table 5.3). By 24 h the RFUs generated from the oxidation of H_2DCFDA in cultures with these BTHs were equal or lower than the values in control cultures. For the three non-cytotoxic BTHs as defined in section 5.3.1 and Fig 5.1, MBTHS elevated ROS slightly and DBTH and NNA caused no change (Table 5.3). These compounds were tested at the highest concentrations possible before they began to come out of solution into the culture medium.

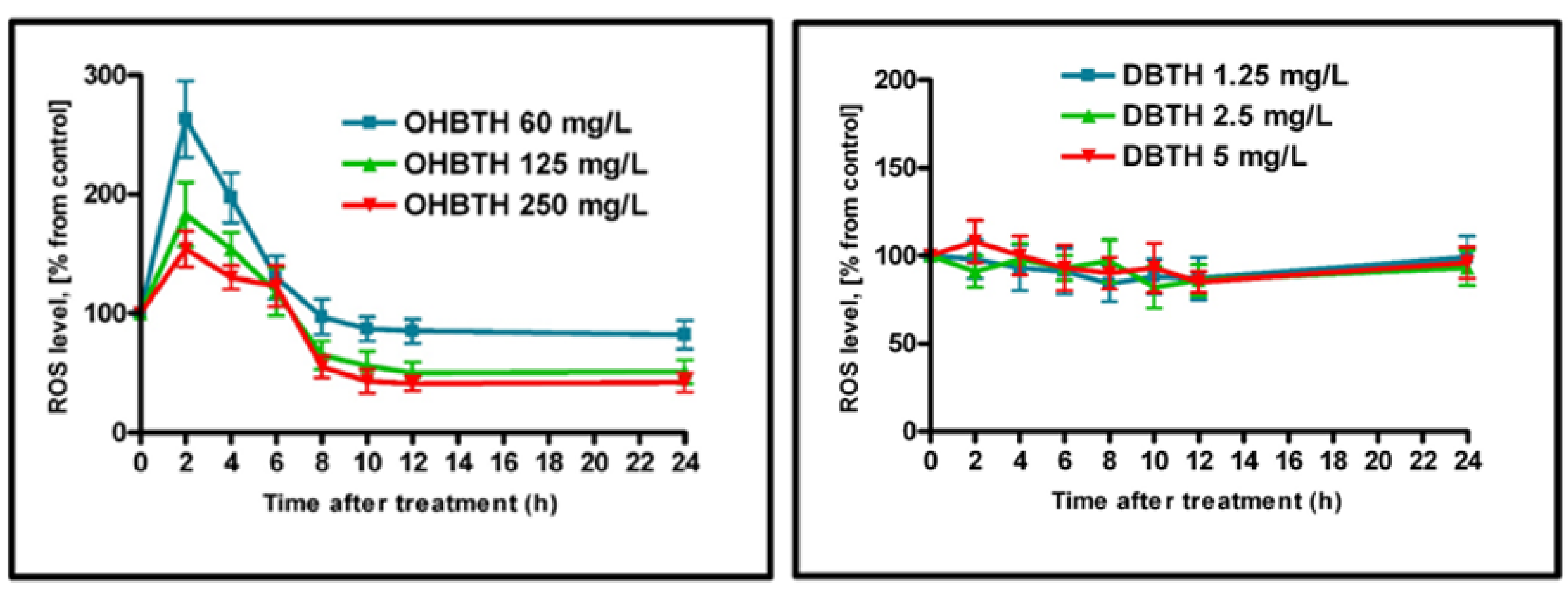


Fig 5.2 Change in ROS levels in RTgill-W1 cultures with time after the addition of OHBTH and DBTH.

OHBTH was added to give 60, 125, and 250 mg/L, which were respectively non-cytotoxic, cytotoxic, and very cytotoxic concentrations. DBTH was added to give 1.25, 2.5, and 5.0 mg/L. DBTH was always non-cytotoxic. H_2DCFDA was used to measure ROS over 24 h (X axis) and the results expressed as a % of the zero time values, which were 100% (Y axis).

TABLE 5.3 Change in ROS as measured with H2DCFDA in RTgill-W1 exposed over 24 h to BTHs

BTHs	Oxidative stress[1]	Duration of oxidative stress[2]	H_2DCFDA reading as a % of time zero Mean ± SD (n=3)	
			Value at 2 h[3] (always highest)	**Value at 24 h** (end of experiment)
VY				
Noncytotoxic (60 mg/L)	Yes	0-8 h	575 % ± 63 %	117 % ± 10 %
Cytotoxic (I25 mg/L)	Yes	0-8 h	686 % ± 96 %	88% ± 11 %
Very cytotoxic (250 mg/L)	Yes	0-8 h	1132 % ± 203 %	76 % ± 8 %
2ABTH				
Noncytotoxic (125 mg/L)	Yes	0-6 h	234 % ± 31 %	103 % ± 11 %
Cytotoxic (250 mg/L)	Yes	0-6 h	257 % ± 23 %	46 % ± 7 %
Very cytotoxic (500 mg/L)	Yes	0-4 h	302 % ± 16 %	24 % ± 8 %
BTH				
Noncytotoxic (150 mg/L)	Yes	0-2 h	145 % ± 18 %	55 % ± 10 %
Cytotoxic (300 mg/L)	No	none	103 % ± 26 %	43 % ± 12 %
Very cytotoxic (600 mg/L)	No	none	54 % ± 8 %	22 % ± 9 %
OHBTH				
Noncytotoxic (60 mg/L)	Yes	0-4 h	263 % ± 29 %	82 % ± 13 %
Cytotoxic (I25 mg/L)	yes	0-4 h	183 % ± 26 %	56 % ± 8 %
Very cytotoxic (250 mg/L)	yes	0-4 h	156 % ± 14 %	43 % ± 12 %
NaMBTH				
Noncytotoxic (25 mg/L)	yes	0-6 h	258 % ± 21 %	119 % ± 15 %
Cytotoxic (50 mg/L)	yes	0-6 h	271 % ± 39 %	78 % ± 12 %
Very cytotoxic (100 mg/L)	yes	0-6 h	258 % ± 26 %	38 % ± 11 %
ZincMBTH				
Noncytotoxic (30 mg/L)	yes	0-6 h	283 % ± 46 %	103 % ± 11 %
Cytotoxic (60 mg/L)	yes	0-2 h	264 % ± 33 %	36 % ± 12 %
Very cytotoxic (125 mg/L)	yes	0-2 h	136 % ± 12 %	25 % ± 8 %
2MBTH				
Noncytotoxic (25 mg/L)	yes	0-6 h	261 % ± 43 %	95 % ± 14 %
Cytotoxic (50 mg/L)	yes	0-6 h	254 % ± 28 %	73 % ± 12 %
Very cytotoxic (100 mg/L)	yes	0-6 h	264 % ± 39 %	54 % ± 9 %
SO				
Noncytotoxic (7.5 mg/L)	yes	0-6 h	310 % ± 37 %	56 % ± 9 %
Cytotoxic (15 mg/L)	yes	0-6 h	397 % ± 46 %	47 % ± 6 %
Very cytotoxic (30 mg/L)	yes	0-6 h	464 % ± 83 %	49 % ± 9 %
DTDC				
Noncytotoxic (0.05 mg/L)	yes	0-2 h	165 % ± 22 %	73 % ± 13 %
Cytotoxic (0.1 mg/L)	yes	0-2 h	136 % ± 13 %	78 % ± 10 %
Very cytotoxic (0.25/mg/L)	no	none	114 % ± 16 %	46 % ± 9 %
MBTHS				
Noncytotoxic (250mg/L)	yes	0-2 h	152 % ± 8 %	98 % ± 13 %
DBTH				
Noncytotoxic (5 mg/L)	no	none	108 % ± 13 %	96 % ± 14 %
NNA				
Noncytotoxic (250 mg/L)	no	none	102 % ± 9 %	97 % ± 6 %

[1]Oxidative stress meant that when the mean RFUs were subjected to a one way analysis of variance (ANOVA) $p < 0.05$.

[2]Duration of oxidative stress meant at these range of time points the RF weres signifcantly greater than the control as judge by Dunnett multiple comparison test ($p < 0.05$).

[3]The highest % increase relative to the control was always at 2 h.

5.3.3 Cell death mechanisms

RTgill-W1 cultures undergoing cell killing by the BTHs showed no signs of apoptosis. When cultures were examined after 6 h of exposure to the different BTHs, the proportion of dead cells, defined as staining with both PE-Annexin V and 7-AAD, ranged between 9 and 30 %. However, very few early apoptotic cells, defined as staining with PE-Annexin V and not with 7-AAD, were seen. In fact the highest percentage of early apoptotic cells was only 3.7 % ± 1.8 (n=3) and was found in cultures with SO at 30 mg/L. The results for 24 h exposures are summarized in Table 5.4. Again the percentage of cells in the early stage of apoptosis was low, less than 7 % in all cases. H33258 nuclear staining of cultures after 6 or 24 h exposures revealed no fragmented nuclei. Likewise DNA laddering was not detected after exposures of 12 and 24 h to any of the BTHs (data not shown).

As viewed by phase contrast microscopy, cells appeared to die by necrosis, but inhibitors of oxidative stress and necroptosis could not protect them. Cytoplasmic swelling was seen in cultures at high BTH concentrations and nuclei appeared smaller and condensed (Fig 5.3). The inhibitors, NAC at up to 20 mM, necrostanin-1 up to 100 μM and IM-54 up to 20 μM had no effect by themselves on the appearance of cultures and on cell viability. When the inhibitors were added to RTgill-W1 cultures 1 h before BTHs were also added, the BTHs continued to cause cytoplasmic swelling and loss of cell viability, with the dose-response curves largely unchanged (Fig. 5. 4).

Table 5.4 Effect of exposing RTgill-W1 for 24 h to BTHs on the % of cells in early apoptosis

Treatment	Early apoptosis cells[1]	Dead cells[2]
VY (125 mg/L)	0.4 % ± 0.2 %	40.1 % ± 3.5 %
VY (250 mg/L)	0.6 % ± 0.2 %	49.6 % ± 2.4 %
2ABTH (125 mg/L)	0.4 % ± 0.1 %	14.8 % ± 3.2 %
2ABTH (250 mg/L)	1.1 % ± 0.5 %	52.3 % ± 5.7 %
BTH (150 mg/L)	4.6 % ± 2.1 %	22.8 % ± 4.5 %
BTH (300 mg/L)	3.5 % ± 0.9 %	87.2 % ± 6.5 %
OHBTH (150 mg/L)	6.6 % ± 2.3 %	19.6 % ± 3.7 %
OHBTH (300 mg/L)	3.2 % ± 1.3 %	72.1 % ± 5.8 %
NaMBTH (50 mg/L)	1.5 % ± 0.3 %	44.6 % ± 6.7 %
NaMBTH (100 mg/L)	0.6 % ± 0.2 %	60.5 % ± 5.2 %
ZincMBTH (60 mg/L)	6.2 % ± 2.1 %	19.5 % ± 4.6 %
ZincMBTH (125 mg/L)	1.6 % ± 0.8 %	86.7 % ± 4.8 %
2MBTH (50 mg/L)	1.8 % ± 0.6 %	46.8 % ± 7.2 %
2MBTH (100 mg/L)	1.6 % ± 0.4 %	73.2 % ± 5.7 %
SO (15 mg/L)	4.3 % ± 1.2 %	48.4 % ± 5.9 %
SO (30 mg/L)	0.7 % ± 0.2 %	82.3 % ± 4.1 %
DTDC (0.1 mg/L)	0.6 % ± 0.1 %	21.7 % ± 6.4 %
DTDC (0.25 mg/L)	2.3 % ± 0.7 %	73.4 % ± 7.8 %
Control (no treatment)	1.2 % ± 0.4 %	1.8 % ± 0.5 %
Positive control (PES)[3]	34.3 % ± 1.7 %	2.2 % ± 0.9 %

[1]Early apoptosis cells: PE-Annexin V positive and 7-AAD negative

[2]Dead cells: PE-Annexin V and 7-AAD positive

[3]Positive control is culture treated with PES 5.25ug/ml for 6 h (Zeng et al., 2014).

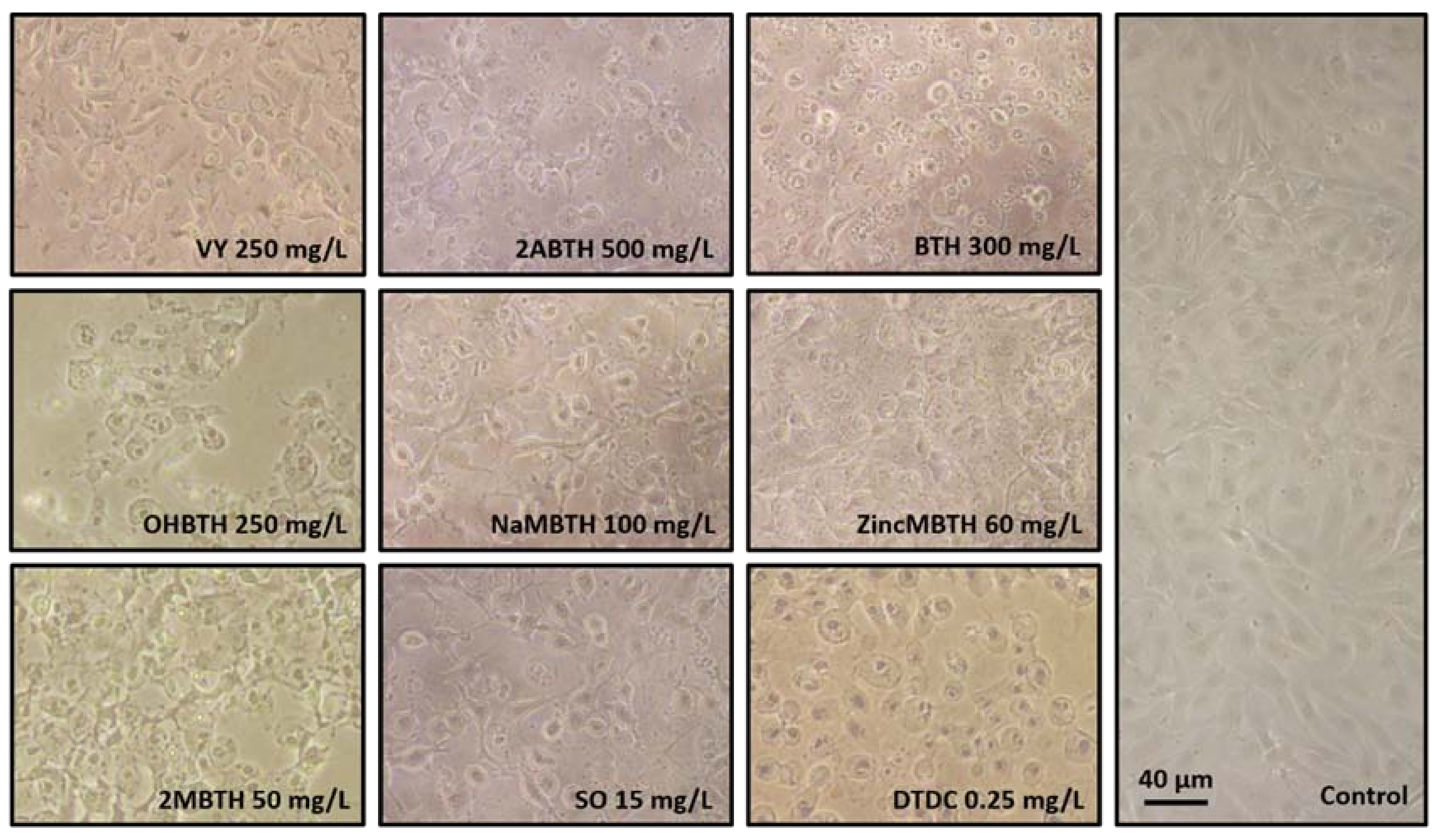


Figure 5.3 Effects of 24 h exposures to high concentrations of BTHs on appearance of RTgill-W1 cultures.

RTgill-W1 cells were treated with VY, 2ABTH, BTH, OHBTH, NaMBTH, ZincMBTH, 2MBTH, SO and DTDC for 24 h in L-15 without FBS. Control is RTgill-W1 cells only treated with DMSO. Pictures were taken at 200X magnification.

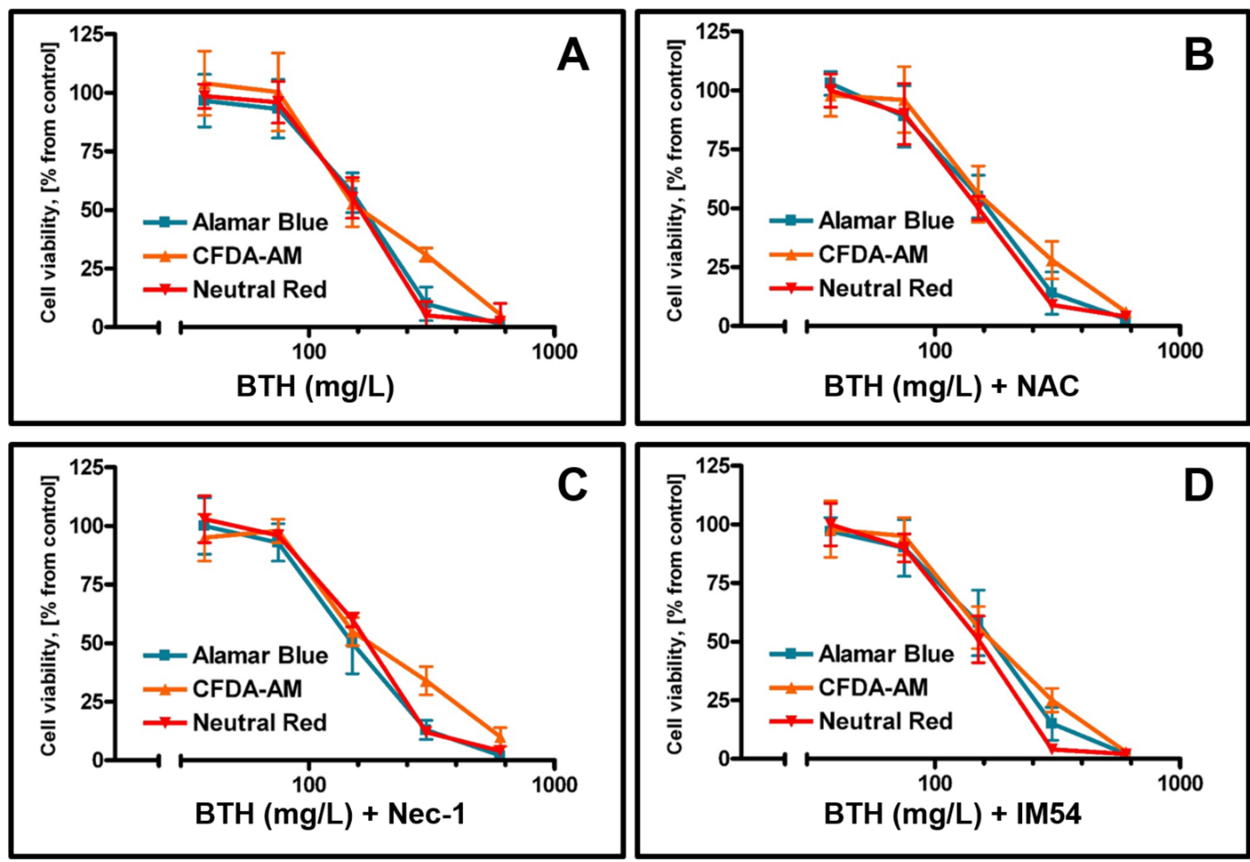


Figure 5.4 Effect of three inhibitors on the killing of RTgill-W1 by BTH.

Varying concentrations of BTH (X axis) were added to RTgill-W1 cultures (panel A, control) or to RTgill-W1 cultures that already had for 1 h and continued to have for the remainder of the experiment 5 mM NAC (panel B), 10 μM Necrostatin-1 (Panel C) and 10 μM IM-54 (panel D). After 24 h the cell viability was measured with Alamar Blue, CFDA-AM, and Neutral Red and expressed as a percentage of viability in control cultures (Y axis).

5.3.4 Genotoxicity of BTHs as determined with the comet assay

The BTHs did not appear to be genotoxic, except possibly BTH. Exposure of RTgill-W1 for 1 day to BTH, 2ABTH, OBTH, ZnMBTH, MBTH and SO increased the number of cells with comet assay scores of 1, 2, 3 and 4 (Fig. 5.5). However, the increases in the DNA damage index were only seen at cytotoxic concentrations as defined in Fig 5.1. At non cytotoxic concentrations the scores were less than 1, indicating no DNA damage. The other BTHs after 1 day of exposure gave scores of less than 1, regardless of whether the test concentrations were cytotoxic or not. Exposures to high but not cytotoxic concentrations were extended to 12 days. These concentrations were DBTH (5 mg/L), NNA (250 mg/L), MBTHS (250 mg/L), VY (15 mg/L), 2ABTH (30 mg/L), BTH (30 mg/L), OHBTH (15 mg/L), NaMBTH (12.5 mg/L), ZincMBTH (5 mg/L), 2MBTH (12.5 mg/L), SO (3 mg/L) and DTDC (0.025 mg/L) and with one exception led to DNA damage scores of less than 1. The exception was BTH, which gave a score of between 1 and 2. When BTH was tested at a lower concentration (15 mg/L), the score was less than 1. Therefore as judged from the application of the alkaline comet assay to RTgill-W1 cultures, only BTH caused DNA damage at non-cytotoxic concentrations and only after 12 days at the highest tested concentration (30 mg/L).

5.3.5 Cytochrome P4501A induction

Most BTHs induced P4501A, also referred to as CYP1A. This was demonstrated by western blotting with RTL-W1 because this cell line responded well to the classic cytochrome P4501A inducer, TCDD. When protein extracts from control and TCDD-treated RTL-W1 cultures were separated on SDS-PAGE electrophoresis and transferred to membranes, a commercial mouse monoclonal antibody to the P4501A of fish stained strongly a band at the expected size of P4501A in extracts from TCDD cultures but stained nothing in extracts from control cultures (Fig 5.6). When RTL-W1 were exposed for 48 h to the BTHs, CYP1A was detected after 48 h exposures to NaMBTH, ZnMBTH, SO, VY, MBTHS, NNA, 2ABTH, and OBHTH. The lowest concentrations at which P4501A induction was seen ranged from 7.5 to 75 mg/L. No induction was seen with 2MBTH, BTH, DTDC and DBTH. These were all tested in the 7.5-75 mg/L concentration range except for DTDC, which because of its cytotoxicity was tested at 0.1 mg/L.

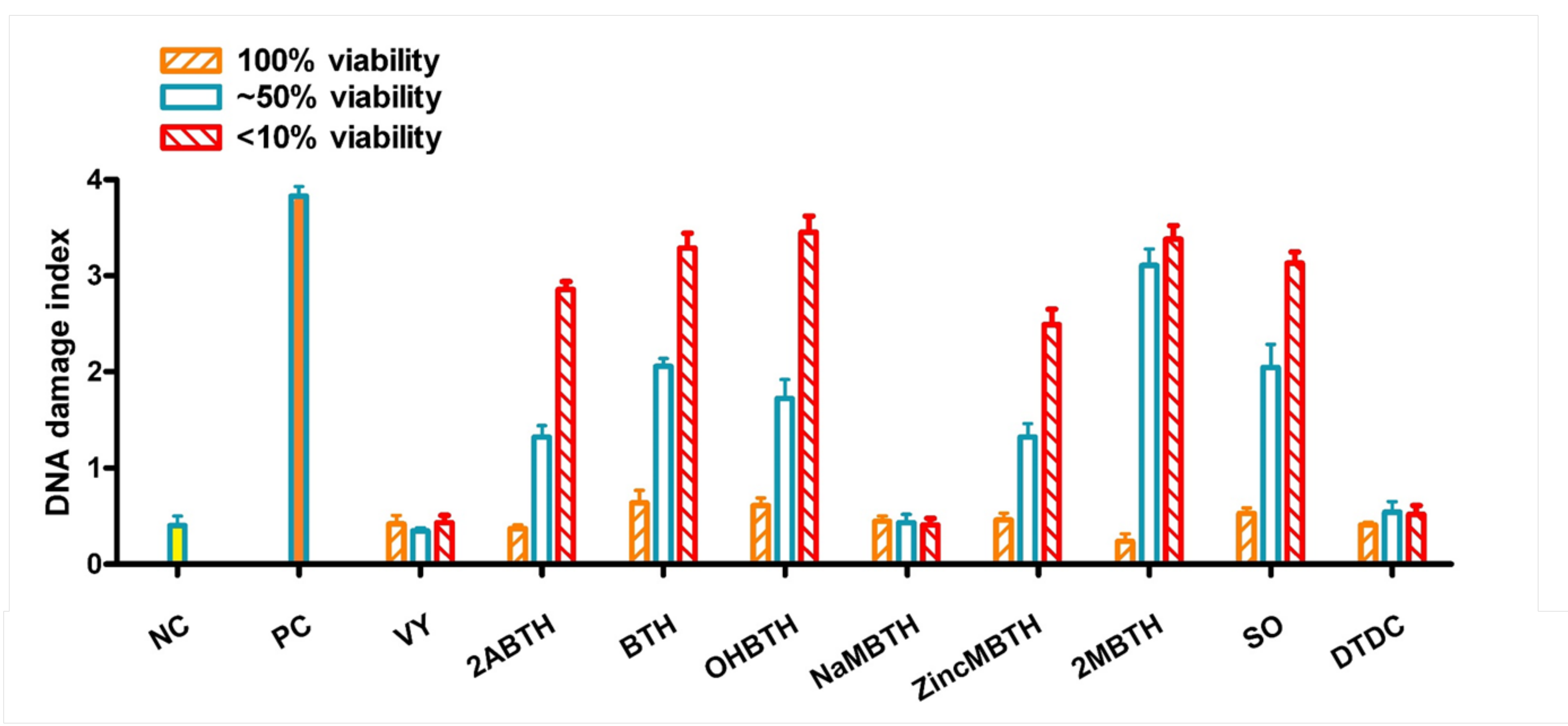


Figure 5.5 RTgill-W1 comet assay scores after exposure to different BTHs for 1 day.

RTgill-W1 cultures were exposed to BTHs at three concentrations, non-cytotoxic (100 % cell viability), cytotoxic (~50 % cell viability), and very cytotoxic (<15 % cell viability), before the comet assay was performed and scored manually to give a DNA damage index ranging from 0 to 4 (Y axis). Negative control (NC) cultures were only treated with DMSO and nearly all nuclei were condensed and round, indicating no DNA damage, and scored as type 0. Positive control (PC) cultures were treated with 200 µM of H_2O_2 for 10min and nearly all nuclei had intense comet tails and scored as type 4. VY was tested at 60, 125 and 250 mg/L; 2ABTH, at 125, 250 and 500; BTH, at 150, 300 and 600 mg/L; OHBTH, at 75, 150 and 300 mg/L; NaMBTH, at 25, 50 and 100 mg/L; ZincMBTH, 30, 60 and 125 mg/L); 2MBTH, 25, 50 and 100 mg/L; SO, 7.5, 15 and 30 mg/L; DTDC, 0.05, 0.1 and 0.25 mg/L.

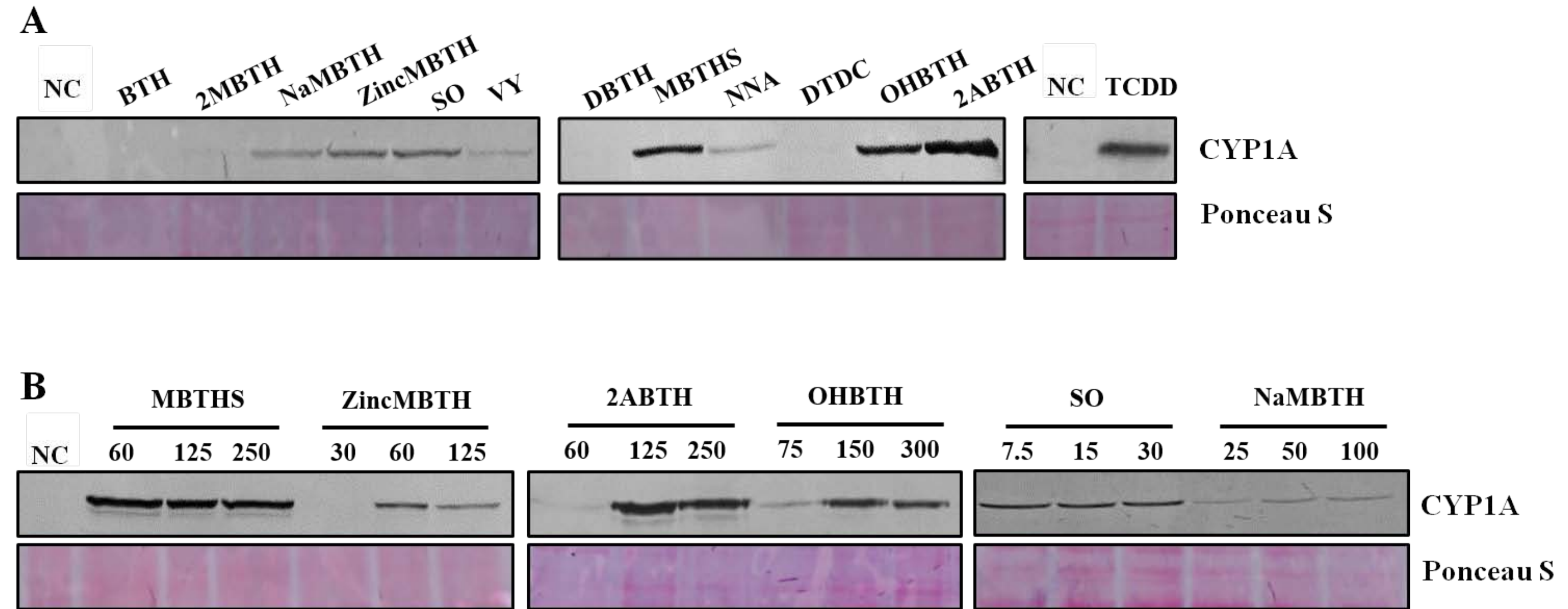


Figure 5.6 Western blotting for cytochrome P4501A in RTL-W1 after 48 h exposures to BTHs

SDS PAGE was used to separate polypeptides in 25 μg of cell extract followed by western blotting with a monoclonal antibody to fish Cytochrome P4501A to identify the CYP1A. Ponceau S staining was used as loading control. Extracts were prepared from RTL-W1 cultures that had been treated for 48 h with DMSO (NC), 97.6 pM TCDD (PC), BTH (30 mg/L), 2MBTH (50 mg/L), NaMBTH (50 mg/L), ZincMBTH (60 mg/L), SO (15 mg/L), VY (125 mg/L), DBTH (5 mg/L), MBTHS (125 mg/L), NNA (125 mg/L), DTDC (0.1 mg/L), OHBTH (150 mg/L) or 2ABTH (125 mg/L) (A) or MBTHS (60, 125, 250 mg/L), ZincMBTH (30, 60, 125 mg/L), 2ABTH (60, 125, 250 mg/L), OHBTH (75, 150, 300 mg/L), SO (7.5, 15, 30 mg/L) or NaMBTH (25, 50, 100 mg/L).

5.4 Discussion

For the first time with animal cells in vitro, benzothiazoles (BTHs) have been systematically investigated for their toxic potential. Two rainbow trout epithelial cell lines, RTgill-W1 and RTL-W1, have been used.

5.4.1 Comparing cytotoxicity of BTHs

Nine of twelve BTHs were identified as cytotoxic to rainbow trout cells, adding to the very limited and scattered toxicology literature on these compounds. The cytotoxicity was demonstrated with three cell viability indicator dyes: alamar Blue for energy metabolism, CFDA AM for plasma membrane integrity, and neutral red for lysosomal activity. Regardless of the exposure time or endpoint, the most toxic of the BTHs was DTDC, which was reported as being toxic to rodents (Booth & McDonald, 1982) but appears not to have been examined in other vertebrates. Next but much less toxic was SO, for which no toxicology reports could be found. VY and OHBTH were cytotoxic to RTgill-W1 and previously have been found to be toxic to respectively fathead minnow (Little & Lamb, 1982) and water fleas (Nawrocki et al., 2005). Rainbow trout cells were killed by 2ABTH and for rodents this compound was toxic at high oral doses and inhibited embryo development (Vigorita et al., 1990). NNA, DBTH, and MBTHS were not cytotoxic to rainbow trout cells and appear not to have previously been subjects for toxicology studies.

The most toxicological information is available for BTH and 2-mercaptobenzothiazole (2MBTH) and the zinc and sodium salts of 2MBTH and the results with RTgill-W1 fit a pattern of toxicity only at high concentrations. BTH was cytotoxic to the RTgill-W1, with EC_{50}s for 12 day exposures with the three endpoints being approximately 100 mg/L. For exposure of medaka to BTH, the 48 h LC_{50} was 110 mg/L (Yoshitada et al., 1986). The BTH EC_{50} for Daphnia magna was 50 mg/L (Hendriks et al., 1994). In other studies, BTH was reported to be cytotoxic to primary brain cell cultures from sheepshead minnow and tilapia and two fish epithelial cell lines, FHM from fathead minnow and CCO from catfish (Evans et al., 2000) and impeded the growth of several microbes, including bacteria (Reemtsma et al., 1995; De Wever& Verachtert, 1997) and protists (Yoshioka et al., 1986). Exposure of RTgill-W1 to the three 2MBTHs for 12

days caused approximately 50 % cell death in the 10 to 20 mg/L range. For 2MBTH, the 96 hours LC_{50} for rainbow trout was 1.3-6.2 mg/L and the approximate 48 hours fatal concentration for goldfish was 2 mg/L (Verschueren, 1983; Milanova et al., 2001). The human cell line HaCT lost viability at greater than 407 mg/L of 2MBTH (He et al., 2011). Growth inhibition of microbes has been seen at high concentrations. For several bacteria, growth was inhibited by 2MBTH to about 50 % at 42 mg/L and 100 % at about 135 mg/L (Folinova et al., 1978). For 15 Candida strains, growth was inhibited by 50 % at concentrations between 1 and 78 mg/L of 2MBTH (Bujdakova et al., 1993).

The cytotoxicity of the BTHs was broadly similar in the two cell lines, RTgill-W1 and RTL-W1, with two exceptions. Based on the EC_{50}s for the three cell viability endpoints, BTH was approximately seven times more cytotoxic to RTL-W1. On the other hand, for all the other BTHs, RTgill-W1 was approximately two times more susceptible to all the other BTHs. Possibly xenobiotic capabilities and antioxidant defense mechanisms differ between the two cell lines. RTL-W1 is known to be capable of xenobiotic metabolism (Schirmer et al, 1999) and to express glutathione peroxidases (GPx1 and GPx4), important components of antioxidant defenses (Pacitti et al., 2013). Possibly, xenobiotic metabolism in RTL-W1 might generate cytotoxic products from BTH but inactivate the cytotoxicity of the other BTHs.

The cytotoxicity of BTHs also can be compared in the context of their chemical properties. Studies of quantitative structure-activity relationships (QSAR) have shown that the toxic potency of narcotic toxicants is associated with their Octanol-Water Partition Coefficient (Kow). For RTgill-W1, Tanneberger et al (2013) showed that the baseline toxicity of organic compounds could be predicted from the QSAR model for metabolic activity: $\log EC_{50}$ (mM) = -0.96 (± 0.09) log Kow + 1.57 (± 0.28). The 24 h EC_{50} values of BTH, 2ABTH, VY, OHBTH, ZincMBTH and 2MBTH obtained from RTgill-W1 were generally similar or higher than the predicted baseline EC_{50}s. This suggests that these BTHs might act as narcotics or baseline toxicants which elicit toxicity by accumulating in the lipid bilayer of cell membranes and disrupting cell membrane functions (Escher et al., 2002). At physiological pH, 2MBTH and OHBTH were found to be hydrophobic, making the lipid bilayer of cell membranes a possible primary target (De Wever & Verachtert, 1997).

5.4.2 Oxidative stress and BTHs

The nine cytotoxic BTHs caused a transitory oxidative stress in RTgill-W1 cultures, whereas the three non-cytotoxic BTHs caused slight or no oxidative stress. In general terms, oxidative stress arises when ROS production and antioxidant defenses are imbalanced (Orrenhius et al., 2011). Thus BTHs might have modulated ROS production and/or ROS defenses. One of the major ROS sources is mitochondrial respiration, and BTR and TT have been noted to alter electron flow during oxidative phosphorylation in protozoa and duckweed (Cornell et al., 2000; Castro et al., 2004; Seeland et al., 2012). Therefore, perhaps like these BTRs, the BTHs might have initially stimulated mitochondrial processes in RTgill-W1 cultures, transitorily elevating ROS. Later, as cellular functions became impaired and as the cells began to die, the ROS levels dropped. Alternatively, the cytotoxic BTHs might have impaired antioxidant mechanisms in RTgill-W1, such as p53 and hsp70 (Zeng et al., 2014). As ROS participate as regulators of intracellular signalling (Finkel 2011), BTHs might subtly alter normal physiological processes, even if only temporarily. The possible roles of ROS in cell death and genotoxicity of BTHs are discussed below.

5.4.3 Cell death mechanisms with BTHs

As the BTRs of chapter 4, the cell death induced by the BTHs was likely due to a process best described by the term, uncontrolled necrosis (Feoktistova & Leverkus, 2015). Cell death lacked the hallmarks of apoptosis: the BTHs failed to cause nuclear fragmentation, DNA laddering, and externalization of phosphatidyl serine. Instead, BTHs induced cell swelling, nuclei condensation and plasma membrane rupture. These are characteristics of necrosis. However, necrostatin-1, which inhibits the controlled necrotic process, necroptosis (Xie et al., 2013), did not block the cytotoxicity of BTHs to RTgill-W1.

The oxidative stress generated by the BTHs appeared not to be involved in the cell death mechanism. Firstly, among the cytotoxic BTHs, the potency to kill cells did not correlate well with the ability to elevate ROS. The most cytotoxic compound was DTDC but this compound caused very little oxidative stress. VY caused the most oxidative stress but was one of the least

cytotoxic compounds. For most BTHs, the elevated ROS levels after 2 h of exposure were often the same after exposure to either non-cytotoxic or cytotoxic concentrations. Secondly, compounds that ameliorate oxidative stress did not prevent cell death. NAC, which is an antioxidant (Zhang et al., 2011), blocked the killing of RTgill-W1 by oxidative stress in a previous study (Zeng et al., 2014), but failed to block the cytotoxicity of the BTHs. IM-54, an inhibitor of oxidative stress induced necrosis (Sodeoka & Dodo, 2010), failed to block RTgill-W1 cell killing. Therefore, as with the BTRs (Chapter 4), the oxidative stress arising from BTHs did not appear to be responsible for killing the cells.

5.4.4 Genotoxicity of BTHs

Of the twelve BTHs that were tested on RTgill-W1 with the alkaline comet assay, only BTH appeared to be unequivocally genotoxic. This is because the comet assay was positive after RTgill-W1 had been exposed to BTH for 24 h and 12 days. Overproduction of ROS often lead to DNA damage (Orrenius et al., 2011), BTH might be expected to be a strong inducer of oxidative stress, but among the compounds that caused oxidative stress BTH was one of the weakest. In contrast to the results with the comet assay, other studies have found no evidence of BTH being genotoxic. BTH was not mutagenic in two different assays: these were the bacterial Ames test and the mouse lymphoma assay (MLA) with the leukaemia cell line, L5178Y tk+/- (Seifried et al., 2006).

The comet assay gave equivocal results with five BTHs: SO, 2ABTH, OHBTH, 2MBTH, and ZincMBTH. This is because as judged with the comet assay, they caused DNA strand breaks after 24 h exposures at cytotoxic concentrations but not after exposures of 12 days at non-cytotoxic concentrations. DNA strand breaks often occur because of cytotoxicity (Hilliard et al., 1998; Storer et al, 1996). As mentioned in Chapter 4, the guideline for in vitro genotoxicity testing suggests that when the putative genotoxicant decreases cell viability by more than 30%, false positives might arise (Tice et al., 2000). For these five BTHs, the DNA damage at 24 h was detected at concentrations that caused the viability, as judged with AB and NR, to be diminished to this level or lower. When the comet assay was applied on cultures after 12 days at concentrations that were not cytotoxic, no DNA damage was detected. Therefore these

compounds might not cause DNA strand breaks. Of these compounds, only 2MBTH appears to have been studied previosuly for genotoxicity. In the bacterial Ames test with and without S9 extract 2MBTH was found not to be mutagenic in either form of the test (Zeiger et al., 1987; Yamaguchi et al., 1991). However, studies with mammals suggest that 2MBTH might be genotoxic. Although in rats no significant covalent binding of 2MBTH to DNA was observed (Brewster et al., 1989), 2MBTH was mutagenic in the MLA with a rat liver S9 (NTP, 1998).

As evaluated with the comet assay, RTgill-W1 did not appear to undergo DNA damage after exposure for either 24 h or 12 days to six BTHs: DTDC, VY, NaMBTH DBTH, NNA, MBTHS, DTDC and. This group includes the most cytotoxic of the test compounds, DTDC, and the most potent inducer of oxidative stress, VY. Although DTDC has not been studied previously for genotoxicity, VY was negative in the Ames test without or with an S9 extract but positive when tested in the MLA with an S9 extract (National Cancer Institute). The sodium salt of 2MBTH appears not to have been examined previously for genotoxicity. The remaining three compounds of this group, MBTHS, DBTH and NNA, were the only compounds that caused no cytotoxicity and almost no oxidative stress. Thus a weak generalization is that BTHs not killing cells and not causing oxidative stress are negative in the comet assay. These compounds have been gauged in a few reports with different genetic tests but the results provide no clear answer as to their genotoxicity. DBTH was mutagenic in the bacterial Ames test for some workers (Zeiger et al., 1987). Yet others found that DBTH was not mutagenic in the Ames test but did induce genetic damage to mammalian cells (Crebelli et al., 1984; Hinderer et al., 1983). NNA was not mutagenic in either bacterial or mammalian cell tests (B G Chemie, 1994).

5.4.5 Cytochrome P4501A induction by BTHs

Some BTHs induced Cytochrome P4501A (CYP1A) in RTL-W1, suggesting that they might activate the aryl hydrocarbon receptor (AhR) pathway. CYP1A is the prototypical AhR-regulated gene product (Guyot et al., 2013). The AhR is present in RTL-W1 (Billard et al., 2002) and many PHHs and some polycyclic aromatic hydrocarbons (PAHs) induce P4501A in this cell line (Clemons et al., 1994; Bols et al., 1999). Recently several new classes of chemicals have been found to bind and activate the AhR in mammals (Murray et al., 2014). In Chapter 4 all seven

BTHs were shown to induce CYP1A. Now, eight of twelve BTHs have been shown to induce CYP1A in RTL-W1. As the AhR is involved in many cellular processes and has roles in tumorigenesis (Murray et al., 2014), BTHs as well as BTRs could elicit toxicological responses in fish by acting through the AhR.

Only a few other studies have noted the involvement of BTHs with AhR. In recombinant yeast assays, BTH was found to activate the human AhR, with an EC_{50} of 10.2 mg/L (Noguerol et al., 2006). When a recombinant mouse hepatoma cell line, Hepa1c1c7, was used in a chemical-activated luciferase gene expression assay (CALUX), 2MBTH was identified as an Ah receptor agonist (He et al., 2011), and in the human cell line, HaCT, 2MBTH induced the expression of mRNA for cytochrome P4501A1 (CYP1A1) (McKim et al., 2010). However, at the protein level, neither BTH nor 2MBTH were found to be CYP1A inducers in RTL-W1. In the case of BTH, the compound was more cytotoxic to RTL-W1 than to RTgill-W1, perhaps providing insufficient exposure time for CYP1A induction. In the case 2MTBH, the zinc and sodium salts of 2MBTH did induce CYP1A in RTL-W1, so perhaps 2MBTH was less available to cells in the culture system and unable to reach intracellular concentrations necessary for induction. DTDC and DDBTH did not induce P4501A.

CYP1A is a xenobiotic metabolizing enzyme that detoxifies some chemicals and activates others (Guyot et al., 2013), including benzo[a] pyrene in RTL-W1 (Schirmer et al., 2000). However, whether CYP1A contributes to the cytotoxicity of the BTHs to RTgill-W1 and RTL-W1 appears unlikely for several reasons. Firstly the cytotoxicity was seen in both cell lines but induction occurs only in RTL-W1 and in the case of RTL-W1 generally CYP1A induction takes 24 to 48 h (Bols et al., 1999) but cytotoxicity was seen at 24 h. Secondly, the most cytotoxic compound both in RTgill-W1 and RTL-W1 was DTDC and was not an inducer. This does not rule the involvement of other xenobiotic enzymes in the cytotoxicity of BTHs in some circumstances. As mentioned earlier, the greater cytotoxicity of BTH in RTL-W1 might be due to xenobiotic metabolism generating cytotoxic products.

CHAPTER 6

General summary and future research

6.1 General Summary

This thesis has used rainbow trout cell lines, primarily RTgill-W1, and two inhibitors of p53 to investigate the operation of this important signal transduction system in fish, and evaluated the toxicity of two classes of emerging contaminants, benzotriazoles (BTR) and benzothiazoles (BTH), to fish. The results have been summarized in the Thesis Abstract. Here the results are summarized around larger themes that have emerged from this research.

A. The responses of RTgill-W1 to the p53 inhibitors, 2-phenylethynesulfonamide (PES) and pifithrin-α (PFT-α), suggest that p53 functions in rainbow trout cells to mediate several basic cellular functions. The experiments with PES suggest that p53, possibly together with HSP70, controls the cellular levels of reactive oxygen species (ROS) through regulation of metabolism and of anti-oxidant mechanisms. This is because PES temporarily elevated ROS in RTgill-W1. PES had not previously been observed to increase ROS levels in any cell cultures system, but recently PES was found to elevate ROS levels in human cancer cell lines as well (Mattiolo et al., 2014). With both the cancer cell lines and RTgill-W1, the burst of ROS triggered cell death pathways. However, the cell death process was necrotic with the cancer cell lines and apoptotic with RTgill-W1. The experiments with PFT-α suggest that as with mammalian cells p53 controls the ploidy level in RTgill-W1 cells. As many environmental toxicants act through ROS and as dysregulation of ploidy is a hallmark of tumour development, a further focus on p53 in fish is warranted in aquatic toxicology.

B. The response of RTgill-W1 to PFT-α raised the difficulty of interpreting experiments with p53 inhibitors due to their possible off-target actions but possibly revealed the importance of p53 in regulating microtubules in fish cells. PFT-α caused a transient rise in the mitotic index and the disruption of cytoskeletal microtubules in RTgill-W1, suggesting that p53 might be regulating the assembly and disassembly of microtubules in the fish cells. PFT-α has not been observed to target microtubules in mammalian cells. The action of PFT-α on RTgill-W1 microtubules might be due to the inhibitor directly interacting with tubulin, which would be an off-target action, or to the inhibitor blocking the transcriptional activity of p53, which be an on-

target action. The results reinforce the view that experiments with inhibitors must be interpreted with caution and other approaches should be used as well.

C. Most BTRs and BTHs elicited toxicological responses in the rainbow trout cell lines but at concentrations that suggest these compounds are not of an immediate environment concern. The concentrations of BTR that were identified as being cytotoxic to RTgill-W1 were broadly similar to the concentrations that were identified as being toxic to a variety of test organisms (Table.1.2) and much higher than any concentration that has been measured in the environment (Table.1.1). Regardless of the endpoint, exposure periods or test system, the cytotoxicity and toxicity of BTRs occurred at concentrations above 15 mg/L (see Tables.4.1 & 4.2). This is more than 4000 fold higher than the highest concentration measured in the environment (Table.1.1). The same generalizations can be made for BTHs, although with these compounds less toxicology data is available on aquatic organisms (Table.1.3). The BTRs and the BTHs elicited cellular responses over the same broadly similar range, with one exception, DTDC. DTDC was approximately 100 more cytotoxic than the most cytotoxic of the other compounds. However DTDC did not induce CYP1A and has a low volume of usage and yet to be recorded in the environment. On the other hand, MBTHS was not cytotoxic but did induce CYP1A. Therefore, BTRs and BTHs should continue to be studied for their toxicology and to be monitored in the environment because the situation could change as the commercial uses of these compounds changes.

6.2 Future research directions

Many research directions are suggested from the work of this thesis. Three that are cell biology oriented and three that are toxicology focused are briefly discussed below.

A. Explore other ways of manipulating p53 in fish cell cultures

Although some ideas about the functions of p53 in RTgill-W1 have been obtained with p53 inhibitors, other approaches should be tried in order to delineate the functions of this master regulator in fish. With mammalian cells two basic experimental strategies have been used. One is to silence p53 expression in cell lines through techniques such as RNA interference (RNAi) (Ma et al., 2006). The opposite approach is to overexpress p53 in cell lines (Woodworth et al., 1994). A different approach has been to use the variation that can occur spontaneously in cell lines. About a half of all human cancers have mutations in p53, with the majority impairing the sequence specific DNA-binding activity of the p53 protein. When human tumours are used as a source of cell lines, the p53 status of the cell lines has been found to vary considerably and has been used to explore p53 functions. Many human tumour cell lines have non-functional p53 but some have different p53 levels. For example some have higher p53 levels (Xu et al., 1994), whereas others, like HCT-116 397.2, are completely deficient in p53 (Gestl & Bottger. 2012). Interestingly in some human cancer lines, the mutations in p53 have led to the protein having properties not found in wild-type p53 and are referred to as having gain-of-function activities (Bossi et al., 2006). Perhaps screening the hundreds of existing fish cell lines might identify some lacking p53 or having an altered p53 status. With these some or all of these approaches, the roles of p53 in fish cells could be studied and delineated.

B. Study the involvement of p53 in regulating microtubule dynamics

The disruption of microtubules (MT) in RTgill-W1 by PFT-α suggests that p53 could be involved in regulating MT assembling and disassembly. As PFT-α appears to do this only in fish cells, the regulation of MT through p53 might be more prominent and more easily studied in fish cells. In all cells, MT transition between stable and dynamic forms, most notably in mitotic spindle formation (Drewes et al., 1998). To date, most information about this has been obtained

with mammalian cells. In these cells the dynamic instability of microtubules is influenced by a variety of different proteins acting through different mechanisms. Among these microtubule-regulating proteins are kinesins, stathmin, and microtubule-associated proteins (MAPs). For at least some members of these protein families, evidence exists for p53 either mediating their induction or suppression (Ahn et al., 1999; Galmarini et al., 2003; Johnsen et al., 2000; Murphy et al., 1996; Utrera et al., 1998). Other angles to explore would be whether p53 regulates tubulin expression and physically associates with tubulin. Under some circumstances, p53 in human cells has been found to be involved in β-tubulin expression (Change et al., 2006) to localize with microtubules (Giannakakou et al., 2002). Overall the interplay between p53 and microtubules is poorly understood and an advantageous system for exploring it might be fish cells.

C. Study the possible involvement of p53 with AhR in regulating P450A1

An interesting avenue to explore might be the involvement of p53 with the aryl hydrocarbon receptor (AhR) in regulating cytochrome P450 1A1 (CYP1A1) induction. Two recent papers point to the possibility of a connection. When p53-null and p53 wild type mice were compared, the loss of p53 was found to increase the level of Aha1, a co-chaperone of Hsp90 (Okayama et al., 2014). Aha1 associates with Hsp90, increasing Hsp90 ATPase activity. ATPase activity causes a conformational change in hsp90 that is needed to induce conformational change in a substrate or client protein, such as AhR. When p53 was silenced in mammalian cell lines, Aha1 amounts were elevated; Hsp90 ATPase activity increased; and CYP1A1 levels enhanced (Okayama et al., 2014). Overexpressing p53 suppressed CYP1A1 levels (Okayama et al., 2014). An interesting preliminary experiment to do would be to see if exposing RTL-W1 to the p53 inhibitor, PFT-α, or p53 stabilizer, CP-31398, would enhance or suppress the expression of P450 1A1 in response to t 2,3,7,8,-tetrachloro-[p]-dioxin (TCDD) and to BTRs and BTHs. If expression were to be altered, this would point to the master regulator p53 being involved in xenobiotic metabolism and provide another reason for investigating p53 in aquatic toxicology.

D. Explore the possible genotoxicity of BTRs and BTHs with other techniques

Further genetic testing should be done to determine whether BTH indeed causes DNA damage and to confirm that the other eleven BTHs do not. A few different genotoxicity tests have received regulatory approval and new tests continue to be developed. The International Conference on Harmonization (ICH) has recommended several in vitro genotoxicity tests for regulatory use (Kirland, 2011). These are the Ames test, the mouse lymphoma assay (MLA), micronucleus assay (MN), and chromosome aberration assay (CA). The Ames test and the MLA are mutation assays, detecting frame shifts and base substitutions. Inasmuch as some chemicals are not mutagenic but their metabolites are, these assays can be run with a S9 fraction that supplies the xenobiotic enzymes for potentially generating mutagens from the chemical under study. The MN and CA measure chromosome loss and chromosome breakage (Fenech, 2000). Each of these assays have been used to study BTHs, but usually only in one report with one or two assays for one or two BTHs and never systematically. There is a need to study all 12 of the BTHs with one or more of these assays as has been done here with the comet assay in order to see whether patterns emerge. Genotoxicity might be observed with just some BTHs or some BTHs might just cause certain kinds of DNA damage. A problem with animal cell assays is the problem of high frequencies of 'false positives'. This has led to the development of additional tests that are still under consideration for regulatory approval (Garcia-Canton et al., 2012). One of these is the γH2AX assay (Garcia-Canton et al., 2012). H2AX is a member of the H2AX family and becomes rapidly phosphorylated at serine 139 in response to double strand DNA breaks to give γH2AX. The γH2AX can be detected by immunoblotting with a monoclonal antibody that is specific for the phosphorylated form of H2AX. Application of this and other new genotoxicity tests to the BTHs should also help answer the question of whether none, all, or just some of the BTHs are genotoxic.

E. Establish the relationship between in vitro versus for in vivo for BTRs and BTHs

The in vitro cell assays in this work are relatively inexpensive alternatives to in vivo tests and allow many compounds to be rapidly screened for potential toxicity in vivo. However, in vitro toxicity data often underestimate the in vivo toxicity, and in some cases by an order to 3 orders of magnitude (Schirmer, 2006, Tanneberger et al., 2013). The underestimation is mainly due to

the more extensive non specific binding of toxicants to extracellular protein and well plate plastic in cell based assay. A generally lower metabolic capability of cell lines also limits the sensitivity of in vitro assays, since toxicants can be metabolized in vivo to more toxic or less toxic compounds (Kramer et al., 2009). A number of techniques have been developed to improve the correlation between in vitro and in vivo bioassays, including cell assays free of serum, mode of action based assays and cell assays with modified culture environment more closely resemble the in vivo exposure (Castano et al., 2003, Schirmer, 2006). However, there is so little in vivo data for fish with the BTRs and BTHs, some in vivo studies are perhaps warranted, especially for rainbow trout, a species commonly used for environmental regulatory purposes. For just a few of the BTHs and BTRs of this work, establishing the relationship between their toxicity to rainbow trout and to rainbow trout cells in culture would improve the confidence with which the in vitro results could be interpreted and would encourage the screening of the many BTRs and BTHs, for which there is no toxicity data.

F. Use the results on BTRs and BTHs to develop environmental monitoring strategies and to understand their environmental significance

An increase in environmental monitoring of BTRs and BTHs should expanded to include many more BTRs and BTHs. In this thesis, most BTHs appeared to be toxic to the rainbow trout cells at the same concentrations as most of BTRs and at concentrations that BTHs have been toxic in other test systems from microorganism to humans, limited as these studies maybe. Thus these compounds do not seem to target particular organisms but act on all. Fish appear not to be exceptionally susceptible, although they might be more frequently exposed to BTRs and BTHs than terrestrial vertebrates. When the full suite of toxicological responses is considered, especially CYP1A induction, only DBTH of the 19 BTRs and BTHs failed to cause any toxicological responses. Thus nearly all these compounds should be considered potentially toxic, although only at very high doses. As the cost of monitoring so many compounds might be prohibitive, some focus will likely have to be adopted.

When the results are considered along with the volume of production and likelihood to be released in the environment, the BTHs that likely should receive the most environmental

attention are BTH and 2MBTH, along with the zinc and sodium salts of 2MBTH. The concentrations of these compounds that elicited toxicological consequences in the rainbow trout cells were between 10 and 100 mg/L. These concentrations are about five hundred to one thousand fold higher than have been reported in the environment and in humans. For example, BTH was found at up to 0.181 mg/L in the urine of human volunteers in Japan (Asimakopoulos et al., 2013). In water samples from the Pearl River Delta, China, BTH values as high as 0.000476 mg/L were noted (Ni et al., 2008). Therefore, one way to look at the results with the fish cells is that fish and humans are unlikely to be at risk with the concentrations that have been found in the environment to date. However, efforts should be made to keep the BTHs at these low concentrations in the environment.

The concentrations of the BTHs and BTRs in the environment should be considered the context of other environmental contaminants to understand more completely their ecotoxicological impact. Of the cellular toxicology mechanisms that were observed in this thesis for the BTHs and BTRs, activation of the AhR and the induction of P4501A might be pivotal in understanding their true impact. Although the BTHs and the BTRs activated the AhR and induced CYP1A in RTL-W1 at very high concentrations, many other environmental contaminants, such as some dioxins, furans, polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) induce CYP1A in fish cells (Clemons et al, 1994; Bols et al., 1999). Thus whether BTHs and BTRs interact with them in an additive, inhibitory or synergistic manner will be of environmental importance. As just one example, if they were to act synergistically with PCBs, the current low environmental concentrations might be more significant and would depend on the concentration of the PCBs at a particular site.

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