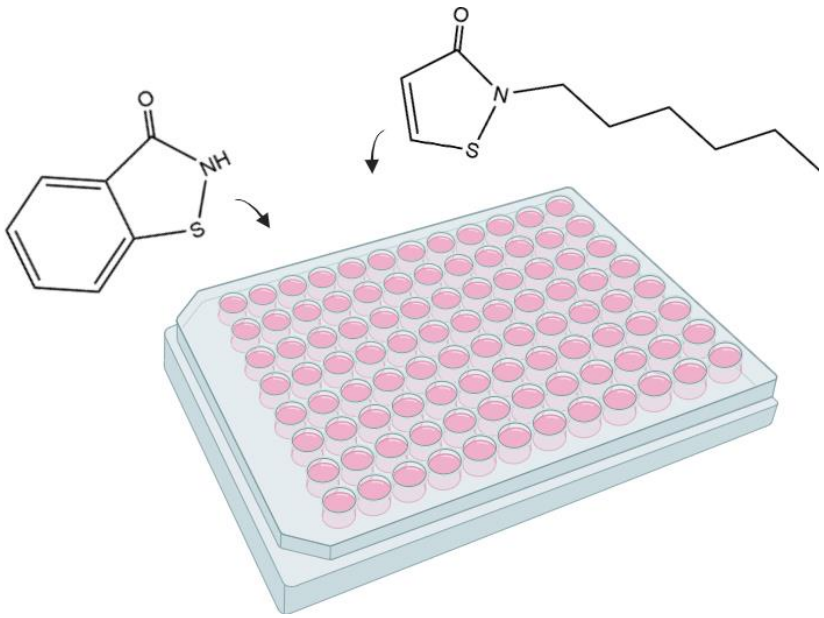
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

EXPLORING THE METABOLISM AND MIXTURE EFFECTS OF THE ISOTHIAZOLINONE BIOCIDES BIT AND OIT IN PLHC-1 CELLS



Oskar Blomqvist

Aline Carvalho Nejstgaard

Degree project for Master of Science (120 hec) with a major in Biotechnology (Chalmers)

ES2540, Master's Thesis in Ecotoxicology, 30 hec

Second cycle

Semester/year: Spring 2024

Supervisor: Malin Celander, Department of Biological and Environmental Sciences

Examiner: Lennart Bornmalm, Department of Marine Sciences

Master's thesis 2024

Exploring the metabolism and mixture effects of the isothiazolinone biocides BIT and OIT in PLHC-1 cells

OSKAR BLOMQVIST
ALINE CARVALHO NEJSTGAARD

UNIVERSITY OF GOTHENBURG

CHALMERS
UNIVERSITY OF TECHNOLOGY

This thesis was conducted at the Department of
Biological and Environmental Sciences
University of Gothenburg
Gothenburg, Sweden 2024

Exploring the metabolism and mixture effects of the isothiazolinone biocides BIT and OIT in PLHC-1 cells
OSKAR BLOMQVIST
ALINE CARVALHO NEJSTGAARD

Contact information: oskar.jblomqvist@hotmail.com and aline.c.nejstgaard@gmail.com

Supervisor: Malin Celander, Department of Biological and Environmental Sciences, University of Gothenburg
Co-supervisors: Anne Farbrot and Anders Blom, Occupational and Environmental Medicine, Sahlgrenska Academy
Examiner: Lennart Bornmalm, Department of Marine Sciences, University of Gothenburg

Master's Thesis 2024
Department of Biological and Environmental Sciences
University of Gothenburg
SE-405 30 Gothenburg
Telephone +46 31 786 0000

Cover: Illustration of PLHC-1 cells being exposed to BIT and OIT in a 96-well plate. Created in BioRender.

Typeset in LaTeX
Gothenburg, Sweden 2024

Exploring the metabolism and mixture effects of the isothiazolinone biocides BIT and OIT in PLHC-1 cells

OSKAR BLOMQVIST, ALINE CARVALHO NEJSTGAARD
Department of Biological and Environmental Sciences
University of Gothenburg

Abstract

The powerful antimicrobial properties of isothiazolinones make them a common preservative in both industrial and consumer products. However, methylated derivatives of isothiazolinones have been associated with concerning allergenic effects leading to stricter regulations. As a consequence, industries have turned to more lipophilic isothiazolinones, such as benzisothiazolinone (BIT) and octylisothiazolinone (OIT), which are less regulated. Similar effects have however been reported for exposures of BIT and OIT, highlighting the importance of expanding the knowledge about these compounds, to enforce stricter regulation and potentially controlling the isothiazolinones as a group. This study aimed to investigate the toxicodynamic and toxicokinetic responses in a fish liver cell model system, *Poeciliopsis lucida* hepatocellular carcinoma (PLHC-1) following exposure to BIT and OIT, alone and in mixture. Results from the ethoxyresorufin-O-deethylase (EROD) assay revealed no apparent induction of the common detoxification enzyme CYP1A after exposure to BIT and OIT. However, more data is needed for a more robust evaluation of whether the enzyme is involved in their metabolism. Analysis of cultured exposure media in high performance liquid chromatography (HPLC) demonstrated cellular biotransformation of BIT into a more polar metabolite. A rapid cellular uptake of OIT was observed, however no potential OIT metabolite could be detected, neither extracellularly nor intracellularly. A cytotoxic assessment was also conducted on exposed PLHC-1 cells investigating the mitochondrial activity and membrane integrity, common markers for cellular viability. Significant toxic effects were observed on both markers after exposure to 10 µM OIT, while no toxic effect was observed for the same concentration of BIT. However, co-exposure to these concentrations of OIT and BIT, displayed an even greater toxicity, indicating a synergistic mixture effect between the two. While this study provides valuable insights into the toxicodynamic and toxicokinetic profiles of BIT and OIT, highlighting the importance of considering mixture effects in assessing their potential health and environmental impacts, additional research is needed to fully understand their specific metabolic pathways and interaction mechanisms, to assess their long-term exposure effects.

Keywords: Isothiazolinones, Benzisothiazolinone, BIT, octylisothiazolinone, OIT, PLHC-1, CYP1A, metabolism, toxicodynamic, toxiokinetic

Popular scientific summary

Isothiazolinones are a type of chemical additive that acts as a preservative in products to prevent microbial growth and spoilage. They are widely used and can be found in common household products, such as dish soaps, cleaning products, shampoos, laundry detergents and wall paints. However there have been reports raising concerns about their potential threat to marine life and ecosystems when released into the environment. Isothiazolinones have also been reported to cause skin allergies in humans, which raises even more concerns of their use as preservatives in consumer products. Two isothiazolinones that in recent years are being increasingly used as preservatives are BIT and OIT, however the research about them is limited. Given the extensive use of these isothiazolinones, and the limited knowledge of their toxic effects, it is important to investigate how they are broken down and their potential adverse health impact.

This study investigated how a type of fish liver cell was affected by BIT and OIT, as well as how they were broken down in the cells. It was found that an enzyme (CYP1A) that is commonly involved in the clearance of toxic substances is unlikely to be involved in breaking down BIT and OIT. It was also determined that OIT is more toxic than BIT at lower concentrations. However, mixtures of BIT and OIT proved to be even more toxic than when the cells were exposed to either chemical alone. This is an important finding, since both chemicals usually co-exist when released into the environment, highlighting the importance of restricting their presence in common household products. However more research is needed on the topic, specifically regarding the breakdown of BIT and OIT to fully understand how they affect biological systems.

Acknowledgements

We would like to thank all the people who have helped us throughout this Master's Thesis project. Starting with our supervisor **Malin Celander**, who has guided us through this project and always shown excitement in our progress and ideas. Thank you for inviting us into your research group and letting us undertake this interesting project. We also want to thank **Annika Lindkvist** who has helped us in the lab and taught us the laboratory techniques. We also want to thank all other members of the Ecotoxicology lab in Natrium, thank you for always being so helpful and understanding. Lastly we want to thank **Anders Blom** and **Anne Farbrot** at the Department of Occupational and Environmental Medicine for showing a great interest in our project and who have helped us greatly with everything related to the HPLC analyses. It has been a pleasure working with you all and we have gained a great deal of valuable knowledge and experience.

We also want to thank **Felicia**, our friends and families for being so supportive and encouraging during these five years of studies at Chalmers University of Technology, especially during the Thesis project this spring.

Oskar Blomqvist and Aline C. Nejstgaard, Gothenburg, June 2024

List of Acronyms

Below is the list of acronyms that have been used throughout this thesis listed in alphabetical order:

AB	alamarBlue™ dye
ABC	ATP-Binding Casette (transporter proteins)
ACD	Allergic contact dermatitis
AhR	Aryl hydrocarbon Receptor
ANOVA	Analysis of variance
AUC	Area Under Curve
BIT	Benzisothiazolinone
BNF	β-Naphthoflavone
CFDA-AM	5-Carboxy Fluorescein Diacetate - Acetoxymethyl Ester dye
CMIT	Chloromethylisothiazolinone
CYP	Cytochrome P450 (enzyme superfamily)
CYP1A	Cytochrome P450 1A
DCOIT	Dichlorooctylisothiazolinone
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ER	7-Ethoxyresorufin
EROD	Ethoxyresorufin-O-Deetylase
FBS	Fetal Bovine Serum
FU	Fluorescent unit
HPLC-UV	High Performance Liquid Chromatography with Ultraviolet detection
MeOH	Methanol
MEM	Minimum Essential Medium
MIT	Methylisothiazolinone
OIT	Octylisothiazolinone
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate Buffered Saline
PLHC-1	*Poeciliopsis lucida* hepatocellular carcinoma (cell line)
ROS	Reactive oxygen species
SD	Standard deviation

Contents

List of Figures

1
Introduction

One of the main threats for producers worldwide is microbial contamination of their products. This not only applies to food products, but also to non-trivial industries such as construction and cosmetics. Biocides are chemicals that act as preservatives in products by killing harmful pathogens in order to protect both people and animals [2]. There are different types of biocidal chemicals and they can be found in a variety of products, for example disinfectants, medicines, hygiene products and waste water. Despite their positive antimicrobial effects, some biocides may pose a threat to both the environment and to animal and human health.

Isothiazolinones are a type of biocide commonly used in cosmetics and other at-home cleaning and hygiene products such as soaps and laundry detergents [3]. Because of their powerful antimicrobial properties, isothiazolinones are also added to paints, boat coatings, and marine coatings to prevent spoilage. However, the potent biocidal nature of isothiazolinones raises concerns about potential ecotoxicological hazards and implies further studies of the compounds. It has also been reported that some isothiazolinones causes allergic reactions upon contact with skin, proposing further toxic concerns [4, 5, 6]. Two of these biocides are benzisothiazolinone (BIT) and octylisothiazolinone (OIT), which will be investigated in this project.

While BIT and OIT are extensively used in consumer products, there is still a notable gap in the knowledge of their definitive effects on humans and animals, specifically concerning their uptake and metabolism. The objective of this thesis is to lessen this knowledge gap and contribute to a broader understanding of the impacts of these isothiazolinones.

1.1 Aim and clarification of project

The aim of this project was to investigate the hepatocellular metabolism and mixture effects of the isothiazolinones BIT and OIT. More specifically, the toxicodynamic and toxicokinetic properties of BIT and OIT in PLHC-1 cells were evaluated.

The aim has been clarified and formulated into two main research questions with correlating subquestions.

- What are the toxicokinetic properties of the isothiazolinones BIT and OIT in PLHC-1 cells?
  - Could the common phase I enzyme CYP1A be involved in the metabolism of the isothiazolinones BIT and OIT in PLHC-1 cells?
  - Are any metabolites formed and what can be said about their properties?

- What are the toxicodynamic properties of the isothiazolinones BIT and OIT in PLHC-1 cells?
  - What are the cellular responses of exposed PLHC-1 cells, with focus on mitochondrial activity and cell membrane integrity?

To achieve this aim, a series of experimental assays have been conducted using the PLHC-1 cell line as model system. The ethoxyresorufin-O-deethylase (EROD) assay was implemented in order to investigate potential involvement of the cytochrome P450 (CYP) 1A enzyme in the metabolism of BIT and OIT. Further, high performance liquid chromatography with UV detection (HPLC-UV) was utilized to investigate both extracellular and intracellular contents over time following exposure of PLHC-1 cells to BIT and OIT, aiming to reveal the uptake of the isothiazolinones and the formation of potential metabolites. Lastly, a cytotoxicity assay analyzing the mitochondrial activity and membrane integrity was performed to assess the overall cytotoxic effect of exposure to the isothiazolinones, both individually and in mixture.

2

Theory

The powerful antimicrobial properties of isothiazolinones make them common preservatives in both industrial and consumer products. However, given their suggested allergenic and toxic effects, further studies are necessary to thoroughly evaluate their potential adverse impacts. Understanding the metabolism of these compounds is essential, as biotransformation processes influence their toxicity and persistence in biological systems. This evaluation is necessary for assessing the safety and environmental impact of isothiazolinones.

2.1 Isothiazolinones

Isothiazolinones are known for and widely used thanks to their potent antimicrobial properties. They are characterized by a pentagonal heterocyclic thiazole ring, containing both sulfur, nitrogen and a carbonyl oxygen. The attached side chains determines the properties of each isothiazolinone derivative [3]. The most commonly prevalent isothiazolinones in consumer products are benzisothiazolinone (BIT), octylisothiazolione (OIT), dichloroocthylisothiazolinone (DCOIT), methylisothiazolinone (MIT), and chloromethylisothiazolinone (CMIT), whose chemical structures can be found in Figure 2.1. These isothiazolinone biocides are both used alone or in mixture with each other in products. It is previously known that exposure of chemicals in mixture may pose an amplification of the toxic response in organisms compared to when exposed alone [7, 8, 9]. This is most prevalent in the aquatic environment where organisms are usually exposed to multiple chemicals at a time.

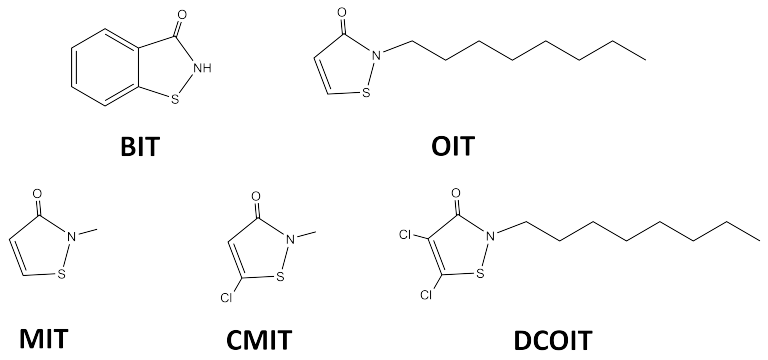


Figure 2.1: Chemical structures of benzisothiazolinone (BIT), octylisothiazolione (OIT), dichloroocthylisothiazolinone (DCOIT), methylisothiazolinone (MIT) and chloromethylisothiazolinone (CMIT). Created in ChemDraw.

Isothiazolinone biocides enter the target microorganisms (e.g. fungi and bacteria) through diffusion across the cell wall or membrane. Upon entering the cells, their mode of action has been described as reacting with the nucleophilic groups of cellular components through the electron-deficient sulfur atom in their thiazole structure [10]. More specifically, they block the enzymatic activity of cellular proteins by binding to thiols from cysteine found in the active sites of the enzymes, ultimately causing cell death. It was found in a study by Arning and colleagues (2008) that the isothiazolinones CMIT and DCOIT strongly decreased the intracellular levels of glutathione (GSH), while simultaneously increasing levels of oxidised glutathione (GSSG) in the human hepatoblastoma cell line HepG2 [11]. As a result of this the cellular thiol reduction potential was severely shifted, consequently leading to morphological changes of the cells and cell death. This effect was also amplified by inhibition of glutathione reductase (GR) caused by CMIT and DCOIT. Their non-chlorinated derivatives MIT and OIT were also assessed in the same study. However they did not display as high effects on the HepG2 cells as their chlorinated analogues [11].

Despite being known for causing skin sensitization [3], isothiazolinones are among the most common ingredients used in consumer products in Sweden and their presence have increased drastically since 2002 [12]. Figure 2.2 illustrates the number of consumer products in Sweden where BIT, CMIT and MIT could be found between 2002-2022. According to the Swedish Chemical Agency's report on the most common substances in consumer products from 2022, BIT appeared as the second-most common substance in terms of number of products it appears in, only surpassed by water.

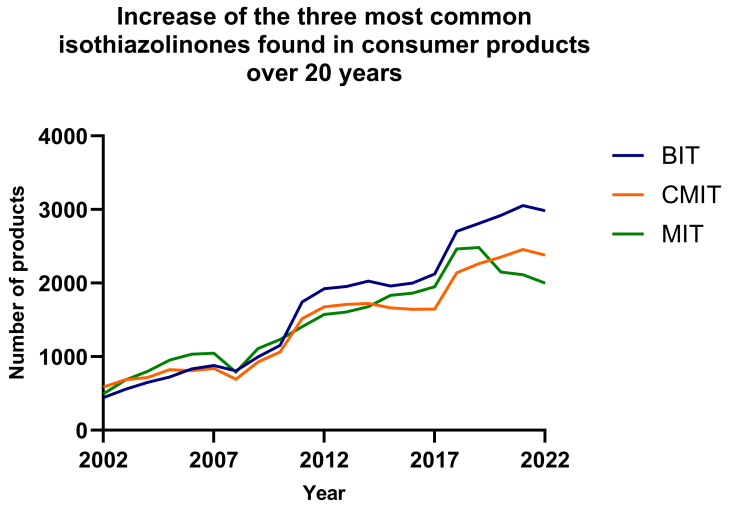


Figure 2.2: Number of consumer products in Sweden where BIT, CMIT and MIT were used from 2002-2022. The data is acquired from the Swedish Chemical Agency.

In 2015 the limit of allowed MIT in cosmetics in the European Union was drastically decreased [13], which in 2017 led to a 50% decrease in reported cases of MIT

contact allergy [14]. The regulation also led to a decline in the appearance of MIT in consumer products in Sweden indicated with the green graph in Figure 2.2. An issue following the banning or regulation of a chemical is that the industry usually replaces it with a similar, but less regulated, chemical [15]. Although DCOIT, BIT and OIT are banned in cosmetics in the EU, the usage of them in other consumer products are still significantly increasing.

In a study where researches had induced skin sensitization towards MIT in mouse subjects by pre-treating them with MIT for three days, it was found that subsequent exposure to BIT or OIT triggered the same allergic reactions as when the mice were exposed to a second dose of MIT [16]. The authors therefore suggests that MIT-sensitized individuals may also react to other isothiazolinones apart from MIT. This cross-reactivity was also investigated in a separate study where allergy patch tests were performed on human patients. The study found that the subjects that presented allergic reactions to OIT also showed strong allergic reactions to MIT, while the same concurrent reactions could not be shown for BIT [17]. The authors propose the structural similarity of OIT and MIT to be the reason behind their cross-reactivity among patients. Thus BIT's lack of cross-reactivity with OIT and MIT might be explained by its aromatic ring, which OIT and MIT does not have.

The isothiazolinone DCOIT, which is present in marine coatings, has previously been claimed to quickly degrade in the environment. However recent research suggests otherwise [18]. Due to its lipophilic nature it agglomerates with marine particles which deposits into the sediment of the seabed and acts as a DCOIT reservoir. There it can be stored for an extensive time period and interact with the present biological entities. Accumulation of DCOIT has been found in several marine environments around the world, including Korean harbours [19, 20], Japanese ports and bays [21, 22, 23, 24], Malaysian and Indonesian coastlines [25, 26], and marinas in Spain, Sweden and Greece [27, 28, 29, 30]. The structural similarities of OIT and DCOIT may suggest that OIT could display similar accumulation in marine sediments.

2.1.1 Benzisothiazolinone and octylisothiazolinone

1,2-Benzisothiazol-3(2H)-one and 2-Octyl-4-isothiazolin-3-one, more commonly referred to as benzisothiazolinone (BIT) and octylisothiazolinone (OIT) are widely used isothiazolinone biocides [3]. BIT is frequently used as a preservative in different personal care and household products such as dish soaps, cleaning products, paints and coatings [31, 32], while OIT is more prevalent in industry and leather products [33], however also occurs in similar products as BIT. Due to being such frequently used industrial chemicals, they are likely to eventually end up in aquatic environments during their lifetime.

Because of its low log K_{ow} value of 0.7, BIT is unlikely to bioaccumulate in aquatic organisms [34, 35]. However, there are some data suggesting moderate toxicity to freshwater fish and invertebrates, as well as high toxicity to green algae [34]. OIT however, has a log K_{ow} value of 3.3 [33] which may be responsible for it being

bioaccumulative and highly toxic to aquatic environments [36]. In a study done on zebrafish it was also shown that larvae development, hatchability and hormonal systems were all negatively affected by exposure to OIT [37], highlighting the importance of minimizing the release of OIT biocides into aquatic environments.

Depending on the dose and time of exposure, both BIT and OIT have been shown to cause skin sensitization and lead to allergic contact dermititis (ACD) in humans [38, 39, 40]. While ACD caused by an occupational exposure of OIT was most common [40], it has also been reported for patients in case studies where subjects displayed allergic reactions caused by OIT in compression stockings [41] as well as leather products (belt, shoes, furniture) [42].

Given the extensive use of BIT and OIT in everyday consumer products, both humans and the environment is frequently exposed to the chemicals. Considering their suggested allergenic and toxic effects, further studies on the isothiazolinones are necessary to thoroughly evaluate their potential adverse impacts.

2.2 Detoxification mechanisms

As the main concerns regarding the isothiazolinones BIT and OIT lies in their potential environmental and health impacts, understanding the metabolic transformation of these compounds is essential for assessing their toxicity and potential risks. Elimination of xenobiotic (foreign) substances, both in humans and other animals, relies on the combined processes of metabolism and transport [43]. The detoxification or elimination process of drugs and other xenobiotic substances, such as BIT and OIT, takes place primarily in the liver and can be divided into three main phases: phase I (functionalization), phase II (conjugation), and phase III (elimination). Phases I and II collectively comprise the biotransformation processes of xenobiotic substances [44]. Here, typically non-polar and lipophilic substances are chemically altered, yielding more hydrophilic products to facilitate their clearance from the system. These products are then transported out of the system in phase III.

2.2.1 Phase I - Functionalization

Phase I includes reactions such as oxidation, reduction, and hydrolysis where reactive or polar groups (e.g. -OH, -COOH, -NH_2, -SH) are introduced or exposed within the compound [45]. The Cytochrome P450 (CYP) enzyme superfamily plays a crucial role in phase I biotransformation, specifically dominating in the catalysation of oxidation processes [44]. If the phase I metabolites are polar enough, they can be excreted immediately [46]. However, in many cases the elimination of phase I products is not rapid enough, leading them to undergo further reaction with endogenous substrates in phase II.

2.2.2 Phase II - Conjugation

In the phase II biotransformation reactions, the polarity of phase I products is generally further enhanced by the conjugation of a bulky polar endogenous molecule through reactions such as glucuronidation, glutathione conjugation, sulfation, and amino acid conjugation [44, 46]. The phase II reactions are catalyzed by a number of enzyme families including UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), and sulfotransferases (SULTs).

2.2.3 Phase III - Elimination

Lastly, in the elimination phase III the polar products from the biotransformation processes are transported out of the liver cells to be excreted, primarily via urine and bile [47]. Key players in this phase includes ATP-Binding Cassette (ABC) transporter proteins, such as P-glycoprotein (P-gp), and Multidrug resistance-associated proteins (MRPs) [48]. Which specific metabolic pathways that are involved in the biotransformation of the isothiazolinones BIT and OIT are however not determined, and is something this thesis aims to investigate further.

2.2.4 Cytochrome P450 1A (CYP1A)

As mentioned, members of the cytochrome P450 (CYP) enzyme family are important in the detoxification of xenobiotic substances, specifically in the phase I biotransformation reactions. CYP enzymes are heme-containing proteins that primarily catalyze monooxygenation reactions, where a singular oxygen atom is introduced in the substrate, increasing its polarity [49]. Isoforms of these enzymes are expressed in a wide variety of organisms, including mammals and other vertebrates such as fish [50]. Some of the most well-studied isoforms belong to the cytochrome P4501A (CYP1A) subfamily, which are important in the metabolism of environmental organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) and planar halogenated aromatic hydrocarbons (PHAHs) [51]. In mammals, this subfamily includes two isoforms, CYP1A1 and CYP1A2, while most fish generally possess only one CYP1A gene [50, 51]. Although the fish CYP1A shares significant similarity with both mammalian isoforms, both in terms of gene sequence and catalytic function, it appears to be more closely related to CYP1A1 [50, 51].

Besides being important in the metabolism of environmental organic contaminants, the CYP1A gene is also inducible by these compounds through activation of the aryl hydrocarbon receptor (AhR) [51, 1]. This is the most well-studied CYP isoform in fish liver [1], and induction of CYP1A serves as a common biomarker for environmental contamination in aquatic systems, specifically for detecting exposure to substances that bind and activate the AhR, such as aromatic hydrocarbons [52]. The simplified pathway of this induction starts with the binding and activation of the AhR in the cytosol [1]. The activated AhR is then translocated to the nucleus where it up-regulates the CYP1A gene expression. This pathway can be visualized in Figure 2.3, starting with the ligand-binding of the target substance to the AhR, and ending with the expressed CYP1A enzyme.

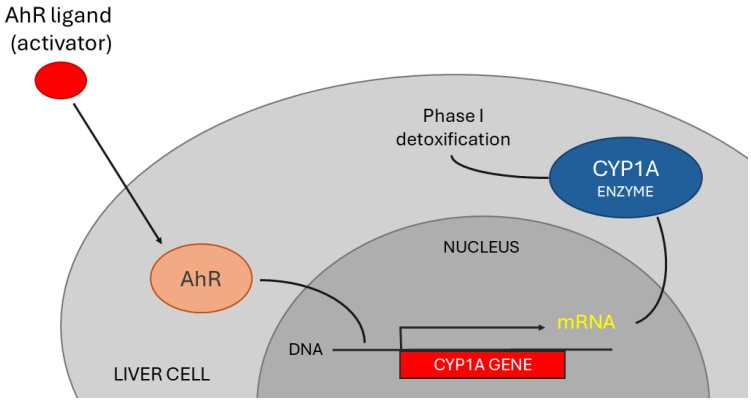


Figure 2.3: Simplified pathway of CYP1A induction through AhR ligand-binding and activation. An AhR ligand activates the cytosolic AhR which translocates to the nucleus where it up-regulates the CYP1A gene, ultimately leading to increased levels of CYP1A enzyme. Adapted from Edenius, M. et al. (2024) [1].

2.3 The *Poeciliopsis lucida* hepatocellular carcinoma (PLHC-1) cell line

In line with the principles of the 3 R's (Replacement, Reduction, Refinement), animal experiments are commonly replaced with cell line-based studies. One such example is the *Poeciliopsis lucida* hepatocellular carcinoma (PLHC-1) cell line which is frequently used as a fish liver model in toxicology studies for screening of environmental stressors [53, 54, 55], making it relevant for studying BIT and OIT. This cell line is known to express CYP enzymes, in particular the CYP1A through activation of the AhR. Although there are studies suggesting PLHC-1 cells exhibit lower phase I and phase II biotransformation activities as compared to primary cultured fish hepatocytes, CYP1A and glutathione-S-transferase prove to be better conserved [56]. The cell line is also known to express toxicologically relevant Pgp- and MRP-related ABC transporter proteins important in phase III elimination [57]. The presence of all three fundamental components of the detoxification pathway in the PLHC-1 cell line, underlines its suitability as an *in vitro* model for toxicology research.

3
Methods

The methods performed in this study were carefully selected in order to adequately fulfill the aim of the thesis. Using the EROD assay, the activity of CYP1A, and its potential involvement in the metabolism of BIT and OIT, was examined. Mitochondrial activity and membrane integrity of the PLHC-1 cells, which gives insight into the cellular response and toxicity of the isothiazolinones, was investigated using a cytotoxicity assay. Lastly, HPLC analyses of cultured exposure media was used for investigating cellular uptake and transformation of the isothiazolinones, while intracellular analyses explored the cellular contents and whether BIT and OIT accumulated within the cells.

3.1 Chemicals

The majority of the chemicals and materials used in this Master's Thesis project were already purchased before the initiation of the project, however if they ran out or additional material was needed it was purchased. 1,2-Benzisothiol-2(2H)-one (BIT) (Sigma 75169-100MG), 2-Octyl-4-isothiazolin-3-one (OIT) (Sigma 46078-250MG-R), 7-ethoxyresorufin (ER, Resorufin ethyl ether) (Sigma E-3763-1MG), Bovine Serum Albumin (BSA) (Sigma A-7906), β-Naphthoflavone (BNF) (Sigma N3633-1G), Dimethyl sulfoxide (DMSO) (Sigma D8418-50ML), Fluorescamine (Sigma F-9015), HEPES free acid (Sigma M-3375), Minimum Essential Medium Eagle (MEM) powder (Sigma A-M-0643-1L), Resorufin (Sigma 73144-20MG) were purchased from Merck, Sweden. 5-Carboxyfluorescein Diacetate Acetoxymethyl Ester (CFDA-AM) (C1354), alamarBlue™ Cell Viability Reagent (AB) (DAL1025), Fetal bovine serum (FBS) (GIBCO 10270-106) and trypsin (GIBCO 25050-014) were purchased from Fisher Scientific, Sweden. All other chemicals were purchased from Supelco or VWR in Sweden.

3.2 Cell line, cell culturing and exposure conditions

For all cell experiments throughout this project, the *Poeciliopsis lucida* hepatocellular carcinoma, PLHC-1 (ATCC® CRL-2406™), cell line was used. The cells were therefore continuously cultured in 75 cm^2 cell culture flasks (Sarstedt) and passaged each week to exchange the cell media and dilute the cells to ensure optimal viability of the cells. The cells were grown at 30°C in Eagle's Minimum Essential Medium

(MEM), supplemented with 5.96 g/L HEPES free acid, 2.2 g/L sodium bicarbonate, and 5% (v/v) fetal bovine serum (FBS).

To passage the PLHC-1 cells, the old media in the flasks was first removed using vacuum aspiration. Subsequently, 5 mL of 0.2% EDTA (w/v) in PBS was added, covering the cells, and was left to incubate for 2-3 minutes before also being aspirated. As the PLHC-1 cell line is adherent, the cells were harvested by adding 1.5 mL of 0.25% trypsin (w/v) in PBS which was allowed to incubate for approximately 1 minute. After incubation with trypsin, the flask was gently tapped to dislodge the cells from the bottom, and 10 mL of fresh media was added to stop the trypsin activity. This was done by gently pipetting up and down for optimal separation of the cells. Thereafter, approximately 20 µL of the cell suspension was added to a Bürker chamber and the cell density was counted under a microscope. Typically, a 1:20 dilution of the cell suspension was performed for passaging the cells. However if the cell density was very low, a 1:10 dilution was used instead.

Prior to all experiments, a portion of the cell suspension was also taken and diluted to appropriate concentrations. This diluted cell suspension was then seeded onto multi-well tissue culture plates and left to incubate overnight. For analysis of CYP1A enzyme activity (EROD assay) and analysis of extracellular BIT and OIT, 3 x 10^5 cells per well were seeded onto 48-well plates (Costar). For analysis of membrane integrity and mitochondrial activity (cytotoxicity assay) 1 x 10^5 cells per well were seeded onto 96-well plates (Sarstedt). For analysis of intracellular BIT and OIT (extraction method) 3 x 10^6 cells per well were seeded onto 6-well plates (Costar).

After overnight incubation the old media in the wells was replaced with fresh media and the cells were exposed to different treatment concentrations and mixtures of BIT and OIT. The chemicals were either dissolved in the media before it was added to the wells or added directly to the wells with fresh media, depending on the assay. For the EROD assay and analysis of extracellular BIT and OIT, the media was replaced (500 µL per well) and the chemicals were added directly to the fresh media in the wells. Similarly, for the extraction assay the cell media was first replaced (2 mL per well) before the chemicals were added directly to the wells. Due to the smaller volumes in the cytotoxicity assay (100 µL per well), the chemicals were instead diluted in the fresh media before being added to the wells to ensure consistency in the exposure concentration over replicates. The concentrations investigated were: 1 µM, 5 µM and 10 µM BIT and OIT, alone and in mixture, with 4 technical replicates per treatment (n=4), unless stated otherwise. The concentrations were chosen based on detection sensitivity of the methods while still avoiding lethal doses.

An overview of the experimental steps prior to analysis is presented in Figure 3.1. The time of exposure varied between the different assays and the analytical steps are described in detail in each separate section.

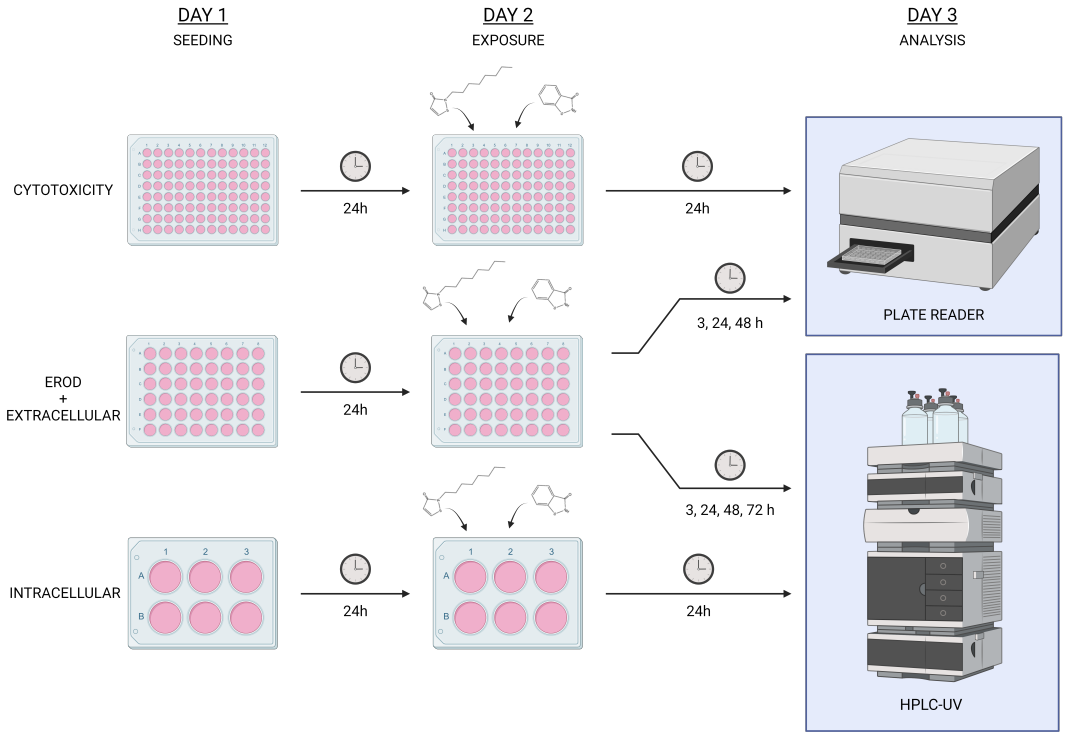


Figure 3.1: Overview of the experimental steps prior to analysis for all assays. On day 1, PLHC-1 cells were seeded onto different sized multi-well plates depending on the assay (96 wells for cytotoxicity, 48 wells for EROD and extracellular analysis, and 6 wells for intracellular analysis), before they were left to incubate overnight. On day 2, the cell media was replaced and the cells were exposed to BIT, OIT, and BNF (EROD assay). The cells were exposed to the chemicals for different time durations depending on the assay. On day 3 analysis was performed. The analytical steps are described in each section for the respective assay. Created in BioRender.

3.3 Analysis of CYP1A activity (EROD assay)

To investigate CYP1A activity following exposure to the chemicals BIT and OIT, the ethoxyresorufin-O-deethylase (EROD) reaction was used. In this reaction, 7-ethoxyresorufin is converted to the fluorescent product resorufin, which is catalyzed by CYP1A enzymes [58]. This product can be fluorescently measured, and its formation is proportional to the CYP1A activity. Therefore, the terms CYP1A activity and EROD activity will be used interchangeably throughout the report. To standardize the EROD activity to specific units [pmol resorufin formed/mg protein/minute], the total protein content in each well was measured using a fluorescamine protein dye and a protein standard of bovine serum albumin (BSA). Fluorescamine reacts with the primary amines on proteins and peptides, forming complexes that are fluorescently detectable [59]. By introducing the dye to the cell samples in each well, the protein content could be assessed and compared to a BSA

standard also treated with fluorescamine. The protein content is also an indication of cell viability and proliferation post-exposure.

The first step of the assay involved preparation of all solutions needed for the experiment. To prepare the 7-ethoxyresorufin (ER) work solution, 7-ethoxyresorufin dissolved in methanol was added gradually to a 50 mM sodium phosphate buffer (ER buffer) and the absorbance of the solution was measured until it reached an absorbance of 0.06. The fluorescamine solution for protein measurement was prepared by adding fluorescamine powder to acetonitrile (3 mg fluorescamine/ 10 mL acetonitrile). In a 48-well plate, a resorufin standard was prepared at concentrations of 0, 3.125, 6.25, 12.5, 25, 50, and 100 pmol/well. Similarly, a BSA standard was prepared at concentrations 0, 6.25, 12.5, 25, 50, and 100 µg/well. For both standards, each concentration was prepared in duplicate.

The chemically exposed cells (3, 24, or 48 h) in the 48-well plates were washed once with 500 µL phosphate buffered saline (PBS), followed by the addition of 200 µL of ER work solution to each well. The plates were immediately placed in a plate reader (SpectraMax® Gemini™ EM, Molecular Devices, California) and the fluorescence was measured (Ex/Em: 530/590) every minute for 10 minutes for a time-resolved measurement of resorufin production. After the 10 minute measurement, 100 µL of the fluorescamine solution was added to both the chemically exposed cells, as well as to the BSA standard plate. The plates were covered with aluminum foil and left to incubate for 10 minutes on a shaker before being placed in a plate reader for an endpoint measurement (Ex/Em: 405/460). Endpoint measurement was also performed for the resorufin standard (Ex/Em: 530/590 nm).

Cells exposed to 0.4% DMSO (final concentration in each treatment) in medium (MEM, 5% FBS) were used as a negative vehicle control, while 1µM of the known CYP1A enzyme inducer β-naphthoflavone (BNF) was used as a positive control.

3.3.1 Data calculations

After obtaining the raw data from the fluorescence measurements, data processing was performed in Excel. Linear regression was applied to both the BSA and resorufin standards to generate calibration curves. The same procedure was then employed for the ten data points measuring resorufin production over a duration of ten minutes yielding an EROD slope for each well with exposed cells.

Protein content in all wells with exposed cells was calculated using the BSA standard curve, where the measured fluorescence values (for fluorescamine) correlated to known protein concentrations in the BSA plate. The EROD activity in each well was then calculated using Equation 3.1:

$$\text{EROD activity} = \frac{\text{EROD slope}}{\text{Resorufin standard} \times \text{Protein concentration}} \tag{3.1}$$

Where the EROD slope was obtained from the linear regression of the fluorescent counts measured at the 10 time points, the resorufin standard was calculated based on the linear regression from the resorufin standard curve, and the protein concentration represented the calculated protein concentration in each well.

3.4 Analysis of membrane integrity and mitochondrial activity (cytotoxicity assay)

To assess potential adverse effects on the cells due to exposure to BIT and OIT, a cytotoxicity assay was performed. This was achieved using two different fluorescent probes for investigation of mitochondrial activity and membrane integrity.

Cell membrane integrity was analysed using the dye 5-Carboxy Fluorescein Diacetate - Acetoxymethyl Ester (CFDA-AM). CFDA-AM is non-fluorescent in its original form, but upon entering viable cells it is converted into its fluorescent form, fluorescein, by esterases [60]. Only cells with intact cell membranes provide the environment necessary for the esterases to function properly, making it possible to distinguish between viable cells and cells with compromised membrane integrity.

Mitochondrial activity was assessed using alamarBlue™ (AB). Here, the cell-permeable and non-fluorescent active ingredient, resazurin (blue), is hydrolysed to the highly fluorescent resorufin (red) in the mitochondria [61, 60]. A lower fluorescent signal corresponds to lower mitochondrial activity, a characteristic of damaged or non-viable cells.

The two fluorescent dyes were simultaneously added to the chemically exposed cells (24 h) through a cytotoxicity solution of 1.25% (v/v) AB and 4 µM CFDA-AM in L-15/ex buffer. Prior to addition of the cytotoxicity solution, the cells in the 96-well plates were washed twice with L-15/ex buffer. Thereafter, 100 µL of cytotoxicity solution was added to each well and the plate was left to incubate on a shaker for 45-60 minutes. The procedure was performed with the lights turned off and both the cytotoxicity solution and the plates were covered with aluminum foil as alamarBlue™ is highly light-sensitive. After incubation, the plates were placed in a plate reader (SpectraMax® Gemini™ EM, Molecular Devices, California) where both fluorescent probes could be simultaneously measured thanks to their different excitation and emission spectras (485/535 nm for fluorescein and 532/590 nm for resorufin).

As for the EROD assay, cells exposed to 0.4% DMSO in medium (MEM, 5%FBS) were used as a negative vehicle control, and wells without cells and only the cytotoxicity solution were used as a blank.

3.4.1 Data calculations

Again, raw data obtained from fluorescent measurements were primarily handled in Excel before being analysed in GraphPad Prism. For both mitochondrial activity (alamarBlue™, AB) and membrane integrity (CFDA-AM), the cytotoxicity of the cells in each well after treatment was calculated using Equation 3.2:

$$\%\text{Cytotoxicity (AB or CFDA-AM)} = \left(\frac{\text{FU}_{\text{exposed cells}} - \text{FU}_{\text{blank}}}{\text{FU}_{\text{vehicle control cells}} - \text{FU}_{\text{blank}}} \right) \times 100 \quad (3.2)$$

Where, $\text{FU}_{\text{exposed cells}}$ is the measured fluorescence in each well in arbitrary fluorescent units (FU), FU_{blank} is the mean value of fluorescent units measured in the wells with only cytotoxicity solution and no cells, and $\text{FU}_{\text{vehicle control cells}}$ is the mean value of fluorescent units measured in the DMSO control wells. This resulted in a percentage of cytotoxicity measured for each replicate in every treatment, compared to the negative vehicle control.

3.5 Analysis of extracellular BIT and OIT

In this assay it was investigated how the concentrations of BIT and OIT in cultured media were affected when cells had been exposed for a range of time durations (3, 24, 48 and 72 h). It also investigated whether the two chemicals affected the cellular uptake of one-another, and if potential cell-made metabolites could be detected.

This assay was usually performed in combination with the EROD assay described in chapter 3.3. After cells had been seeded onto 48-well plates and exposed to the treatments for the different time durations (3, 24, 48 and 72 h), 500 µL of the cultured media was harvested from the wells and transferred to 1.5 mL microcentrifuge tubes. The EROD assay was then performed on the exposed cells left on the plate, while the analysis of extracellular BIT and OIT was performed on the harvested media. If this assay was not performed in combination with the EROD assay, cells were still seeded onto 48-well plates and exposed in the same way as described in chapter 3.2. Post sampling, the media samples were centrifuged in a tabletop centrifuge at room temperature and 11000 RPM for 5 minutes. 250 µL of the supernatant was collected and transferred to 1.8 mL glass vials with 250 µL inserts purchased from Agilent Technologies. If the analyses were performed on a different day than when the samples were collected, they were stored in -20°C.

The extracellular concentrations of BIT and OIT were analysed using a HPLC system with UV detection (Agilent 1260 system with binary pump, 1260 DAD VL+ detector, HiP-ALS SL+ autosampler, column oven and degasser, Agilent Technologies, Santa Clara), equipped with a Kromasil column (4.6 x 100 mm 2.5u EternityXT-PhenylHexyl, Nouryon, Bohus) and a protective pre-column. The injection volume of samples was set to 20 µL, the temperature of the column unit was kept at 40°C at all times and the UV detector used two detection wavelengths (275 nm and 320 nm, using 10 nm bandwidth and no background correction). The system uses two

mobile phases, A and B. Phase A was composed of 95% water and 5% methanol, while phase B was composed of 100% methanol. Both phases were pH-adjusted using 4 mL/L acetic acid. The system used the following 22 minute mobile phase gradient program presented in Table 3.1 for analysis.

Table 3.1: The mobile phase gradient program used for HPLC analysis in this project.

Time (min)	A (%)	B (%)	Flow (ml/min)
0	99	1	1.3
13	5	95	1.3
16	5	95	2.0
17	99	1	1.5
20	99	1	1.3

The chromatograms obtained from the analyses were saved and analysed using Clarity Chromatography Software 8.7 (DataApex, Prague). The retention times of BIT and OIT in the system were determined by performing standard curves with increasing concentrations of BIT and OIT in cell culture media. Due to the nature of the mobile phase gradient program, hydrophilic compounds are expected to have a shorter retention time than lipophilic compounds. The extracellular concentrations of BIT and OIT were then examined by calculating the area under the curve (AUC) responses on the chromatograms, performed by the Clarity Software. Samples of all tested exposure concentrations of BIT and OIT dissolved in MEM media (non-cultured) was used as controls in this assay.

3.6 Analysis of intracellular BIT and OIT

This assay investigated the intracellular concentrations of BIT and OIT in PLHC-1 cells that has been exposed for 24 h and is based on a method for extracting intracellular benzo[a]pyrene and clotrimazole previously described by Edenius and colleagues [1].

PLHC-1 cells were seeded onto 6-well plates and exposed as described in chapter 3.2. After 24 hour exposure 500 µL of the exposure media was sampled for extracellular analysis of BIT and OIT. The remaining media from each well were then removed and each well was washed once with 2 mL of phosphate buffered saline pH 7.4 (PBS). The cells were detached from the bottom of the wells using 200 µL 0.25% (w/v) trypsin and a sterile cell scraper (Sarstedt) before being transferred to 1.5 microcentrifuge tubes. 200 µL HPLC-grade acetone was added and each sample was vortexed. 200 µL heptane was added and the samples were vortexed again before being sonicated in an ultrasonic waterbath for 10 min. The samples were then centrifuged in a tabletop centrifuge at room temperature and 11000 RPM for 5 minutes. 100 µL of the top layer of the supernatant (consisting of the intracellular components dissolved in heptane) was collected and transferred to 1.8 mL glass vials with 250 µL inserts purchased from Agilent Technologies. The heptane was then

allowed to evaporate in a fume hood overnight. 100 µL methanol was added to each vial before being analysed in the same HPLC system as described in chapter 3.5. Chromatograms were analysed in Clarity software where the AUC of the peaks was calculated. Samples of all tested exposure concentrations of BIT and OIT dissolved in methanol was used as controls in this assay.

3.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism 10.2.3 software. Data for measurements with at least 4 replicates were analysed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison posthoc test. For measurements with less than 4 replicates, means and standard deviations are presented. Differences were considered statistically significant when $p \leq 0.05$.

4

Results

4.1 Analysis of CYP1A enzyme activity (EROD assay)

The CYP1A activity in the PLHC-1 cells was assessed using the EROD assay as previously described in chapter 3.3. The goal was to fluorescently measure the resorufin production in the treated cells after addition of 7-ethoxyresorufin, and compare it to a resorufin standard to determine the specific amount of produced resorufin. However, the stock solution from which the resorufin standard was prepared was found to be of insufficient quality, resulting in an unreliable expected concentration. Therefore, the unit of the CYP1A activity was not standardized to [pmol resorufin formed/min/mg protein] as originally intended, but is instead expressed as [FU/min/mg protein], where FU represents the fluorescent units measured in the plate reader. This was decided to be sufficient for evaluating different trends between treatments as the goal was not to specifically quantify resorufin production.

Additionally, the known CYP1A inducer BNF was utilized as a positive control for the EROD assay to ensure the cells properly expressed the CYP1A enzyme and that it was inducible. This treatment was not always successful, thus only experiments where the BNF-treated cells exhibited a noticeably higher CYP1A activity compared to the negative vehicle control group were included, unless stated otherwise.

The result of a 24-hour exposure experiment is demonstrated in Figure 4.1. In this experiment, the CYP1A activity of the cells following treatment with 10 µM BIT alone and in combination with either 1 µM BNF or 5 µM OIT was investigated. Only the positive control with 1 µM BNF and the mixture of 10 µM BIT with 1 µM BNF exhibited a visible CYP1A activity compared to the negative vehicle control.

Further, the PLHC-1 cells were exposed to the treatments: 1 µM BIT alone; 1 µM OIT alone; 1 µM BIT in mixture with 1 µM OIT; 1 µM BIT in mixture with 5 µM OIT; 1 µM BIT in mixture with 1 µM BNF; and 1 µM OIT in mixture with 1 µM BNF for 3, 24, and 48 hours. Results from this time-course experiment are presented in Figure 4.2. As shown in Figure 4.2a, no apparent effect on CYP1A activity could be observed for any of the treatments, including the BNF control, after 3 hours.

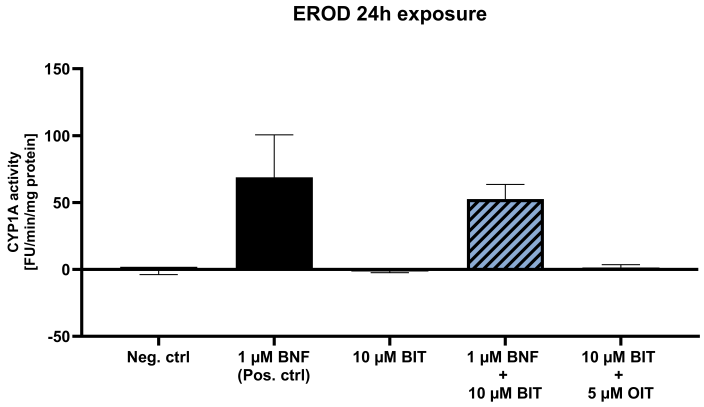
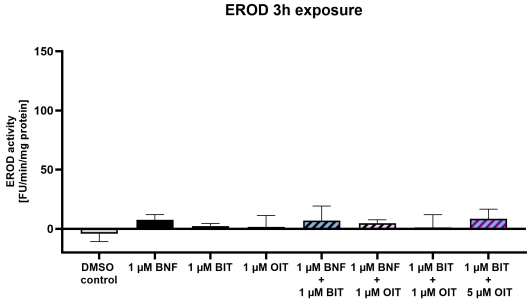
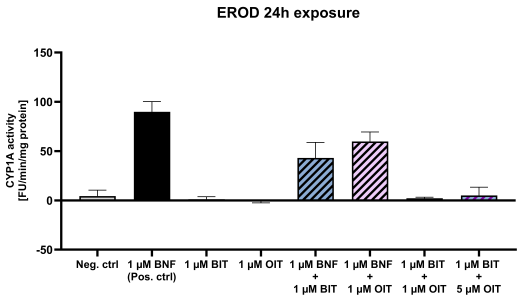


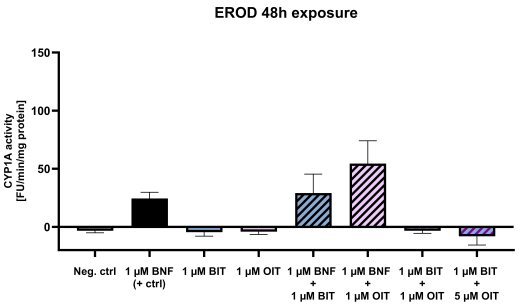
Figure 4.1: CYP1A activity expressed in terms of EROD activity in fluorescent units (FU) per minute per mg protein for a 24h exposure experiment in PLHC-1 cells. Cells were treated with 0.4% DMSO (Negative vehicle control), 1 µM BNF (Positive control), 10 µM BIT alone and in combination with 1 µM BNF or 5 µM OIT (n=4). Data is expressed as mean ± SD.



(a) CYP1A activity in PLHC-1 cells after 3h exposure



(b) CYP1A activity in PLHC-1 cells after 24h exposure



(c) CYP1A activity in PLHC-1 cells after 48h exposure

Figure 4.2: CYP1A activity expressed in terms of EROD activity in fluorescent units (FU) per minute per mg protein for a) 3h, b) 24h, and c) 48h exposure experiments in PLHC-1 cells. Cells were treated with: 0.4% DMSO (Negative vehicle control); 1 µM BNF (Positive control); 1 µM BIT; 1 µM OIT; 1 µM BIT + 1 µM BNF; 1 µM OIT + 1 µM BNF; 1 µM BIT + 1 µM OIT; and 1 µM BIT + 5 µM OIT (n=4). Data is expressed as mean ± SD.

After 24 hours of exposure, shown in Figure 4.2b, a noticeable effect on the CYP1A activity was observed only for the BNF control, as well as for the cells treated with 1

µM BIT or 1 µM OIT in combination with 1 µM BNF. Moreover, a seemingly lower CYP1A activity was observed for the cells that were co-treated with 1 µM BNF and 1 µM OIT or 1 µM BIT, compared to those treated with 1 µM BNF alone. Similar to the 24h exposure, only cells exposed to 1 µM BNF alone or in combination with 1 µM BIT or 1 µM OIT exhibited any observable CYP1A activity after 48 hours, as illustrated in Figure 4.2c. However in this case, co-treatment with 1 µM BNF and 1 µM OIT instead display a slightly higher CYP1A activity compared to treatment with 1 µM BNF alone.

In summary, no treatment except for those containing BNF resulted in an observable increase of CYP1A activity in the PLHC-1 cells when compared to the negative control group.

4.2 Analysis of membrane integrity and mitochondrial activity (cytotoxicity assay)

The cytotoxic effect of BIT and OIT exposure on the PLHC-1 cell line was investigated using the fluorescent probes alamarBlue™ and CFDA-AM to analyze the mitochondrial activity and membrane integrity, respectively. The output for each treatment has been normalized to the vehicle control and the cytotoxicity of each treatment is expressed as a percentage of the control group. The vehicle control represents healthy cells and is normalized to 100%. Values lower than 100% signifies lower mitochondrial activity or lower membrane integrity, while higher values indicate the opposite.

The treatments investigated in this assay includes the vehicle control (0.4% DMSO), BIT at concentrations 1 µM, 5 µM, and 10 µM, OIT at concentrations 1 µM, 5 µM, and 10 µM, as well as the combination of BIT and OIT at these concentrations. For all experiments the duration of exposure to the treatments was 24 hours.

Exposure for 24 hours to BIT alone at concentrations 1 µM, 5 µM, and 10 µM resulted in minimal cytotoxic effects on both mitochondrial activity and membrane integrity as demonstrated by Figure 4.3. While exposure to 1 µM BIT exhibited a statistically significant increase in mitochondrial activity compared to the control group, the magnitude of the difference is small.

For 24h exposure to OIT alone, no significant effect was observed on the mitochondrial activity for concentrations of 1 µM or 5 µM as shown in Figure 4.4. A statistically significant increase in membrane integrity was however observed at the same concentrations when compared to the control group. Further, exposure to 10 µM OIT displayed a slight decrease in membrane integrity, and a more distinct and highly significant decrease in mitochondrial activity as compared to the control.

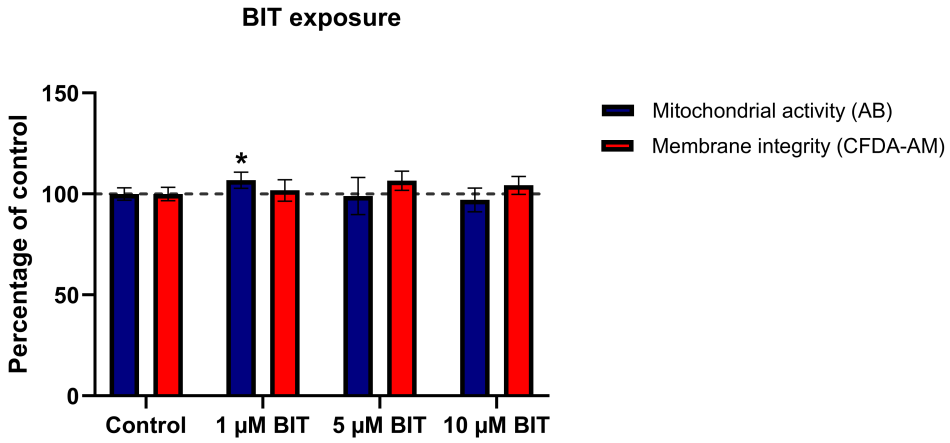


Figure 4.3: Cytotoxicity measured in terms of mitochondrial activity (alamarBlue™, AB) and membrane integrity (CFDA-AM) and expressed as percentage of vehicle control, for a 24h exposure experiment in PLHC-1 cells. Cells were treated with 0.4% DMSO (Negative vehicle control), and BIT at concentrations 1 µM, 5 µM, and 10 mM (n=12). Data is combined from three repeated experiments with 4 technical replicates for each treatment, expressed as mean ± SD. Significance was calculated with one-way ANOVA followed by Tukey's multiple comparison test. * $p \leq 0.05$

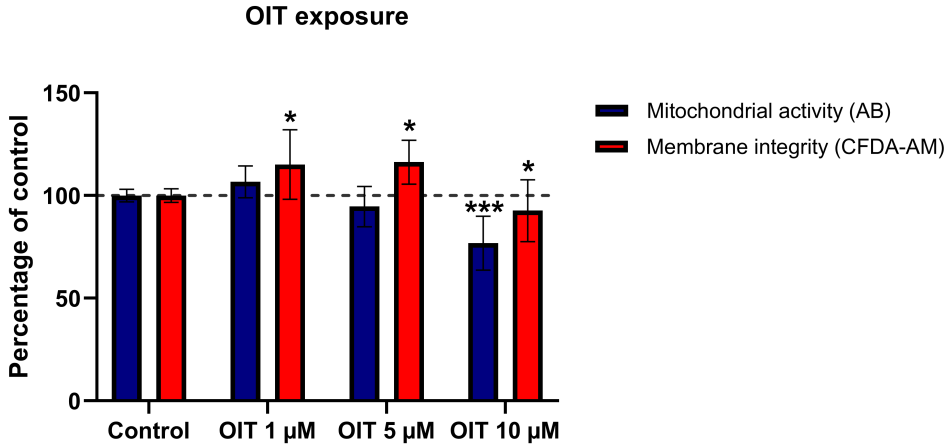


Figure 4.4: Cytotoxicity measured in terms of mitochondrial activity (alamarBlue™, AB) and membrane integrity (CFDA-AM) and expressed as percentage of vehicle control, for a 24h exposure experiment in PLHC-1 cells. Cells were treated with 0.4% DMSO (Negative vehicle control), and OIT at concentrations 1 µM, 5 µM, and 10 mM (n=12). Data is combined from three repeated experiments with 4 technical replicates for each treatment, expressed as mean ± SD. Significance was calculated with one-way ANOVA followed by Tukey's multiple comparison test, * $p \leq 0.05$, *** $p \leq 0.001$.

To investigate potential mixture effects of BIT and OIT, experiments were conducted where the two were combined at different concentrations. Figure 4.5 displays the cytotoxic effect when BIT was kept constant at 10 µM and combined with increasing concentrations of OIT. The combination of 10 µM BIT with 10 µM OIT exhibited a large significant decrease in both mitochondrial activity and membrane integrity, while no other treatment showed a significant cytotoxic effect compared to the control group.

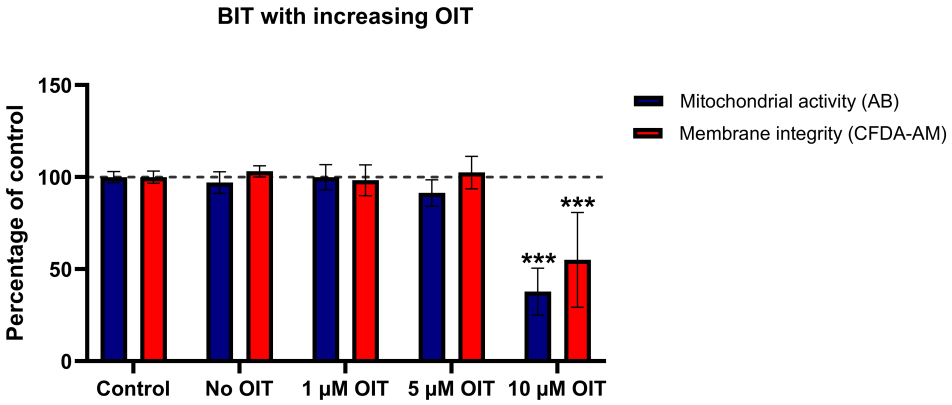


Figure 4.5: Cytotoxicity measured in terms of mitochondrial activity (alamarBlue™, AB) and membrane integrity (CFDA-AM) and expressed as percentage of vehicle control, for a 24h exposure experiment in PLHC-1 cells. Cells were treated with 0.4% DMSO (Negative vehicle control), 10 µM BIT alone and in mixture with OIT at concentrations 1 µM, 5 µM, and 10 mM (n=12). Data is combined from three repeated experiments with 4 technical replicates for each treatment, expressed as mean ± SD. Significance was calculated with one-way ANOVA followed by Tukey's multiple comparison test. *** $p \leq 0.001$.

Corresponding experiments were conducted where OIT was instead kept at a constant concentration of 5 µM while combined with increasing concentrations of BIT (1 µM, 5 µM, 10 µM). Treatment with 5 µM OIT without BIT as well as 5 µM OIT in combination with 1 µM BIT showed a significant increase in membrane integrity compared to the control group. However no other treatment in these experiments exhibited a significant difference in neither mitochondrial activity nor membrane integrity compared to the control.

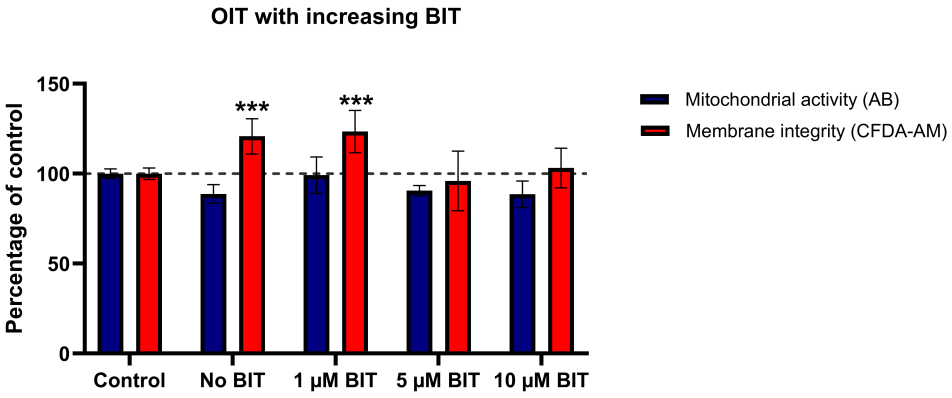


Figure 4.6: Cytotoxicity measured in terms of mitochondrial activity (alamarBlue™, AB) and membrane integrity (CFDA-AM) and expressed as percentage of vehicle control, for a 24h exposure experiment in PLHC-1 cells. Cells were treated with 0.4% DMSO (Negative vehicle control), 5 µM OIT alone and in mixture with BIT at concentrations 1 µM, 5 µM, and 10 mM (n=8). Data is combined from two repeated experiments with 4 technical replicates for each treatment, expressed as mean ± SD. Significance was calculated with one-way ANOVA followed by Tukey's multiple comparison test. *** $p \leq 0.001$.

To further investigate potential mixture effects of BIT and OIT, the cytotoxic effect

for the combination of BIT and OIT at a concentration of 10 µM was compared to exposure of the two compounds separately at the same concentration as shown in Figure 4.7. As previously observed, treatment with 10 µM OIT alone displayed a significant decrease in mitochondrial activity, while no significant cytotoxic effect was observed for treatment with 10 µM BIT alone compared to the control group. However, when the cells were exposed to the combination of the two, a very significant decrease in both mitochondrial activity as well as membrane integrity was observed, surpassing the effect observed with either of the compounds alone.

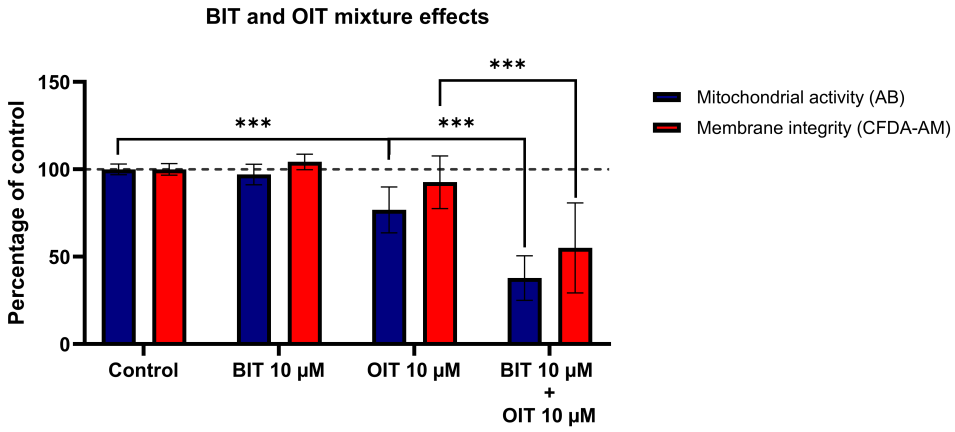


Figure 4.7: Cytotoxicity measured in terms of mitochondrial activity (alamarBlue™, AB) and membrane integrity (CFDA-AM) and expressed as percentage of vehicle control, for a 24h exposure experiment in PLHC-1 cells. Cells were treated with 0.4% DMSO (Negative vehicle control), 10 µM BIT and 10 µM OIT alone and in mixture (n=12). Data is combined from three repeated experiments with 4 technical replicates for each treatment, expressed as mean ± SD. Significance was calculated with one-way ANOVA followed by Tukey's multiple comparison test. *** $p \leq 0.001$.

4.3 Analysis of extracellular BIT and OIT

As previously described, the extracellular concentrations of BIT and OIT were analysed through sampling of the cell media following exposure. The media samples were then run through an HPLC system with UV detection at two wavelengths (275 nm and 320 nm), which gave an output in the form of a chromatogram for each sample. By comparing chromatograms, controls and external standards, the peaks of interest (BIT, OIT, and possible metabolites) could be identified. The integral of these peaks, also known as area under the curve (AUC), was subsequently calculated and correlates to the concentration of analyte in the sample. The results of the extracellular BIT and OIT analysis will hence be presented in arbitrary AUC units. As the goal was to only compare differences between treatments, determining absolute concentrations was deemed unnecessary.

In Figure 4.8 below is a visualisation of an example chromatogram (DMSO control) obtained from the HPLC system used in this project. Since every sample is analysed at both wavelengths, all chromatograms have two lines (output signals) which corresponds to the two wavelengths used for detection.

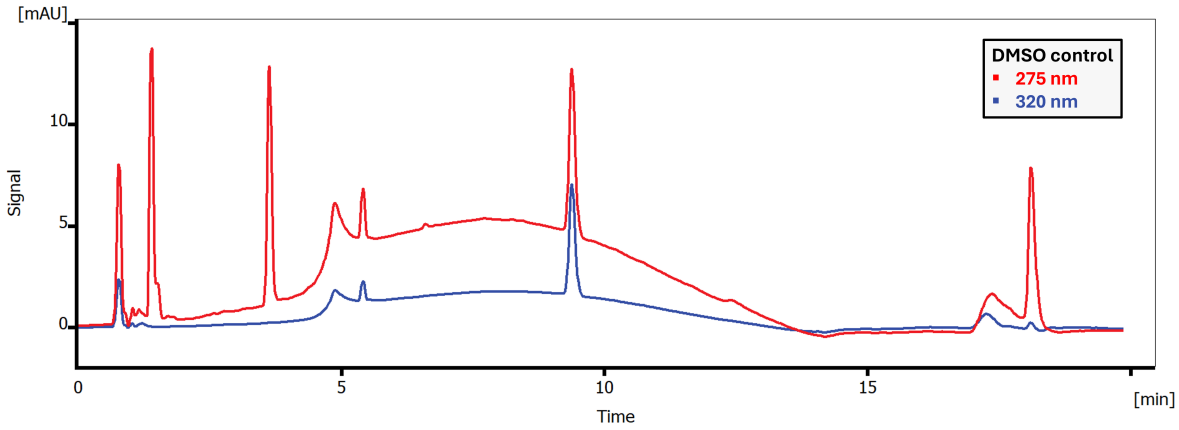


Figure 4.8: Example of a chromatogram obtained from the HPLC system in use. This sample consisted of cultured cell media containing the 0.4% DMSO vehicle control and was sampled after 24h exposure. The red line corresponds to the output signal at 275 nm detection, while the blue line corresponds to detection at 320 nm.

Using an external standard consisting of four concentrations of OIT (0.5, 1, 5 and 10 µM) dissolved in DMSO, the retention time of OIT in this HPLC system was determined at 12 minutes. The standard test also proved that the system could detect concentrations of OIT between 0.5-10 µM successfully. Figure 4.9 illustrates the determination of the retention time of OIT. It was also confirmed that the optimal wavelength of detection for OIT was 275 nm in this system, since no quantifiable peak was appearing on the signal corresponding to 320 nm.

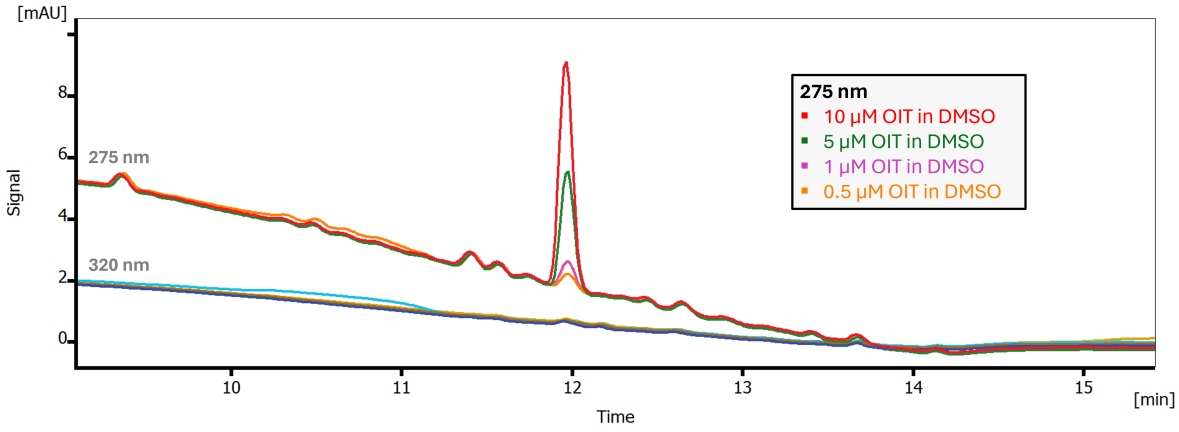


Figure 4.9: Determination of the retention time of OIT in the HPLC system. The image is zoomed in on the region of the chromatograms where the standard peaks appeared.

A previous Master thesis project (not yet published) performed in the research group also analysed extracellular BIT, however in a different HPLC system [62]. In samples where cells had been exposed to BIT the student could describe a peak on the chromatogram at a slightly shorter retention time compared to that of BIT. This bump was hypothesised to be a metabolite of BIT as it was only visible in the chromatograms obtained from media samples where cells had been exposed to BIT. This

proposed metabolite was further investigated in this project. It was found that in this HPLC system, the retention time for BIT was around 7 minutes, while for the potential metabolite it was around 6.3 minutes. Figure 4.10 illustrates an experiment where the retention time for BIT in this system was determined, as well as confirming that the peak of the proposed metabolite was also visible. It can also be seen that BIT is better detected at 320 nm, while the BIT metabolite was best detected at 275nm. Since new mobile phases were prepared once they ran out (batches of 1 L), deviations in the preparations of them slightly affected the retention times of peaks in the chromatograms, however not to an extent of not being identifiable.

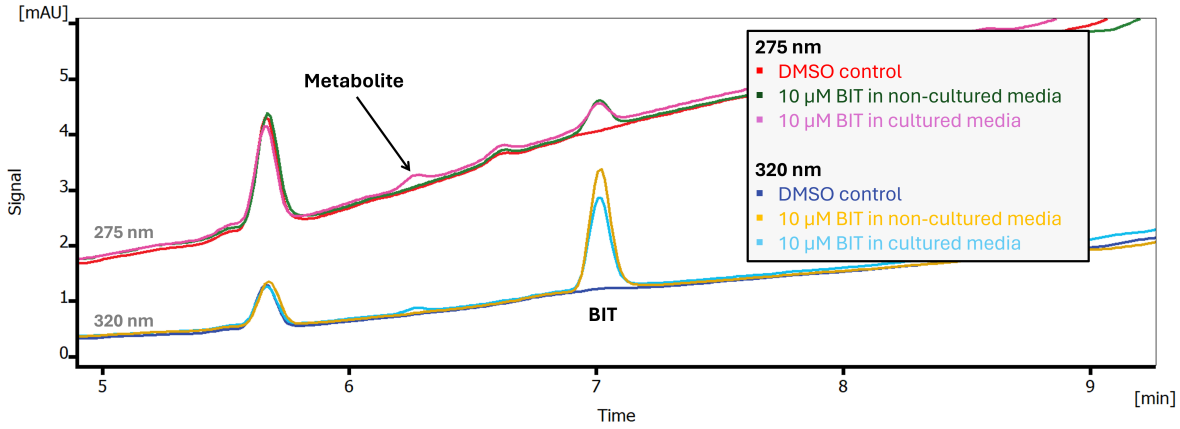
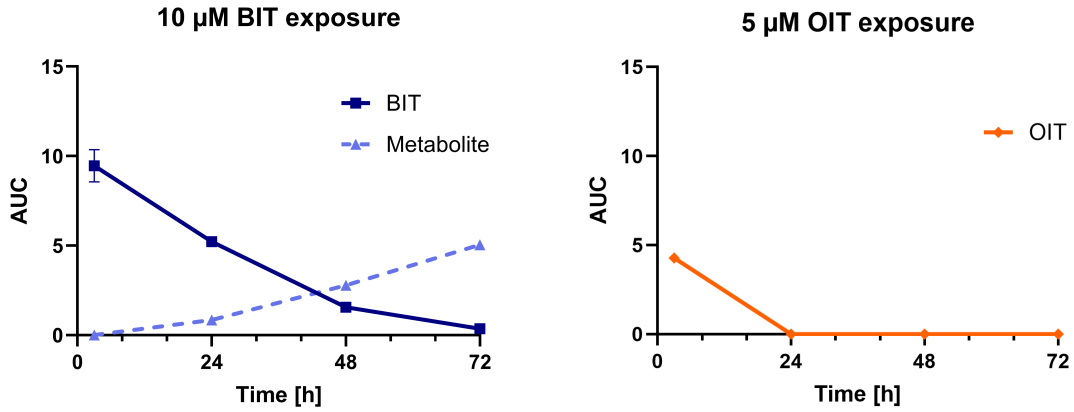


Figure 4.10: An initial chromatogram to determine the retention time of BIT in the HPLC system as well as confirming the presence of the presumed metabolite. The image is zoomed in on the region of the chromatograms where the BIT peaks appeared.

When the retention times for BIT and OIT were determined the data acquisition began, where the isothiazolinones were analysed extracellularly over time. It was possible to detect a decrease (represented by AUC) of both extracellular BIT and OIT over time in cultured media samples, however extracellular OIT decreased considerably faster compared to BIT. These decreases are visualised using media samples where cells have been exposed to 10 µM BIT and 5 µM OIT respectively and can be seen in Figure 4.11. In media samples from 10 µM BIT exposed cells, it was also possible to detect an increase of AUC for the peak hypothesised to be the metabolite of BIT over time, also included in Figure 4.11a. It is however important to note that the results illustrated in Figure 4.11a is a representation from one of the experimental repeats. However similar trends were noted between the repeats and are visualised in Figure A.1 in Appendix A. A peak for a potential OIT metabolite was never found, as no chromatograms from cultured media samples with exposures of the concentrations of OIT (1 µM, 5 µM, 10 µM) contained such a peak in this HPLC system.



(a) BIT and BIT metabolite in cultured media.

(b) OIT in cultured media.

Figure 4.11: AUC for BIT, BIT metabolite and OIT peaks in cultured media over time (3, 24, 48, 72h) after exposure with 10 µM BIT or 5 µM OIT (n=2). Data is from one experimental repeat with 2 technical replicates per treatment, expressed as mean ± SD.

Analysis of extracellular BIT and OIT in media samples where cells had been exposed to 10 µM BIT in combination with different concentrations of OIT (1, 5 and 10 µM) were also performed. As described above and shown in Figure 4.11b, extracellular OIT was not detectable in exposures >3 hours (in this experimental setup), regardless of its exposure concentration or if in mixture with BIT. However, it was noted that in cultured media samples exposed to a mixture of 5 µM OIT and 10 µM BIT (for 3 hours) it was not possible to detect extracellular OIT. This means that the cellular uptake of OIT was drastically increased when in mixture with BIT, as opposed to when cells were exposed to OIT alone, see Figure 4.12.

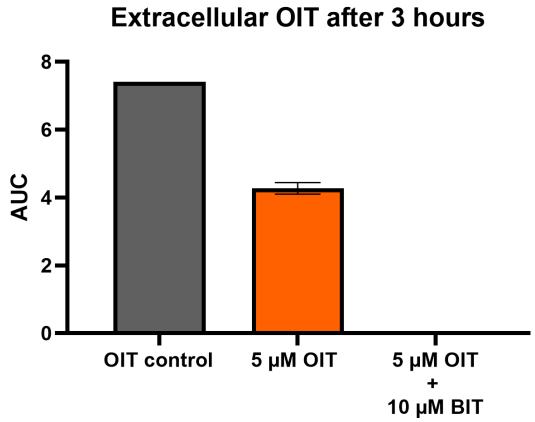
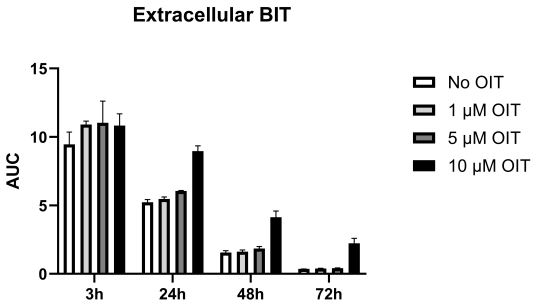


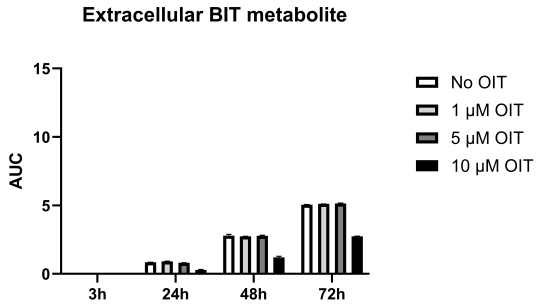
Figure 4.12: AUC values for peaks of extracellular OIT in media samples from treatments of 5 µM OIT, 5 µM OIT in combination with 10 µM BIT and 10 µM OIT in combination with 10 µM BIT. The exposure time was 3 hours. The gray bar represents the control of 5 µM OIT in non-cultured media. Data is from one experimental repeat with 2 technical replicates per treatment, expressed as mean ± SD.

The mixture effects of BIT and OIT mixes were also investigated in a time study. Since OIT could not be detected in cultured media for time points >3 hours, the

following analysis focused only on changes in amount of extracellular BIT and BIT metabolite in cultured media. A comparison with exposures to 10 μM BIT alone were also performed to assess whether the presence of OIT in the exposure mixture had an impact on the cellular clearance of BIT and excretion of metabolite. The AUC of the peaks corresponding to BIT and the presumed metabolite were calculated for each time point and treatment (10 μM BIT alone and in combination with 1, 5 or 10 μM OIT) and are presented in Figure 4.13. The AUC values of the BIT and BIT metabolite peaks were not affected by the presence of 1 μM or 5 μM OIT in the exposure mixtures; as the AUC values for BIT and the metabolite were similar when exposed to only 10 μM BIT over time, represented by the white bars in Figure 4.13a and 4.13b. For each time point, except 3 hour exposure, the AUCs of extracellular BIT were largest when cells had been exposed to a mixture of 10 μM BIT in combination with 10 μM OIT. The opposite effect was observed for the AUCs of the metabolite, which were lower in samples cultivated with this mixture. This would mean that only the exposure mixture containing the highest concentration of OIT had an effect on the presence of extracellular BIT and BIT metabolite in cultured media over time. It is important to note that this time study with BIT and OIT mixtures was only performed once (with two replicates), the results presented in Figure 4.13 should thus be interpreted with caution.



(a) BIT in cultured media after exposure to mixtures of BIT and OIT.



(b) BIT metabolite in cultured media after exposure to mixtures of BIT and OIT.

Figure 4.13: AUC for BIT and BIT metabolite peaks in cultured media with exposures of 10 μM BIT in combination with no, 1 μM, 5 μM or 10 μM OIT. Sampling took place after 3, 24, 48 and 72 hours of exposure. The white bars represents cultured media samples with only 10 μM BIT exposure (no OIT) and the gray bars represents cultured media samples with 10 μM BIT exposure in combination with 1, 5 or 10 μM OIT. Data is from one experimental repeat with 2 technical replicates per treatment, expressed as mean ± SD.

To further asses potential involvement of the CYP1A enzyme in the clearance of extracellular BIT, cells were treated with a mixture of 10 μM BIT and 1 μM BNF, the known CYP1A inducer, for 24 hours. The BIT and BIT metabolite peak sizes were then calculated in media samples where cells had been exposed to either 10 μM BIT alone or the combination of 10 μM BIT and 1 μM BNF. Figure 4.14 below is a compilation of three experimental replicates (each consisting of two technical replicates) where the AUC of BIT and metabolite peaks have been calculated in cultured media samples with 10 μM BIT (+/-) 1 μM BNF exposure(s). It can be noted that the presence of 1μM BNF did not increase the clearance of extracellular

BIT, but rather decreased it significantly. The figure also displays the AUC values for the BIT metabolite produced in the samples, however there are no significant difference in the amount of excreted metabolite between the samples.

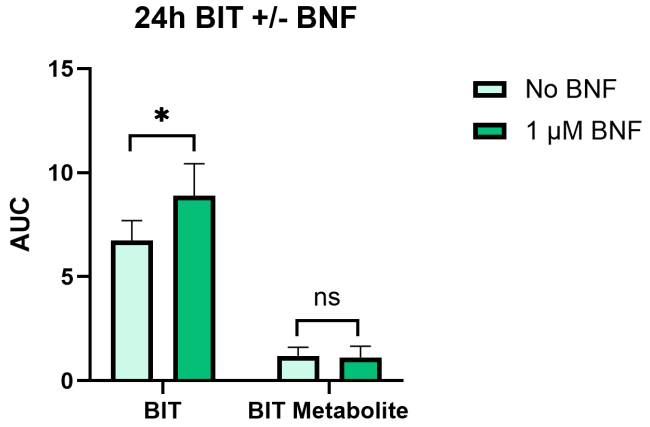


Figure 4.14: AUC for extracellular BIT and BIT metabolite in cultured media samples from 24 hour exposures of 10 µM BIT (+/-) 1 µM BNF (n=6). Data is combined from three repeated experiments with 2 technical replicates for each treatment. Significance was measured with one-way ANOVA followed by Tukey's multiple comparison test. ns $p > 0.05$, * $p < 0.05$.

4.4 Analysis of intracellular BIT and OIT

The analysis of intracellular BIT and OIT involved extracting the cellular contents of cells exposed to BIT and OIT followed by anlysis in the same HPLC system used for analysing extracellular BIT and OIT. The purpose was to investigate whether it was possible to extract OIT and BIT from inside of the treated cells after 24 hour exposure. The method did not successfully extract any detectable amounts of BIT and OIT the two times it was performed. The samples containing intracellular BIT and OIT produced chromatograms that were challenging to interpret, even when compared to controls and external standards. They did not display detectable peaks on expected retention times. Figure 4.15 illustrates two chromatograms obtained from the extraction procedure, one obtained from 10 µM BIT exposure and the other from 5 µM OIT exposure. As can be seen there seems to be no apparent distinction between the two extraction samples as they look identical. This means that also the BIT metabolite was not detectable intracellularly since the region around its expected retention time had identical peaks on both chromatograms. If the BIT metabolite was present in the samples there should have been a distinction between the chromatograms from samples of cells exposed to the different isothiazolinones. When comparing the samples to the external standards of 10 µM BIT and 10 µM OIT dissolved in methanol it was not possible to identify any peaks corresponding to BIT and OIT either. These chromatograms can be found in Figure A.2 and A.3 in Appendix A.

The chromatograms of extraction samples from exposures with 10 µM BIT in combination with 5 µM OIT also looked similar to the chromatograms in Figure 4.15.

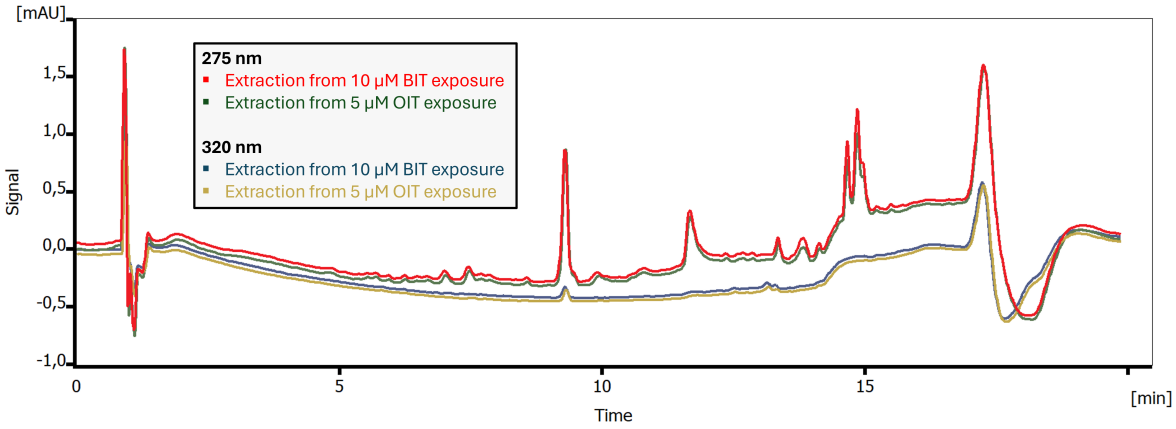


Figure 4.15: Chomatograms of extraction samples from cells treated with 10 µM BIT or 5 µM OIT for 24 hours.

5
Discussion

Due to the extensive use of the isothiazolinone biocides BIT and OIT, their reported skin sensitizablility and release into the environment, it is important to review their (eco)toxicological properties. Following, the results from each assay performed in this study will be thoroughly evaluated and discussed.

5.1 CYP1A enzyme activity (EROD)

As induction of CYP1A serves as a common biomarker for for exposure to the enzyme's target compounds, such as polycyclic aromatic hydrocarbons (PAHs), investigating how exposure to BIT and OIT affects the CYP1A activity is a reasonable approach to detect potential involvement of the enzyme in metabolizing the isothiazolinones. The results of this study showed no noticeable increase in CYP1A activity in the PLHC-1 cells when they were exposed to BIT and OIT, alone or in mixture. However, due to the limited amount of data, it is not possible to say if other concentrations of the compounds could have produced a different outcome.

As briefly mentioned in the results section, problems with the positive BNF control resulted in data that was decided unadequate to present and draw conclusions from. Previous studies on PLHC-1 cells display a significant induction of CYP1A activity when treated with 1 µM BNF [51], however this induction was not always observed in experiments within this project. At times it worked as expected, and other times it did not display sufficient CYP1A induction for it to be used as a positive control. To investigate the source of this problem, different approaches for troubleshooting were performed. Experiments were conducted investigating the quality of the BNF, as well as the performance of the instrument. It was eventually discovered that the settings on the plate reader were not optimal for sensitive resorufin detection in the EROD assay, likely being the reason for inconsistent fluorescent measurements of the positive control. As discussed in the results section, the resorufin standard proved to be of insufficient quality, where the fluorescent output was considerably lower than expected for the standard concentrations. Besides acting as a parameter in the calculations of EROD activity (for resorufin quantification), the resorufin standard also works as an indication of the limit of detection for fluorescent measurement with the instrument. Without this standard, it is not possible to determine if the resorufin produced by the cells reaches a quantifiable amount. However, the results obtained during this project can still give some hint about if BIT or OIT could induce CYP1A activation similar to the BNF. As the CYP1A activity appeared to

be higher in the BNF-treated cells compared to the negative control group, it seems as though the CYP1A activity could be induced by the BNF. If BIT and OIT were as potent as BNF in the activation of the AhR, a similar trend would presumably be observed. Nonetheless, smaller differences in fluorescence can not be interpreted with high certainty, both due to the absence of a resorufin standard as well as the instrument settings. From the few results obtained in this assay, no comparable effect as observed for BNF-treated cells was observed after treatment with BIT or OIT, alone or in mixture. However, if there would have been a slight CYP1A activity after exposure to these isothiazolinones, it is not certain that this effect would be detectable with our setup. As a consequence of these issues the data for CYP1A induction after treatment with BIT and OIT is limited in this study, and statistical testing of these results was determined unsuitable. Thus similar experiments should be performed with well-functioning controls, standards and correct instrumental settings to supplement this data.

Slight differences in the CYP1A activity could be observed depending on if the cells were exposed to BNF alone or in mixture with BIT or OIT. The induction of CYP1A activity for these mixtures was likely due to the presence of BNF rather than the isothiazolinones, as no activity was observed without BNF. The induction of CYP1A activity from isothiazolinone mixtures with BNF was at times higher than the BNF control, while other times the opposite was observed. The inconsistency of these results might be due to differences in cell density between treatments and experiments, but could also suggest an interaction between BNF and the isothiazolinones. However, due to the lack of resorufin standard, no definite conclusion can be drawn from these results.

While no induction of CYP1A enzyme was observed after exposure to BIT and OIT, its involvement in their metabolism cannot be ruled out based on these results.

5.2 Mitochondrial activity and membrane integrity (cytotoxicity)

Because of the likely exposure to isothiozolinones, such as BIT and OIT, for both humans and the marine ecosystems, the cytotoxic effect of these compounds is of importance to study. The current data on how BIT and OIT, especially in mixture, affect mitochondrial activity and membrane integrity in hepatocytes is limited. Thus, the results from this study will contribute to a broader knowledge about these effects. At the concentrations investigated in this study, no apparent toxic effect was observed on the cells when exposed to BIT alone. Similar results were observed in a study by Kim and colleagues (2021) on mouse brain endothelial cells (bEND.3) where no effect on mitochondrial activity or membrane integrity was observed after 24 hours of 10 µM BIT exposure [63].

For exposures to OIT at concentrations of 1 µM and 5 µM there was a statistically significant increase in membrane integrity when compared to the control, while ex-

posure to 10 µM OIT significantly decreased the membrane integrity. A reason for this could potentially be an effect referred to as hormesis. This term is primarily used in toxicology to describe the biphasic dose-response, where a low dose of a toxin results in a positive or beneficial effect, while a higher dose causes a negative or toxic effect [64]. Although this is only a hypothesis, it might be an explanation for the positive effect observed on the membrane integrity following exposure to the lower concentrations of OIT. Further, treatment with 10 µM OIT significantly decreased the mitochondrial activity. In a study performed by Kim and colleagues (2021), it was found that 24 hour exposure of 5 µM OIT resulted in no significant difference in mitochondrial activity or membrane integrity in bEND.3 cells, when compared to a control group [65]. However, exposure to 25 µM OIT resulted in a significant decrease in both cellular properties. Their results suggest that the toxic concentration of OIT lies within the range of 5 to 25 µM, which is further reinforced by the results of our study. However it is important to point out the different cell types used in these studies.

As both BIT and OIT are extensively used in similar products and are likely to end up in the same environment, their potential mixture effects are of high interest. In experiments of co-exposure to BIT and OIT, it was found that an increasing concentration of OIT resulted in greater cytotoxic effects rather than when the BIT concentration was increased. This suggests that in mixture with BIT, it is still the presence of OIT that determines the magnitude of toxicity in terms of mitochondrial activity and membrane integrity. Interestingly, exposure to 10 µM OIT proved to have an even greater toxic effect when combined with the non-toxic concentration of 10 µM BIT, as compared to 10 µM OIT alone. These results suggests a synergistic mixture effect on cytotoxicity where the combination of BIT and OIT at these concentrations display a greater toxic effect than either compound alone. Such behaviour might be a potential threat in the environment, where these compounds are likely to co-exist.

CYP enzymes are known to cause formation of reactive oxygen species (ROS) upon uncoupling of the catalytic cycle [66]. In phase I metabolism, CYP enzymes increase the polarity of xenobiotic substances by adding functional groups like -OH or -COOH. However, if uncoupled, these groups can react further leading to the formation of potent ROS such as $O2^{-}$ or $H2O_2$. There are several features that affects the formation of ROS in the CYP catalytic cycle, such as pH, oxygen presence, concentration of substrate and even which specific CYP enzyme derivative is metabolising the compound. It is known that ROS can modify cellular macromolecules such as DNA, lipids and proteins, and has also been linked to development of disease and cancer in humans [67]. In addition, ROS is believed to be highly reactive with mitochondrial DNA, ultimately leading to mitochondrial dysfunction upon formation of ROS [68]. Although the results obtained from the EROD assay do not suggest involvement of CYP1A, it can still not be ruled out. The involvement of other CYP enzymes in the metabolism of both BIT and OIT is also likely given their important role in phase I biotransformation. Due to the likelihood of ROS production during the catalytic cycle of CYPs, this could be a potential explanation for the decreased

mitochondrial activity in the cell samples exposed to the higher concentration of OIT. However, there are many other possible explanations for the decreased cellular functions upon treatment with isothiazolinones. The biocidal mechanism of action that BIT and OIT have towards microorganisms may very well cause similar effects in other organisms and cell types, such as PLHC-1 cells, though to a lesser extent. The specific mechanism for the toxic effects that are observed after exposure to OIT, alone and in combination with BIT, would have to be further specified but the toxic nature is well demonstrated in our results.

5.3 Cellular uptake and excretion of BIT and OIT (extracellular)

Measuring the concentrations of extracellular compounds over time can give an indication of the cellular metabolism of these compounds, in this case BIT and OIT. As mentioned in chapter 4.3, the number of replicates for HPLC analysis were few which is a result of several constraints. HPLC analysis is both time-consuming and resource-demanding, limiting the number of samples that could be analysed. This was further restricted by the limited availability of the instrument. This project aimed to do a preliminary screening of BIT and OIT metabolism rather than performing a precise quantitative analysis. Therefore, it was decided that assessing different exposure concentrations was more valuable than increasing the number of replicates per treatment.

As the results showed, BIT was detectable in the cell media at all time points, while OIT was only detectable after 3 hours of exposure. This indicates a more rapid cellular uptake of OIT compared to BIT, which could be a consequence of their different chemical properties. The OIT molecule contains a long hydrophobic carbon chain, resulting in a log K_{ow} of 3.3, while BIT has a much lower log K_{ow} of 0.7. As shown in a study by McKeage and colleagues (2000) on cellular uptake of different drugs in a human ovarian carcinoma cell line, the uptake rate of the drugs increased with increasing lipophilicity [69]. Similarly, the lipophilic properties of OIT likely resulted in a more rapid transport over the cell membrane, as compared to the less lipophilic BIT. What was also observed in our study, was an even faster uptake of OIT when the cells were co-exposed to BIT and OIT. The cause of this observation is not quite clear, but could however be of interest to further investigate.

As previously observed in a Master's thesis project [62], a potential BIT metabolite was detected in an HPLC experiment. This potential metabolite was further observed in this project as a peak in the chromatogram appearing just before the BIT peak. In time-resolved experiments, this proposed metabolite peak increased over time, while the BIT peak decreased, displaying an inverse correlation. These findings further supports the proposed metabolism of BIT in PLHC-1 cells. The shorter retention time of the proposed metabolite compared to BIT in the HPLC system suggests an increased hydrophilicity, potentially resulting from biotransformation during phase I, phase II, or both. The fact that the presumed metabolite is better

detected at a shorter wavelength than BIT, is an indication of structural differences between the two. Both the aromatic and the thiazole ring in the BIT molecule contribute to a conjugated system, resulting in increased electron delocalization. Larger conjugated systems absorb light at higher wavelengths [70], indicating that the degree of conjugation in the proposed metabolite is reduced. This reduction in conjugation could be due to the opening of one of the ring structures. However, it is more likely that the thiazole ring is opened because of the high reactivity of the sulfur atom. The common denominator for several studies on BIT in different reaction pathways, is the opening of the thiazole ring [71, 72, 73], which further reinforces this theory. To properly investigate the nature of the proposed metabolite, further analytical methods should be utilized. Such methods could, for example, be mass spectrometry techniques including LC-MS and GC-MS, which could provide detailed information about molecular weight, fragmentation pattern and ultimately the structure of the metabolite.

It was also investigated whether the presence of OIT affected the metabolism of BIT in the PLHC-1 cells. It was found that only concentrations of 10 µM OIT (in combination with 10 µM BIT) exhibited a considerable effect on BIT uptake and metabolite production. What has already been observed in the cytotoxicity assay, is that the combination of BIT and OIT at these concentrations resulted in a significant toxic effect. Similar results were also observed in the total protein content determination of treatments in the EROD assay (found in Figure A.4 in Appendix A), where this combination resulted in decreased protein concentration (low cell proliferation). The high concentration of BIT left in the cell media, as well as the low concentration of BIT metabolite may therefore only be a result of lower cell density as a result of toxicity rather than an interaction between the BIT and OIT.

The potential involvement of the CYP1A enzyme in the metabolism of BIT and OIT was primarily investigated in the EROD assay, however it was further assessed in the HPLC analyses. As OIT was not detectable at other timepoints than after 3 hours of exposure, and the induction of CYP1A activity was at a maximum after around 24 hours exposure to BNF (see Fig. 4.2), this analysis focused on BIT metabolism. Co-exposure to BIT and BNF was compared to exposure to BIT only, and no differences in extracellular amounts of metabolite was observed between the two treatments. Thus, an induction of CYP1A enzyme does not seem to have an effect on the production of the metabolite. It was however observed that when in combination with BNF, a higher concentration of BIT was detectable in the cell media, suggesting less cellular uptake of BIT. It was also ruled out that this decreased cellular uptake of BIT was due to a lower cell density from these culturing samples, as there were no apparent difference in the protein contents after 24 hour exposure to the two treatments (see Figure A.4 in Appendix A). Hence, the reason for this response is not entirely clear. It could however be a result of the lipophilic nature of the BNF molecule (log K_{ow} of 4.65 [74]). Similar to the case with OIT, the lipophilicity of BNF likely results in a more rapid cellular uptake compared to the more hydrophilic BIT, which potentially hinders the uptake of BIT. However, no difference in the amount of metabolite was detected in the cell media for cells exposed

to BIT alone or in co-exposure with BNF, suggesting enough BIT has entered the cells in order to produce a similar amount of metabolite in both cases. This could indicate that either the metabolism of BIT or the excretion of BIT metabolite is slower than the cellular uptake of BIT. However, as this effect was not observed when BIT was in combination with the lipophilic OIT, other possible explanations should be considered.

5.4 Extraction of intracellular BIT and OIT

As previously discussed, OIT was undetectable in the media at time points >3 hours, and thus hypothesized to quickly enter the cells. Additionally, no OIT metabolite could be detected at any time point. Therefore, an attempt to extract the cellular content was performed to investigate whether OIT and/or a potential metabolite could be detected intracellularly. However, this was not successfully accomplished with the performed assay since no peaks corresponding to the BIT and OIT standards were identified on the chromatograms obtained from the cell extractions. When extracting the intracellular content using heptane, the samples are expected to not only contain the isothiazolinones but also other non-polar molecules, such as different lipids and proteins. Because of the complex composition of the samples, the resulting chromatograms proved to be more challenging to interpret and may contribute to the inability to distinguish any isothiazolinone peaks. Why OIT was not detectable is unclear, however the lack of metabolite may be a result of a structural change of the molecule. As previously discussed, the thiazole ring may be opened during biotransformation. Upon this ring-opening of the OIT molecule, the degree of conjugation would be decreased, or even lost, in the OIT metabolite. Therefore, it would most likely not be detectable at the detection wavelengths used in this HPLC system.
Overall, the results obtained from the analysis of intracellular BIT and OIT suggests that the method needs further perfection and development in order to adequately investigate the possibility of intracellular detection of the isothiazolinones.

6

Conclusions and future perspectives

This study explored the metabolism and mixture effects of the isothiazolinone biocides BIT and OIT, in PLHC-1 cells. Given their widespread use and potential impact on environment and human health, their toxicodynamic and toxicokinetic properties are crucial to establish.

The findings regarding CYP1A activity, revealing no apparent increase when PLHC-1 cells were exposed to BIT and OIT alone or in mixture, does not suggest involvement of the enzyme in their metabolism. However, more robust data is necessary to entirely rule it out. Involvement of other CYP enzymes can also not be excluded, based on the current study.

Extracellular analyses provided insights into the metabolism of BIT, revealing the presence of a probable metabolite with increased polarity and structural differences compared to BIT. However further analysis is necessary to determine its definite chemical structure. No OIT or OIT metabolite was detected intracellularly, thus requiring refining of the analytical procedure, such as further development of the extraction method and HPLC system.

At the concentrations investigated in this study, only OIT displayed a significant toxic effect represented by impaired mitochondrial activity and membrane integrity in the PLHC-1 cells. Cellular uptake of OIT proved to be considerably faster than that of BIT, which may be a contributing factor to its toxicity. Indication of synergistic toxic effects were also observed for BIT and OIT at the highest concentrations examined, underscoring the importance of studying mixture effects in environmental contexts.

In summary, this study contributes valuable insights into the toxicodynamic and toxicokinetic profile of BIT and OIT, emphasizing the importance of considering mixture effects and metabolic pathways in assessing their potential adverse health and environmental effect. Future research should focus on further exploring metabolic pathways and interaction mechanisms of BIT and OIT, refining detection methodologies, and assessing the long-term effects of their exposure.

To investigate other enzymes potentially involved in the metabolism of BIT and OIT, co-exposure with compounds known to inhibit (or induce) other detoxicifica-

tion enzymes could be implemented to evaluate how production of the presumed BIT metabolite is affected. Potential enzyme inhibitors could include: clotrimazole, inhibitor of CYP1A and CYP3A (phase I) [75]; fluoxetine, inhibitor of CYP2C9 and CYP2D6 (phase I) [76]; and chlorpromazine, inhibitor of glutathione S-transferases (GSTs, phase II) [77]. While the EROD assay is suitable for investigating how well 7-ethoxyresorufin is converted to the fluorescent resorufin by the CYP1A enzyme, it does not directly assess the AhR activation and up-regulation of the CYP1A gene. A high CYP1A activity may be influenced by factors other than enzyme quantity, thus mRNA quantification would give an indication if this activity could be connected to increased expression of the enzyme or if it could be a result of some other interaction that increases the efficiency of the enzyme. Coversely, a low CYP1A activity does not necessarily mean that the gene is not up-regulated as there could be simultaneous process of gene induction and inhibition of CYP1A enzyme activity. Therefore, analysing the transcription of the CYP1A enzyme through mRNA quantification, would be a good complementary assay to investigate if BIT and OIT may still interact with the AhR receptor and induce CYP1A transcription. Quantification of mRNA levels however do not take into account if the RNA is translated into the CYP1A enzyme, or if post-translational modification occurs.

As the collection and composition of enzymes and other cellular functions vary in liver cells between species, it is important to expand the study to include other cell lines in the future. The human hepatocellular carcinoma (HepG2) cell line is one such example that could help in explaining the behaviour and faith of the isothiazolinones further in the human body.

Bibliography

[1] Maja Edenius, Anne Farbrot, Anders Blom, and Malin C Celander. Delayed clearance of the pro-carcinogen benzo [a] pyrene in PLHC-1 cells when co-exposed to the antifungal drug clotrimazole and effects on the CYP1A biomarker. *Toxicology in Vitro*, 95:105767, 2024.

[2] Jean-Yves Maillard. Resistance of bacteria to biocides. *Microbiology spectrum*, 6(2):10–1128, 2018.

[3] Vânia Silva, Cátia Silva, Pedro Soares, E Manuela Garrido, Fernanda Borges, and Jorge Garrido. Isothiazolinone biocides: chemistry, biological, and toxicity profiles. *Molecules*, 25(4):991, 2020.

[4] David A Basketter, Rosemary Rodford, Ian Kimber, Ian Smith, and Jan E Wahlberg. Skin sensitization risk assessment: a comparative evaluation of 3 isothiazolinone biocides. *Contact Dermatitis*, 40(3):150–154, 1999.

[5] Olivier Aerts, An Goossens, Julien Lambert, and Jean-Pierre Lepoittevin. Contact allergy caused by isothiazolinone derivatives: an overview of non-cosmetic and unusual cosmetic sources. *European Journal of Dermatology*, 27:115–122, 2017.

[6] Anne Herman, Olivier Aerts, Laurence de Montjoye, Isabelle Tromme, Annie Goossens, and Marie Baeck. Isothiazolinone derivatives and allergic contact dermatitis: a review and update. *Journal of the European Academy of Dermatology and Venereology*, 33(2):267–276, 2019.

[7] Nagamany N Nirmalakhandan, V Arulgnanendran, M Mohsin, B Sun, and Fernando Cadena. Toxicity of mixtures of organic chemicals to microorganisms. *Water Research*, 28(3):543–551, 1994.

[8] Elisabete Silva, Nissanka Rajapakse, and Andreas Kortenkamp. Something from "nothing"- eight weak estrogenic chemicals combined at concentrations below noecs produce significant mixture effects. *Environmental science & technology*, 36(8):1751–1756, 2002.

[9] Åsa Arrhenius, Frederick Grönvall, Martin Scholze, Thomas Backhaus, and Hans Blanck. Predictability of the mixture toxicity of 12 similarly acting congeneric inhibitors of photosystem II in marine periphyton and epipsammon communities. *Aquatic toxicology*, 68(4):351–367, 2004.

[10] John O Morley, A Jayne Oliver Kapur, and Michael H Charlton. Structure–

activity relationships in 3-isothiazolones. *Organic & Biomolecular Chemistry*, 3(20):3713–3719, 2005.

[11] Jürgen Arning, Ralf Dringen, Maike Schmidt, Anette Thiessen, Stefan Stolte, Marianne Matzke, Ulrike Bottin-Weber, Birgit Caesar-Geertz, Bernd Jastorff, and Johannes Ranke. Structure–activity relationships for the impact of selected isothiazol-3-one biocides on glutathione metabolism and glutathione reductase of the human liver cell line Hep G2. *Toxicology*, 246(2-3):203–212, 2008.

[12] Swedish Chemicals Agency (KEMI). The most common substances in consumer products - number of products. Quantity in tonnes (2022). Report, Swedish Chemicals Agency (KEMI), 2022. Table 8 from the report.

[13] European Commission. COMMISSION REGULATION (EU) 2017/1224 of 6 July 2017 amending Annex V to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products. *Comm Regul No 752/2014*, 15(1223):1–71, 2017.

[14] Wolfgang Uter, Kristiina Aalto-Korte, Tove Agner, Klaus E Andersen, Andreas J Bircher, Richard Brans, Magnus Bruze, TL Diepgen, Caterina Foti, A Giménez Arnau, et al. The epidemic of methylisothiazolinone contact allergy in Europe: follow-up on changing exposures. *Journal of the European Academy of Dermatology and Venereology*, 34(2):333–339, 2020.

[15] Carola Lidén, Niklas Andersson, and Ian R White. Preservatives in non-cosmetic products: Increasing human exposure requires action for protection of health. *Contact Dermatitis*, 87(5):389–405, 2022.

[16] Jakob Ferløv Schwensen, C Menné Bonefeld, Claus Zachariae, C Agerbeck, TH Petersen, Carsten Geisler, UE Bollmann, K Bester, and Jeanne Duus Johansen. Cross-reactivity between methylisothiazolinone, octylisothiazolinone and benzisothiazolinone using a modified local lymph node assay. *British Journal of Dermatology*, 176(1):176–183, 2017.

[17] Kristiina Aalto-Korte and Katri Suuronen. Patterns of concomitant allergic reactions in patients suggest cross-sensitization between octylisothiazolinone and methylisothiazolinone. *Contact Dermatitis*, 77(6):385–389, 2017.

[18] Lianguo Chen and James CW Lam. Seanine 211 as antifouling biocide: A coastal pollutant of emerging concern. *Journal of Environmental Sciences*, 61: 68–79, 2017.

[19] Nam Sook Kim, Won Joon Shim, Un Hyuk Yim, Sang Hee Hong, Sung Yong Ha, Gi Myung Han, and Kyung-Hoon Shin. Assessment of TBT and organic booster biocide contamination in seawater from coastal areas of South Korea. *Marine pollution bulletin*, 78(1-2):201–208, 2014.

[20] Un-Jung Kim, In-Seok Lee, Minkyu Choi, Jeong-Eun Oh, et al. Assessment of organotin and tin-free antifouling paints contamination in the korean coastal area. *Marine Pollution Bulletin*, 99(1-2):157–165, 2015.

[21] Hiroya Harino, Masaaki Kitano, Yoshiaki Mori, Kazuhiko Mochida, Akira Kakuno, and Satoshi Arima. Degradation of antifouling booster biocides in water. *Journal of the Marine Biological Association of the United Kingdom*, 85 (1):33–38, 2005.

[22] Hiroya Harino, Yoshiaki Mori, Yoshitaka Yamaguchi, Kiyoshi Shibata, and Tetsuya Senda. Monitoring of antifouling booster biocides in water and sediment-from the port of Osaka, Japan. *Archives of environmental contamination and toxicology*, 48:303–310, 2005.

[23] Kazuhiko Mochida, Takeshi Hano, Toshimitsu Onduka, Hideki Ichihashi, Haruna Amano, Mana Ito, Katsutoshi Ito, Hiroyuki Tanaka, and Kazunori Fujii. Spatial analysis of 4, 5-dichloro-2-n-octyl-4-isothiazolin-3-one (Sea-Nine 211) concentrations and probabilistic risk to marine organisms in Hiroshima Bay, Japan. *Environmental pollution*, 204:233–240, 2015.

[24] Sayaka Eguchi, Hiroya Harino, and Yoshikazu Yamamoto. Assessment of antifouling biocides contaminations in Maizuru Bay, Japan. *Archives of environmental contamination and toxicology*, 58:684–693, 2010.

[25] Hiroya Harino, Takaomi Arai, Madoka Ohji, Ahmad Bin Ismail, and Nobuyuki Miyazaki. Contamination profiles of antifouling biocides in selected coastal regions of Malaysia. *Archives of environmental contamination and toxicology*, 56:468–478, 2009.

[26] Hiroya Harino, Zainal Arifin, Inneke FM Rumengan, Takaomi Arai, Madoka Ohji, and Nobuyuki Miyazaki. Distribution of antifouling biocides and perfluoroalkyl compounds in sediments from selected locations in Indonesian coastal waters. *Archives of Environmental Contamination and Toxicology*, 63:13–21, 2012.

[27] Hernando M Dolores, Luis Piedra, Angel Belmonte, Ana Aguera, and Amadeo R Fernandez-Alba. Determination of traces of five antifouling agents in water by gas chromatography with positive/negative chemical ionisation and tandem mass spectrometric detection. *Journal of Chromatography A*, 938(1-2): 103–111, 2001.

[28] K Martinez and D Barceló. Determination of antifouling pesticides and their degradation products in marine sediments by means of ultrasonic extraction and HPLC–APCI–MS. *Fresenius' journal of analytical chemistry*, 370:940–945, 2001.

[29] James W Readman. Development, occurrence and regulationof antifouling paint biocides: historical review and future trends. *Antifouling paint biocides*, pages 1–15, 2006.

[30] Vasilios A Sakkas, Ioannis K Konstantinou, Dimitra A Lambropoulou, and Triantafyllos A Albanis. Survey for the occurrence of antifouling paint booster biocides in the aquatic environment of Greece. *Environmental Science and Pollution Research*, 9:327–332, 2002.

[31] Mari-Ann Flyvholm. Preservatives in registered chemical products. *Contact dermatitis*, 53(1):27–32, 2005.

[32] Elena Garcia-Hidalgo, Dovilé Schneider, Natalie von Goetz, Christiaan Delmaar, Michael Siegrist, and Konrad Hungerbühler. Aggregate consumer exposure to isothiazolinones via household care and personal care products: Probabilistic modelling and benzisothiazolinone risk assessment. *Environment international*, 118:245–256, 2018.

[33] Australian Industrial Chemicals Introduction Scheme. Octylisothiazolinone preservatives and industrial biocides: Environment tier II assessment, 2019.

[34] Environmental Protection Agency (EPA). Reregistration Eligibility Decision (RED) for Benzisothi-azolin-3-one. Technical report, Environmental Protection Agency (EPA), 2005.

[35] Biocidal Products Committee (BPC). Opinion on the application for approval of the active substance: 1,2-BENZISOTHIAZOL-3-(2H)-ONE (BIT). Technical Report 287, ECHA/BPC, 2021. Product type: 13.

[36] Iuliana Paun, Florinela Pirvu, Vasile Ion Iancu, and Florentina Laura Chiriac. Occurrence and transport of isothiazolinone-type biocides from commercial products to aquatic environment and environmental risk assessment. *International Journal of Environmental Research and Public Health*, 19(13):7777, 2022.

[37] Sujin Lee, Ji-Su Lee, Younglim Kho, and Kyunghee Ji. Effects of methylisothiazolinone and octylisothiazolinone on development and thyroid endocrine system in zebrafish larvae. *Journal of Hazardous Materials*, 425:127994, 2022.

[38] Rachel M Novick, Mindy L Nelson, Kenneth M Unice, James J Keenan, and Dennis J Paustenbach. Estimation of the safe use concentrations of the preservative 1, 2-benzisothiazolin-3-one (BIT) in consumer cleaning products and sunscreens. *Food and chemical toxicology*, 56:60–66, 2013.

[39] JP Thyssen, N Sederberg-Olsen, JF Thomsen, and T Menné. Contact dermatitis from methylisothiazolinone in a paint factory. *Contact Dermatitis*, 54(6): 322–324, 2006.

[40] Anja P Mose, Simon Frost, Ulf Öhlund, and Klaus E Andersen. Allergic contact dermatitis from octylisothiazolinone. *Contact Dermatitis*, 69(1):49–52, 2013.

[41] Nils Hamnerius, Ann Pontén, and Martin Mowitz. Textile contact dermatitis caused by octylisothiazolinone in compression stockings. *Contact Dermatitis*, 78(6):419–421, 2018.

[42] Olivier Aerts, Hans Meert, Elien Romaen, Julie Leysen, Lucretia Matthieu, Sandra Apers, Julien Lambert, and An Goossens. Octylisothiazolinone, an additional cause of allergic contact dermatitis caused by leather: case series and potential implications for the study of cross-reactivity with methylisothiazolinone. *Contact Dermatitis*, 75(5):276–284, 2016.

[43] Barbara Döring and Ernst Petzinger. Phase 0 and phase III transport in various organs: combined concept of phases in xenobiotic transport and metabolism. *Drug metabolism reviews*, 46(3):261–282, 2014.

[44] Daniel Schlenk, Malin Celander, Evan P Gallagher, Stephen George, Margaret James, Seth W Kullman, Peter van den Hurk, and Kristie Willett. Biotransformation in fishes. *The toxicology of fishes*, 1, 2008.

[45] Mingzhe Zhao, Jingsong Ma, Mo Li, Yingtian Zhang, Bixuan Jiang, Xianglong Zhao, Cong Huai, Lu Shen, Na Zhang, Lin He, et al. Cytochrome P450 enzymes and drug metabolism in humans. *International journal of molecular sciences*, 22(23):12808, 2021.

[46] Maria Almira Correia. *Drug biotransformation*, chapter 4. McGraw Hill Education New York, NY, USA, 2018.

[47] Christopher J Kennedy and Keith B Tierney. Xenobiotic protection/resistance mechanisms in organisms. In *Environmental toxicology: selected entries from the encyclopedia of sustainability science and technology*, pages 689–721. Springer, 2012.

[48] Changjiang Xu, Christina Yong-Tao Li, and Ah-Ng Tony Kong. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Archives of pharmacal research*, 28:249–268, 2005.

[49] Zinhle Edith Chiliza, José Martínez-Oyanedel, and Khajamohiddin Syed. An overview of the factors playing a role in cytochrome P450 monooxygenase and ferredoxin interactions. *Biophysical Reviews*, 12(5):1217–1222, 2020.

[50] Heather MH Goldstone and John J Stegeman. A revised evolutionary history of the CYP1A subfamily: gene duplication, gene conversion, and positive selection. *Journal of molecular evolution*, 62:708–717, 2006.

[51] Malin Celander, Jessica Bremer, Mark E Hahn, and John J Stegeman. Glucocorticoid-xenobiotic interactions: Dexamethasone-mediated potentiation of cytochrome P4501A induction by β-naphthoflavone in a fish hepatoma cell line (PLHC-1). *Environmental Toxicology and Chemistry: An International Journal*, 16(5):900–907, 1997.

[52] Thomas D Bucheli and Karl Fent. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical reviews in environmental science and technology*, 25(3):201–268, 1995.

[53] Elisabet Pérez-Albaladejo, Alejandra Solís, Ilaria Bani, and Cinta Porte. Plhc-1 topminnow liver cells: An alternative model to investigate the toxicity of plastic additives in the aquatic environment. *Ecotoxicology and Environmental Safety*, 208:111746, 2021.

[54] PH Krone, SR Blechinger, TG Evans, JA Ryan, EJ Noonan, and LE Hightower. Use of fish liver plhc-1 cells and zebrafish embryos in cytotoxicity assays. *Methods*, 35(2):176–187, 2005.

[55] Elvira Mennillo, Aina O Adeogun, and Augustine Arukwe. Quality screening of the lagos lagoon sediment by assessing the cytotoxicity and toxicological responses of rat hepatoma h4iie and fish plhc-1 cell-lines using different extraction approaches. *Environmental research*, 182:108986, 2020.

[56] Rémi Thibaut, Sabine Schnell, and Cinta Porte. Assessment of metabolic capabilities of PLHC-1 and RTL-W1 fish liver cell lines. *Cell biology and toxicology*, 25:611–622, 2009.

[57] Roko Zaja, Roberta Sauerborn Klobučar, and Tvrtko Smital. Detection and functional characterization of Pgp1 (ABCB1) and MRP3 (ABCC3) efflux transporters in the PLHC-1 fish hepatoma cell line. *Aquatic toxicology*, 81(4):365–376, 2007.

[58] John R Petrulis, Guosheng Chen, Sally Benn, Jon LaMarre, and Nigel J Bunce. Application of the ethoxyresorufin-O-deethylase (EROD) assay to mixtures of halogenated aromatic compounds. *Environmental Toxicology: An International Journal*, 16(2):177–184, 2001.

[59] Sidney Udenfriend, Stanley Stein, Peter Boehlen, Wallace Dairman, Willy Leimgruber, and Manfred Weigele. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science*, 178(4063):871–872, 1972.

[60] Stephanie K Bopp and Teresa Lettieri. Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. *BMC pharmacology*, 8:1–11, 2008.

[61] Thermo Fisher Scientific. Alamarblue® assay, US Patent No. 5 501 959, Feb. 1995.

[62] Isabella Marchini. Metabolism of the lipophilic isothiazolinones BIT and DCOIT in the fish liver cell line PLHC-1. Master's thesis, University of Gothenburg, 2024. Unpublished Master's Thesis.

[63] Donghyun Kim, Eun-Hye Kim, and Ok-Nam Bae. Comparative study of two isothiazolinone biocides, 1, 2-benzisothiazolin-3-one (BIT) and 4, 5-dichloro-2-n-octyl-isothiazolin-3-one (DCOIT), on barrier function and mitochondrial bioenergetics using murine brain endothelial cell line (bEND. 3). *Journal of Toxicology and Environmental Health, Part A*, 84(22):932–943, 2021.

[64] Mark P Mattson. Hormesis defined. *Ageing research reviews*, 7(1):1–7, 2008.

[65] Donghyun Kim, Eun-Hye Kim, Sungbin Choi, Kyung-Min Lim, Lu Tie, Arshad Majid, and Ok-Nam Bae. A commonly used biocide 2-n-octyl-4-isothiazolin-3-oneinduces blood–brain barrier dysfunction via cellular thiol modification and mitochondrial damage. *International Journal of Molecular Sciences*, 22(5):2563, 2021.

[66] Alex Veith and Bhagavatula Moorthy. Role of cytochrome P450s in the generation and metabolism of reactive oxygen species. *Current opinion in toxicology*, 7:44–51, 2018.

[67] Eugene G Hrycay and Stelvio M Bandiera. Involvement of cytochrome P450 in reactive oxygen species formation and cancer. *Advances in Pharmacology*, 74: 35–84, 2015.

[68] Chunyan Guo, Li Sun, Xueping Chen, and Danshen Zhang. Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural regeneration research*, 8(21):2003–2014, 2013.

[69] Mark J McKeage, Susan J Berners-Price, Peter Galettis, Richard J Bowen, Wandy Brouwer, Li Ding, Li Zhuang, and Bruce C Baguley. Role of lipophilicity in determining cellular uptake and antitumour activity of gold phosphine complexes. *Cancer chemotherapy and pharmacology*, 46:343–350, 2000.

[70] John McMurry. *Organic Chemistry*, chapter 14.8. Cengage Learning, Boston, MA, USA, 9th edition, 2016.

[71] PJ Collier, A Ramsey, RD Waigh, KT Douglas, P Austin, and P Gilbert. Chemical reactivity of some isothiazolone biocides. *Journal of Applied Bacteriology*, 69(4):578–584, 1990.

[72] Zsuzsanna Varga, Edith Nicol, and Stéphane Bouchonnet. Photodegradation of benzisothiazolinone: Identification and biological activity of degradation products. *Chemosphere*, 240:124862, 2020.

[73] Ting Wang, Qian-Yuan Wu, Wen-Long Wang, Zhuo Chen, Bing-Tian Li, Ang Li, Zi-Ye Liu, and Hong-Ying Hu. Self-sensitized photodegradation of benzisothiazolinone by low-pressure UV-C irradiation: Kinetics, mechanisms, and the effect of media. *Separation and Purification Technology*, 189:419–424, 2017.

[74] Yuji Fujita, Mitsuhiro Yonehara, Masashi Tetsuhashi, Tomomi Noguchi-Yachide, Yuichi Hashimoto, and Minoru Ishikawa. β-Naphthoflavone analogs as potent and soluble aryl hydrocarbon receptor agonists: Improvement of solubility by disruption of molecular planarity. *Bioorganic & medicinal chemistry*, 18(3):1194–1203, 2010.

[75] Viktoriia Burkina, Vladimir Zlabek, and Galia Zamaratskaia. Clotrimazole, but not dexamethasone, is a potent in vitro inhibitor of cytochrome P450 isoforms CYP1A and CYP3A in rainbow trout. *Chemosphere*, 92(9):1099–1104, 2013.

[76] Brian Gilani and Manouchkathe Cassagnol. *Biochemistry, Cytochrome P450*. StatPearls Publishing, Treasure Island (FL), 2023.

[77] Fikret Türkan, Mehmet Harbi Calimli, Gülşah Saydan Kanberoğlu, and Muhammet Karaman. Inhibition effects of isoproterenol, chlorpromazine, carbamazepine, tamoxifen drugs on glutathione S-transferase, cholinesterases enzymes and molecular docking studies. *Journal of Biomolecular Structure and Dynamics*, 39(9):3277–3284, 2021.

A

Appendix: Additional results

Appendix A presents some additional results obtained from the project, needed to interpret and understand the explanations and reasoning in chapter 4 and 5.

The extracellular amount of BIT and BIT metabolite was analysed in a time study twice (two experimental repeats), one consisting of two technical replicates (presented in chapter 4.3) and the other one with no technical replicates (presented in Figure A.1). The two experimental repeats presented similar trends, decreased extracellular BIT over time while extracellular metabolite increased, however displayed similar absolute values of the arbitrary AUC unit. Therefore, they should not be combined into one dataset and this is also the reason for the complementary data in Figure A.1.

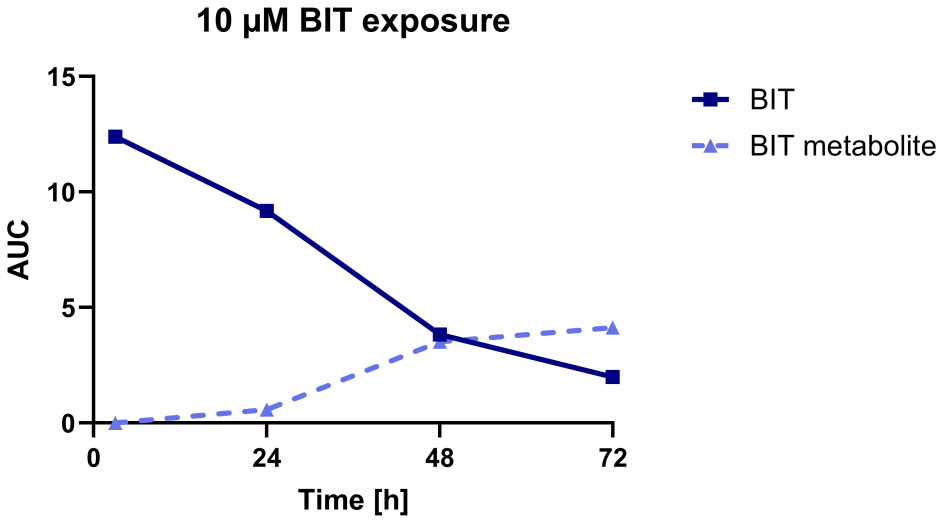


Figure A.1: AUC for BIT and BIT metabolite peaks over time (3, 24, 48, 72h) after exposure with 10 µM BIT. The data is from one experimental repeat containing no technical replicates.

Below, in Figure A.2, is a chromatogram obtained from the analysis of intracellular BIT, overlayed with the chromatogram of a 10 µM BIT standard dissolved in MeOH. The standard was used to determine the retention time of BIT in the environment of the cell extraction samples. As indicated by the arrow, the red chromatogram appears to display a peak corresponding to BIT. However, this is not a BIT peak as the peak also appeared on the chromatograms of extraction samples from cells exposed to OIT (i.e. not BIT). Also, since the peak is only appearing on the 275

nm detection wavelength, and not the 320 nm, further implies that the peak does not represent intracellular BIT.

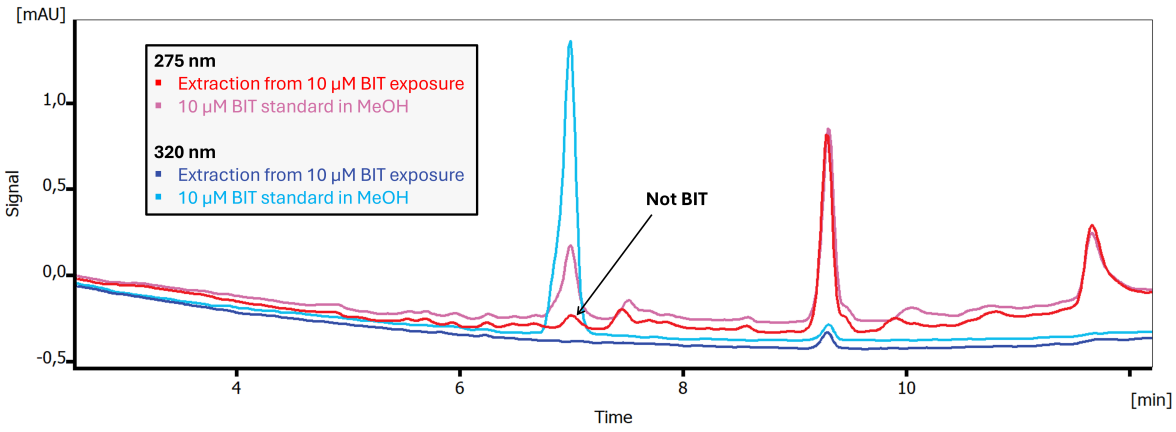


Figure A.2: Chomatograms of extraction samples from cells treated with 10 µM BIT for 24 hours. The cromatogram is compared to a 10 µM BIT standard dissolved in MeOH to indicate the retention time of BIT. As can be seen, the expected retention time of BIT in the system is around 7 minutes as visualized by the light blue peak from 320 nm detection. The arrow indicates a peak on the red chromatogram that could be missinterpreted as presence of intracellular BIT, however this was ruled out. The image is zoomed in on the region of the chromatograms where the BIT standard peaks appeared.

The retention time of OIT was also determined in the system using a standard of 10 µM OIT dissolved in MeOH. Indicated by the brown chromatogram in Figure A.3, the retention time was around 12 minutes. The lack of peak at 12 minutes on the green chromatogram indicates no presence of intracellular OIT in the extraction samples.

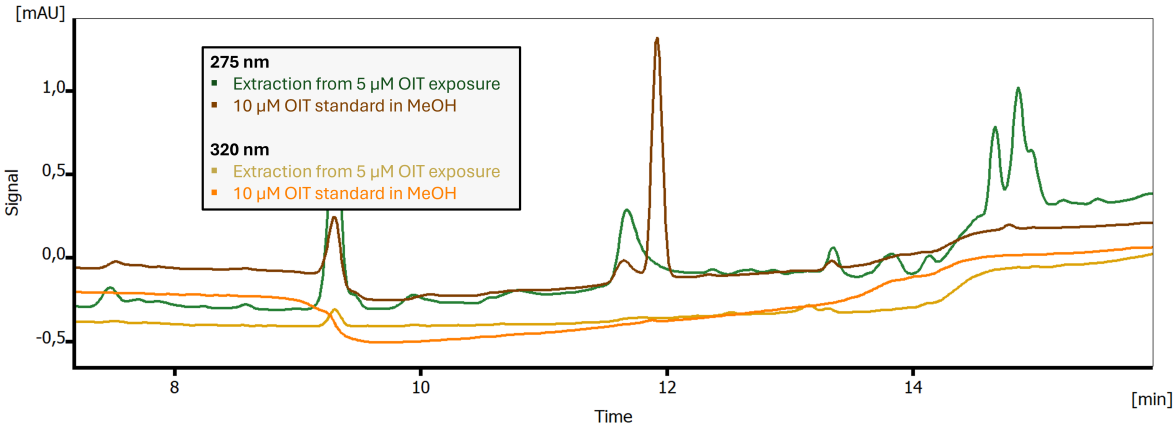


Figure A.3: Chomatograms of extraction samples from cells treated with 5 µM OIT for 24 hours. The cromatogram is compared to a 10 µM OIT standard dissolved in MeOH to indicate the retention time of OIT. As can be seen, the expected retention time of OIT in the system is around 12 minutes as visualized by the brown peak from 275 nm detection. The image is zoomed in on the region of the chromatograms where the OIT standard peaks appeared.

In the EROD assay, the protein content in every well was determined following exposure. The protein content for each 24h treatment was then normalised to protein

contents of vehicle control wells, so they could be comparable between exposure treatments. The protein contents of some 24h treatments are displayed in Figure A.4 and presented in percentage of the control.

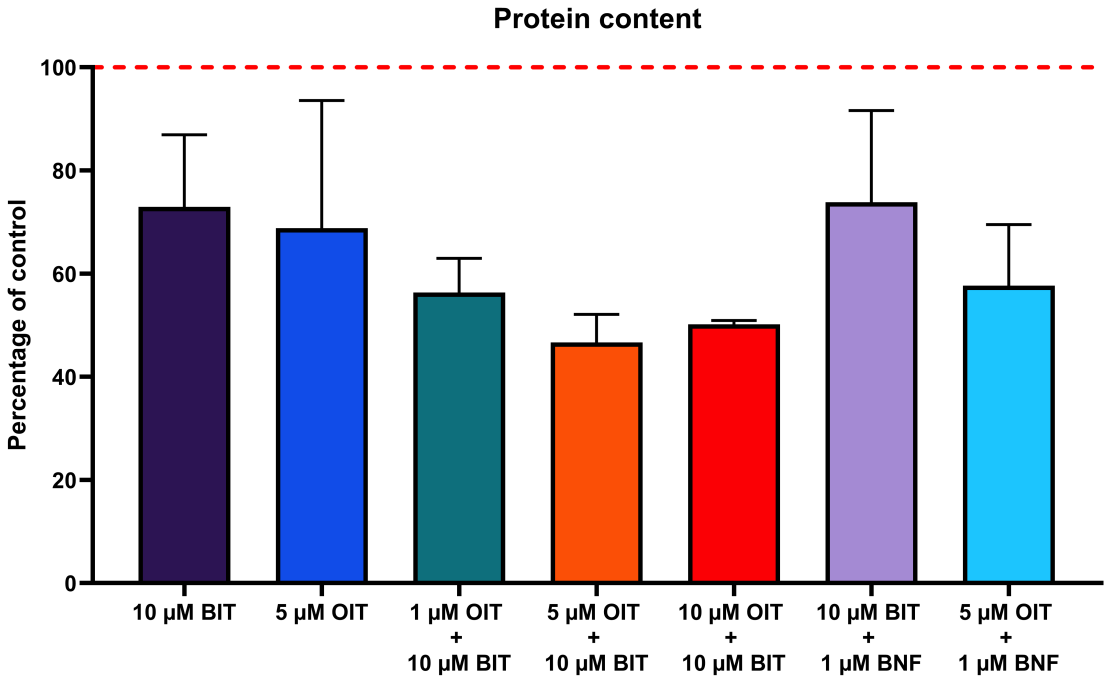


Figure A.4: Collected data on protein content from the EROD assay after 24h exposure of some treatments with BIT and OIT. For each culturing plate, the protein contents in each treatment well have been normalized to the protein content for the DMSO vehicle control wells. The protein contents for each treatment is therefore presented as a percentage of the protein content in wells with vehicle control treated cells. The protein content of the DMSO vehicle control is set to 100%, indicated by the dashed red line. The treatments include 10 µM BIT (n=16), 5 µM OIT (n=12), 1 µM OIT + 10 µM BIT (n=8), 5 µM OIT + 10 µM BIT (n=12), 10 µM OIT + 10 µM BIT (n=4), 1 µM BNF + 10 µM BIT (n=12) and 1 µM BNF + 5 µM OIT (n=8). The data is combined from up to three separate experimental repeats, each with four technical replicates, and expressed as mean ± SD.